MycoKeys 94: 1–16 (2022)
doi: 10.3897/mycokeys.94.91340
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Two new *Botryosphaeria* (Botryosphaeriales, Botryosphaeriaceae) species in China

Jing-E Sun[1], Chao-Rong Meng[1], Alan J. L. Phillips[2], Yong Wang[1]

1 *Department of Plant Pathology, Agricultural College, Guizhou University, Guiyang, 550025, China*
2 *Faculty of Sciences, Biosystems and Integrative Sciences Institute (BioISI), University of Lisbon, Campo Grande, 1749-016 Lisbon, Portugal*

Corresponding author: Yong Wang (yongwangbis@aliyun.com)

Academic editor: Huzefa Raja | Received 7 August 2022 | Accepted 2 November 2022 | Published 11 November 2022

Citation: Sun J-E, Meng C-R, Phillips AJL, Wang Y (2022) Two new *Botryosphaeria* (Botryosphaeriales, Botryosphaeriaceae) species in China. MycoKeys 94: 1–16. https://doi.org/10.3897/mycokeys.94.91340

Abstract

Five ascomycetous strains were isolated from dead branches and leaves of *Salix* (Salicaceae) and *Osmanthus fragrans* (Oleaceae), respectively. BLAST searches with ITS sequences in GenBank suggested a high degree of similarity to *Botryosphaeria dothidea*. To accurately identify these strains, we further analysed their morphological characteristics of asci, ascospores, all conidiophore cells and conidia. Phylogenetic relationships, based on ITS, *rpb2*, *tef1* and *tub2* gene sequences, confirmed our strains represented two novel species, which are introduced here as *B. salicicola* and *B. osmanthuse* **spp. nov.**

Keywords

Ascomycetes, molecular analyses, morphology, new species, new woody host

Introduction

The genus *Botryosphaeria* (Botryosphaeriales, Botryosphaeriaceae) was established by Cesati and Notaris (1863) and is widely distributed throughout many geographical and climatic regions of the world, with the exception of polar regions (Phillips et al. 2013). Species of *Botryosphaeria* are reported in many woody plants as endophytes, saprobes and pathogens (Crous et al. 2006; Liu et al. 2012; Phillips et al. 2013; Ariyawansa et al. 2016; Dissanayake et al. 2016; Slippers et al. 2017). Some species of *Botryosphaeria* are aggressive pathogens that pose a significant threat to agricultural and forest ecosystems (Slippers and Wingfield 2007). *Botryosphaeria dothidea* is known to cause serious diseases,

such as Apple ring rot (Slippers and Wingfield 2007; Marsberg et al. 2017). Moreover, according to the database of the common names of plant diseases in Japan, 14 species of the genus *Botryosphaeria* cause diseases on 30 plant species (Yukako et al. 2021).

Botryosphaeria has been considered as one of the hot topics in fungal taxonomy for a long time, based on its universality, including areas and hosts (from 1863 to 2022) (Cesati and Notaris 1863; Shoemaker 1964; Pennycook and Samuels 1985; Slippers et al. 2004; Slippers and Wingfield 2007; Liu et al. 2012; Phillips et al. 2008, 2019; Xu et al. 2015; Ariyawansa et al. 2016; Zhou et al. 2016, 2017; Li et al. 2018, 2020; Vu et al. 2019; Chen et al. 2020; Chu et al. 2021; Yukako et al. 2021). More than 300 species epithets are listed in MycoBank (https://www.mycobank.org, 17 October 2022), but only about 7% of *Botryosphaeria* species currently have associated DNA sequences data. In the past, species in *Botryosphaeria* were defined, based on morphological characters alone or on host association, but studies have shown these are inadequate characters to identify species (Shoemaker 1964; Pennycook and Samuels 1985; Slippers et al. 2004). With the advent of DNA sequencing methods, the nomenclature and identification of *Botryosphaeria* species have significantly improved (Phillips et al. 2013).

Some species of *Botryosphaeria* are aggressive pathogens in China, mainly distributed in the southwest, such as *B. fabicerciana*, *B. fujianensis*, *B. fusispora*, *B. kuwatsukai*, *B. dolichospermatii*, *B. pseudoramosa* and *B. wangensis* as shown in Table 4. In this study, five strains were isolated during surveys of fungi on new woody hosts (Salicaceae and Oleaceae) in Guizhou and Guangxi Provinces, China. Combining morphology and phylogenetic analyses, these isolates represent two novel *Botryosphaeria* species, which are described and illustrated here. The discovery of new species within this genus is important to help researchers better understand the diversity and ecology of *Botryosphaeria*.

Materials and methods

Sampling, fungal isolation and morphological observation

Fungi were isolated from dry branches of *Salix* (Salicaceae) and diseased leaf pieces of *Osmanthus fragrans* (Oleaceae) collected in forest parks in Guizhou and Guangxi Provinces, China, respectively. Samples were placed in envelopes and returned to the laboratory as described by Senanayake et al. (2020). Fruiting bodies (including asci, ascospores, conidiophore cells and conidia) on natural substrates were observed using a Zeiss Scope 5 compound microscope Axioscope 5 (Carl Zeiss Microscopy GmbH, Jena, Germany) with the microscope techniques of differential interference contrast light (DIC) and photographed using an AxioCam 208 colour (Carl Zeiss Microscopy GmbH, Jena, Germany) camera and saved as JPG files. Approximately 30 measurements of new species were made of each feature using the ZEN 3.0 (blue edition) (Jena, Germany) software.

Pure cultures were obtained using a single spore isolation method as described in Senanayake et al. (2020). The germinated spores were transferred to fresh potato dextrose agar (PDA) plates and incubated at 25 °C for 14 days. Type specimens were deposited in the Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University

(**HGUP**). Ex-type cultures were deposited in the Culture Collection at the Department of Plant Pathology, Agriculture College, Guizhou University, P.R. China (**GUCC**). Taxonomic information of the new species was submitted to MycoBank (www.mycobank.org).

DNA extraction, PCR and sequencing

Mycelium growing on PDA for seven days was scraped off with a sterile scalpel. Total DNA was extracted with a (Biomiga#GD2416, San Diego, California, USA) BIOMIGA Fungus Genomic DNA Extraction Kit (GD2416) following the manufacturer's protocol. Four loci (ITS, *rpb2*, *tef1* and *tub2*) were amplified with the respective forward and reverse primers (Table 1). PCR cycling conditions were followed according to Yukako et al. (2021). For ITS: initial denaturation (94 °C, 5 min), 40 cycles of amplification (denaturation 94 °C, 45 s; annealing 48 °C, 30 s; and extension 72 °C, 90 s) and final extension (72 °C, 2 min); for *tef1*: initial denaturation (94 °C, 5 min), 40 cycles of amplification (denaturation 94 °C, 30 s; annealing 52 °C, 30 s; and extension 72 °C, 45 s) and final extension (72 °C, 2 min); for *tub2*: initial denaturation (94 °C, 5 min), 40 cycles of amplification (denaturation 94 °C, 30 s; annealing 52 °C, 30 s; and extension 72 °C, 60 s) and final extension (72 °C, 2 min); and for *rpb2*: initial denaturation (95 °C, 5 min), touch-down amplification (5 cycles of 95 °C for 45 s, 60 °C for 45 s and 72 °C for 120 s; 5 cycles of 95 °C for 45 s, 58 °C for 45 s and 72 °C for 120 s; and 30 cycles of 95 °C for 45 s, 54 °C for 45 s and 72 °C for 120 s) and final elongation at 72 °C for 8 min. PCR products were sequenced by SinoGegoMax (Beijing, China).

Table 1. Primers used in this study.

Used genes	Primer	Direction	Sequence (5'–3')	Reference
tef1	EF1-688	Forward	CGGTCACTTGATCTACAAGTGC	Alves et al. (2008)
	EF1-1251	Reverse	CCTCGAACTCACCAGTACCG	
ITS	ITS1	Forward	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	
tub2	BT-2a	Forward	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson (1995)
	BT-2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	
rpb2	fRPB2-5f2	Forward	GATGATAGAGATCATTTTGG	Liu et al. (1999)
	fRPB2-7cR	Reverse	CCCATAGCTTGTTTACCCAT	

Phylogenetic analyses

Newly-generated sequences were deposited in GenBank. All the taxa used in the phylogenetic analyses are provided in Table 2. These sequences were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) and available sequences of species in the genus containing ex-type or representative isolates were downloaded from GenBank and previous publications (Li et al. 2018, 2020; Vu et al. 2019; Chen et al. 2020; Chu et al. 2021; Yukako et al. 2021). Alignments for the individual locus matrices were generated with the online version of MAFFT v. 7.307 (Katoh et al. 2019). Ambiguous sequences at the start and the end were deleted and

the alignments edited with MEGA6 (Tamura et al. 2013) for maximum alignment and minimum gaps. Sequence matrix v. 1.7.8 was used to concatenate the aligned sequences (Vaidya et al. 2011). *Neoscytalidium dimidiatum* (CBS 145.78 and CBS 251.49) and *Cophinforma atrovirens* (MFLUCC 11-0425 and MFLUCC 11-0655) were used as outgroup. Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI) were used to place the newly-discovered strains into a phylogenetic framework and estimate phylogenetic relationships with other *Botryosphaeria* spp.

ML analysis was performed using IQ-TREE (Nguyen et al. 2015; Trifinopoulos et al. 2016) on the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at, 17 October 2022). The MP analysis was implemented to test the discrepancy of the ITS, *rpb2*, *tef1* and *tub2* sequence datasets with PAUP v. 4.0b10 (Swofford 2002). Gaps were treated as missing data, which were interpreted as uncertainty of multistate taxa. Phylogenetic trees were generated using the heuristic search option with tree bisection re-connection (TBR) branch swapping. "Maxtrees" was set to 5000, the tree length (TL), consistency index (CI), homoplasy index (HI), retention index (RI) and rescaled consistency index (RC) were calculated. Bayesian Inference analysis was made with MrBayes 3.2.6 (Ronquist et al. 2012) based on a best substitution model for ITS: GTR+G, *rpb2*: K2P+I, *tef1*: HKY+G and *tub2*: HKY+G. BI was performed using six Markov Chain Monte Carlo runs for 5,000,000 generations, sampling every 1000 generations. The first 25% resulting trees were discarded as burn-in phase of each analysis.

MP, ML bootstrap support values greater than 70% and BI posterior probability values greater than 0.90 were denoted at the nodes and separated by "/". Bootstrap values less than 70% and BI posterior probability values less than 0.90 were labelled with "_".

Results

The MP, ML and Bayesian analyses resulted in trees with similar topologies and the MP tree is shown in Fig. 1. The combined data matrix of ITS–*rpb2*–*tef1*–*tub2* consisted of 1805 characters (ITS: 466, *rpb2*: 716, *tef1*: 286 and *tub2*: 337), of which 1579 characters were constant and 13 variable characters were parsimony uninformative. Maximum Parsimony analysis of the remaining 213 parsimony informative characters produced a tree with the following parameters: TL = 291; CI = 0.862; HI = 0.137; RI = 0.931; and RC = 0.803.

In the phylogenetic tree (Fig. 1), the isolates from this study formed two distinct, well-supported clades and, thus, were considered to represent two previously unknown species. *Botryosphaeria osmanthuse* GUCC 21433, GUCC 21433.1 and GUCC 21433.2 without the DNA base differences in four loci amongst strains (ITS, *rpb2*, *tef1* and *tub2*) form an independent branch with strong support (ML = 85, PP = 0.94) sister to *B. puerensis*. *Botryosphaeria salicicola* (GUCC 21230 and GUCC 21230.1) clustered sister to *B. corticis*, *B. fabicerciana*, *B. fusispora*, *B. fujianensis*, *B. kuwatsukai* and *B. rosaceae*, although with weak-supports (ML = 75). These two novel taxa were also supported by DNA base pair differences (Table 3).

Table 2. Taxa used for molecular phylogenetic analyses and their GenBank accession numbers. (T) = ex-type strains.

Species	Strain	Host	Country	GenBank accession numbers			
				ITS	*tef1*	*tub2*	*rpb2*
Botryosphaeria agaves	CBS 133992[T]	*Agave* sp.	Thailand	JX646791	JX646856	JX646841	N/A
B. agaves	MFLUCC 10-0051	*Agave* sp.	Thailand	JX646790	JX646855	JX646840	N/A
B. auasmontanum	CMW 25413[T]	*Pinus* sp.	Namibia	KF766167	N/A	N/A	N/A
B. corticis	CBS 119047[T]	*Vaccinium corymbosum*	USA	DQ299245	EU017539	EU673107	N/A
B. corticis	ATCC 22927	*Vaccinium* sp.	USA	DQ299247	EU673291	EU673108	N/A
B. dothidea	CBS 115476[T]	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927	N/A
B. dothidea	CBS 110302	*Vitis vinifera*	Portugal	AY259092	AY573218	EU673106	N/A
B. fabicerciana	CBS 127193[T]	*Eucalyptus* sp.	China	HQ332197	HQ332213	KF779068	N/A
B. fabicerciana	CMW 27121	*Eucalyptus* sp.	China	HQ332198	HQ332214	KF779069	N/A
B. fujianensis	CGMCC 3.19099[T]	*Vaccinium uliginosum*	China	MH491973	MH491977	MH562330	N/A
B. fujianensis	BJFUCC 180226-3	*Vaccinium uliginosum*	China	MW251380	MW251388	MW251379	N/A
B. fusispora	MFLUCC 10-0098[T]	*Entada* sp.	Thailand	JX646789	JX646854	JX646839	N/A
B. fusispora	MFLUCC 11-0507	*Caryota* sp.	Thailand	JX646788	JX646853	JX646838	N/A
B. guttulata	CGMCC3.20094[T]	N/A	China	MT327839	MT331606	N/A	N/A
B. guttulata	GZCC 19-0188	N/A	China	MT327833	MT331601	N/A	N/A
B. kuwatsukai	CBS 135219[T]	*Malus domestica*	China	KJ433388	KJ433410	N/A	N/A
B. kuwatsukai	LSP 5	*Pyrus* sp.	China	KJ433395	KJ433417	N/A	N/A
B. dolichospermatii	CGMCC 3.19096[T]	*Vaccinium uliginosum*	China	MH491970	MH491974	MH562327	N/A
B. dolichospermatii	CGMCC 3.19097	*Vaccinium uliginosum*	China	MH491971	MH491975	MH562328	N/A
B. minutispermatia	GZCC 16-0013[T]	Dead wood	China	KX447675	KX447678	N/A	N/A
B. minutispermatia	GZCC 16-0014	Dead wood	China	KX447676	KX447679	N/A	N/A
B. osmanthuse	**GUCC 21433[T]**	**GUCC 21433**	**China**	**OL854215**	**OP650906**	**OP669376**	**OP650903**
B. osmanthuse	**GUCC 21433.1**	***Osmanthus fragrans***	**China**	**OL854216**	**OP650907**	**OP669377**	**OP650904**
B. osmanthuse	**GUCC 21433.2**	***Osmanthus fragrans***	**China**	**OL854217**	**OP650908**	**OP669378**	**OP650905**
B. pseudoramosa	CERC 2001[T]	*Eucalyptus hybrid*	China	KX277989	KX278094	KX278198	MF410140
B. pseudoramosa	CERC 2983	*Melastoma sanguineum*	China	KX277992	KX278097	KX278201	MF410143
B. puerensis	CSF6052 T	*Eucalyptus urophylla*	China	MT028569	MT028735	MT028901	MT029057
B. qingyuanensis	CERC 2946[T]	*Eucalyptus hybrid*	China	KX278000	KX278105	KX278209	MF410151
B. qingyuanensis	CERC 2947	*Eucalyptus hybrid*	China	KX278001	KX278106	KX278210	MF410152
B. quercus	MFLUCC:14-0459 T	*Quercus* sp.	Italy	KU848199	N/A	N/A	N/A
B. ramosa	CBS 122069[T]	*Eucalyptus camaldulensis*	Bell Australia	EU144055	EU144070	KF766132	N/A
B. ramosa	CGMCC 3.18004	*Acacia* sp.	China	KX197073	KX197093	KX197100	N/A
B. rosaceae	CGMCC 3.18007[T]	*Malus* sp.	China	KX197074	KX197094	KX197101	N/A
B. rosaceae	CGMCC 3.18008	*Amygdalus* sp.	China	KX197075	KX197095	KX197102	N/A
B. salicicola	**GUCC 21230[T]**	***Salix***	**China**	**OL854218**	**OP669379**	**OP750032**	**N/A**
B. salicicola	**GUCC 21230.1**	***Salix***	**China**	**OL854219**	**OP669380**	**OP750033**	**N/A**
B. scharifii	CBS 124703[T]	*Mangifera indica*	Iran	JQ772020	JQ772057	N/A	N/A
B. sinensia	CGMCC 3.17722[T]	*Populus* sp.	China	KT343255	N/A	N/A	N/A
B. tenuispora	MUCC 2900	*Aucuba japonica*	Japan	LC585276	LC585148	LC585172	N/A
B. tenuispora	MUCC 237[T]	*Leucothoe fontanesiana*	Japan	LC585278	LC585150	LC585174	LC585196
B. wangensis	CERC 2298[T]	*Cunninghamina deodara*	China	KX278002	KX278107	KX278211	MF410153
B. wangensis	CERC 2299	*Cunninghamina deodara*	China	KX278003	KX278108	KX278212	MF410154
Cophinforma atrovirens	MFLUCC 11-0425 T	*Eucalyptus* sp.	Thailand	JX646800	JX646865	JX646848	N/A
C. atrovirens	MFLUCC 11-0655	*Eucalyptus* sp.	Thailand	JX646801	JX646866	JX646849	N/A
Neoscytalidium dimidiatum	CBS 145.78[T]	*Homo sapiens*	United Kingdom	KF531816	KF531795	KF531796	N/A
N. dimidiatum	CBS 251.49	*Juglans regia*	USA	KF531819	KF531797	KF531799	N/A

Note: Newly generated sequences are indicated in bold.

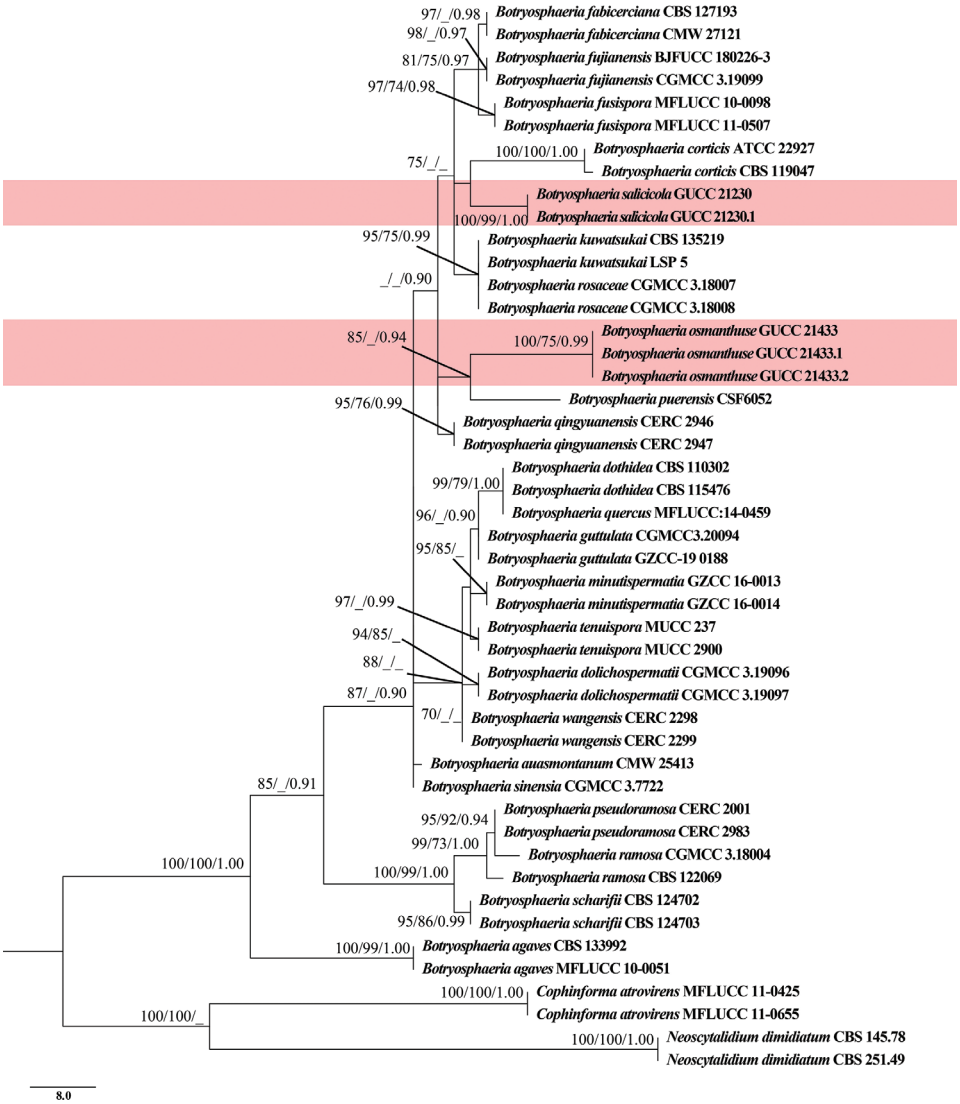

Figure 1. Trees resulting from MP analysis of the combined ITS, *rpb2*, *tef1* and *tub2* sequence alignment for forty-three isolates in *Botryosphaeria*. RAxML and MP bootstrap support values (ML, MP ≥ 70%) and Bayesian posterior probability (PP ≥ 0.90) are denoted on the nodes (ML/MP/PP). The tree was rooted to *Neoscytalidium dimidiatum* (CBS 145.78 and CBS 251.49) and *Cophinforma atrovirens* (MFLUCC 11-0425 and MFLUCC 11-0655). The new species are highlighted in pale red. The scale bar indicates 8.0 expected changes per site.

Taxonomy

Botryosphaeria salicicola **J. E. Sun, C. R. Meng & Yong Wang bis, sp. nov.**
MycoBank No: 843685
Figs 2a–i

Etymology. In reference to the host from which the fungus was first isolated.

Diagnosis. *Botryosphaeria salicicola* is characterised by oval to broadly fusiform ascospores (25.2 × 10.8; L/W = 2.3 vs. 22.7× 7.8 µm, L/W = 2.9) and cylindrical to clavate asci (65–170 × 20–30 µm), with moderate growth rate.

Type. China, Guizhou Province, Guiyang City, 26°65'N, 106°63'W, from branches of *Salix* sp., 20 June 2020, C.R. Meng, HGUP 21230 (holotype), ex-type culture GUCC 21230.

Description. Saprobic on dead branches of *Salix*. **Teleomorph:** ***Ascomata*** superficial, becoming erumpent at maturity, aggregated, thick-walled, wall composed of dark brown, thick-walled ***textura angularis***, becoming thinner-walled and hyaline towards the inner layers, 160 µm diam. ***Hamathecium*** comprising hyaline, septate, branched, 2–3.5 µm wide filamentous pseudoparaphyses. ***Asci*** 65–170 × 20–30 µm, 8-spored, bitunicate, cylindrical, to clavate, stipitate. ***Ascospores*** 22–26 × 9.0–13 µm (average = 25.2 × 10.8 µm, n = 20, L/W = 2.3), irregularly biseriate in the ascus, hyaline, guttulate, smooth with granular contents, aseptate, oval to broadly fusiform, widest in the middle or upper third of the ascospore, tapering to the obtuse base and apex. **Anamorph:** Not observed.

Culture characteristics. Ascospores germinate on PDA within 24 hours at room temperature (25 °C). Colonies with white fluffy mycelium on PDA (90 mm), after 7 days becomes grey-black at the bottom of centre, olivaceous-grey at the bottom of edge, white mycelium, raised, fluffy, dense filamentous.

Table 3. The DNA base differences in four loci between the two new species and closely-related species.

Species	Strain number	ITS (1–458 characters)	*tef1* (459–703 characters)	*tub2* (704–1039 characters)	*rpb2* (1040–1754 characters)
Botryosphaeria salicicola	GUCC 21230	0	0	0	–
	GUCC 21230.1	0	0	0	–
B. corticis	CBS 119047	10 (gap: 2)	11 (gap: 6)	6 (gap: 0)	–
	ATCC 22927	10 (gap: 2)	11 (gap: 6)	6 (gap: 0)	–
B. fabicerciana	CBS 127193	4 (gap: 3)	8 (gap: 2)	3 (gap: 1)	–
	CMW 27121	4 (gap: 3)	8 (gap: 2)	3 (gap: 1)	–
B. fujianensis	CGMCC 3.19099	4 (gap: 3)	8 (gap: 2)	4 (gap: 1)	–
	BJFUCC 180226-3	4 (gap: 3)	8 (gap: 2)	4 (gap: 1)	–
B. fusispora	MFLUCC 10-0098	4 (gap: 3)	10 (gap: 3)	3 (gap: 1)	–
	MFLUCC 11-0507	4 (gap: 3)	10 (gap: 3)	3 (gap: 1)	–
B. kuwatsukai	CBS 135219	4 (gap: 4)	7 (gap: 2)	–	–
	LSP 5	4 (gap: 4)	7 (gap: 2)	–	–
B. rosaceae	CGMCC 3.18007	4 (gap: 4)	7 (gap: 2)	2 (gap: 0)	–
	CGMCC 3.18008	4 (gap: 4)	7 (gap: 2)	2 (gap: 0)	–
B. dothidea	CBS 115476	8 (gap: 2)	12 (gap: 4)	3 (gap: 1)	–
	CBS 110302	8 (gap: 2)	12 (gap: 4)	3 (gap: 1)	–
Species	**Strain number**	**ITS (1–456 characters)**	***tef1* (471–702 characters)**	***tub2* (703–1034 characters)**	***rpb2* (1035–1750 characters)**
Botryosphaeria osmanthuse	GUCC 21443	0	0	0	0
	GUCC 21443.1	0	0	0	0
	GUCC 21443.2	0	0	0	0
B. puerensis	CSF6052	1 (gap: 1)	13 (gap: 4)	8 (gap: 0)	8 (gap: 0)
B. dothidea	CBS 115476	5 (gap: 1)	9 (gap: 2)	12 (gap: 0)	–
	CBS 110302	5 (gap: 1)	9 (gap: 2)	12 (gap: 0)	–

Figure 2. *Botryosphaeria salicicola* (GUCC 21230, holotype) **a–c** ascomata on natural substrate **d** section through ascomata **f** mature asci **g** ascospores **h** colony on PDA (left: above, right: reverse). Scale bars: 400 μm (**b**); 200 μm (**c**); 50 μm (**d**); 40 μm (**e**); 20 μm (**f, g**); 15 mm (**h**).

Distribution. China, Guizhou Province, Guiyang City.

Other material examined. China, Guizhou Provice, Guiyang City, 26°65'N, 106°63'W, from dead branches of *Salix*, 20 June 2020, C.R. Meng, HGUP 21230, living culture GUCC 21230.1.

Notes. NCBI BLAST searches of ITS sequences from our strains suggested a high degree of similarity (99–100%) to *Botryosphaeria dothidea*. However, *B. salicicola* and

B. dothidea show distant phylogenetic relationships in the phylogeny. *Botryosphaeria salicicola* has longer asci (65–170 × 20–30 μm vs. 63–125 × 16–20 μm) than *B. dothidea* and longer ascospores (25.2 × 10.8; L/W = 2.3 vs. 22.7× 7.8 μm, L/W = 2.9) (Slippers et al. 2004). The phylogenetic analyses indicate that *Botryosphaeria salicicola* forms an independent branch with respect to *B. corticis*, *B. fabicerciana*, *B. fusispora*, *B. fujianensis*, *B. kuwatsukai* and *B. rosaceae*. Comparing the morphological characteristics shows that *B. corticis* has longer ascospores than *B. salicicola* (29.3 × 11.6 μm vs. 25.2 × 10.8 μm) (Phillips et al. 2006); *B. fusispora* has shorter asci than *B. salicicola* (77.5–112.5 × 20–25 μm vs. 65–170 × 20–30 μm) (Liu et al. 2012); *B. rosaceae* has longer ascomata than *B. salicicola* (170–290 μm vs. 160 μm) (Zhou et al. 2017). The sexual morphs of *B. fabicerciana* (Chen et al. 2011), *B. fujianensis* (Chu et al. 2021) and *B. kuwatsukai* (Xu et al. 2015) are unknown.

Botryosphaeria osmanthuse **J. E. Sun, C. R. Meng & Yong Wang bis, sp. nov.**
MycoBank No: 843684
Figs 3a–i

Etymology. In reference to the host from which the fungus was first isolated.

Diagnosis. *Botryosphaeria osmanthuse* is characterised by aseptate narrowly fusiform conidia (16.0–20.5 × 5.0–6.0 μm (average = 17.0 × 5.3 μm, n = 45, L/W = 3.2) and short-length conidiogenous cells (8.5–10.5 × 2.3–2.8 μm), with moderate growth rate.

Type. China, Guangxi Province, Nanning City, 22°51'N, 108°19'E, from leaves of *Osmanthus fragrans*, 20 October 2017, C.R. Meng, HGUP 21433 (holotype), ex-type living culture GUCC 21433.

Description. Saprobic on living leaves of *Osmanthus fragrans*. **Teleomorph:** Not observed. **Anamorph:** ***Conidiomata*** up to 200 μm diam., covered with hyphae, black, globose, ostiolate, solitary, separate, uniloculate, immersed to semi-immersed. ***Conidiomatal wall*** composed of thick-walled, dark brown cells of ***textura angularis***, becoming thin-walled and hyaline towards the inner region. ***Conidiophores*** reduced to conidiogenous cells. ***Conidiogenous cells*** 8.5–10.5 × 2.3–2.8 μm (average = 10 × 2.5 μm, n = 20), holoblastic, discrete, hyaline, cylindrical to lageniform, phialidic with periclinal thickening. ***Paraphyses*** not were seen. ***Conidia*** 16.0–20.5 × 5.0–6.0 μm (average = 17.0 × 5.3 μm, n = 45, L/W = 3.2), hyaline, thin-walled, smooth with granular contents, unicellular, aseptate narrowly fusiform, base subtruncate to bluntly rounded.

Culture characteristics. Conidia germinate on PDA within 24 hours at room temperature (25 °C) with germ tubes produced from both ends of the conidia. Colonies with white fluffy mycelium on PDA (90 mm), after 7 days becomes raised, fluffy, white mycelium, dense filamentous.

Distribution. China, Guangxi Province, Nanning City.

Other material examined. China, Guangxi Province, Nanning City, 22°51'N, 108°19'E, from living leaves of *Osmanthus fragrans*, 20 October 2017, C.R. Meng, HGUP 21433, living culture GUCC 21433.1 and GUCC 21433.2.

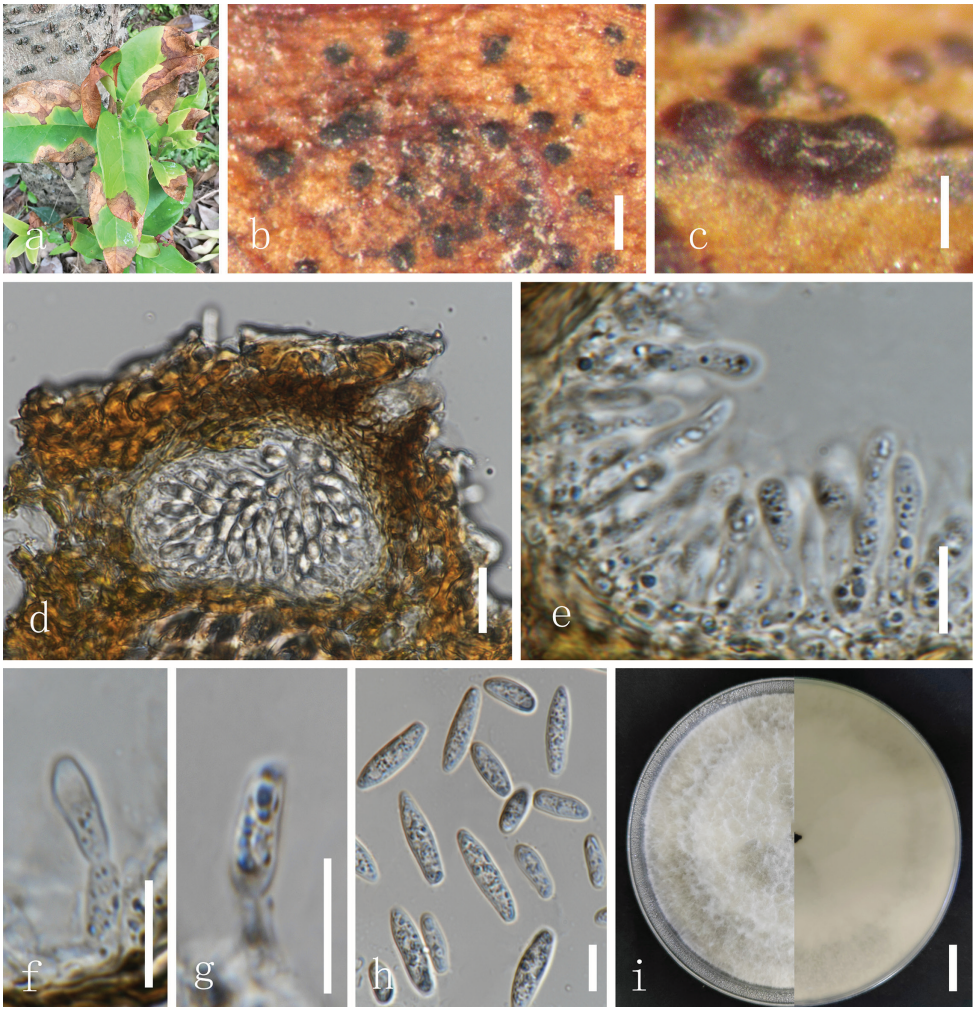

Figure 3. *Botryosphaeria osmanthuse* (GUCC 21433, holotype) **a–c** colonies on natural substrate **d** section through conidiomata **e–g** conidiophores and conidia **h** conidia **i** colony on PDA (left: above, right: reverse). Scale bars: 300 μm (**b**); 140 μm (**c**); 50 μm (**d**); 20 μm (**e**); 10 μm (**f–h**); 15 mm (**i**).

Notes. NCBI BLAST searches of ITS sequences from our strains suggest a high degree of similarity (99–100%) to *Botryosphaeria dothidea*. However, DNA bases in the two loci (*tef1* and *tub2*) showed a high amount of difference between *B. osmanthuse* and *B. dothidea*. *Botryosphaeria osmanthuse* shows close phylogenetic affinity to *B. puerensis* (Fig. 1). Comparing the morphological characteristics, conidia of *B. osmanthuse* (av. 17.0 × 5.3; L/W = 3.2) are narrower and shorter than *B. puerensis* (av. 26.8 × 6.4; L/W = 4.2) (Li et al. 2020). *Botryosphaeria osmanthuse* was first isolated from *Osmanthus fragrans* (Oleaceae), while *B. puerensis* has been reported from *Eucalyptus urophylla* (Myrtaceae).

Discussion

Two new species of *Botryosphaeria*, *B. salicicola* and *B. osmanthuse*, are described and illustrated from southern China in this paper. Previously reported *Botryosphaeria* species in China are listed in Table 4. Thirteen *Botryosphaeria* species were described from nine different areas in southern China, covering three climatic zones (northern sub-tropical zone, central sub-tropical zone and warm temperate zone) along an altitudinal gradient (Hui 2021). Most species, such as *B. fabicerciana*, *B. fujianensis*, *B. fusispora*, *B. kuwatsukai*, *B. dolichospermatii*, *B. minutispermatia*, *B. pseudoramosa*, *B. qingyuanensis* and *B. wangensis*, often caused serious diseases on their hosts (Xu et al. 2015; Ariyawansa et al. 2016; Zhou et al. 2016, 2017; Li et al. 2018, 2020; Vu et al. 2019; Chen et al. 2020; Chu et al. 2021). Geographical and climatic regions have a large influence on the taxonomy, ecological distribution and pathogenicity of *Botryosphaeria* species (Phillips et al. 2013).

Table 4. List of Chinese *Botryosphaeria* strains.

Species	Strain	Host/ Natural substrate	Regions	Fungi	References
Botryosphaeria fabicerciana	CBS 127193	*Eucalyptus* sp.	Fujian	Pathogens	Li et al. (2018)
	CMW 27094	*Eucalyptus* sp.	Fujian	Pathogens	Li et al. (2018)
	CMW 27121	*Eucalyptus* sp.	Fujian	Pathogens	Li et al. (2018)
	CERC 2930	*Eucalyptus* sp.	Yunnan	Pathogens	Li et al. (2018)
	CERC 3446	*Eucalyptus* sp.	Guangdong	Pathogens	Li et al. (2018)
	CERC 2912	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2018)
	CERC 2913	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2018)
B. fujianensis	CGMCC 3.19099	*Vaccinium uliginosum*	Fujian	Pathogens	Chu et al. (2021)
	BJFUCC 180226-3	*V. uliginosum*	Fujian	Pathogens	Chu et al. (2021)
	BJFUCC 180226-4	*V. uliginosum*	Fujian	Pathogens	Chu et al. (2021)
B. fusispora	CSF6063	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
	CSF6178	*E. globulus*	Yunnan	Pathogens	Li et al. (2020)
	CSF5872	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
	CSF5950	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
	CSF6160	*E. globulus*	Yunnan	Pathogens	Li et al. (2020)
	CSF6056	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
B. guttulata	CGMCC3.20094	Decaying branch	Guizhou	Saprobes	Chen et al. (2020)
	GZCC 19-0186	Decaying branch	Guizhou	Saprobes	Chen et al. (2020)
	GZCC 19-0188	Decaying branch	Guizhou	Saprobes	Chen et al. (2020)
B. kuwatsukai	CBS 135219	*Malus domestica*	Unknown	Pathogens	Xu et al. (2015)
	LSP 5	*Pyrus* sp.	Unknown	Pathogens	Xu et al. (2015)
B. dolichospermatii	CGMCC 3.19096	*V. uliginosum*	Fujian	Pathogens	Chu et al. (2021)
	CGMCC 3.19097	*V. uliginosum*	Fujian	Pathogens	Chu et al. (2021)
	GZCC 16-0013	Dead wood	Guizhou	Saprobes	Ariyawansa et al. (2016)
	GZCC 16-0014	Dead wood	Guizhou	Saprobes	Ariyawansa et al. (2016)
B. pseudoramosa	CERC 2001	*E. hybrid*	Guangxi	Pathogens	Li et al. (2018)
	CERC 2982	Unknow	Guangxi	Pathogens	Li et al. (2018)
	CERC 2983	*Melastoma sanguineum*	Guangxi	Unsure	Li et al. (2018)
	CGMCC 3.18739	*Eucalyptus* sp.	Guangxi	Unsure	Li et al. (2018)
	CERC 3462	*Eucalyptus* sp.	Guangxi	Unsure	Li et al. (2018)
	CERC 2019	*E. urophylla* & *E. grandis*	Guangxi	Unsure	Li et al. (2018)
	CERC 2987	*Me. sanguineum*	Guangxi	Unsure	Li et al. (2018)
	CERC 3455	*Eucalyptus* sp.	Guangxi	Unsure	Li et al. (2018)
	CERC 2988	*Me. sanguineum*	Guangxi	Unsure	Li et al. (2018)

Species	Strain	Host/ Natural substrate	Regions	Fungi	References
B. qingyuanensis	CERC 2946	*E. hybrid*	Guangdong	Pathogens	Li et al. (2018)
	CERC 2947	*E. hybrid*	Guangdong	Pathogens	Li et al. (2018)
B. ramosa	CGMCC 3.18004	*Acacia* sp.	Hainan	Unsure	Vu et al. (2019)
	CGMCC 3.18006	*Myrtaceae*	Guangdong	Unsure	Vu et al. (2019)
B. rosaceae	CGMCC 3.18007	*Malus* sp.	Shandong	Unsure	Zhou et al. (2017)
	CGMCC 3.18008	*Amygdalus* sp.	Shandong	Unsure	Zhou et al. (2017)
	CGMCC3.18009	*Malus* sp.	Shandong	Unsure	Zhou et al. (2017)
	CGMCC3.18010	*Pyrus* sp.	Shandong	Unsure	Zhou et al. (2017)
	CFCC 82350	*Malus* sp.	Unknown	Unsure	Zhou et al. (2017)
	CGMCC3.18011	*Pyrus* sp.	Shandong	Unsure	Zhou et al. (2017)
B. sinensia	CGMCC 3.17722	*Populus* sp.	Henan	Unsure	Zhou et al. (2016)
	CGMCC 3.17723	*Morus* sp.	Henan	Unsure	Zhou et al. (2016)
	CGMCC 3.17724	*Juglans regia*	Henan	Unsure	Zhou et al. (2016)
	CFCC 82346	*J. regia*	Beijing	Unsure	Zhou et al. (2016)
	CFCC 82255	*Ma. pumila*	Beijing	Unsure	Zhou et al. (2016)
B. wangensis	CERC 2298	*C. deodara*	Henan	Pathogens	Li et al. (2018)
	CERC 2299	*C. deodara*	Henan	Pathogens	Li et al. (2018)
	CGMCC 3.18744	*C. deodara*	Henan	Pathogens	Li et al. (2018)
	CERC 2300	*C. deodara*	Henan	Pathogens	Li et al. (2018)
	CSF5820	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
	CSF5733	*Eucalyptus* sp.	Yunnan	Pathogens	Li et al. (2020)
	CSF5944	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
	CSF5971	*E. urophylla* & *E. grandis*	Yunnan	Pathogens	Li et al. (2020)
	CSF5781	*E. globulus*	Yunnan	Pathogens	Li et al. (2020)
	CSF6174	*E. globulus*	Yunnan	Pathogens	Li et al. (2020)
	CSF5737	*Eucalyptus* sp.	Yunnan	Pathogens	Li et al. (2020)
B. archontophoenicis	HKU (M) 3539	*Archontophoenix alexandrae*	Hong Kong	Saprobes	Index Fungorum and mycobank
B. brunneispora	HKU (M) 3987	*Trachycarpus fortune*	Hubei	Unsure	Index Fungorum and mycobank
B. cunninghamiae	N/A	*Cunninghamia lanceolata*	China	Saprobes	Index Fungorum and mycobank
B. puerensis	HMAS 255719	*E. urophylla* & *E. grandis*	China	Pathogens	Index Fungorum and mycobank
B. qinlingensis	BJFC S1576	*Quercus aliena var. acuteserrata*	Shaanxi	Unsure	Index Fungorum and mycobank
B. yedoensis	N/A	*Prunus yedoensis*	Taiwan	Unsure	Index Fungorum and mycobank

Botryosphaeria species have been known to exist in many woody plants (Crous et al. 2006; Liu et al. 2012; Phillips et al. 2013; Ariyawansa et al. 2016; Dissanayake et al. 2016; Slippers et al. 2017). *Botryosphaeria dothidea*, the type species of the genus (Slippers and Wingfield 2007), is known from numerous hosts (Phillips et al. 2013; Marsberg et al. 2017) and was isolated from an *Asphondylia* gall on *Lamiaceae* in Italy and Poland (Zimowska et al. 2020). Other species of *Botryosphaeria* have often been isolated from many wood plants (Table 2). Amongst them, *B. fabicerciana*, *B. fusispora*, *B. kuwatsukai*, *B. pseudoramosa*, *B. rosaceae*, *B. wangensis* and *B. puerensis* often exist in many economic plants, such as *Eucalyptus* sp., *Pyrus* sp., *Malus* sp., *Citrus* sp. and *Vaccinium* sp. (Phillips et al. 2006; Lazzizera et al. 2008; Zhou et al. 2017; Li et al. 2018, 2020; Chen et al. 2020). Our strains were isolated from the *Salix* (Salicaceae) and *O. fragrans* (Oleaceae) of woody plants. In contrast, the few hosts or natural substrates of the known species belong to the Salicaceae and Oleaceae.

Acknowledgements

This research is supported by the following projects: National Natural Science Foundation of China (No. 31972222, 31660011), Program of Introducing Talents of Discipline to Universities of China (111 Program, D20023), Talent project of Guizhou Science and Technology Cooperation Platform ([2017]5788-5, [2019]5641, [2019]13), Guizhou Science, Technology Department of International Cooperation Base project ([2018]5806), the project of Guizhou Provincial Education Department ([2020]001), and Guizhou Science and Technology Innovation Talent Team Project ([2020]5001). Alan JL Phillips acknowledges the support from UIDB/04046/2020 and UIDP/04046/2020 Centre grants from FCT, Portugal (to BioISI).

References

Ariyawansa HA, Hyde KD, Liu JK, Wu SP, Liu ZY (2016) Additions to Karst Fungi 1: *Botryosphaeria minutispermatia* sp. nov., from Guizhou Province, China. Phytotaxa 275(1): 35–44. https://doi.org/10.11646/phytotaxa.275.1.4

Alves A, Crous PW, Correia A, Phillips AJL (2008) Morphological and molecular data reveal cryptic species in *Lasiodiplodia theobromae*. Fungal Diversity 28: 1–13.

Cesati V, Notaris GD (1863) Schema diclassificazione degli sferiacei italici aschigeri piu'o meno appartenenti al genere Sphaerianell'antico significato attribuitoglide Persoon. Commentario della Società Crittogamologica Italiana 14: 177–240.

Chen SF, Pavlic D, Roux J, Slippers B, Xie YJ, Wingfield MJ, Zhou XD (2011) Characterization of Botryosphaeriaceae from plantation-grown *Eucalyptus* species in South China. Plant Pathology 60(4): 739–751. https://doi.org/10.1111/j.1365-3059.2011.02431.x

Chen YY, Dissanayake AJ, Liu ZY, Liu JK (2020) Additions to Karst Fungi 4: *Botryosphaeria* spp. associated with woody hosts in Guizhou province, China including *B. guttulata* sp. nov. Phytotaxa 454(3): 186–202. https://doi.org/10.11646/phytotaxa.454.3.2

Chu RT, Dou ZP, He W, Zhang Y (2021) Two novel species of *Botryosphaeria* causing stem canker of blueberries from China. Junwu Xuebao 40: 473–486. https://doi.org/10.13346/j.mycosystema.200333

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Phillips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the Botryosphaeriaceae. Studies in Mycology 55: 235–253. https://doi.org/10.3114/sim.55.1.235

Dissanayake AJ, Phillips AJL, Li XH, Hyde KD (2016) Botryosphaeriaceae: Current status of genera and species. Mycosphere: Journal of Fungal Biology 7(7): 1001–1073. https://doi.org/10.5943/mycosphere/si/1b/13

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Microbiology 61: 1323–1330. https://doi.org/10.1128/aem.61.4.1323-1330.1995

Hui QH (2021) Division of climatic zones during the peak of the Holocene Warm Period in China. Journal of Qinghai Normal University 3: 60–65. [Natural Science]

Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20(4): 1160–1166. https://doi.org/10.1093/bib/bbx108

Lazzizera C, Frisullo S, Alves A, Phillips AJL (2008) Morphology, phylogeny and pathogenicity of *Botryosphaeria* and *Neofusicoccum* species associated with drupe rot of olives in southern Italy. Plant Pathology 57(5): 948–956. https://doi.org/10.1111/j.1365-3059.2008.01842.x

Li GQ, Liu FF, Li JQ, Liu QL, Chen SF (2018) Botryosphaeriaceae from *Eucalyptus* plantations and adjacent plants in China. Persoonia 40(1): 63–95. https://doi.org/10.3767/persoonia.2018.40.03

Li GQ, Slippers B, Wingfield MJ, Chen SF (2020) Variation in Botryosphaeriaceae from *Eucalyptus* plantations in Yunnan Province in southwestern China across a climatic gradient. IMA Fungus 11(1): 22. https://doi.org/10.1186/s43008-020-00043-x

Liu YJ, Whelen S, Hall BD (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Molecular Biology and Evolution 16: 1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092

Liu JK, Phookamsak R, Doilom M, Wikee S, Li YM, Ariyawansha H, Boonmee S, Chomnunti P, Dai DQ, Bhat JD, Romero AI, Zhuang WY, Monkai J, Gareth Jones EB, Chukeatirote E, Ko TWK, Zhao YC, Wang Y, Hyde KD (2012) Towards a natural classification of Botryosphaeriales. Fungal Diversity 57(1): 149–210. https://doi.org/10.1007/s13225-012-0207-4

Marsberg A, Kemler M, Jami F, Nagel JH, Smidt AP, Naidoo S, Wingfield MJ, Crous PW, Spatafora JW, Hesse CN, Robbertse B, Slippers AB (2017) *Botryosphaeria dothidea*: A latent pathogen of global importance to woody plant health. Molecular Plant Pathology 18(4): 477–488. https://doi.org/10.1111/mpp.12495

Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ (2015) IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution 32(1): 268–274. https://doi.org/10.1093/molbev/msu300

Pennycook SR, Samuels GJ (1985) *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (kiwifruit) in New Zealand. Mycotaxon 24: 445–458.

Phillips AJL, Oudemans PV, Correia A, Alves A (2006) Characterisation and epitypification of *Botryosphaeria corticis*, the cause of blueberry cane canker. Fungal Diversity 21: 141–155.

Phillips AJL, Alves A, Pennycook SR, Johnston PR, Ramaley A, Akulov A, Crous PW (2008) Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the Botryosphaeriaceae. Persoonia 21(1): 29–55. https://doi.org/10.3767/003158508X340742

Phillips AJL, Alves A, Abdollahzadeh J, Slippers B, Wingfield MJ, Groenewald JZ, Crous PW (2013) The Botryosphaeriaceae: Genera and species known from culture. Studies in Mycology 76: 51–167. https://doi.org/10.3114/sim0021

Phillips AJL, Hyde KD, Alves A, Liu JK (2019) Families in Botryosphaeriales: A phylogenetic, morphological, and evolutionary perspective. Fungal Diversity 94(1): 1–22. https://doi.org/10.1007/s13225-018-0416-6

Ronquist F, Teslenko M, Mark VD, Ayres P, Darling DL, Höhna A, Larget S, Liu B, Suchard LMA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and

model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Senanayake IC, Rathnayake AR, Marasinghe DS, Calabon MS, Gentekaki E, Lee HB, Hurdeal VG, Pem D, Dissanayake LS, Wijesinghe SN, Bundhun D, Nguyen TT, Goonasekara ID, Abeywickrama PD, Bhunjun CS, Jayawardena RS, Wanasinghe DN, Jeewon R, Bhat DJ, Xiang MM (2020) Morphological approaches in studying fungi: Collection, examination, isolation, sporulation and preservation. Mycosphere: Journal of Fungal Biology 11(1): 2678–2754. https://doi.org/10.5943/mycosphere/11/1/20

Shoemaker RA (1964) Conidial states of some *Botryosphaeria* species on *Vitis* and *Quercus*. Canadian Journal of Botany 42(9): 1297–1301. https://doi.org/10.1139/b64-122

Slippers B, Crous PW, Denman S, Coutinho T (2004) Combined Multiple Gene Genealogies and Phenotypic Characters Differentiate Several Species Previously Identified as *Botryosphaeria dothidea*. Mycologia 96: 83–101. https://doi.org/10.1080/15572536.2005.11833000

Slippers B, Wingfield MJ (2007) Botryosphaeriaceae as endophytes and latent pathogens of woody plants: Diversity, ecology and impact. Fungal Biology Reviews 21(2–3): 90–106. https://doi.org/10.1016/j.fbr.2007.06.002

Slippers B, Crous PW, Jami F, Groenewald JZ, Wingfield MJ (2017) Diversity in the Botryosphaeriales: Looking back, looking forward. Fungal Biology 121(4): 307–321. https://doi.org/10.1016/j.funbio.2017.02.002

Swofford DL (2002) PAUP*: Phylogenetic analysis using parsimony (and other methods), version 4.0 b10. MA: Sinauer Associates. Sunderland, UK.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Research 44(W1): W232–W235. https://doi.org/10.1093/nar/gkw256

Vaidya G, Lohman DJ, Meier R (2011) SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27(2): 171–180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

Vu D, Groenewald M, Vries MD, Stielow B, Eberhardt U, Al-Hatmi A, Groenewald JZ, Cardinali G, Houbraken J, Boekhout T, Crous PW, Robert V, Verkley GJM (2019) Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. Studies in Mycology 92(1): 135–154. https://doi.org/10.1016/j.simyco.2018.05.001

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Xu C, Wang C, Ju LL, Zhang R, Biggs AR, Tanaka E, Li B, Sun GY (2015) Multiple locus genealogies and phenotypic characters reappraise the causal agents of apple ring rot in China. Fungal Diversity 71(1): 215–231. https://doi.org/10.1007/s13225-014-0306-5

Yukako H, Yuho A, Atsuko S, Nami U, Chiharu N (2021) Taxonomical study of noteworthy species of *Botryosphaeria* in Japan. Mycobiology 49(2): 122–132. https://doi.org/10.1080/12298093.2021.1895486

Zhou YP, Dou ZP, He W, Zhang Y, Zhang Y (2016) *Botryosphaeria sinensia* sp. nov., a new species from China. Phytotaxa 245(1): 43–50. https://doi.org/10.11646/phytotaxa.245.1.4

Zhou YP, Zhang M, Dou ZhP, Zhang Y (2017) *Botryosphaeria rosaceae* sp. nov. and *B. ramosa*, new botryosphaeriaceous taxa from China. Mycosphere: Journal of Fungal Biology 8(2): 162–171. https://doi.org/10.5943/mycosphere/8/2/2

Zimowska B, Okoń S, Becchimanzi A, Krol ED, Nicoletti R (2020) Phylogenetic characterization of *Botryosphaeria* strains associated with *Asphondylia* galls on species of Lamiaceae. Diversity (Basel) 12(2): 41. https://doi.org/10.3390/d12020041

MycoKeys 94: 17–35 (2022)
doi: 10.3897/mycokeys.94.96301
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Soil-borne *Calonectria* (Hypocreales, Nectriaceae) associated with *Eucalyptus* plantations in Colombia

Nam Q. Pham[1], Seonju Marincowitz[2], ShuaiFei Chen[2,3,4], Carlos A. Rodas[2,5], Michael J. Wingfield[1]

1 *Department of Plant and Soil Sciences, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa* **2** *Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa* **3** *Research Institute of Fast-growing Trees (RIFT), Chinese Academy of Forestry (CAF), Zhanjiang, Guangdong Province, China* **4** *China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), Zhanjiang, Guangdong Province, China* **5** *Forestry Health Protection Programme, SmurfitKappa Colombia, Yumbo, Colombia*

Corresponding author: Nam Q. Pham (Nam.Pham@fabi.up.ac.za)

Academic editor: N. Wijayawardene | Received 16 October 2022 | Accepted 31 October 2022 | Published 30 November 2022

Citation: Pham NQ, Marincowitz S, Chen SF, Rodas CA, Wingfield MJ (2022) Soil-borne *Calonectria* (Hypocreales, Nectriaceae) associated with *Eucalyptus* plantations in Colombia. MycoKeys 94: 17–35. https://doi.org/10.3897/mycokeys.94.96301

Abstract

Eucalyptus spp. are widely planted in Colombia as an important component of a growing paper and pulp industry. Leaf and shoot blight caused by *Calonectria* spp. was one of the first disease problems to emerge in these plantations. A survey of *Eucalyptus* plantations in four forestry regions of Colombia during 2016 resulted in a large number of *Calonectria* isolates from soil samples collected in the understories of trees having symptoms of *Calonectria* leaf and shoot blight. The aim of this study was to identify and resolve the phylogenetic relationships for these isolates using DNA sequence comparisons of six gene regions as well as morphological characters. From a collection of 107 isolates, seven *Calonectria* species residing in three species complexes were identified. Two of these represented undescribed species, namely *C. exiguispora* **sp. nov.** and *C. guahibo* **sp. nov.** *Calonectria parvispora* and *C. spathulata* were the most commonly isolated species, each of which accounted for approximately 30% of the isolates. The results suggest that Colombia has a wide diversity of *Calonectria* spp. and that these could challenge *Eucalyptus* plantation forestry in the future.

Keywords
Calonectria leaf and shoot blight, *Cylindrocladium*, multi-gene phylogeny, taxonomy, two new taxa

Introduction

Colombian plantation forestry is based primarily on non-native *Pinus* and *Eucalyptus* species, which have been widely deployed as an important component of the growing wood and paper industry. These plantations are based on short rotations, and in the case of *Eucalyptus*, clonal propagation has been established rapidly during the course of the last decade. There are currently approximately 540 000 ha of commercially managed plantations, of which *Eucalyptus* makes up a substantial component (20%) of this resource (MADR; https://www.minagricultura.gov.co/).

As plantation forestry has grown globally, damage due to insect pests and microbial pathogens has become increasingly important (Wingfield et al. 2008, 2015; Paine et al. 2011). Relevant diseases of planted *Eucalyptus* in Colombia include stem canker caused by species of Cryphonectriaceae and Botryosphaeriaceae (van der Merwe et al. 2001; Rodas et al. 2009), wilt and dieback caused by *Ceratocystis neglecta* (Rodas et al. 2008), Myrtle rust caused by *Austropuccinia psidii* (Rodas et al. 2015; Granados et al. 2017), as well as leaf and shoot blight caused by *Calonectria* species (Rodas et al. 2005). Of these, Calonectria leaf and shoot blight was amongst the first disease problems to emerge (Rodas et al. 2005; Rodas and Wingfield 2020).

Species of *Calonectria* (Hypocreales, Nectriaceae) have a wide distribution globally, especially in tropical and sub-tropical regions (Crous 2002; Lombard et al. 2010b; Marin-Felix et al. 2017). These fungi represent some of the most aggressive pathogens of agricultural, forestry, horticultural and ornamental plants (Crous 2002; Lombard et al. 2010b). *Calonectria* spp. are best known as root, shoot and foliar pathogens and can be associated with various disease symptoms, including damping-off, seedling blight, leaf and shoot blight, leaf spot, stem lesions, collar and root rot, fruit rot, and cutting rot (Sharma et al. 1984; Mohanan and Sharma 1985; Crous et al. 1991, 1998; Ferreira et al. 1995; Crous 2002; Old et al. 2003; Lombard et al. 2010b; Lopes et al. 2018).

In Colombia, the first outbreak of Calonectria leaf and shoot blight in *Eucalyptus* plantations occurred in 1998, where *Calonectria spathulata* was shown to be the predominant pathogen (Rodas et al. 2005). High humidity and abundant free moisture in this region result in conditions highly conducive to disease outbreaks (Crous 2002; Rodas et al. 2005). Infections by *Calonectria* spp. have consequently resulted in severe defoliation and significant negative impacts on the growth of susceptible genotypes (Rodas et al. 2005).

Calonectria spp. are typically soil-borne fungi and many of these move between the soil environment and the leaf canopy of host trees (Crous 2002; Li et al. 2022). Previous studies of Calonectria leaf and shoot blight on *Eucalyptus* in Colombia considered only isolates from infected leaves (Rodas et al. 2005; Rodas and Wingfield 2020). In

order to provide a more comprehensive overview of *Calonectria* species associated with *Eucalyptus* in Colombia, soil samples were collected from *Eucalyptus* plantations in Colombia, resulting in a large number of isolates. The aim of this study was to identify and resolve the phylogenetic relationships for these isolates using multi-gene DNA sequence comparisons as well as morphological characteristics.

Materials and methods

Sampling and fungal isolations

During 2016, surveys of *Eucalyptus* plantations were conducted in different forestry farms located across four provinces of Colombia, namely, Cauca, Risaralda, Valle del Cauca, and Vichada (Fig. 1; Suppl. material 2). Soil samples were taken in the understories of *Eucalyptus* trees having symptoms of Calonectria leaf and shoot blight. In addition, random soil samples were collected from the native vegetation surrounding the *Eucalyptus* plantations in these regions. Soils were packed in plastic bags and transferred to the laboratory for isolation. The samples were baited with germinating alfalfa (*Medicago sativa*) seeds following the method recommended by Crous (2002).

A dissection microscope was used to locate conidiophores and conidia typical of *Calonectria* on the infected alfalfa sprouts. These were lifted from the infected tissues using a sterile hypodermic needle and transferred to Petri dishes containing 2% (w/v) malt extract agar (MEA; 20 g malt extract, Biolab, Midrand, South Africa; 20 g Difco agar, Becton Dickinson, Maryland, USA; 1 L deionised water). Primary isolations were incubated for 3–7 d at 25 °C to allow fungal growth. Single hyphal tips were cut from the fungal colonies, transferred to fresh MEA plates, and incubated at 25 °C to obtain pure cultures. These cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative cultures, including the ex-type strains of novel taxa, were deposited in the CMW-IA (the culture collection of Innovation Africa, University of Pretoria, Pretoria, South Africa). Dried-down specimens of sporulating cultures were deposited in the PRU (H.G.W.J. Schweickerdt Herbarium of the University of Pretoria, Pretoria, South Africa).

DNA extraction, PCR amplification and sequencing

Prepman Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the total genomic DNA from 7-d-old isolates grown on 2% MEA, following the manufacturer's suggested protocols. A fragment of the actin (*ACT*), calmodulin (*CMDA*), histone H3 (*HIS3*), translation elongation factor 1-alpha (*TEF1*), β-tubulin (*TUB2*), and DNA-directed RNA polymerase II second largest subunit (*RPB2*) gene regions were amplified using the primers ACT-512F and

ACT-783R (Carbone and Kohn 1999), CAL-228F and CAL-2Rd (Carbone and Kohn 1999; Groenewald et al. 2013) CYLH3F and CYLH3R (Crous et al. 2004), EF1-728F and EF2 (O'Donnell and Cigelnik 1997; Carbone and Kohn 1999), T1 and CYLTUB1R (O'Donnell and Cigelnik 1997; Crous et al. 2004), and fRPB2-5F and fRBP2-7cR (Liu et al. 1999), respectively.

The PCR reactions and conditions were the same as those used by Pham et al. (2019) and Liu et al. (2020). ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to purify the Amplicons. Cleaned-up amplified fragments were sequenced in both directions using an ABI PRISM 3100 DNA sequencer (Thermo Fisher Scientific, Waltham, MA, USA) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria. Geneious Prime 2022.1.1 was used to assemble and edit the raw sequences (https://www.geneious.com). Sequences obtained in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov).

Phylogenetic analyses

The sequences generated in this study were compared with those for previously published species of *Calonectria* sourced from the GenBank database (http://www.ncbi.nlm.nih.gov/) and subjected to phylogenetic analyses. Alignments of all sequences were assembled using the online version of MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013) and then confirmed manually in MEGA v. 7 (Kumar et al. 2016). Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed on data sets for each individual gene region and the combined data set. The most appropriate models were obtained using the software jModeltest v. 1.2.5. (Posada 2008). ML analyses were conducted using RaxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Stamatakis 2014) with a default GTR substitution matrix and 1,000 rapid bootstraps. BI analyses were performed using MrBayes v. 3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway v. 3.3. Four Markov Chain Monte Carlo (MCMC) chains were run from a random starting tree for five million generations, and trees were sampled every 100th generation. The first 25% of trees sampled were eliminated as burn-in, and the remaining trees were used to determine the posterior probabilities. Sequences for two isolates (CBS 109167 and CBS 109168) of *Curvicladiella cignea* were used as the outgroup taxa in all phylogenetic analyses. Phylogenetic trees were viewed using MEGA v. 7 (Kumar et al. 2016).

Morphology

The isolates were grown on synthetic nutrient-poor agar (SNA) (Nirenberg 1981) or together with alfalfa sprouts to induce the production of the asexual structures. Fruiting structures were initially mounted in water and replaced with 85% lactic acid for observation. Crosses between single hyphal tip isolates on minimal salt agar (MSA)

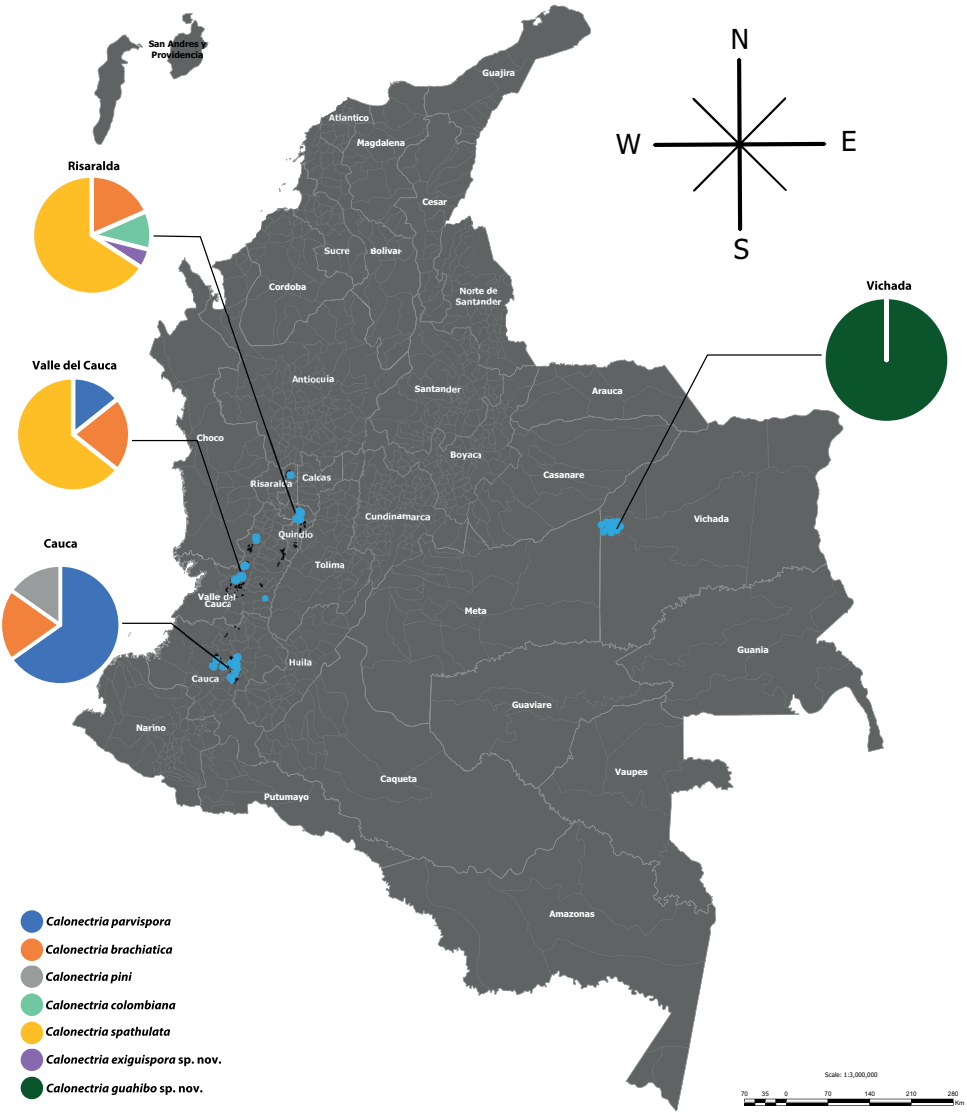

Figure 1. Geographic location of the sampling sites in Colombia, indicated as blue dots on the map, and the diversity of *Calonectria* spp. isolated from each region.

were made to induce the production of a sexual state, as described by Pham et al. (2019). Nikon microscopes (Eclipse Ni, SMZ 18, Tokyo, Japan) were used to study the morphological characteristics. Images were captured using a Nikon DS-Ri2 camera mounted on the microscopes using the NIS-Elements BR program. Up to fifty measurements were made of all characteristic structures whenever possible. Dimensions were presented as minimum-maximum and with average ± standard deviation for the key morphological characteristics.

Colony characteristics were observed on 6-d and 30-d-old cultures on 2% MEA. Colours were described using the charts of Rayner (1970). Three replicates for each species were prepared to determine the optimum growth temperature. A mycelial plug (5 mm diam) from the margins of actively growing 4 d-old cultures was transferred to the centres of Petri dishes containing MEA. These cultures were grown at temperatures ranging from 5–35 °C at 5 °C intervals. Colony diameters perpendicular to each other were measured when colony growth reached the edges of Petri dishes at an optimum temperature, and averages were computed.

Results

Fungal isolates

A total of 107 isolates having morphological characteristics typical of *Calonectria* spp. were obtained from the soil samples (Suppl. material 2). Of these, 46 were from Cauca, 38 from Risaralda, 14 from Valle del Cauca, and nine from Vichada. Up to four different *Calonectria* spp. were detected in each of these regions (Figs 1, 2).

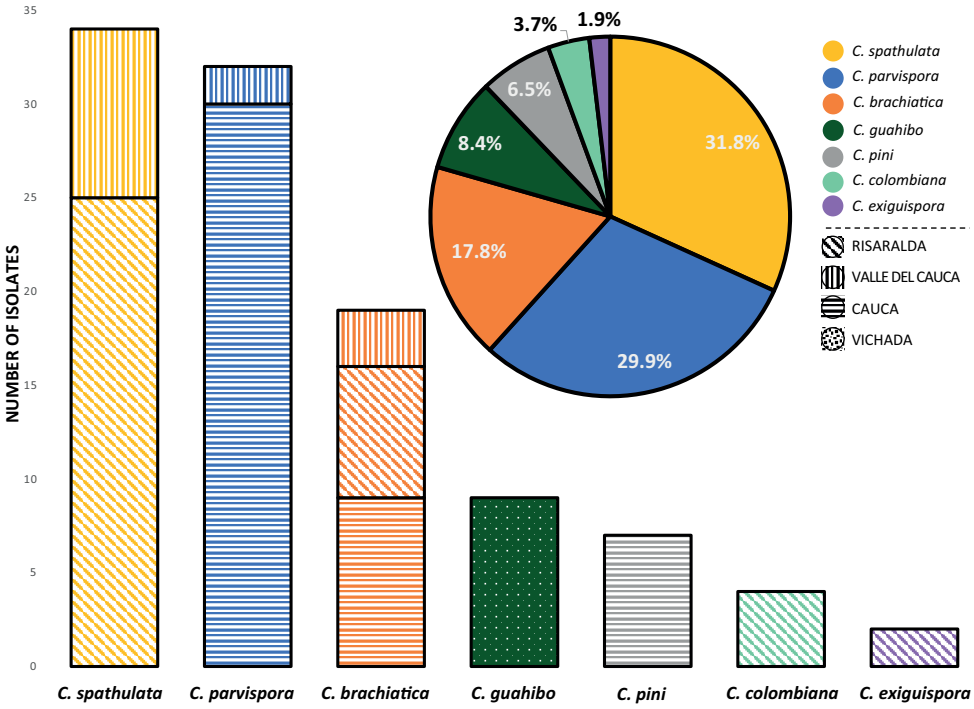

Figure 2. Relative occurrence of *Calonectria* species associated with *Eucalyptus* plantations in Colombia. Different species are represented by different colours. Isolates obtained from different regions are represented by different patterns in the bar chart.

Two of the most commonly isolated species each accounted for approximately 30% of the isolates (Fig. 2). The remaining isolates represented 1.9–17.8% of any one species (Fig. 2). All isolates were fast growing on SNA and MEA, producing abundant aerial mycelia, and scarce numbers of sclerotia, chlamydospores or fruiting structures in 3–4 w.

Phylogenetic analyses

Sequence data were generated for all 107 isolates, which were approximately 250 bp for the *ACT* gene region, 660 bp for the *CMDA*, 430 bp for the *HIS3*, 1000 bp for the *RPB2*, 500 bp for the *TEF1*, and 560 bp for the *TUB2*. For the phylogenetic analyses of each individual data set, the HKY+G model was selected for *ACT*, the GTR+G model for *CMDA*, the GTR+G for *HIS3*, the TIM2ef+G for *RPB2*, the TPM1uf+G for *TUB2*, and the TPM3uf+I+G for *TEF1*. The ML tree for each individual gene region with bootstrap support values of ML and posterior probabilities of BI are presented in Suppl. material 1.

The combined sequence data set used in the phylogenetic analyses included 191 ingroup taxa and 3 315 characters, including alignment gaps. Concatenated sequence alignments of the six gene regions together with closely related *Calonectria* species were deposited in Zenodo (10.5281/zenodo.7195911). Topologies of the trees resulting from the ML and BI analyses were concordant and showed similar phylogenetic relationships between taxa. The ML tree with bootstrap support values for the ML and the posterior probabilities obtained from BI is presented in Fig. 3. Isolates considered in this study were all in the Prolate Group (Liu et al. 2020) and resided in either the *C. brassicae*, *C. candelabrum* or *C. pteridis* species complex.

The majority of the isolates resided in the *C. brassicae* species complex. Fifty-eight isolates in this complex clustered in three different clades. Of these, 32 isolates grouped in the same clade with the ex-type isolate of *C. parvispora*, 19 isolates clustered together with *C. brachiatica*, and seven with *C. pini.*

In the *C. candelabrum* species complex, 40 isolates clustered in three groups. Of these, four isolates grouped together with *C. colombiana*, 34 isolates with *C. spathulata*, and two isolates resided in a well-supported clade (ML/BI = 100/1.00) distinct from any known species in this complex and thus represent a novel taxon.

The remaining nine isolates residing in the *C. pteridis* species complex were closely related to *C. gordoniae* but formed an independent clade (ML/BI = 100/1.00) distinct from *C. gordoniae*, as well as other species in this complex. These isolates represent an undescribed taxon in *Calonectria*.

Taxonomy

Based on phylogenetic analyses and morphological observations, isolates collected from soils in *Eucalyptus* plantations and their adjacent native vegetation in Colombia represented five previously described species, namely, *C. brachiatica*, *C. colombiana*,

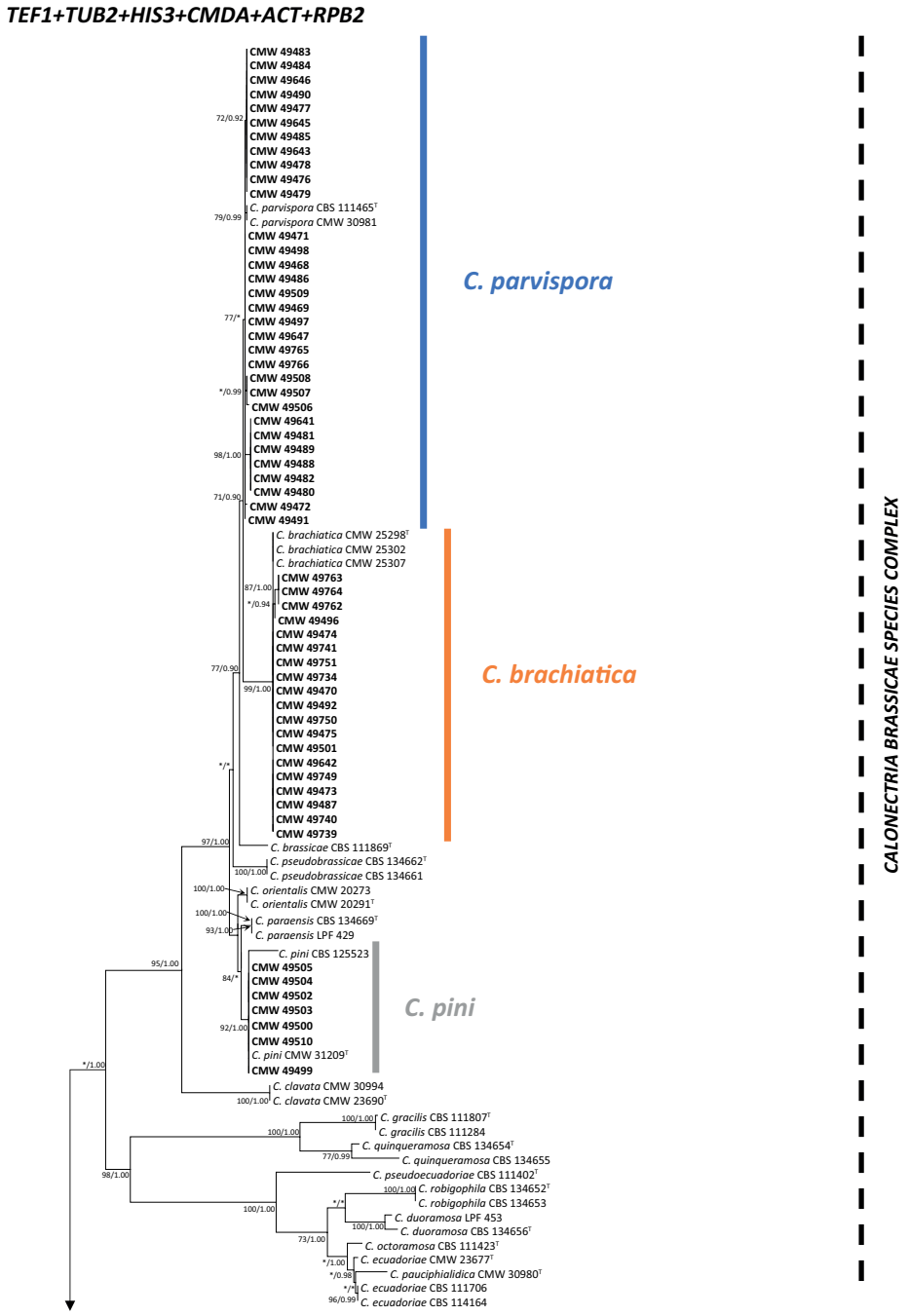

Figure 3. Phylogenetic tree based on maximum likelihood (ML) analysis of a combined DNA data set of *ACT*, *CMDA*, *HIS3*, *RPB2*, *TEF1* and *TUB2* sequences for *Calonectria* spp. Bootstrap values ≥ 70% for ML analyses and posterior probabilities values ≥ 0.90 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Bootstrap values < 70% or probabilities values < 0.90 are marked with "*", and nodes lacking the support values are marked with "–". Isolates representing ex-type material are marked with "T". *Curvicladiella cignea* (isolate CBS 109167 and CBS 109168) represents the outgroup.

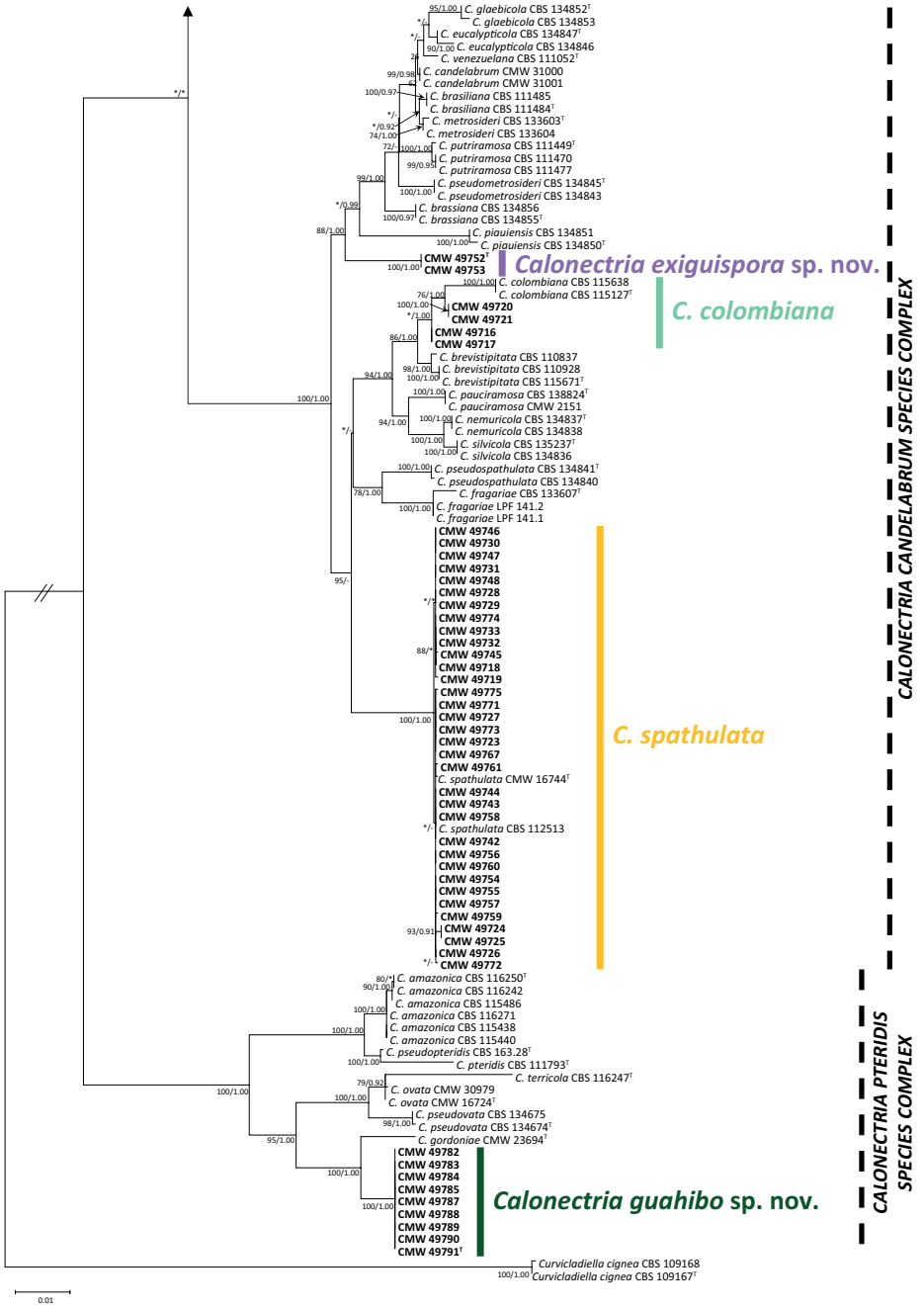

Figure 3. Continued.

C. parvispora, *C. pini* and *C. spathulata*, and two novel species. One of these novel taxa resided in the *C. candelabrum* species complex and the other in the *C. pteridis* species complex. Descriptions for these species are provided as follows.

Calonectria exiguispora **N.Q. Pham, Marinc. & M.J. Wingf., sp. nov.**
MycoBank No: 846456
Figs 4, 6A, B

Etymology. "exiguus" (Latin) = small + "spora" (Latin) = spores, referring to the small macroconidia produced by this species.

Diagnosis. Phylogenetically close to *C. piauiensis* and *C. brassianae* but differs in having smaller macroconidia.

Type. Colombia: Risaralda, Quinchía. Soils in *Eucalyptus* plantation. August 2016. C.A. Rodas. (***Holotype*** PRU(M) 4501, stored in a metabolically inactive state; ***ex-holotype*** CMW 49752, CMW-IA 160). GenBank: OP796405 (*ACT*); OP822275 (*CMDA*); OP822382 (*HIS3*); OP822489 (*RPB2*); OP822168 (*TEF1*); OP822596 (*TUB2*).

Description. ***Sexual morph*** not observed. ***Conidiophores*** scarce on SNA, consisting of conidiogenous apparatus and stipes, branched or simple. ***Stipes*** infrequent, elongated, septate, 75–273 µm long, 2–5 µm wide near base, tapering towards apex, simple or occasionally dichotomously branched, mostly being part of conidiogenous apparatus; ***vesicles*** terminal, slightly inflate to ellipsoidal, 2–5 µm wide. ***Conidiogenous apparatus*** hyaline, simple or branched in 1–3 (–4) tiers, uncommonly developing from stipes; ***main axis*** upright, septate, 20–275 × 3–7 µm; ***branches*** doliiform to cylindrical, primary branches 8–39 × 3–7 µm, secondary branches 8–24 × 2–6 µm, tertiary branches 10–23 × 2–5 µm, quarternary branches 10–14 × 3–4 µm. ***Conidiogenous cells*** holoblastic, hyaline, discrete, cylindrical to tapered above, often constricted near base, with periclinal thickening, 8–20 × 3–5 (11.8 ± 2.71 × 3.2 ± 0.5) µm. ***Macroconidia*** hyaline, cylindrical, round at apex, 1-septate, septum sub-median or median, guttulate, 21–40 × 3–4 (30.9 ± 4.09 × 3.5 ± 0.23) µm. ***Chlamydospores*** present, scarce, in clumps or in chains. ***Mega-*** and ***microconidia*** not observed.

Colonies on 2% MEA in the dark for 6 d, white on surface, pale luteous in reverse, with moderate amount of aerial mycelium, with entire edges. Optimal growth temperature at 25 °C reaching 65.2 mm in 6 d, followed by 20 °C (57.3 mm), 15 °C (39.8 mm), 10 °C (19.7 mm), 5 °C (8.2 mm), no growth at 30 °C and 35 °C. Colonies kept at 30 °C and 35 °C being relocated to 25 °C for another 6 d revived (30 °C) and showed no growth (35 °C). Colonies on 2% MEA in the dark for 30 d, white to umber on surface, umber to dark brick in reverse, with flat mycelia.

Distribution. Colombia.

Material examined. Colombia: Risaralda, Quinchía. Soils in *Eucalyptus* sp. plantation. August 2016. C.A. Rodas. (PRU(M) 4502, stored in a metabolically inactive state; culture CMW 49753, CMW-IA 161).

Notes. *Calonectria exiguispora* is a member of the *C. candelabrum* species complex (Liu et al. 2020). It shares some characteristics with other species in the complex, such as 1-septate macroconidia and 'ellipsoidal to obpyriform' shape vesicle. However, it can be distinguished from most species in the complex by its smaller conidial dimensions (21–40 × 3–4 µm, avg. 30.9 × 3.5 µm) except for *C. brevistipitata* (29–35 × 3–4 µm, avg. 31 × 3.5 µm, isolated from Mexican soil) and *C. stipitate* (27–37 × 3–6, avg. 32 × 4 µm,

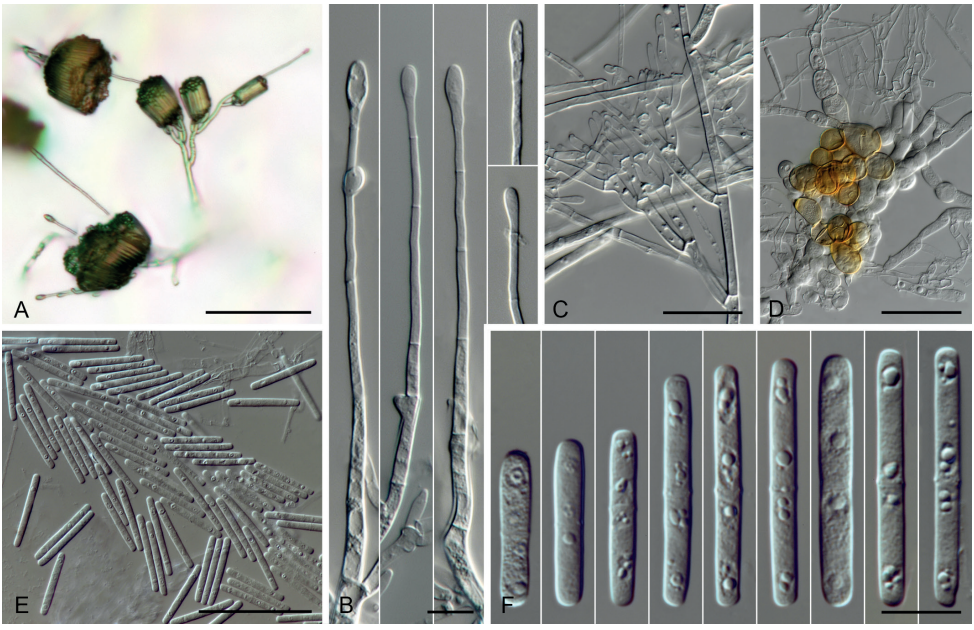

Figure 4. Micrographs of *Calonectria exiguispora* sp. nov. (ex-holotype: CMW 49752 = CMW-IA 160). **A** conidiophores formed on SNA **B** stipes and vesicles **C** conidiogenous apparatus **D** chlamydospores **E** conidia (× 400) **F** conidia (×1 000). Scale bars: 100 µm (**A**); 50 µm (**D, E**); 25 µm (**C**); 10 µm (**B, F**).

isolated from Colombian *Eucalyptus* sp.) (Lombard et al. 2016). Nevertheless, these two species are distantly related to *C. exiguispora* (Fig. 3). Recently Liu et al. (2020) reduced *C. stipitata* to synonymy with *C. spathulata*, the conidial dimensions of which range from 48–100 × 4–6 µm (avg. 80 × 6 µm). They regarded the smaller conidial dimensions of *C. "stipitata"* as representing intraspecific variation. *Calonectria exiguispora* is phylogenetically closely related to *C. piauiensis* and *C. brassianae*, which were isolated from soils associated with *Eucalyptus brassiana* trees in Brazil (Alfenas et al. 2015). These two species, however, have much larger conidial dimensions: *C. piauiensis* (38–60 × 3–5 µm, avg. 49 × 4.5 µm) and *C. brassianae* (35–65 × 3–5 µm, avg. 53 × 4 µm) (Alfenas et al. 2015). It can be differentiated from its most closely related species by sequences of *ACT*, *CMDA*, *HIS3*, *RPB2*, *TEF1* and *TUB2* gene regions.

Calonectria guahibo **N.Q. Pham, Marinc. & M.J. Wingf., sp. nov.**
MycoBank No: 846457
Figs 5, 6E, F

Etymology. Name refers to the indigenous people, Guahibo, native to Vichada, Colombia.

Diagnosis. Closely related to *C. gordoniae* but differs in having smaller macroconidia.

Type. Colombia: Vichada, Cumaribo. Soils in *Eucalyptus* plantation. August 2016. C.A. Rodas. (***Holotype*** PRU(M) 4503, stored in a metabolically inactive

state; ***ex-holotype*** CMW 49791, CMW-IA 162). GenBank: OP796480 (*ACT*); OP822350 (*CMDA*); OP822457 (*HIS3*); OP822564 (*RPB2*); OP822243 (*TEF1*); OP822671 (*TUB2*).

Description. ***Sexual morph*** not observed. ***Conidiophores*** scarce on SNA, composed of conidiogenous apparatus and stipes. ***Stipes*** part of conidiogenous apparatus, elongated, septate, 81–223 µm long, 2–5 µm wide near base, tapering towards apex, simple, infrequently branched; ***vesicles*** slightly inflated to clavate, 2–5 µm wide. ***Conidiogenous apparatus*** hyaline, branched irregularly in 2–3 (–4) tiers; main axis upright, septate, 25–83 × 4–6 µm; ***branches*** doliiform to cylindrical, primary branches 11–23 × 4–6 µm, secondary branches 7–16 × 3–5 µm, tertiary branches 9–11 × 3–4 µm. ***Conidiogenous cells*** holoblastic, hyaline, discrete, cylindrical to ovoid, tapering towards apex, with perclinal thickening, 6–12 × 2–4 (9.3 ± 1.46 × 3.0 ± 0.52) µm. ***Macroconidia*** hyaline, cylindrical with round ends, 1-septate, straight, septum median or sub-median, 26–42 × 3–4 (31.7 ± 3.59 × 3.2 ± 0.19) µm. ***Chlamydospores*** present in clumps or in chains. ***Mega-*** and ***microconidia*** not observed.

Colonies on 2% MEA after 6 d in the dark, growing circular, with fluffy aerial mycelia, above white to pale luteous towards centre, reverse luteous to umber towards centre. Optimal growth temperature at 30 °C reaching 61 mm, followed by 25 °C (57.5 mm), 20 °C (48.3 mm), 15 °C (21.8 mm), and no growth at 5, 10, and 35 °C. Colonies kept at 5, 10, and 35 °C revived after being relocated to 25 °C. Colonies on 2% MEA in the dark for 30 d, with cottony mycelia filled entire Petri dish, above saffron to umber with patches of white, reverse dark brick to sepia.

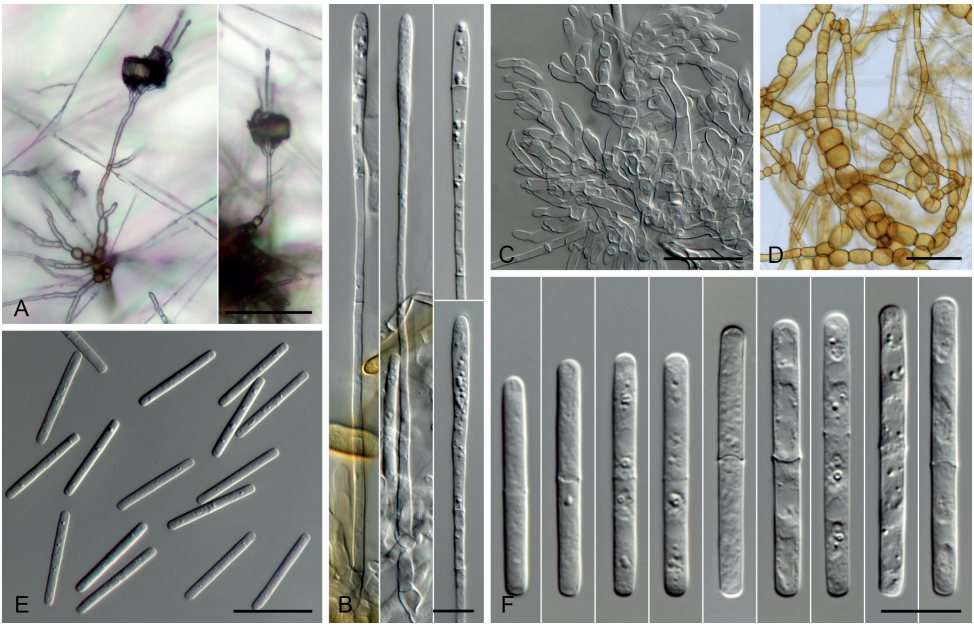

Figure 5. Micrographs of *Calonectria guahibo* sp. nov. (ex-holotype: CMW 49791 = CMW-IA 162). **A** conidiophores formed on SNA **B** stipes and vesicles **C** conidiogenous apparatus **D** chlamydospores **E** conidia (× 400) **F** conidia (×1 000). Scale bars: 100 µm (**A**); 50 µm (**D**); 25 µm (**C, E**); 10 µm (**B, F**).

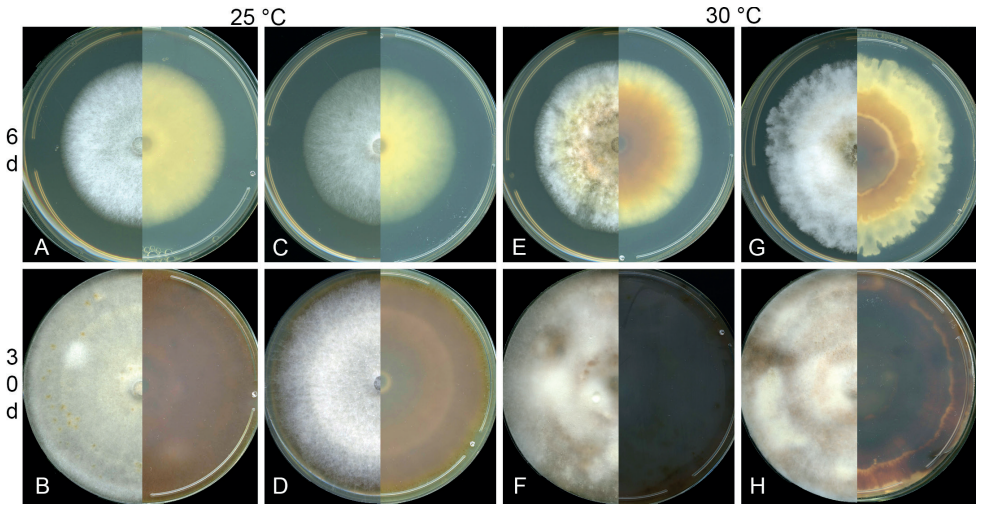

Figure 6. Culture morphology of *Calonectria exiguispora* (**A–D**) at 25 °C and *C. guahibo* (**E–H**) at 30 °C in the dark for 6 d (**A, C, E, G**) and 30 d (**B, E, F, H**) at its optimum temperature **A, B** CMW 49752 (ex-holotype) **C, D** CMW 49753 **E, F** CMW 49791 (ex-holotype) **G, H** CMW 49782.

Distribution. Colombia.

Material examined. Colombia: Vichada, Cumaribo. Soils in *Eucalyptus* sp. plantation. August 2016. C.A. Rodas, *CMW 49782*.

Notes. *Calonectria guahibo* forms part of the *C. pteridis* species complex as a sister taxon to *C. gordoniae*. *Calonectria gordoniae* was reported from Florida, USA, causing leaf spots and blotches on loblolly bay (*Gordonia lasianthus*) and is known to produce macroconidia (45–81 × 4–6 µm, avg. 61.7 × 5 µm) and microconidia (20–42 × 3–4 µm, avg. 32.5 × 3.6 µm) (Leahy et al. 2000). Leahy et al. (2000) reported slightly curved conidia which were not observed in *C. guahibo*. *Calonectria guahibo* can be distinguished by its smaller conidia (26–42 × 3–4 µm, avg. 31.7 × 3.2 µm) from other closely related species, *i.e. C. ovata* (50–110 × 4–6 µm, avg. 70 × 5 µm) (Victor et al. 1997), *C. pseudovata* (55–50 × 4–7, avg. 69 × 5 µm) (Alfenas et al. 2015), and *C. terricola* (40–53 × 3–6 µm, avg. 46 × 4.5 µm) (Lombard et al. 2016). It can be differentiated from its most closely related species by sequences of *ACT*, *CMDA*, *HIS3*, *RPB2*, *TEF1* and *TUB2* gene regions.

Discussion

A relatively large number of *Calonectria* species were discovered from soils collected in *Eucalyptus* plantations in four forestry regions of Colombia. All of the isolates were identified based on DNA sequence comparisons for six gene regions and supported by morphological characteristics. Seven species residing in three species complexes were identified. These include five previously described species, *C. brachiatica*, *C. parvispora* and *C. pini* in the *C. brassicae* species complex, and *C. colombiana* and *C. spathulata*

in the *C. candelabrum* species complex and two novel taxa for which the names *C. exiguispora* and *C. guahibo* have been provided.

Calonectria parvispora was one of the most commonly isolated species (29.9%) and was recovered from two forestry regions (Fig. 1). This species has previously been found in soils collected from Brazil and Colombia (Marin-Felix et al. 2017), but this is the first record of this species from soils associated with *Eucalyptus*. Interestingly, *C. brachiatica* and *C. pini* in the *C. brassicae* species complex were also found, which were previously isolated from *Pinus* cuttings displaying collar and root rot symptoms in Colombian nurseries (Lombard et al. 2009; Lombard et al. 2010a). *Calonectria pini* was previously collected in Valle da Cauca (Lombard et al. 2010a), and its appearance in this study suggests that it has a relatively wide distribution in Colombia.

Calonectria exiguispora, described in this study, has extended the total number of species of the *C. candelabrum* species complex to 20 (Liu et al. 2020; Sanchez-Gonzalez et al. 2022). In addition, two previously described species in the *C. candelabrum* species complex, *C. colombiana* and *C. spathulata*, were also found. The latter species represented the majority of the isolates (31.8%). This is relevant because *C. spathulata* is a well-known pathogen commonly associated with leaf and shoot blight on *Eucalyptus*, and it has been reported from plantations in tropical regions of South America (Crous and Kang 2001; Crous 2002; Rodas et al. 2005). In this study, *C. spathulata* was also isolated from soils collected in natural rainforests surrounding *Eucalyptus* plantations in Risaralda, where the first outbreak of the disease occurred. It is possible that it is native to this area, but further studies, including those at a population genetics level, would be required to resolve that question.

Calonectria guahibo represents a new addition to the *C. pteridis* species complex, which now includes eight species (Liu et al. 2020), all of which have 1-septate macroconidia and clavate or ovate vesicles. *Calonectria guahibo* appears to have a limited distribution, with all isolates obtained from soils collected in plantations in Vichada, and interestingly, it was the only species found in this region. Although the *C. pteridis* species complex incorporates some of the most important pathogens of *Eucalyptus* (Crous 2002; Graça et al. 2009; Alfenas et al. 2013, 2015), nothing is known regarding the pathogenicity of the newly described *C. guahibo*.

Many previous reports of *Calonectria* spp. are considered to be of dubious significance because identifications were mostly based on morphology. It is now well-recognised that multi-gene markers together with morphological comparisons are required to identify these fungi with confidence. Consequently, this study has provided a more comprehensive understanding of the species diversity and distribution of *Calonectria* in Colombian *Eucalyptus* plantations. This should contribute to the establishment of an effective management strategy for the diseases caused by these fungi in plantations and nurseries.

Results of previous investigations and the present study have shown that soils associated with commercially propagated *Eucalyptus* spp. in tropical and subtropical regions represent a niche that is remarkably rich in species of *Calonectria* (Alfenas et al. 2015; Lombard et al. 2015; Li et al. 2017; Pham et al. 2019, 2022; Wu and Chen 2021). New species of these important fungi will most likely emerge when more extensive

surveys are extended for the remaining areas in Colombia in the future. Further studies should also be conducted to determine the relative importance of the many *Calonectria* spp. residing in the soils associated with *Eucalyptus* plantations in the country.

Acknowledgements

We acknowledge the financial support of the DST/NRF Centre of Excellence in Plant Health Biotechnology (CPHB), South Africa. We are also grateful to members of the staff of SmurfitKappa Colombia for assistance in collecting soil samples.

References

Alfenas RF, Pereira OL, Freitas RG, Freitas CS, Dita MA, Alfenas AC (2013) Mass spore production and inoculation of *Calonectria pteridis* on *Eucalyptus* spp. under different environmental conditions. Tropical Plant Pathology 38(5): 406–413. https://doi.org/10.1590/S1982-56762013000500005

Alfenas RF, Lombard L, Pereira OL, Alfenas AC, Crous PW (2015) Diversity and potential impact of *Calonectria* species in *Eucalyptus* plantations in Brazil. Studies in Mycology 80(1): 89–130. https://doi.org/10.1016/j.simyco.2014.11.002

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91(3): 553–556. https://doi.org/10.1080/00275514.1999.12061051

Crous PW (2002) Taxonomy and pathology of *Cylindrocladium* (*Calonectria*) and allied genera. American Phytopathological Society (APS Press), St. Paul, Minnesota.

Crous PW, Groenewald JZ, Risède JM, Simoneau P, Hywel-Jones NL (2004) *Calonectria* species and their *Cylindrocladium* anamorphs: Species with sphaeropedunculate vesicles. Studies in Mycology 50: 415–430.

Crous PW, Kang J-C (2001) Phylogenetic confirmation of *Calonectria spathulata* and *Cylindrocladium leucothoes* based on morphology, and sequence data of the β-tubulin and ITS rRNA genes. Mycoscience 42(1): 51–57. https://doi.org/10.1007/BF02463975

Crous PW, Phillips AJL, Wingfield MJ (1991) The genera *Cylindrocladium* and *Cylindrocladiella* in South Africa, with special reference to forest nurseries. South African Forestry Journal 157(1): 69–85. https://doi.org/10.1080/00382167.1991.9629103

Crous PW, Wingfield MJ, Mohammed C, Yuan ZQ (1998) New foliar pathogens of *Eucalyptus* from Australia and Indonesia. Mycological Research 102(5): 527–532. https://doi.org/10.1017/S0953756297005091

Ferreira FA, Alfenas AC, Moreira AM, Demuner NL (1995) A foliar eucalypt disease in tropical regions of Brazil caused by *Cylindrocladium pteridis*. Fitopatologia Brasileira 20: 107–110.

Graça RN, Alfenas AC, Maffia LA, Titon M, Alfenas RF, Lau D, Rocabado JMA (2009) Factors influencing infection of eucalypts by *Cylindrocladium pteridis*. Plant Pathology 58(5): 971–981. https://doi.org/10.1111/j.1365-3059.2009.02094.x

Granados GM, McTaggart AR, Barnes I, Rodas C, Roux J, Wingfield MJ (2017) The pandemic biotype of *Austropuccinia psidii* discovered in South America. Australasian Plant Pathology 46(3): 267–275. https://doi.org/10.1007/s13313-017-0488-x

Groenewald JZ, Nakashima C, Nishikawa J, Shin HD, Park JH, Jama AN, Groenewald M, Braun U, Crous PW (2013) Species concepts in *Cercospora*: Spotting the weeds among the roses. Studies in Mycology 75: 115–170. https://doi.org/10.3114/sim0012

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Leahy RM, Schubert TS, El-Gholl NE (2000) *Cylindrocladium gordoniae* sp. nov. Mycotaxon 76: 77–83.

Li JQ, Wingfield MJ, Liu QL, Barnes I, Roux J, Lombard L, Crous PW, Chen SF (2017) *Calonectria* species isolated from *Eucalyptus* plantations and nurseries in South China. IMA Fungus 8(2): 259–286. https://doi.org/10.5598/imafungus.2017.08.02.04

Li JQ, Wingfield MJ, Barnes I, Chen SF (2022) *Calonectria* in the age of genes and genomes: Towards understanding an important but relatively unknown group of pathogens. Molecular Plant Pathology 23(7): 1060–1072. https://doi.org/10.1111/mpp.13209

Liu YJ, Whelen S, Hall BD (1999) Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerse II subunit. Molecular Biology and Evolution 16(12): 1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092

Liu QL, Li JQ, Wingfield MJ, Duong TA, Wingfield BD, Crous PW, Chen SF (2020) Reconsideration of species boundaries and proposed DNA barcodes for *Calonectria*. Studies in Mycology 97: 100106. https://doi.org/10.1016/j.simyco.2020.08.001

Lombard L, Rodas CA, Crous PW, Wingfield BD, Wingfield MJ (2009) *Calonectria* (*Cylindrocladium*) species associated with dying *Pinus* cuttings. Persoonia 23(1): 41–47. https://doi.org/10.3767/003158509X471052

Lombard L, Crous PW, Wingfield BD, Wingfield MJ (2010a) Phylogeny and systematics of the genus *Calonectria*. Studies in Mycology 66: 31–69. https://doi.org/10.3114/sim.2010.66.03

Lombard L, Crous PW, Wingfield BD, Wingfield MJ (2010b) Species concepts in *Calonectria* (*Cylindrocladium*). Studies in Mycology 66: 1–13. https://doi.org/10.3114/sim.2010.66.01

Lombard L, Chen SF, Mou X, Zhou XD, Crous PW, Wingfield MJ (2015) New species, hyperdiversity and potential importance of *Calonectria* spp. from *Eucalyptus* in South China. Studies in Mycology 80(1): 151–188. https://doi.org/10.1016/j.simyco.2014.11.003

Lombard L, Wingfield MJ, Alfenas AC, Crous PW (2016) The forgotten *Calonectria* collection: Pouring old wine into new bags. Studies in Mycology 85(1): 159–198. https://doi.org/10.1016/j.simyco.2016.11.004

Lopes UP, Alfenas RF, Zambolim L, Crous PW, Costa H, Pereira OL (2018) A new species of *Calonectria* causing rot on ripe strawberry fruit in Brazil. Australasian Plant Pathology 47(1): 1–11. https://doi.org/10.1007/s13313-017-0532-x

Marin-Felix Y, Groenewald JZ, Cai L, Chen Q, Marincowitz S, Barnes I, Bensch K, Braun U, Camporesi E, Damm U, de Beer ZW, Dissanayake A, Edwards J, Giraldo A, Hernández-Restrepo M, Hyde KD, Jayawardena RS, Lombard L, Luangsa-ard J, McTaggart AR, Rossman AY, Sandoval-Denis M, Shen M, Shivas RG, Tan YP, van der Linde EJ, Wingfield MJ, Wood AR, Zhang JQ, Zhang Y, Crous PW (2017) Genera of phytopathogenic fungi: GOPHY 1. Studies in Mycology 86(1): 99–216. https://doi.org/10.1016/j.simyco.2017.04.002

Mohanan C, Sharma JK (1985) *Cylindrocladium* causing seedling diseases of *Eucalyptus* in Kerala, India. Transactions of the British Mycological Society 84(3): 538–539. https://doi.org/10.1016/S0007-1536(85)80019-X

Nirenberg HI (1981) A simplified method for identifying *Fusarium* spp. occurring on wheat. Canadian Journal of Botany 59(9): 1599–1609. https://doi.org/10.1139/b81-217

O'Donnell K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7(1): 103–116. https://doi.org/10.1006/mpev.1996.0376

Old KM, Kenneth M, Wingfield MJ (2003) A manual of diseases of eucalyptus in South-East Asia. CIFOR.

Paine TD, Steinbauer MJ, Lawson SA (2011) Native and exotic pests of eucalyptus: A worldwide perspective. Annual Review of Entomology 56(1): 181–201. https://doi.org/10.1146/annurev-ento-120709-144817

Pham NQ, Barnes I, Chen SF, Liu FF, Dang QN, Pham TQ, Lombard L, Crous PW, Wingfield MJ (2019) Ten new species of *Calonectria* from Indonesia and Vietnam. Mycologia 111(1): 78–102. https://doi.org/10.1080/00275514.2018.1522179

Pham NQ, Marincowitz S, Chen SF, Yaparudin Y, Wingfield MJ (2022) *Calonectria* species, including four novel taxa, associated with *Eucalyptus* in Malaysia. Mycological Progress 21(1): 181–197. https://doi.org/10.1007/s11557-021-01768-8

Posada D (2008) jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25(7): 1253–1256. https://doi.org/10.1093/molbev/msn083

Rayner RW (1970) A mycological colour chart. Commonwealth Mycological Institute, Kew, UK.

Rodas CA, Wingfield MJ (2020) Important Insect Pests and Diseases of *Pinus* and *Eucalyptus* in Colombia. Smurfit Kappa, Santiago de Cali, Colombia, 180 pp.

Rodas CA, Lombard L, Gryzenhout M, Slippers B, Wingfield MJ (2005) *Cylindrocladium* blight of *Eucalyptus grandis* in Colombia. Australasian Plant Pathology 34(2): 143–149. https://doi.org/10.1071/AP05012

Rodas CA, Roux J, van Wyk M, Wingfield BD, Wingfield MJ (2008) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. Fungal Diversity 28: 73–84.

Rodas CA, Slippers B, Gryzenhout M, Wingfield MJ (2009) Botryosphaeriaceae associated with *Eucalyptus* canker diseases in Colombia. Forest Pathology 39(2): 110–123. https://doi.org/10.1111/j.1439-0329.2008.00569.x

Rodas CA, Roux J, Maier W, Granados GM, Bolaños MD, McTaggart AR, Wingfield MJ (2015) First report of *Puccinia psidii* on *Corymbia citriodora* and *Eucalyptus* in Colombia. Forest Pathology 45(6): 534–536. https://doi.org/10.1111/efp.12223

Ronquist F, Teslenko M, Van Der Mark P, Ayres D, Darling A, Höhna S, Larget B, Liu L, Suchard M, Huelsenbeck J (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Sanchez-Gonzalez EI, Soares TdPF, Zarpelon TG, Zauza EAV, Mafia RG, Ferreira MA (2022) Two new species of *Calonectria* (Hypocreales, Nectriaceae) causing *Eucalyptus* leaf blight in Brazil. MycoKeys 91: 169–197. https://doi.org/10.3897/mycokeys.91.84896

Sharma JK, Mohanan C, Florence EJM (1984) Nursery Diseases of *Eucalyptus* in Kerala. Forest Pathology 14(2): 77–89. https://doi.org/10.1111/j.1439-0329.1984.tb00156.x

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

van der Merwe NA, Myburg H, Wingfield BD, Rodas CA, Wingfield MJ (2001) Identification of *Cryphonectria cubensis* from Colombia based on rDNA sequence data. South African Journal of Science 97: 295–296.

Victor D, Crous PW, Janse BJH, Wingfield MJ (1997) Genetic variation in *Cylindrocladium floridanum* and other morphologically similar *Cylindrocladium* species. Systematic and Applied Microbiology 20(2): 268–285. https://doi.org/10.1016/S0723-2020(97)80074-4

Wingfield MJ, Slippers B, Hurley BP, Coutinho TA, Wingfield BD, Roux J (2008) Eucalypt pests and diseases: Growing threats to plantation productivity. Southern Forests 70(2): 139–144. https://doi.org/10.2989/SOUTH.FOR.2008.70.2.9.537

Wingfield MJ, Brockerhoff EG, Wingfield BD, Slippers B (2015) Planted forest health: The need for a global strategy. Science 349(6250): 832–836. https://doi.org/10.1126/science.aac6674

Wu WX, Chen SF (2021) Species diversity, mating strategy and pathogenicity of *Calonectria* species from diseased leaves and soils in the *Eucalyptus* plantation in southern China. Journal of Fungi 7(2): 73. https://doi.org/10.3390/jof7020073

Supplementary material 1

Phylogenetic tree based on maximum likelihood (ML) analysis of individual gene region (ACT, CMDA, HIS3, TUB2, TEF1 and RBP2)

Authors: Nam Q. Pham, Seonju Marincowitz, ShuaiFei Chen, Carlos A. Rodas, Michael J. Wingfield

Data type: (word document)

Link: https://doi.org/10.3897/mycokeys.94.96301.suppl1

Supplementary material 2

Collection details and GenBank accessions of isolates included in the phylogenetic analyses

Authors: Nam Q. Pham, Seonju Marincowitz, ShuaiFei Chen, Carlos A. Rodas, Michael J. Wingfield

Data type: table (excel document)

Link: https://doi.org/10.3897/mycokeys.94.96301.suppl2

MycoKeys 94: 37–50 (2022)
doi: 10.3897/mycokeys.94.95858
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

A hotspot of lichen diversity and lichenological research in the Alps: the Paneveggio-Pale di San Martino Natural Park (Italy)

Juri Nascimbene[1*], Gabriele Gheza[1*], Peter O. Bilovitz[2], Luana Francesconi[1], Josef Hafellner[2], Helmut Mayrhofer[2], Maurizio Salvadori[3], Chiara Vallese[1], Pier Luigi Nimis[4]

1 *BIOME Lab, Department of Biological, Geological and Environmental Sciences, Alma Mater Studiorum University of Bologna, Via Irnerio 42, 40126 Bologna, Italy* **2** *Division of Plant Sciences, Institute of Biology, NAWI Graz, University of Graz, Holteigasse 6, 8010 Graz, Austria* **3** *Paneveggio-Pale di San Martino Natural Park, Villa Welsperg, località Castelpietra 2, 38054 Primiero San Martino di Castrozza (Trento), Italy* **4** *Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, 34127 Trieste, Italy*

Corresponding author: Gabriele Gheza (gheza.gabriele@gmail.com)

Academic editor: Thorsten Lumbsch | Received 30 September 2022 | Accepted 10 November 2022 | Published 6 December 2022

Citation: Nascimbene J, Gheza G, Bilovitz PO, Francesconi L, Hafellner J, Mayrhofer H, Salvadori M, Vallese C, Nimis PL (2022) A hotspot of lichen diversity and lichenological research in the Alps: the Paneveggio-Pale di San Martino Natural Park (Italy). MycoKeys 94: 37–50. https://doi.org/10.3897/mycokeys.94.95858

Abstract

A checklist of 916 lichenised taxa is reported from the Paneveggio-Pale di San Martino Natural Park and its surroundings (Trentino-Alto Adige, N Italy), based on 7351 records from: (a) 72 literature sources, (b) eight public and private herbaria and (c) field observations by some of the authors. The study area appears as a hotspot of lichen diversity, hosting 30.1% of the lichen biota of the Alps in a territory that has 0.064% of their total surface area. This is mainly due to its high climatical, geological and orographic heterogeneity, but also to the long history of lichenological exploration, that started in the 19th century with Ferdinand Arnold and is still ongoing. The present work highlights the importance of detailed species inventories to support knowledge of biodiversity patterns, taxonomy and ecology and to properly address conservation issues. *Fuscidea mollis* var. *caesioalbescens*, *Hydropunctaria scabra*, *Protoparmelia badia* var. *cinereobadia* and *Variospora paulii* are new to Italy, 18 other taxa are new to Trentino-Alto Adige.

Keywords

Alps, biodiversity, checklists, conservation, herbarium studies, historical records, lichen inventories

* These authors contributed equally to this work.

Introduction

Basic information on the distribution, ecology and taxonomy of species is fundamental for revealing biodiversity patterns and providing effective conservation guidelines. Field species inventories carried out by specialists (Vondrák et al. 2016, 2018; Spribille et al. 2020) as well as survey of herbaria and literature records (Isocrono et al. 2007; Himelbrant et al. 2018) are fundamental for lichen biodiversity research, sometimes triggering taxonomic advances, including the description of new species (Spribille et al. 2020; Leavitt et al. 2021; Vondrák et al. 2022). Furthermore, temporal continuity of basic biodiversity data from a given region may allow comparison of biodiversity patterns across time, to track the effect of global changes (Hauck et al. 2013).

Unfortunately, basic biodiversity data on lichens are often missing, even for relatively well-explored areas, thus hampering conservation efforts (Hunter and Webb 2002; Rubio-Salcedo et al. 2013). However, some notable exceptions exist, as in the case of the Alps, which are amongst the lichenologically best known areas of the world, thanks to their long-lasting and accurate exploration. To date, 3046 lichenised infrageneric taxa are known from the area (Nimis et al. 2018a), but this number is likely to increase with the widening of exploration and the deepening of taxonomical knowledge.

Within the Alps, the historical region of Tyrol is certainly one of the best-explored, with one of the oldest known "checklists": in their compilative monograph "*Die Flechten (Lichenes) von Tirol, Vorarlberg und Liechtenstein*", Dalla Torre and Sarnthein (1902) summarised a huge amount of information on the lichen biota of the Tyrolean area, mainly based on original papers, based predominantly on multiple field explorations by Ferdinand Arnold (1828–1901) and Ernst Kernstock (1852–1900). These data largely contributed to the present lichen inventory of Trentino-Alto Adige, that is the lichenologically richest region of Italy, with 1573 infrageneric taxa of lichenised fungi reported to date (Nimis and Martellos 2022).

In particular, Arnold intensely explored the area of Paneveggio and Predazzo (Arnold 1879, 1880, 1887, 1897), whose localities are famous amongst lichenologists, due to the many specimens collected there and distributed in several public herbaria, as well as to the new species described from this area. Since 1967, the area of Paneveggio was included in the Paneveggio-Pale di San Martino Natural Park, that extends south of Paneveggio to incorporate almost all the Pale di San Martino dolomitic chain and a metamorphic mountain area at the orographic right side of the Vanoi River. Since its institution, this Park has attracted lichen research thanks to the fame resulting from Arnold's explorations. In particular, since the mid-nineties the administration of the Park promoted a new phase of exploration that focused both on lichen floristics (e.g. Nascimbene and Caniglia 2003; Thor and Nascimbene 2007) and ecology (e.g. Nascimbene and Caniglia 1997, 1999; Nascimbene et al. 2008), expanding the research effort to almost all of the protected area.

In this work, we summarise about 150 years of lichenological exploration of the Paneveggio-Pale di San Martino Natural Park, providing an updated checklist of its lichenised fungi.

Materials and methods

Study area

The Paneveggio Pale di San Martino Natural Park, spanning an elevational gradient of about 2000 m (from 1200 m in Val Canali to 3192 m on Mt. Vezzana) and covering a surface of about 20,000 hectares, includes the typical mountain environments of the Alps, being located in the south-eastern part of the Alpine chain (Fig. 1). As a Natural Park, it includes both core areas under strict protection and buffer areas where some human activities are allowed, for example, logging, tourism and winter recreation activities.

The territory is characterised, from a geological point of view, by a high diversity of substrates. The sedimentary rocks of the Mesozoic emerge on the orographic left of the Cismon Stream, while igneous and metamorphic rocks of the Paleozoic emerge in the western part of the Park. The metamorphic unit is made up of quartz-containing phyllite and mica-schists emerging in the Scanaiol, Arzon and Tognola-Valcicolera Group. Porphyric rocks characterise the Lagorai chain, from Tognazza-Cavallazza group towards the west up to the edge of the Park, including the Bocche-Iuribrutto group. Sedimentary rocks include both well-stratified evaporitic-arenaceous formations originating between late Paleozoic and early Mesozoic (e.g. *Bellerophon* and Werfen formations) and compact dolomitic rocks (Sciliar Dolomite) which can be over a thousand metres thick. These heterogeneous sedimentary rocks characterise the landscape of the Pale di San Martino chain that reach and even exceed 3000 metres (e.g. Cimon della Pala, Mt. Mulaz, Vezzana).

The morphology of the territory influences climatic conditions: the natural barrier formed by the Pale di San Martino and Lagorai mountain ranges interrupts the flow of humid currents coming from the Adriatic Sea, determining very humid, sub-oceanic conditions on the southern slopes and cooler and drier (i.e. more continental) conditions in the northern area beyond Rolle Pass that, thus, represents a climatic border. This is reflected in differences of annual precipitation, that is higher in the southern part (i.e. San Martino di Castrozza 1550 mm/y, Val Canali 1500 mm/y) than in the northern part (i.e. Paneveggio 1180 mm/y and Predazzo 1100 mm/y). Mean annual temperature varies between 8 °C at 1100 m (e.g. Val Canali and Predazzo), 5.5 °C at 1500 m (e.g. San Martino di Castrozza) and -1 °C at 2900 m (Pale di San Martino).

The regional climate influences the distribution of vegetation types, with mixed *Abies alba-Fagus sylvatica*-forests in the montane belt (1000–1800 m) of the southern part and *Picea abies-Larix decidua-Pinus cembra* formations in the montane (1300–1800 m) and subalpine (1800–2300 m) belts of the northern part, including the famous Paneveggio Forest. In the alpine belt (2300–2700 m), primary grasslands prevail, dominated by *Carex curvula* in the acidic part of the Park and by *Sesleria caerulea* and *Carex sempervirens* in the carbonatic part. The nival belt (> 2700 m) hosts pioneer, discontinuous vegetation types, as in the case of chasmophytic assemblages whose composition depends on the geological substrate. Freshwater habitats (springs, rivulets, creeks) and bogs are more frequent in the porphyric-metamorphic part of the Park,

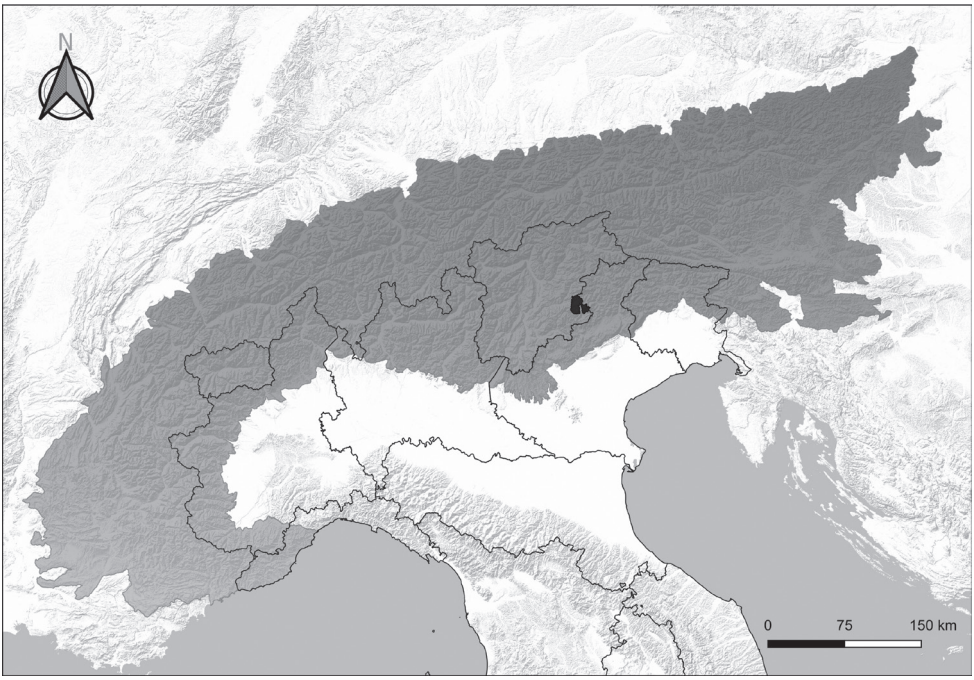

Figure 1. Location of the Paneveggio-Pale di San Martino Natural Park within the Alps.

while in the carbonatic part, superficial waters are rare due to Karst phenomena, being mainly related to snow-ice melting in high elevation ranges and small springs. Overall, the vascular flora is rich (about 1500 species), including several endemic taxa, such as *Campanula morettiana*, *Primula tyrolensis*, *Saxifraga facchinii* and *Rhizobotrya alpina*, that are restricted to the Dolomites.

The data

Between 1878 and 1886, the Bavarian lichenologist Ferdinand Arnold (1828–1901) carefully explored the region of Val di Fiemme, including the area of Paneveggio and Predazzo, for a total of 146 days of fieldwork (Arnold 1887). In summer 1884, he was supported by Hugó Lojka (1844–1887), who explored the Travignolo Valley, leading to several interesting findings (Arnold 1887). The data collected by Arnold, the oldest source on the lichen biota of the study area, are scattered in several main papers (Arnold 1869, 1875, 1879, 1880, 1886, 1887, 1889, 1893, 1896, 1897), that were later summarised in the monograph by Dalla Torre and Sarnthein (1902) (with a few genera treated by Magnus 1905). Arnold distributed a considerable number of *exsiccata* of specimens collected in the Paneveggio-Predazzo area in his "Lichenes exsiccati", whose duplicates can be currently found in various herbaria, for example, CANB, COLO, DUKE, F, FR, GB, GZU, LD, M, NY, O, PC, S, UPS and WIS. Additional numbers were traced in, for example, Flora exs. Austro-hungarica, Lojka, Lichenotheca Universalis, Rehm

- Cladoniae exs. and Zwackh - Lich. exs. Material of some more recent collections has been distributed in Plantae Graecenses. Several of Arnold's specimens are also cited in more recent literature (Suppl. material 1). In this work, we included also Arnold's records referring to localities that are in the surroundings of the protected area (e.g. Predazzo) for two reasons: 1) to valorise the precious work of Arnold in this region; 2) to include species potentially occurring in the protected area since they were collected on similar substrates and under comparable environmental conditions.

In the 20th century, the area of Paneveggio was far less explored by lichenologists. Maria Cengia Sambo (1888–1939) published some records from the area of Passo Rolle (Cengia Sambo 1931), but specimens cited in her work are unfortunately missing to date. Later, the area was explored by Austrian lichenologists from Graz, mainly Josef Poelt (1924–1995) and Josef Hafellner, whose published and unpublished specimens are housed in GZU.

Lichenological research increased again from the late 1990s to the present and is still ongoing. Most of the records collected in this period refer to herbarium specimens and field observations by Juri Nascimbene, only a few of them having already been published (Nascimbene and Caniglia 1997, 1999, 2003; Caniglia et al. 2002; Thor and Nascimbene 2007; Nascimbene et al. 2008, 2021). The latter research, discontinuous over time, derives from an alternation of floristic and ecological studies aimed at investigating the effects of forest management on lichen diversity. While floristic studies covered the entire territory of the Park (although gaps are still present) and a wide array of substrates, the ecological studies focused on the Paneveggio Forest and included epiphytic and lignicolous lichens only. As in the case of Arnold, our checklist also includes some collections from the surroundings of the protected area.

In this work, lichenological exploration is subdivided in three main periods: 1) 19th century: mainly Arnold's collections; 2) 20th century: sporadic collections mainly by lichenologists from Graz; 3) 21th century (including the last five years of the previous century): mainly Nascimbene's work.

Data were retrieved from 72 literature sources (the full list is in Suppl. material 1), eight herbaria (i.e. GB, GZU, LD, M, S, UPS, lichen herbarium of the Paneveggio-Pale di San Martino Natural Park, private lichen herbarium of Juri Nascimbene) and several field observations, mainly by Juri Nascimbene. They were organised into a georeferenced database that to date includes 7351 records. For each record, the following information was retrieved, when possible: current name (updated according to Nimis and Martellos 2022), name of the taxon in the original source, source type, locality, altitude, altitudinal belt, substrate, habitat, collection year and century, collector, identifier. Most of the historical records were incomplete, for example, by lacking detailed information on habitat and substrate. Recent collections and field observations were georeferenced and, whenever the indications of the localities allowed it (namely when a toponym, a habitat or a substrate were mentioned), historical records were georeferenced as well, with an approximation of several hundred metres, due to uncertainty. Recently collected specimens were identified by means of standard lichenological procedures, i.e. observation of morphological and anatomical features and, when needed,

study of secondary metabolites by means of thin-layer chromatography in solvents A, B' and C. For some aquatic specimens, belonging to genera *Hydropunctaria*, *Thelidium* and *Verrucaria*, molecular studies were carried out to achieve a correct identification.

Only lichenised fungi were considered; lichenicolous fungi and non-lichenised fungi usually treated by lichenologists (see Nimis 2016) are not included in this paper. Nomenclature, taxonomy and information on species' traits refer to Nimis and Martellos (2022).

Results

The checklist of the lichenised fungi of the Paneveggio-Pale di San Martino Natural Park includes 916 specific and infraspecific taxa (Suppl. material 1), corresponding to 58.4% of the lichen biota of Trentino-Alto Adige (Nimis and Martellos 2022), 35.2% of Italy (Nimis and Martellos 2022) and 30.1% of the Alps (Nimis et al. 2018a). Most records (4551, 731 taxa) were retrieved from literature, whereas a lesser amount refers to herbarium specimens (1325, 522 taxa) and field observations (1475, 180 taxa).

The species belong to 270 genera (most represented, with more than 20 species each: *Cladonia*, *Lecanora*, *Lecidea s. lat.*, *Rhizocarpon*, *Verrucaria*, *Rinodina*; 128 genera with only one species each), 75 families (most represented, with more than 50 species each: Parmeliaceae, Lecanoraceae, Lecideaceae, Teloschistaceae, Verrucariaceae; 22 families with only one species each) and 26 orders (most represented, with more than 50 species each: Lecanorales, Verrucariales, Caliciales, Lecideales, Teloschistales, Peltigerales).

Chlorolichens are the most frequent group (93.0%), followed by cyanolichens (6.0%) and cephalolichens (1.0%); amongst chlorolichens, most have a chlorococcoid photobiont (88.3%) and only a few a trentepohlioid photobiont (4.7%). Most numerous are crustose forms (68.8%), followed by foliose (15.5%), fruticose (11.2%); squamulose (3.4%) and leprose (1.1%) forms are far less represented. Most taxa reproduce sexually (76.5%), while 23.5% reproduce asexually, mainly by soredia (17.0%), followed by isidia (4.1%) and thallus fragmentation (2.4%).

The number of subcontinental taxa is 22 (2.4%), that of suboceanic taxa 80 (8.7%), while only two taxa can be considered as oceanic (0.2%).

Four taxa are new to Italy, i.e. *Fuscidea mollis* var. *caesioalbescens*, *Hydropunctaria scabra*, *Protoparmelia badia* var. *cinereobadia* and *Variospora paulii*. Eighteen other taxa are new to Trentino Alto Adige, i.e. *Acarospora sphaerospora*, *Bacidina arnoldiana*, *Chrysothrix chlorina*, *Circinaria hoffmanniana*, *Dermatocarpon arnoldianum*, *Gyalecta erythrozona*, *Lecanora bicincta* var. *bicincta*, *Lecanora caesiosora*, *Lempholemma intricatum*, *Miriquidica plumbea*, *Myriolecis agardhiana* subsp. *sapaudica*, *Myriolecis invadens*, *Myriospora myochroa*, *Parmotrema arnoldii*, *Rhizocarpon geographicum* subsp. *arcticum*, *Sarcogyne urceolata*, *Staurothele sapaudica* and *Variospora macrocarpa*. One species, belonging to genus *Lecanora*, still awaits a formal description as new to science (Nascimbene, pers. comm.). In previous, recent publications, several other species from the study area were recorded as new to Italy or Trentino Alto Adige (e.g. Thor and Nascimbene 2007; Nascimbene et al. 2021).

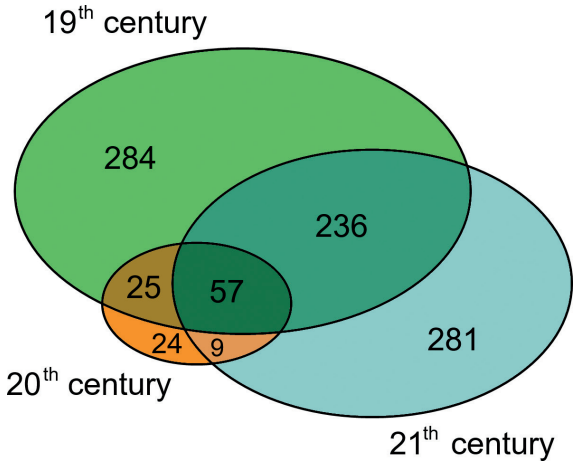

Figure 2. Number of lichen taxa recorded in the three exploration periods and their overlapping.

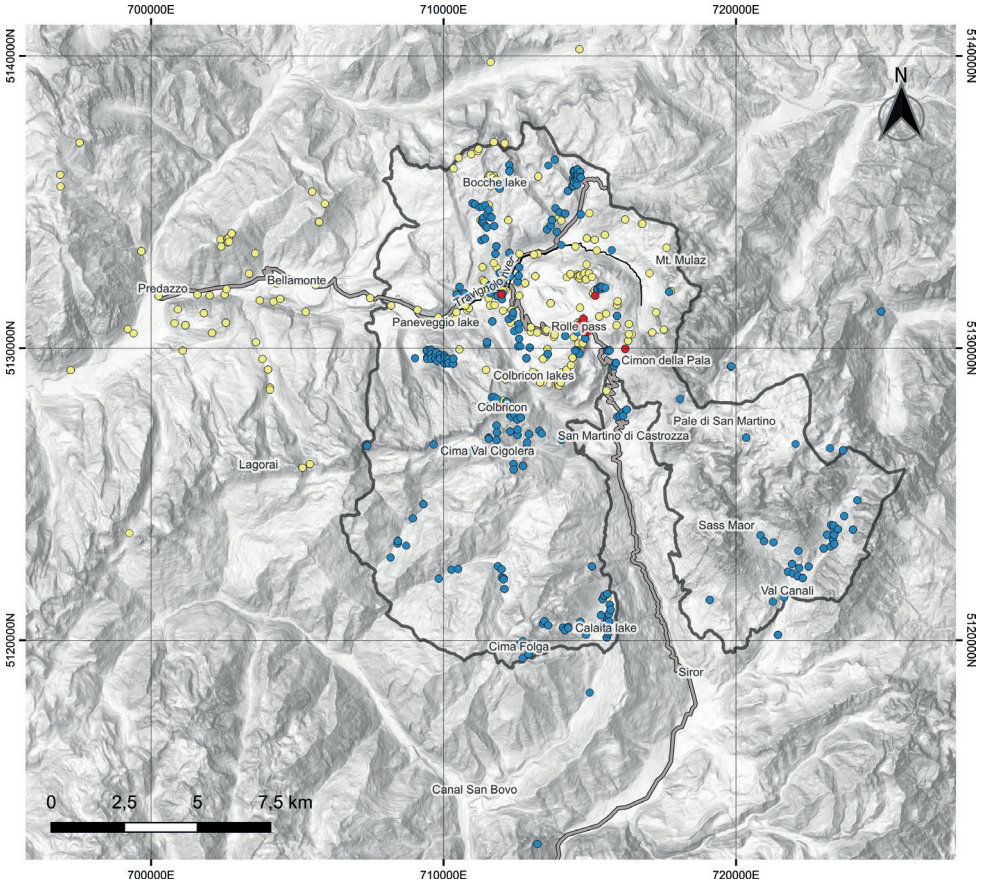

Figure 3. Georeferenced collection sites referred to the 19th (yellow dots), 20th (red dots) and 21st (blue dots) centuries; the continuous black line indicates the borders of the Paneveggio-Pale di San Martino Natural Park.

Ninety-one species are Red-listed: 62 epiphytic lichens (Nascimbene et al. 2013) and 25 terricolous lichens (Gheza et al. 2022), including four species of *Cladonia* subgen. *Cladina* (Ravera et al. 2016).

Only 57 taxa were recorded in all of the three exploration periods, whereas 271 were recorded in two of them, the largest overlap being between the 19th and 21st centuries, sharing 236 species (Fig. 2). Five hundred and ninety species were recorded only in one century (19th: 284, 20th: 24, 21st: 281). Overall, 601 taxa (3794 records) were recorded in the 19th century, 116 (186 records) in the 20th and 585 (3371) in the 21st century.

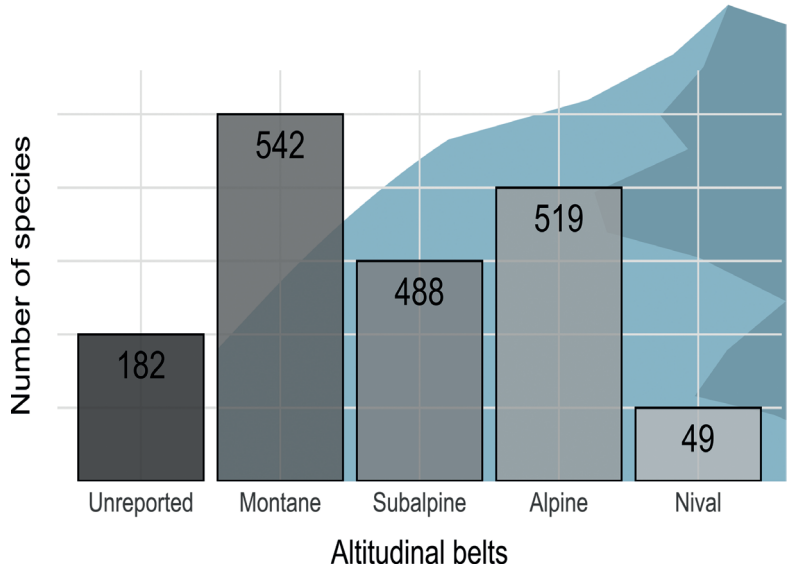

Figure 4. Number of lichen taxa in each altitudinal belt.

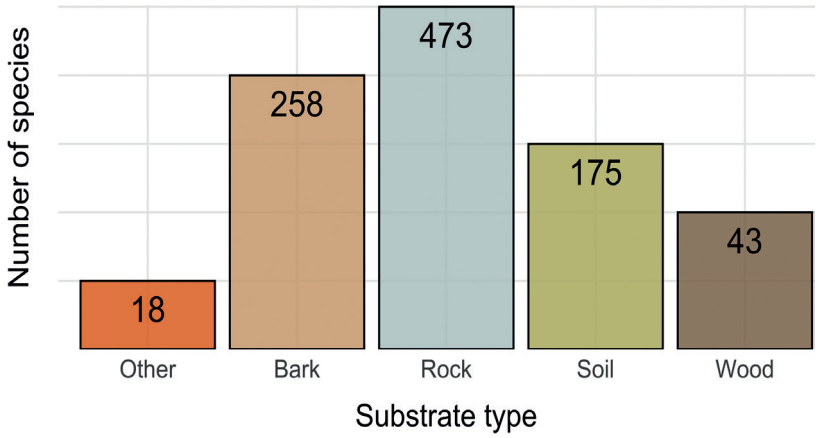

Figure 5. Number of lichen taxa on the main substrate types.

The spatial distribution of historical (Arnold's) and recent (20th and 21th centuries) records reflects the exploration history, with Arnold's localities concentrated in the northern part of the protected area (and its surroundings; Fig. 3) and recent records also scattered in the southern part of the Natural Park, both in the dolomitic and metamorphic areas where, however, some gaps still remain.

The montane belt was the most explored, with 2654 records of 535 taxa, followed by the subalpine, (2296 records of 476 taxa) and the alpine belts (1852 records of 514 taxa) (Fig. 4). The nival belt was the less explored, with 109 records of 49 taxa (Fig. 4).

The highest number of records is from rocks (2351 records, 458 taxa), followed by bark (2003 records, 257 taxa) and soil (665 records, 116 taxa) (Fig. 5). Other substrates, such as deadwood, are less represented. Information about rock type, tree species and soil type was not always available. Amongst saxicolous lichens, most records are from magmatic and metamorphic siliceous rocks (1368 records, 287 taxa), while carbonatic rocks are poorer (873 records, 214 taxa). Epiphytic lichens were mainly collected on *Picea abies* (133 taxa, 730 records), followed by *Abies alba* (61, 174), *Larix decidua* (48, 527), *Pinus cembra* (34, 161), *Alnus incana* (24, 52), *Rhododendron ferrugineum* (24, 48) and *Fraxinus excelsior* (19, 19). Terricolous lichens were mainly from acidic soil (53 taxa), with 25 taxa from carbonatic soil.

Discussion

The Paneveggio-Pale di San Martino Natural Park can be considered as a hotspot of both lichenological research, with more than 150 years of exploration, and of lichen diversity. Almost one third of the lichen biota of both the Alps and Italy occurs in this area, whose surface is ca. 0.06% of their total surface area. This highlights its importance for lichen conservation and lichenological research, with several regionally and nationally new taxa, the occurence of species that still await formal description or of taxa that are known from this area only, as in the case of *Thelidium paneveggiensis*. Moreover, lichen diversity is at least 60% of that of vascular plants, indicating that lichens strongly contribute to the biodiversity of the protected area.

This level of knowledge of the lichen biota is rare in protected areas of the European Alps. Arnold himself stated that, thanks to the repeated and careful investigations he carried out "from the valleys to the highest heights", the upper Val di Fiemme could be considered as the lichenologically best known area of Tyrol at the time (Arnold 1887). A similar situation is perhaps that of the High Tauern National Park, in which over 1100 species have been recorded since the times of Arnold (Türk 2016) on an area which is, however, larger by a factor of ten. In the Italian Alps, other checklists are available, as in the case of a sector of the Stelvio National Park (Nascimbene et al. 2012) or for the Sciliar Natural Park in South Tyrol (Nascimbene 2008), but these are far less exhaustive and the number of species will certainly increase with further exploration. In the case of lichens, not easily detectable and often with a rarefied distribution (Nimis et al. 2018b), it is difficult to provide exhaustive checklists. However,

when exploration is concentrated on relatively small and environmentally heterogeneous areas, the number of species can be surprisingly high (Vondrák et al. 2022). At a national level, in the absence of comparable knowledge on other protected areas, the Paneveggio-Pale di San Martino Natural Park is certainly a priority area for lichen conservation, which should be amongst its main management aims.

This small Natural Park has a great climatic, geological and orographical heterogeneity that likely enhances lichen diversity (Vondrák et al. 2022). For example, Passo Rolle, located in the central part of the study area, is a boundary between oceanic (south) and continental (north) climates, as well as a geological and tectonic boundary. The climatical heterogeneity determines the occurrence of many species with different phytoclimatic affinities, i.e. 22 subcontinental and 82 suboceanic/oceanic taxa. Geological diversity as well plays an important role in shaping and enriching lichen diversity, at least with regard to saxicolous and terricolous species: the checklist includes many specialists of either siliceous or carbonatic rocks and soils, whose co-occurrence in the study area is allowed by the high variety of rock types. Finally, the wide altitudinal range offers favourable conditions for montane, subalpine, alpine and nival species. This also implies different tree species available for epiphytic lichens along the gradient, from broadleaved forests at lower altitudes to coniferous stands in the highest forested belts.

The other component of this lichen hotspot is its exploration history, starting from the 19th century. It should be noticed that, at the times of Arnold, explorations were much more difficult: although he spent a long time in the study area, the investigations carried out in the last decades covered an overall longer timespan and also took into account several areas not explored by Arnold. Nevertheless, a high number of taxa was recorded only either by Arnold or by Nascimbene, but it is hard to say whether the species recorded only in the 19th century could actually have disappeared today. In some cases, the lack of recent records is probably due to merely overlooking the widespread and common taxa in recent surveys, as in the cases of *Athallia pyracea*, *Circinaria calcarea* and *Physconia grisea* that surely still occur. It is also difficult to understand how several widespread or locally common species that likely already occurred at the times of Arnold went unnoticed in historical times and were recorded only in the 21st century, as in the cases of *Athallia cerinella*, *Cladonia symphycarpa*, *Evernia prunastri* and *Lecidella elaeochroma*. On the other hand, some species were recorded only in recent times, because they were described recently (e.g. *Absconditella lignicola*, *Anaptychia bryorum*, *Calicium pinicola* and *Variospora paulii*) or were recognised later as independent taxa (e.g. *Cetrelia cetrarioides*, *C. monachorum* and *C. olivetorum*). Even when the same locality was visited across the three periods, as in the case of Mt. Castellazzo, the overlapping of records was relatively low, differences being mainly related to poorly detectable species, such as small crustose and endolithic lichens and perhaps also the the bias related to the effect of different collectors. Under these circumstances, the checklist is likely more an image of lichen diversity taken with a long exposure time rather than a generalised framework for directly assessing changes of the lichen biota across time, that can be only achieved with resampling of small and clearly localised plots. Only in the case of some easily-detectable species, sensitive to environmental changes (e.g.

Nephroma laevigatum, *Sticta fuliginosa* and *Usnea longissima*) that were not recorded in recent years, we could hypothesise that they actually disappeared due to global changes (i.e., climate, land-use, forest management).

Conclusions

The checklist of the lichens of the Paneveggio-Pale di San Martino Natural Park contributes to a better knowledge of the lichen biota at a broader level than a mere local checklist. It has: (1) a biogeographical value, including a high number of records useful to better elucidate the distribution of many rare and/or poorly known taxa; and (2) a value for biodiversity conservation, providing a framework on which further research can be based. Such detailed floristic information is useful to plan new explorations for assessing the occurrence of the rarest species, which is of paramount importance for planning future conservation actions. Focusing on this topic with a targeted sampling could help to understand the effects of environmental changes in the last 150 years (Hauck et al. 2013), including increased human impact and the ongoing climate change.

Last but not least, this checklist is a remarkable demonstration that even the best-studied areas can still reveal many novelties and should not be considered as "accomplished missions", but should be monitored continuously.

Acknowledgements

We are grateful to Matteo Barcella, Luca Di Nuzzo, Camilla Frascari and Angelica Riciputi for help in managing the dataset and to Holger Thüs for checking (also with molecular approach) specimens of some freshwater lichens.

References

Arnold F (1869) Lichenologische Ausflüge in Tirol. IV. Der Schlern. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 19: 606–656.

Arnold F (1875) Lichenologische Ausflüge in Tirol. XIV. Finsterthal. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 25: 433–496.

Arnold F (1879) Lichenologische Ausflüge in Tirol. XX. Predazzo. Berichtigungen und Nachträge. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 29: 351–394.

Arnold F (1880) Lichenologische Ausflüge in Tirol. XXI. A: Berichtigungen und Nachträge. B: Verzeichniss der Tiroler Lichenen. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 30: 95–154.

Arnold F (1886) Lichenologische Ausflüge in Tirol. XXII. Sulden. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 36: 61–88.

Arnold F (1887) Lichenologische Ausflüge in Tirol. XXIII. Predazzo und Paneveggio. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 37: 81–150.

Arnold F (1889) Lichenologische Ausflüge in Tirol. XXIV. Finkenberg. Nachträge. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 39: 249–266.

Arnold F (1893) Lichenologische Ausflüge in Tirol. XXV. Der Arlberg. Berichtigungen und Nachträge. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 43: 360–407.

Arnold F (1896) Lichenologische Ausflüge in Tirol. XXVI. Pians. XXVII. Galtür. XXVIII. Wolkenstein. XXIX. Plansee. Nachträge. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 46: 101–143.

Arnold F (1897) Lichenologische Ausflüge in Tirol. XXX. Verzeichniss der Lichenen von Tirol. Verhandlungen der kaiserlich-königlichen zoologisch-botanischen Gesellschaft in Wien 47: 353–395. https://doi.org/10.5962/bhl.part.11464

Caniglia G, Corsini L, Dalle Vedove M, De Marco V, Nascimbene J, Rabacchi R, Tonina C (2002) Escursione lichenologica SLI-SBI al Parco Naturale Paneveggio-Pale di San Martino (Trento). Notiziario della Società Lichenologica Italiana 15: 81–86.

Cengia Sambo M (1931) Ecologia dei licheni II. I licheni umicoli dei Passi alpini erbosi. Atti della Società Italiana di Scienze Naturali 70: 279–291.

Dalla Torre KW, Sarnthein Lv (1902) Die Flechten (Lichenes) von Tirol, Vorarlberg und Liechtenstein. In: Dalla Torre KW, Sarnthein Lv (Eds) Flora der gefürsteten Grafschaft Tirol, des Landes Vorarlberg und des Fürstenthumes Liechtenstein. Nach eigenen und fremden Beobachtungen, Sammlungen und den Litteraturquellen. Band 4. Verlag der Wagner'schen Universitäts-Buchhandlung, Innsbruck, [I–XLVI] 1–693.

Gheza G, Di Nuzzo L, Nimis PL, Benesperi R, Giordani P, Vallese C, Nascimbene J (2022) Towards a Red List of the terricolous lichens of Italy. Plant Biosystems 156(3): 824–825. https://doi.org/10.1080/11263504.2022.2065379

Hauck M, de Bruyn U, Leuschner C (2013) Dramatic diversity losses in epiphytic lichens in temperate broad-leaved forests during the last 150 years. Biological Conservation 157: 136–145. https://doi.org/10.1016/j.biocon.2012.06.015

Himelbrant DE, Stepanchikova IS, Kuznetsova ES, Motiejūnaitė J, Konoreva LA (2018) Konevets Island (Leningrad Region, Russia) – a historical refuge of lichen diversity in Lake Ladoga. Folia Cryptogamica Estonica 55: 51–78. https://doi.org/10.12697/fce.2018.55.07

Hunter ML, Webb SL (2002) Enlisting taxonomists to survey poorly known taxa for biodiversity conservation: A lichen case study. Conservation Biology 16(3): 660–665. https://doi.org/10.1046/j.1523-1739.2002.01108.x

Isocrono D, Matteucci E, Ferrarese A, Pensi E, Piervittori R (2007) Lichen colonization in the city of Turin (N Italy) based on current and historical data. Environmental Pollution 145(1): 258–265. https://doi.org/10.1016/j.envpol.2006.03.031

Leavitt SD, Hollinger J, Summerhays S, Munger I, Allen J, Smith B (2021) Alpine lichen diversity in an isolated sky island in the Colorado Plateau, USA—Insight from an integrative biodiversity inventory. Ecology and Evolution 11(16): 11090–11101. https://doi.org/10.1002/ece3.7896

Magnus P (1905) Die Pilze (Fungi) von Tirol, Vorarlberg und Liechtenstein. In: Torre D, von Wilhelm K, Sarnthein L (Eds) Flora der gefürsteten Grafschaft Tirol, des Landes Vorarlberg und des Fürstenthumes Liechtenstein. Innsbruck: Wagner'sche Universitäts-Buchhandlung 3: [I–LIV] 1–716. https://doi.org/10.5962/bhl.title.3876

Nascimbene J (2008) Habitat types and lichen conservation in the Alps: Perspectives from a case study in the Stelvio National Park (Italy). Gredleriana 8: 75–94.

Nascimbene J, Caniglia G (1997) Licheni, indicatori della qualità ambientale degli ecosistemi forestali nei Parchi Naturali di Paneveggio-Pale di S. Martino (Trento) e delle Dolomiti d'Ampezzo (Belluno). Studi Trentini di Scienze Naturali. Acta Biologica 74: 133–142.

Nascimbene J, Caniglia G (1999) Biodiversità dei popolamenti lichenici epifiti negli ecosistemi forestali dei parchi di Paneveggio-Pale di S. Martino (Trento) e delle Dolomiti d'Ampezzo (Belluno). Revue Valdôtaine d'Histoire Naturelle 51(suppl.): 431–436.

Nascimbene J, Caniglia G (2003) Licheni del Parco Naturale Paneveggio-Pale di San Martino. Quaderni del Parco Naturale Paneveggio-Pale di San Martino 3: 1–97.

Nascimbene J, Marini L, Caniglia G, Nimis PL (2008) Lichen diversity on stumps in relation to wood decay in subalpine forests of Northern Italy. Biodiversity and Conservation 17(11): 2661–2670. https://doi.org/10.1007/s10531-008-9344-1

Nascimbene J, Thor G, Nimis PL (2012) Habitat types and lichen conservation in the Alps: Perspectives from a case study in the Stelvio National Park (Italy). Plant Biosystems 146(2): 428–442. https://doi.org/10.1080/11263504.2011.557099

Nascimbene J, Nimis PL, Ravera S (2013) Evaluating the conservation status of epiphytic lichens of Italy: A red list. Plant Biosystems 147(4): 898–904. https://doi.org/10.1080/11263504.2012.748101

Nascimbene J, Gheza G, Hafellner J, Mayrhofer H, Muggia L, Obermayer W, Thor G, Nimis PL (2021) Refining the picture: New records to the lichen biota of Italy. MycoKeys 82: 97–137. https://doi.org/10.3897/mycokeys.82.69027

Nimis PL (2016) The Lichens of Italy. A Second Annotated Catalogue. EUT, Trieste, 739 pp.

Nimis PL, Martellos S (2022 [continuously updated]) ITALIC – The Information System on Italian Lichens. Version 7.0. University of Trieste, Department of Biology. https://italic.units.it/ [accessed on 01/09/2022]

Nimis PL, Hafellner J, Roux C, Clerc P, Mayrhofer H, Martellos S, Bilovitz PO (2018a) The lichens of the Alps – an annotated checklist. MycoKeys 31: 1–634. https://doi.org/10.3897/mycokeys.31.23568

Nimis PL, Martellos S, Spitale D, Nascimbene J (2018b) Exploring patterns of commonness and rarity in lichens: A case study from Italy (Southern Europe). Lichenologist 50(3): 385–396. https://doi.org/10.1017/S0024282917000731

Ravera S, Isocrono D, Benesperi R, Giordani P, Nascimbene J, Tretiach M, Montagnani C (2016) Assessment of the conservation status of the mat-forming lichens *Cladonia* subgenus *Cladina* in Italy. Plant Biosystems 150(5): 1010–1022. https://doi.org/10.1080/11263504.2014.1000422

Rubio-Salcedo M, Martinez I, Carreno F, Escudero A (2013) Poor effectiveness of the Natura 2000 network protecting Mediterranean lichen species. Journal for Nature Conservation 21(1): 1–9. https://doi.org/10.1016/j.jnc.2012.06.001

Spribille T, Fryday AM, Pérez-Ortega S, Svensson M, Tønsberg T, Ekman S, Holien H, Resl P, Schneider K, Stabentheiner E, Thüs H, Vondrák J, Sharman L (2020) Lichens and associated fungi from Glacier Bay National Park, Alaska. Lichenologist 52(2): 61–181. https://doi.org/10.1017/S0024282920000079

Thor G, Nascimbene J (2007) A floristic survey in the Southern Alps: Additions to the lichen flora of Italy. Cryptogamie, Mycologie 28: 247–260.

Türk R (2016) Nationalpark Hohe Tauern – Flechten. Athesia-Tyrolia Druck, Innsbruck, 312 pp.

Vondrák J, Malíček J, Palice Z, Coppins B, Kukwa M, Czarnota P, Sanderson N, Acton A (2016) Methods for obtaining more complete species lists in surveys of lichen biodiversity. Nordic Journal of Botany 34(5): 619–626. https://doi.org/10.1111/njb.01053

Vondrák J, Malíček J, Palice Z, Bouda F, Berger F, Sanderson N, Acton A, Pouska V, Kish R (2018) Exploiting hot-spots; effective determination of lichen diversity in a Carpathian virgin forest. PLoS ONE 13(9): e0203540. https://doi.org/10.1371/journal.pone.0203540

Vondrák J, Svoboda S, Malíček J, Palice Z, Kocourková J, Knudsen K, Mayrhofer H, Thüs H, Schultz M, Košnar J, Hofmeister J (2022) From Cinderella to Princess: An exceptional hotspot of lichen diversity in a long-inhabited central-European landscape. Preslia 94(1): 143–181. https://doi.org/10.23855/preslia.2022.143

Supplementary material 1

A hotspot of lichen diversity and lichenological research in the Alps: the Paneveggio-Pale di San Martino Natural Park (Italy)

Authors: Juri Nascimbene, Gabriele Gheza, Peter O. Bilovitz, Luana Francesconi, Josef Hafellner, Helmut Mayrhofer, Maurizio Salvadori, Chiara Vallese, Pier Luigi Nimis

Data type: occurrences

Link: https://doi.org/10.3897/mycokeys.94.95858.suppl1

MycoKeys 94: 51–71 (2022)
doi: 10.3897/mycokeys.94.95751
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Three new species of *Nigrograna* (Dothideomycetes, Pleosporales) associated with Arabica coffee from Yunnan Province, China

Li Lu[1,2,3], Samantha C. Karunarathna[1], Dong-qin Dai[1], Ruvishika S. Jayawardena[2,3], Nakarin Suwannarach[4], Saowaluck Tibpromma[1]

1 *Center for Yunnan Plateau Biological Resources Protection and Utilization, College of Biological Resource and Food Engineering, Qujing Normal University, Qujing, Yunnan 655011, China* **2** *Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand* **3** *School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand* **4** *Research Center of Microbial Diversity and Sustainable Utilization, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*

Corresponding authors: Nakarin Suwannarach (suwan.462@gmail.com), Saowaluck Tibpromma (saowaluckfai@gmail.com)

Academic editor: C. Bhunjun | Received 28 September 2022 | Accepted 17 November 2022 | Published 12 December 2022

Citation: Lu L, Karunarathna SC, Dai D-q, Jayawardena RS, Suwannarach N, Tibpromma S (2022) Three new species of *Nigrograna* (Dothideomycetes, Pleosporales) associated with Arabica coffee from Yunnan Province, China. MycoKeys 94: 51–71. https://doi.org/10.3897/mycokeys.94.95751

Abstract

Coffee is one of the most important cash crops in Yunnan Province, China. Yunnan is ranked as the biggest producer of high-quality coffee in China. During surveys of microfungi from coffee plantations in Yunnan, six fungal strains that resemble Nigrograna ceae were collected. Multi-gene analyses of a combined SSU-LSU-ITS-*rpb2*-*tef1*-α sequence data matrix were used to infer the phylogenetic position of the new species in *Nigrograna* while morphological characteristics were used to deduce the taxonomic position of the new species. Six fungal strains isolated from decaying branches of *Coffea arabica* represent three new saprobic species in *Nigrograna*. The three new species, *N. asexualis*, *N. coffeae*, and *N. puerensis*, are described with full (macro and micro characteristics) descriptions, illustrations, and a phylogenetic tree that shows the phylogenetic position of new taxa.

Keywords

3 new taxa, *Coffea arabica*, Nigrogranaceae, phylogeny, saprobic fungi, taxonomy

Introduction

Coffee (*Coffea* L.) was first planted in Yunnan Province, China in 1982 (Zhang et al. 2014). To date, about 170 varieties of coffee (Global Biodiversity Information Facility database (GBIF), available at: https://www.gbif.org/species/2895315 (accessed on 07 November 2022)) are available in the world, of which *Coffea arabica* L. is the most popular coffee accounting for 75% of the world's production, while 25% is provided by *C. canephora* Pierre ex A. Froehner, and less than 1% by *C. liberica* W. Bull and other varieties (Sharma 2020). The coffee production in Yunnan Province is approximately 90% of China's total coffee production (Neilson and Wang 2019), while Pu'er is the largest coffee planting area in Yunnan, in terms of the highest yield and the best quality (Li 2014).

Fungal diversity is highly uncertain; the current estimated numbers are between 1.5 to 12 million, of which about 150,000 species have been named and classified (Hawksworth and Lücking 2017; Hyde et al. 2020; Bhunjun et al. 2022). Fungi are important organisms in terrestrial and aquatic ecosystems that are involved in the decomposition and nutrient cycling of dead plant material (Hyde et al. 2020; Bhunjun et al. 2022; Phukhamsakda et al. 2022). Also, saprobic fungi play vital roles in soil food chains, decomposition of plant, and animal materials, and solubilization of phosphorous (Dighton 2003; Pandey et al. 2008). However, coffee saprobic fungi have been poorly investigated (Arias and Abarca 2014; Lu et al. 2022a). Coffee saprobic fungi are distributed in 15 orders, and among them, Pleosporales Luttr. is the most common order (Lu et al. 2022a).

Pleosporales, belonging to Dothideomycetes O.E. Erikss. & Winka, was first proposed by Luttrell (1955), and later it was formally established by Barr (1987). In 2021, it consists of 91 families and 614 genera as the largest order (Hongsanan et al. 2020a; Wijayawardene et al. 2022). They are distributed in terrestrial and aquatic habitats (Zhang et al. 2008; Jiang et al. 2021). The members of Pleosporales are characterized by perithecioid and ostiolar ascomata, with or without periphyses, presence of cellular pseudoparaphyses, bitunicate, with ocular chambers or apical ring asci, various shapes of ascospores, with pigmentation and septation, and sheath present or absent (Zhang et al. 2012; Tennakoon et al. 2021; Yang et al. 2022).

Nigrogranaceae Jaklitsch & Voglmayr (Pleosporales) was proposed as a new family by Jaklitsch and Voglmayr (2016) to accommodate *Nigrograna* Gruyter, Verkley & Crous as the type genus. Liu et al. (2017) estimated that the divergence time of Nigrogranaceae is around 79 (44–124) Mya in crown age and 131 (86–180) Mya in stem age. Nigrogranaceae is monotypic, and they exist as endophytic, human pathogenic, and saprobic lifestyles (Hongsanan et al. 2020b; Zhang et al. 2020; Boonmee et al. 2021). The sexual morph of Nigrogranaceae is characterized by globose and black, ostiolar, clavate, and fissitunicate ascomata, with a short stipe and asci with a knob-like base, fusoid to narrowly ellipsoid, septate, and smooth or faintly verruculose ascospores (Jaklitsch and Voglmayr 2016). The asexual morph is characterized by pycnidia similar to ascomata, filiform and branched conidiophores, ampulliform or lageniform phialides, rod-like to ellipsoid, and hyaline or sub-hyaline conidia (Jaklitsch and Voglmayr 2016).

Nigrograna was introduced by de Gruyter (2012) with *N. mackinnonii* (Borelli) Gruyter, Verkley & Crous (basionym: *Pyrenochaeta mackinnonii* Borelli) as the type species. *Pyrenochaeta mackinnonii* was reported from a mycetoma patient by Borelli (1976), but it was found to be remote from the generic type species *P. nobilis* De Not. (de Gruyter et al. 2010, 2013). Since it was not possible to determine which family in Pleosporales *P. mackinnonii* belongs to, only the new genus *Nigrograna* was introduced to accommodate *P. mackinnonii* and named as *N. mackinnonii* (de Gruyter 2012). Later, *Nigrograna* was used as a synonym of *Biatriospora* K.D. Hyde & Borse, as *N. mackinnonii* is phylogenetically closely related to the type species of *Biatriospora* (*B. marina* K.D. Hyde & Borse) (Ahmed et al. 2014), while Hongsanan et al. (2020a) treated *Biatriospora* and *Nigrograna* as two separate genera. In 2022, *Nigrograna* represents 20 epithets listed in Index Fungorum (2022), and the members have been reported as saprobic, human pathogenic, and endophytic worldwide (Kolařík 2018; Zhao et al. 2018), showing a wide range of hosts (marine and terrestrial habitats) (Hyde et al. 2017; Tibpromma et al. 2017; Dayarathne et al. 2020). The sexual morph of *Nigrograna* is characterized by globose to subglobose and black ascomata, with ostiolar, two-layered peridium, clavate and fissitunicate asci, fusoid to narrowly ellipsoid, straight or curved, septate, and smooth or verruculose ascospores (Jaklitsch and Voglmayr 2016; Zhang et al. 2020). Asexual morph is characterized by globose to subglobose or pyriform pycnidia, filiform and branched conidiophores, hyaline, phialidic, discrete conidiogenous cells, sub-hyaline, aseptate and ellipsoidal conidia (de Gruyter 2012; Jaklitsch and Voglmayr 2016).

In this study, three saprobic *Nigrograna* were collected from *Coffea arabica* branches in Yunnan Province, China. One species was isolated as an asexual morph (*N. asexualis*), while the other two isolated as sexual morphs (*N. coffeae*, *N. puerensis*) are illustrated and described as new species based on morphology and multi-gene phylogenetic analyses and are compared with closely related taxa.

Materials and methods

Collection, morphology and isolation

Coffee branch samples were collected from coffee plantations in Pu'er and Xishuangbanna, Yunnan Province, China. Specimens were put in plastic bags and taken to the mycology laboratory at Qujing Normal University. The vertical sections of fruiting structures were made for microscope studies and photomicrography. Micro-morphological characteristics were observed using a Leica DM2500 compound microscope and photographed with a Leica DMC4500 camera fitted onto the microscope. Color codes in the manuscript followed colorhexa (https://www.colorhexa.com). The measurements were processed in Tarosoft (R) Image Frame Work v. 0.9.7, and photographic plates were made in Adobe Photoshop CC 2018. Single spore isolation was carried out following Senanayake et al. (2020). Herbarium specimens were deposited at Zhongkai University of Agriculture and Engineering (**ZHKU**), while the living cultures growing on potato dextrose agar (**PDA**) were deposited at the culture collection of Zhongkai

University of Agriculture and Engineering (**ZHKUCC**). Faces of fungi (**FoF**) numbers and Index Fungorum (**IF**) numbers were obtained as explained in Jayasiri et al. (2015) and Index Fungorum (2022).

DNA extraction and PCR amplification

Genomic DNA was extracted from the fresh fungal mycelia which were grown on PDA for about two weeks, using Biospin Fungus Genomic DNA Extraction Kit–BSC14S1 (BioFlux, China) following the manufacturer's instructions. Lu et al. (2021) was followed for the Polymerase Chain Reaction (PCR). Five partial gene regions were used in this study viz. the internal transcribed spacer (ITS) region was amplified with the primers ITS4 and ITS5 (White et al. 1990), the 18 s small subunit (SSU) region was amplified by primers NS1 and NS4 (White et al. 1990), the nuclear ribosomal 28 s large subunit (LSU) region was amplified by the primers LROR and LR5 (Vilgalys and Hester 1990), the partial RNA polymerase II subunit (*rpb2*) region was amplified with the primers RPB2-5F and RPB2-7cR (Liu et al. 1999), and the partial translation elongation factor 1-alpha (*tef*1-α) gene was amplified with the primers EF1-983F and 2218R (Rehner and Buckley 2005). Lu et al. (2022b) was followed for the amplification reactions of different primers. Amplified PCR products were sent to Sango Biotechnology Co., Ltd. (Shanghai, China) for sequencing. All sequences generated in this study were deposited in GenBank (Table 1).

Phylogenetic analyses

Phylogenetic analyses of the aligned sequences referred to Dissanayake et al. (2020). Newly generated reverse and forward sequences were assembled with Geneious program (9.1.2) and the preliminary identification was done by the BLASTn search in NCBI (https://www.ncbi.nlm.nih.gov). Additional highly similar sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) based on the BLASTn results and recent publications. Single-gene sequence alignments were made in MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/), edited in trimAl v1.2 (http://trimal.cgenomics.org), and multi-gene alignments were made by Sequence Matrix program (1.7.8) (Vaidya et al. 2011). The sequence datasets used to build the phylogenetic trees are shown in Table 1.

Phylogenetic analyses were conducted with maximum likelihood (ML) and Bayesian inference (BI) algorithms on the CIPRES Science Gateway portal (https://www.phylo.org/) (Miller et al. 2012). The ML tree was run with RAxML-HPC v.8 on XSEDE (Stamatakis 2014), and GTRGAMMA substitution model with 1000 bootstrap iterations. The BI tree was run with MrBayes on XSEDE (3.2.7a) (Ronquist et al. 2012). MrModeltest 2.2 (Nylander 2004) and PAUP v. 4.0b10 (Ronquist and Huelsenbeck 2003) were used to evaluate the best models of evolution, the evolutionary model of SYM+I+G substitution model was selected for LSU, HKY+I+G substitution model was selected for SSU, and GTR+I+G substitution model was selected for ITS, *rpb2* and *tef*1-α. Six simultaneous Markov Chains were run for two million generations

Table 1. Taxa names, strain numbers, and corresponding GenBank accession numbers of the taxa used in the phylogenetic analyses. Newly generated sequences in this study are indicated in bold. The type species are noted with [T] after the species name, while NA indicates the unavailability of data.

Taxon	Strain numbers	ITS	LSU	*rpb2*	SSU	*tef1-α*
Cyclothyriella rubronotata (Berk. & Broome) Jaklitsch & Voglmayr [T]	CBS 141486	KX650544	KX650519	NA	KX650507	KX650574
Cyclothyriella rubronotata	CBS 419.85	NA	GU349002	GU301875	NA	GU371728
Nigrograna antibiotica (M. Kolařík & A. Kubátová) M. Kolařík [T]	CCF 4378	JX570932	KF925327	NA	KF925328	JX570934
Nigrograna antibiotica	CCF 4998	LT221894	NA	LT221895	NA	NA
Nigrograna aquatica W. Dong, H. Zhang & K.D. Hyde [T]	MFLUCC 14-1178	MF399065	MF415392	NA	MF415394	MF498582
Nigrograna aquatica	MFLUCC 17-2318	MT627705	MN913705	NA	NA	NA
Nigrograna asexualis T	**ZHKUCC 22-0214**	**OP450965**	**OP450971**	**OP432241**	**OP450979**	**OP432245**
Nigrograna asexualis	**ZHKUCC 22-0215**	**OP450966**	**OP450972**	**OP432242**	**OP450980**	**OP432246**
Nigrograna cangshanensis Z.L. Luo, H.Y. Su & K.D. Hyde [T]	MFLUCC 15-0253	KY511063	KY511064	NA	KY511065	NA
Nigrograna carollii M. Kolařík [T]	CCF 4484	LN626657	LN626682	LN626662	LN626674	LN626668
Nigrograna chromolaenae Mapook & K.D. Hyde [T]	MFLUCC 17-1437	MT214379	MT214473	NA	NA	MT235801
Nigrograna coffeae [T]	**ZHKUCC 22-0210**	**OP450967**	**OP450973**	**OP432243**	**OP450981**	**OP432247**
Nigrograna coffeae	**ZHKUCC 22-0211**	**OP450968**	**OP450974**	**OP432244**	**OP450982**	**OP432248**
Nigrograna fuscidula (Sacc.) Jaklitsch & Voglmayr [T]	CBS 141556	KX650550	NA	NA	NA	KX650525
Nigrograna fuscidula	CBS 141476	KX650547	NA	KX650576	KX650509	KX650522
Nigrograna fuscidula	MF1a	KX650548	NA	NA	NA	KX650523
Nigrograna fuscidula	MF3	KX650549	NA	NA	NA	KX650524
Nigrograna hydei J.F. Zhang, J.K. Liu & Z.Y. Liu [T]	GZCC 19-0050	MN387225	MN387227	NA	NA	MN389249
Nigrograna impatientis J.F. Zhang, J.K. Liu & Z.Y. Liu [T]	GZCC 19-0042	MN387226	MN387228	NA	NA	MN389250
Nigrograna jinghongensis Wanas. & K.D. Hyde [T]	KUMUCC 21-0035	MZ493303	MZ493317	MZ508421	MZ493289	MZ508412
Nigrograna jinghongensis	KUMUCC 21-0036	MZ493304	MZ493318	MZ508422	MZ493290	MZ508413
Nigrograna kunmingensis T.Y. Du & Tibpromma [T]	ZHKUCC 22-0242	OP456214	OP456379	NA	OP456382	OP471608
Nigrograna kunmingensis	ZHKUCC 22-0243	OP484334	OP456380	NA	OP456383	OP471609
Nigrograna locuta-pollinis F. Liu & L. Cai [T]	CGMCC 3.18784	MF939601	MF939583	MF939610	NA	MF939613
Nigrograna locuta-pollinis	LC11690	MF939603	MF939584	MF939611	NA	MF939614
Nigrograna mackinnonii [T]	CBS 674.75	KF015654	KF015612	KF015703	GQ387552	KF407986
Nigrograna mackinnonii	E5202H	JX264157	KJ605422	JX264156	JX264155	JX264154
Nigrograna mackinnonii	E9303e	JN545759	LN626681	LN626666	LN626678	LN626673
Nigrograna magnoliae Wanas. [T]	MFLUCC 20-0020	MT159628	MT159622	MT159611	MT159634	MT159605
Nigrograna magnoliae	GZCC 17-0057	MF399066	MF415393	NA	MF415395	MF498583
Nigrograna magnoliae	MFLUCC 20-0021	MT159629	MT159623	MT159612	MT159635	MT159606
Nigrograna mycophila Jaklitsch, Friebes & Voglmayr [T]	CBS 141478	KX650553	NA	NA	NA	KX650526
Nigrograna mycophila	CBS 141483	KX650555	NA	KX650577	KX650510	KX650528
Nigrograna mycophila	MF6	KX650554	NA	NA	NA	KX650527
Nigrograna norvegica Jaklitsch & Voglmayr [T]	CBS 141485	KX650556	NA	KX650578	KX650511	NA
Nigrograna obliqua Jaklitsch & Voglmayr [T]	CBS 141477	KX650560	NA	KX650580	NA	KX650531
Nigrograna obliqua	CBS 141475	KX650558	NA	KX650579	KX650512	KX650530
Nigrograna obliqua	MRP	KX650561	NA	KX650581	NA	KX650532

Taxon	Strain numbers	ITS	LSU	*rpb2*	SSU	*tef1-α*
Nigrograna peruviensis (M. Kolařík & R. Gazis) M. Kolařík [T]	CCF 4485	LN626658	LN626683	LN626665	LN626677	LN626671
Nigrograna puerensis [T]	**ZHKUCC 22-0212**	**OP450969**	**OP450975**	**NA**	**OP450983**	**OP432249**
Nigrograna puerensis	**ZHKUCC 22-0213**	**OP450970**	**OP450976**	**NA**	**OP450984**	**OP432250**
Nigrograna rhizophorae Dayar., E.B.G. Jones & K.D. Hyde [T]	MFLUCC 18-0397	MN047085	NA	MN431489	NA	MN077064
Nigrograna rhizophorae	MFLU 19-1234	NA	MN017845	MN431490	NA	MN077063
Nigrograna samueliana Devadatha, V.V. Sarma & E.B.G. Jones [T]	NFCCI-4383	MK358817	MK358812	MK330939	MK358810	MK330937
Nigrograna thymi Mapook, Camporesi & K.D. Hyde [T]	MFLUCC 14-1096	KY775576	KY775573	NA	KY775574	KY775578
Nigrograna yasuniana M. Kolařík [T]	YU.101026	HQ108005	LN626684	LN626664	LN626676	LN626670
Occultibambusa bambusae D.Q. Dai & K.D. Hyde [T]	MFLUCC 13-0855	KU940123	KU863112	KU940170	NA	KU940193
Occultibambusa fusispora Phookamsak, D.Q. Dai & K.D. Hyde	MFLUCC 11-0127	MZ329036	MZ325466	MZ329032	MZ329028	MZ325469
Occultibambusa pustula D.Q. Dai & K.D. Hyde [T]	MFLUCC 11-0502	KU940126	KU863115	NA	NA	NA
Paradictyoarthrinium diffractum Matsush.	MFLUCC13-0466	KP744455	NA	KP744498	NA	NA
Paradictyoarthrinium tectonicola Doilom & K.D. Hyde [T]	MFLUCC 13-0465	KP744456	NA	KP744500	KP753961	KX437763
Seriascoma didymosporum Phookamsak, D.Q. Dai, Karun. & K.D. Hyde [T]	MFLUCC 11-0179	KU940127	KU940196	KU863116	NA	KU940173
Seriascoma honghense H.B. Jiang, Phookamsak & K.D. Hyde [T]	KUMCC 21-0021	MZ329039	MZ325468	MZ329035	NA	MZ325470
Versicolorisporium triseptatum Sat. Hatak., Kaz. Tanaka & Y. Harada [T]	HHUF 28815	NR_119392	NA	NG_042318	NG_060995	NA

and trees were sampled at every 200th generation (resulting in 10,000 trees), and these chains stopped when all convergences met and the standard deviation fell below 0.01. All resulting trees were plotted using FigTree v. 1.4.0 (Rambaut 2014) and the layout of the trees was made by Microsoft Office PowerPoint 2020.

Results

Phylogenetic analyses

Three new species formed a distinct clade in *Nigrograna* with strong statistical support (*N. coffeae* and *N. puerensis* ML = 100%, BIPP = 1.00, and *N. asexualis* ML = 68%, BIPP = 0.97). Multi-locus data (SSU, LSU, ITS, *rpb2* and *tef1-α*) composed of 54 strains (Table 1), and *Cyclothyriella rubronotata* strains CBS 141486 and CBS 419.85 were used as the outgroup taxa. A total of 4485 characters were fed to the phylogenetic analysis after alignment, 1–1047 (SSU), 1048–1956 (LSU), 1957–2477 (ITS), 2478–3510 (*rpb2*) and 3511–4485 (*tef1-α*). The topology of the phylogenetic tree generated by the ML method was highly similar to that by BI, and therefore it was chosen to represent the evolutionary history of *Nigrograna*.

The ML analysis of the combined dataset yielded a best-scoring tree with a final ML optimization likelihood value of -23091.568105. The alignment has 1495

distinct alignment patterns, with 33.58% completely undetermined characters and gaps. Parameters for the GTR + I + G model of the combined SSU, LSU, ITS, *rpb*2 and *tef*1-α were as follows: estimated base frequencies A = 0.247145, C = 0.250645, G = 0.263985, T = 0.238225; substitution rates AC = 1.810004, AG = 4.475190, AT = 1.758134, CG = 1.340389, CT = 10.583215, GT = 1.000; gamma distribution shape parameter α = 0.167006. The phylogenetic tree resulting from RAxML analysis is shown in Fig. 1.

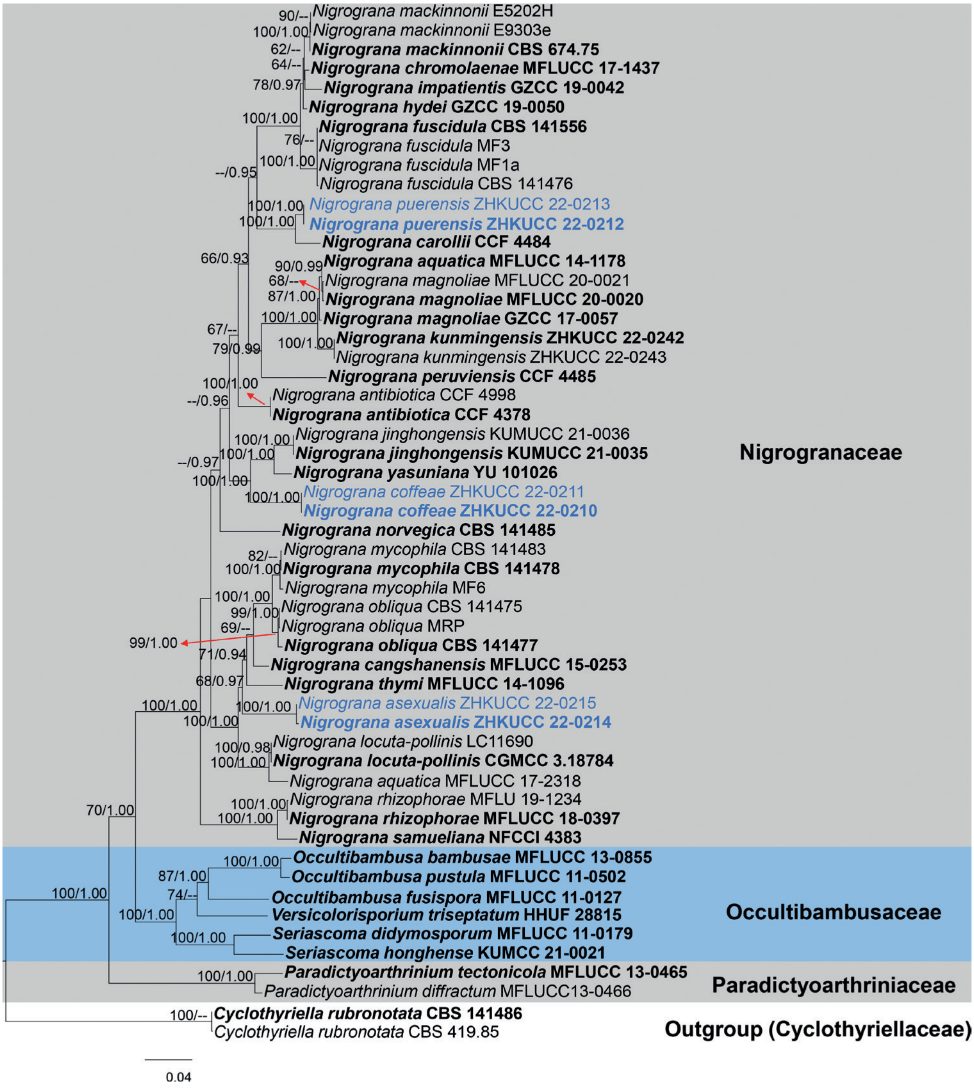

Figure 1. The maximum-likelihood phylogram of *Nigrograna* based on a combined SSU, LSU, ITS, *rpb*2 and *tef*1-α sequence dataset with *Cyclothyriella rubronotata* CBS 141486 and CBS 419.85 as the outgroup taxa (Dayarathne et al. 2020). The maximum-likelihood bootstrap values (ML ≥ 60%, left) and Bayesian Inference Posterior Probability values (BIPP ≥ 0.90, right) are shown above the nodes. Strains derived from the current study are in blue, while type strains are in bold.

Taxonomy

Nigrograna coffeae **L. Lu & Tibpromma, sp. nov.**
Index Fungorum number: IF559425
Facesoffungi Number: FoF12765
Fig. 2

Etymology. Species epithet refers to the host genus "*Coffea*" where the fungus was isolated.

Holotype. ZHKU 22-0121.

Description. ***Saprobic*** on decaying branch of *Coffea arabica*. **Sexual morph:** ***Ascomata*** 90–140 μm high, 140–200 μm wide ($\bar{x}$ = 115 × 168 μm, n = 10), immersed, solitary, black spots on substrate, subglobose to oval, sometimes obpyriform, some with ostiolate. ***Peridium*** 10–15 μm wide, composed of 3–5 layers, hyaline to brown (#937463) cells of ***textura angularis***. ***Hamathecium*** 1.5–3 μm wide, composed of numerous, hyaline, filamentous, septate, branched, pseudoparaphyses. ***Asci*** 50–70 × 7–11 μm ($\bar{x}$ = 58 × 9 μm, n = 20), 8-spored, bitunicate, fissitunicate, clavate to cylindric-clavate, short stalked, some with club-shape pedicel, apically rounded, with a small ocular chamber. ***Ascospores*** 12–16 × 4–5 μm, ($\bar{x}$ = 14.4 × 4.6 μm, n = 30), overlapping uni- to bi-seriately arranged, fusiform, straight or slightly curved, hyaline when immature and become pale brown (#e1af33) to dark-brown (#6e5031) when mature, mostly 1-septate, few 2 or 3-septate, constricted at each septum, with obviously guttulate. **Asexual morph:** Undetermined.

Culture characteristics. Ascospores germinated on PDA within 24 h and germ tubes arising from both ends. Colonies on PDA, reaching 4.5 cm diam. after two months of incubation at room temperature (22–26 °C), initially white (#f2f3f4) becoming grey (#bbbeb2) to dark brown (#6e5031) at maturity, dense, circular, slightly raised, smooth surface, radially fimbriate at the edge, reverse dark green (#3a4543) to brown (#937463).

Material examined. Pu'wen Town, Xishuangbanna, Yunnan Province, China, on a decaying branch of *Coffea arabica*, (22°31'18"N, 101°2'44"E, 856.89 m), 15 September 2021, LiLu, JHPW16 (ZHKU 22-0121, holotype), ZHKUCC 22-0210 = ZHKUCC 22-0211. GenBank number; ITS: OP450967, LSU: OP450973, *rpb2*: OP432243, SSU: OP450981, *tef*1-α: OP432247 (ZHKUCC 22-0210, extype); ITS: OP450968, LSU: OP450974, *rpb2*: OP432244, SSU: OP450982, *tef*1-α: OP432248 (ZHKUCC 22-0211).

Notes. Our phylogenetic analyses showed that *Nigrograna coffeae* forms an independent clade (100% ML, 1.00 BIPP, Fig. 1), and is phylogenetically related to *N. yasuniana* and *N. jinghongensis*. *Nigrograna yasuniana* was reported as endophytes from *Conceveiba guianensis* Aubl. in Ecuador, but there were not enough morphological data, the comparison of base pairs in ITS showed 3.4% differences (15/433 bp), LSU showed 1.5% differences (12/812bp), SSU only showed 0.3% differences (3/1028 bp), *rpb2* showed

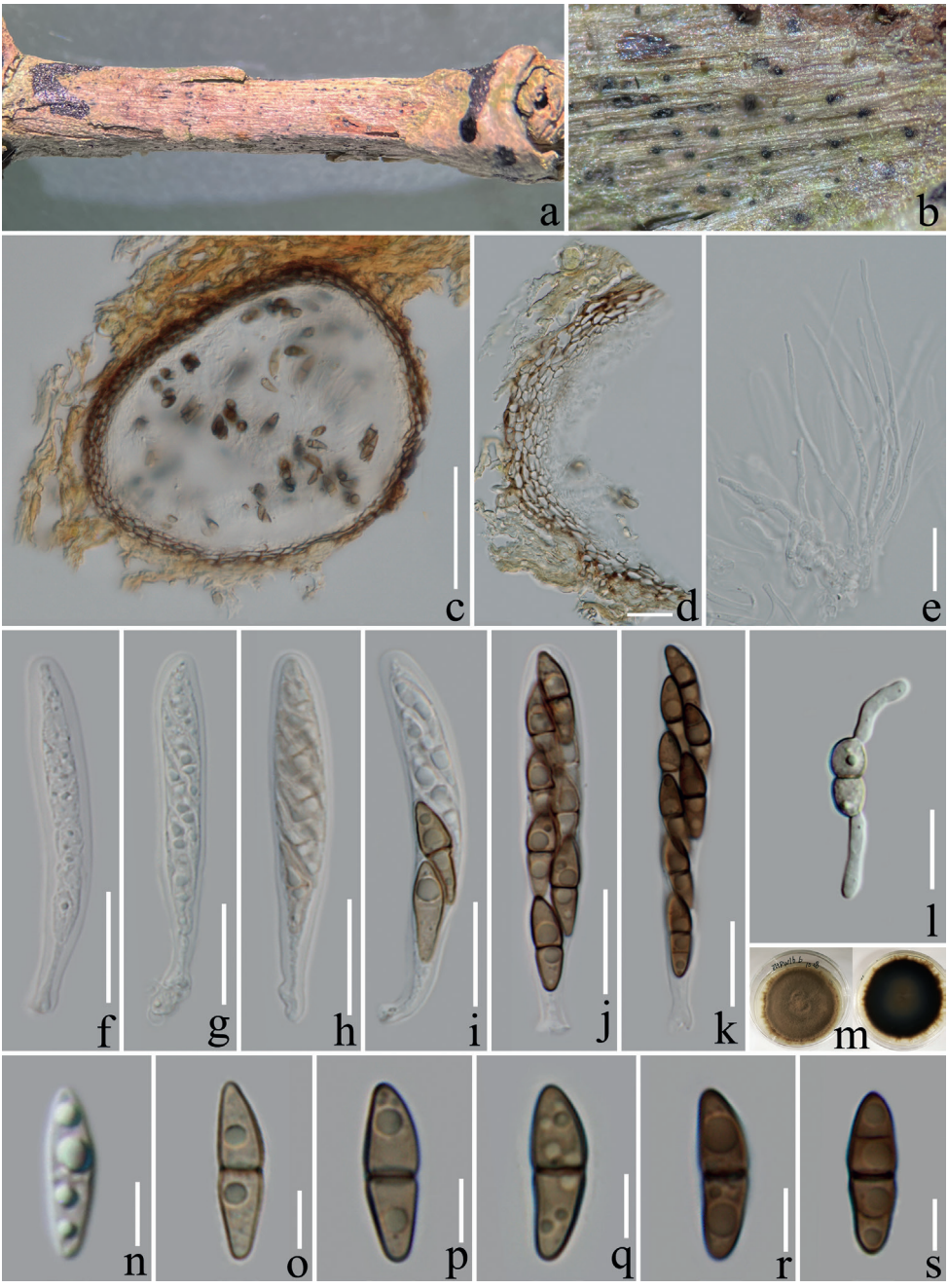

Figure 2. *Nigrograna coffeae* (ZHKU 22-0121, holotype) **a, b** ascomata on the host substrate **c** a vertical section through an ascoma **d** peridium **e** hamathecium **f–k** asci **l** germinated ascospore **m** culture on pda from above and reverse **n–s** ascospores (arrows indicate the septa). Scale bars: 50 μm (**c**); 10 μm (**d–l**); 5 μm (**n–s**).

14% differences (117/829 bp), and *tef*1-α showed 3.2% differences (31/954 bp) (Kolařík et al. 2017). *Nigrograna jinghongensis* was introduced as a saprobic fungus from woody litter in China, and our new isolate shares a similar size (12–16 × 4–5 µm *vs* 12–15 × 4–5.5 µm) and color (hyaline to dark brown *vs* yellowish-brown to brown) of ascospores with *N. jinghongensis* (Boonmee et al. 2021), but there are some significant differences in the size of the ascomata (90–140 µm high, 140–200 µm wide *vs* 300–400 µm high 220–300 µm wide) and the shape of ascospores (fusiform, straight or slightly curved *vs* ellipsoid) (Boonmee et al. 2021). Based on the sequence blast results, ITS, LSU and *rpb*2 gene sequences were similar to *Nigrograna* sp., with 97.5% (MZ270683), 98.4% (MK762716), and 86% (MZ508421) respectively, SSU was similar to *N. mycophila* with 99% (KX650510), and *tef*1-α was similar to *N. yasuniana* with 96.6% (LN626670). Therefore, we introduce our new isolate as a new species *N. coffeae* based on both morphological characteristics and phylogenetic analyses.

Nigrograna puerensis **L. Lu & Tibpromma, sp. nov.**
Index Fungorum number: IF559426
Facesoffungi Number: FoF12766
Fig. 3

Etymology. The specific epithet "*puerensis*" refers to the location Pu'er City, where the type species was collected.

Holotype. ZHKU 22-0122.

Description. ***Saprobic*** on decaying branch of *Coffea arabica*. **Sexual morph:** ***Ascomata*** 90–180 µm high, 90–150 µm wide ($\bar{x}$ = 138 × 115 µm, n = 10), immersed, with only ostiolar necks visible on the host surface or erumpent, solitary, subglobose to ellipsoid, dark brown (#6e5031). ***Peridium*** 10–15 µm wide ($\bar{x}$ = 13 µm, n = 15), outer layer consists of 2–3 layers of ***textura prismatica***, brown (#937463) and thick-walled cells, inner layer hyaline with thin-walled cells. ***Hamathecium*** composed of numerous, 1.5–2 µm wide ($\bar{x}$ = 1.8 µm, n = 20), filamentous, hyaline, septate, pseudoparaphyse. ***Asci*** 50–80 × 8–11 µm ($\bar{x}$ = 66 × 9.5 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical to clavate, short pedicellate, apically rounded, with poorly developed ocular chamber. ***Ascospores*** 15–18 × 4–5 µm, ($\bar{x}$ = 16 × 4.5 µm, n = 30), uni- to bi-seriately arranged, fusoid, apical cell and basal cell acute, and apical cell slightly wider than basal cell, straight or slightly curved, 1-septate, constricted at septum, guttulate, hyaline to yellow-brownish (#daceb8) when young, brownish (#937463) when mature. **Asexual morph:** Undetermined.

Culture characteristics. On PDA, colonies reached up to 4 cm diam. after two months at room temperature (22–26 °C). Colony dense, circular, slightly raised at the center, surface with white aerial mycelium, fluffy, with a serrate edge, grayish (#c9bfb3) to dark brown (#6e5031) from center to edge, reverse dark green (#3a4543) to dark brown (#6e5031).

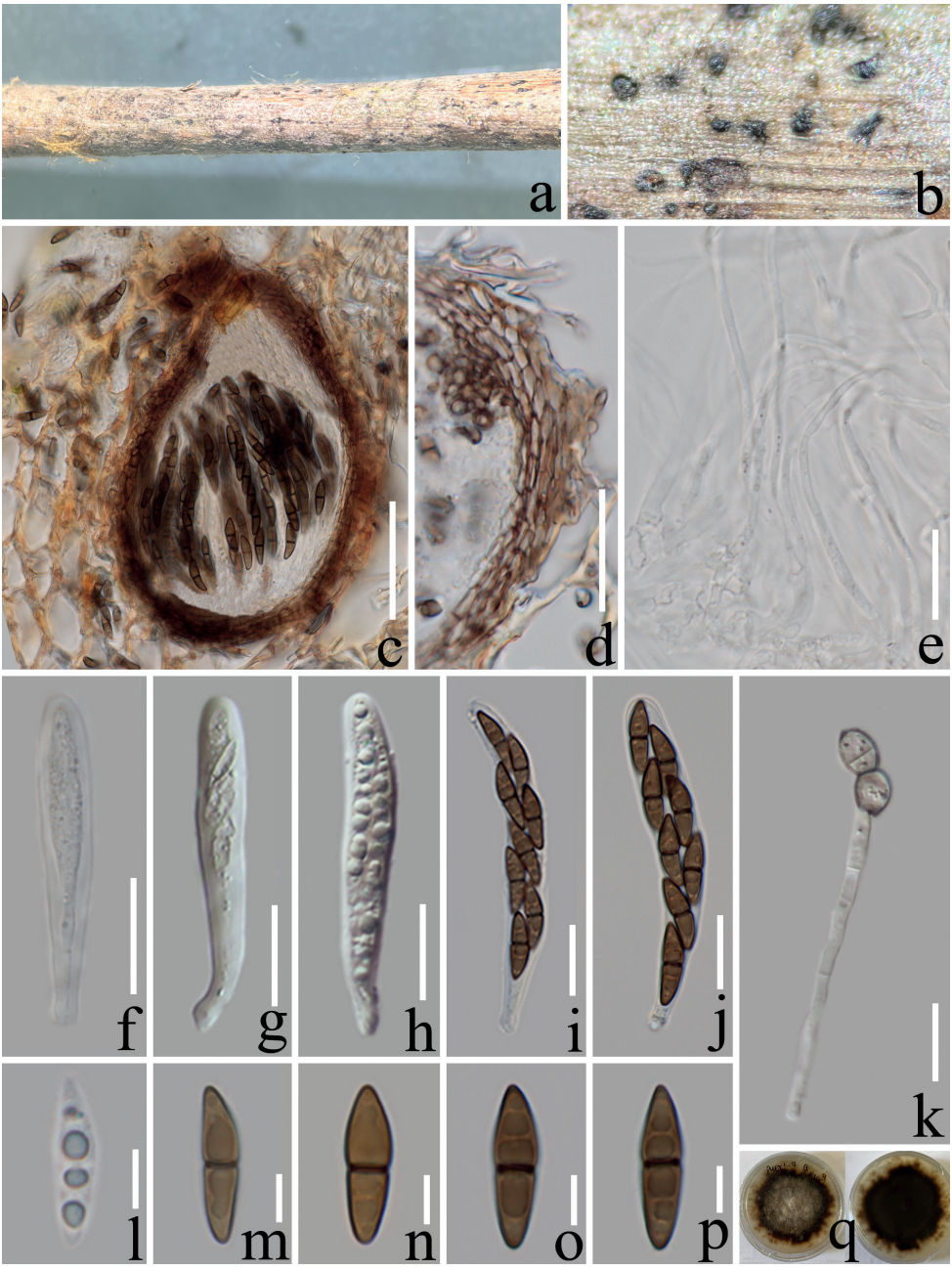

Figure 3. *Nigrograna puerensis* (ZHKU 22-0122, holotype) **a, b** ascomata observed on host substrate **c** a vertical section through an ascoma **d** peridium **e** hamathecium **f–j** asci **k** germinated ascospore **l–p** ascospores **q** culture on PDA from above and reverse. Scale bars: 50 μm (**c**); 30 μm (**d**); 15 μm (**e–k**); 5 μm (**l–p**).

Material examined. Pu'er City, Yunnan Province, China, on a decaying branch of *Coffea arabica*, (22°36'2"N, 101°0'59"E, 1016.43 m), 16 September 2021, LiLu, Puer 1-4 (ZHKU 22-0122, holotype), ZHKUCC 22-0212 = ZHKUCC 22-0213. GenBank number; ITS: OP450969, LSU: OP450975, SSU: OP450983, *tef*1-α: OP432249 (ZHKUCC 22-0212, ex-type); ITS: OP450970, LSU: OP450976, SSU: OP450984, *tef*1-α: OP432250 (ZHKUCC 22-0213).

Notes. *Nigrograna puerensis* clusters with *N. carollii* with significant statistical support from ML 100% and BIPP 1.00. In morphology, our new strains best fit *Nigrograna* by having immersed ascomata, clavate and short pedicellate asci, and pale to brown, fusoid to narrowly ellipsoid, and septate ascospores (Jaklitsch and Voglmayr 2016; Zhang et al. 2020). Blast search results of ITS, LSU and *tef*1-α sequence data revealed that our taxon (ZHKUCC 22-0212) is similar to *N. mackinnonii* (96% MZ270697, 99% KJ605422, and 95% LT797087 respectively), while the similarity of SSU sequence to *N. carollii* is as high as 99%. Based on nucleotide comparisons, our isolate (ZHKUCC 22-0212) differs from *N. carollii* (CCF 4484) by 9/490 bp (1.8%) in ITS, 2/222 bp (1%) in LSU, 2/1306 bp (0.2%) in SSU, and 10/530 bp (2%) in *tef*1-α. Unfortunately, for *N. carollii*, sufficient morphological data was not available to compare with our novel taxon which was isolated as an endophyte on living sapwood of wild *Hevea brasiliensis* Müll. Arg., and *N. mackinnonii* which was isolated as a human pathogen (de Gruyter 2012; Kolařík et al. 2017). In addition, the colony morphology of *N. carollii* on PDA is described as colonies plane, effuse, and light gray (Kolařík et al. 2017), while *N. puerensis* colony surface is seen as white aerial mycelium, fluffy, with a serrate edge, and grayish to dark brown from center to edge. Therefore, based on morphological and phylogenetic analyses, we introduce *N. puerensis* as a distinct new species.

Nigrograna asexualis **L. Lu & Tibpromma, sp. nov.**
Index Fungorum number: IF559427
Facesoffungi Number: FoF12767
Fig. 4

Etymology. The species epithet '*asexualis*' refers to the asexual morph.

Holotype. ZHKU 22-0123.

Description. ***Saprobic*** on decaying branch of *Coffea arabica.* **Sexual morph:** Undetermined. **Asexual morph:** Coelomycetous. ***Pycnidia*** 100–230 μm high, 120–180 μm wide ($\bar{x}$ = 156 × 144 μm, n = 10), globose to subglobose, or pyriform, immersed, solitary, unilocular, dark brown, papillate ostiole, appearing as black spots on host surface. ***Pycnidial wall*** 11–16 μm wide ($\bar{x}$ = 14 μm, n = 15), brown (#937463), the wall with pseudoparenchymatous cells. ***Conidiophores*** arising from the pycnidial wall, up to 46 μm long and 3–4.4 μm wide ($\bar{x}$ = 3.4 μm, n = 25), filiform, septate, hyaline, simple to sparsely branched, with pegs along one or two sides and solitary phialides terminally. ***Phialides*** 3–6 × 1–2 μm ($\bar{x}$ = 4.5 × 1.5 μm, n = 15), variable

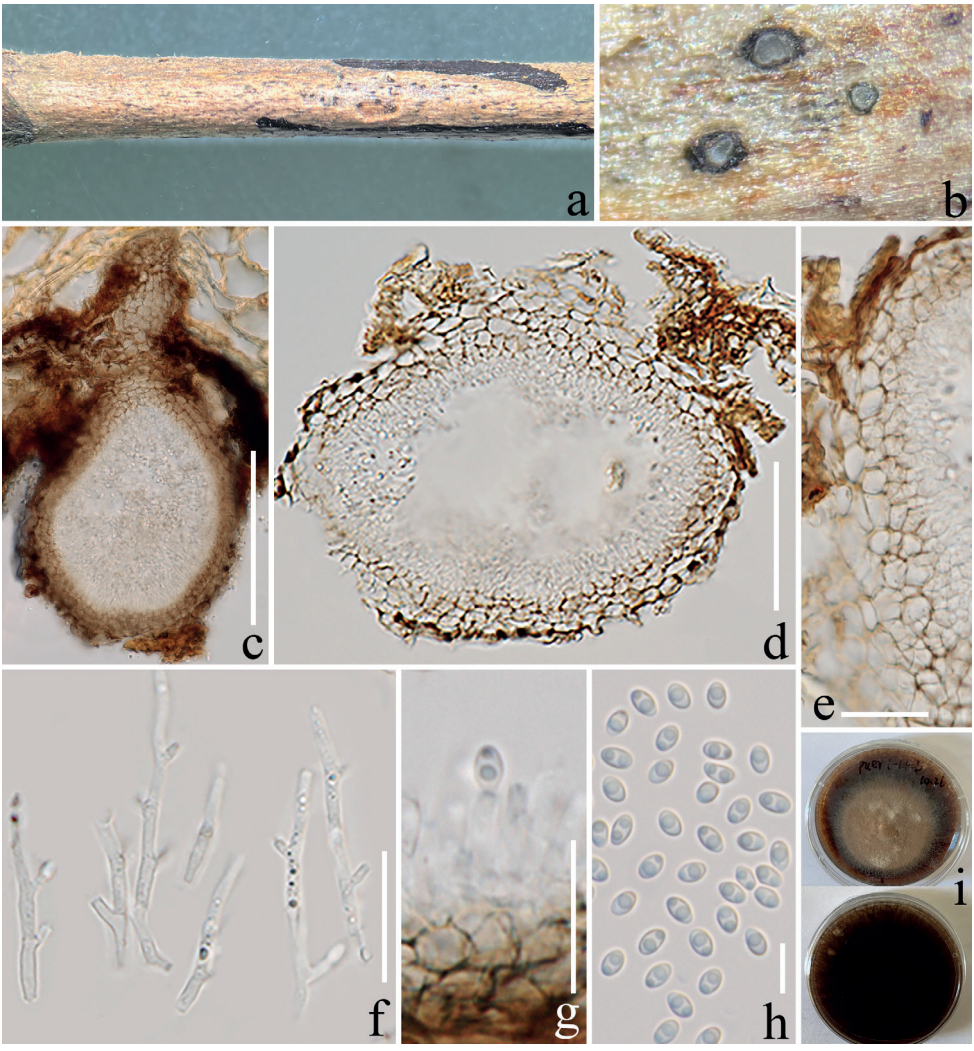

Figure 4. *Nigrograna asexualis* (ZHKU 22-0123, holotype) **a, b** conidiomata on the host substrate **c, d** vertical sections of a conidioma **e** peridium **f, g** conidiophores with phialides **h** conidia **i** culture on PDA from above and reverse. Scale bars: 100 μm (**c**); 50 μm (**d**); 15 μm (**e**); 30 μm (**f**); 20 μm (**g**); 10 μm (**h**).

in shape, phialidic, discrete, ampulliform-lageniform-subcylindrical. ***Conidia*** 5–6.5 × 3–4 μm ($\bar{x}$ = 5.5 × 3.7 μm, n = 30), ellipsoidal, unicellular, aseptate with 1–2 granules, subhyaline, smooth-walled.

Culture characteristics. Conidium germinated on PDA within 24 h. Colonies growing on PDA reaching 5 cm diam. after two months at room temperature (22–26 °C). Colony dense, circular, surface sparsely hairy, radially striate, with a fimbriate edge, yellowish (#eabf83) to pale brown (#e1af33) at the center and dark brown (#6e5031) at the margin, reverse dark brown (#6e5031).

Material examined. Pu'er City, Yunnan Province, China, on a decaying branch of *Coffea arabica*, (22°36'2"N, 101°0'59"E, 1016.43 m), 16 September 2021, LiLu, Puer 1-14 (ZHKU 22-0123, holotype), ZHKUCC 22-0214 = ZHKUCC 22-0215. GenBank number; ITS: OP450965, LSU: OP450971, *rpb2*: OP432241, SSU: OP450979, *tef*1-α: OP432245 (ZHKUCC 22-0214, ex-type); ITS: OP450966, LSU: OP450972, *rpb2*: OP432242, SSU: OP450980, *tef*1-α: OP432246 (ZHKUCC 22-0215).

Notes. In multi-gene phylogeny, *Nigrograna asexualis* formed a separate (68% ML, 0.97 BIPP) and distinct clade within *Nigrograna* (Fig. 1). Morphologically, *N. asexualis* conforms to the morphological characteristics of *Nigrograna* by having hyaline or subhyaline, long and branched conidiophores, solitary phialides, and aseptate, ellipsoidal or cylindrical conidia (Jaklitsch and Voglmayr 2016; Dayarathne et al. 2020; Wanasinghe et al. 2020). Blast results of the sequences show that ITS is similar to *N. fuscidula* with 89% (MH856004), and SSU is similar to *N. mycophila* with 99.8% (KX650510). *Nigrograna asexualis* is different from *N. fuscidula* and *N. mycophila* by its ellipsoidal conidia, but the similarities of these three species are hyaline, 1-celled, smooth-walled conidia forming on philipides (Jaklitsch and Voglmayr 2016). The LSU and *rpb2* sequences of our strain blast results are similar to *N. obliqua*, and the similarities are 98.9% (KX650560) and 87% (KX650579) respectively, but *N. obliqua* lacks the asexual morph (Jaklitsch and Voglmayr 2016). The *tef*1-α sequence of our strain is 95.8% (MF939615) similar to *N. locuta-pollinis*, which was isolated from hive-stored pollen of *Brassica campestris* L. that lacks morphology (Zhao et al. 2018). Therefore, we introduce *N. asexualis* as a distinct new species from coffee in China.

Discussion

Members of *Nigrograna* are distributed worldwide in soil, wood, and other plant debris (Mapook et al. 2020), and the hotspots of *Nigrograna* are reported as Central and South America, where the taxa are also found as human pathogens (Kolařík 2018; Puing et al. 2020). To date, five *Nigrograna* species viz. *N. cangshanensis* (decaying wood, Yunnan), *N. jinghongensis* (dead woody litter, Yunnan), *N. kunmingensis* (dead twigs of *Gleditsia sinensis* Lam., Yunnan), *N. magnoliae* (living branches of *Magnolia denudate* Desr., Yunnan), and *N. locuta-pollinis* (hive-stored pollen, Hubei) have been isolated from different hosts in China (Tibpromma et al. 2017; Zhao et al. 2018; Wanasinghe et al. 2020; Boonmee et al. 2021; Zhou et al. 2022). In this study, three new saprobic fungi were isolated from decaying branches of *Coffea arabica* in Yunnan Province, China, and this is the first report of *Nigrograna* species from coffee.

Species of *Nigrograna* are morphologically very similar and overlapping, hence can be interpreted as cryptic species. Therefore, it is difficult to delimit the species based only on their morphological characteristics (Jaklitsch and Voglmayr 2016; Zhang et al. 2020). In our research, we found that *N. coffeae* and *N. puerensis* have similar morphology, but in phylogeny, they are distributed differently within *Nigrograna*. This confirms the view of Jaklitsch and Voglmayr (2016) that the gene sequences are important and crucial for the identification of taxa at the genus and the species level.

Acknowledgements

Li Lu thanks Mae Fah Luang University for the award of a fee-less scholarship. The Center for Yunnan Plateau Biological Resources Protection and Utilization, College of Biological Resource and Food Engineering, Qujing Normal University is thanked for the facilities provided for the research work. Dr. Shaun Pennycook is thanked for his advice on new fungi names. Dai Dong-qin thanks the National Natural Science Foundation of China (No. NSFC 31760013, 31950410558), and High-Level Talent Recruitment Plan of Yunnan Provinces ("Young Talents" Program). Samantha C. Karunarathna thanks the National Natural Science Foundation of China grant number 32260004 for the support. Nakarin Suwannarach thanks Chiang Mai University, Thailand for financial support. Saowaluck Tibpromma thanks "the most cited article award" for allowing one free publication in MycoKeys.

References

Ahmed SA, van de Sande WWJ, Stevens DA, Fahal A, van Diepeningen AD, Menken SBJ, de Hoog GS (2014) Revision of agents of black-grain eumycetoma in the order of Pleosporales. Persoonia 33: 141–154. https://doi.org/10.3767/003158514X684744

Arias RM, Abarca GH (2014) Fungal diversity in coffee plantation systems and in a tropical montane cloud forest in Veracruz, Mexico. Agroforestry Systems 88: 921–933. https://doi.org/10.1007/s10457-014-9736-z

Barr ME (1987) Prodromus to class Loculoascomycetes. Published by the Author, Amherst, Massachusetts; University of Massachusetts, U.S.A., 168 pp.

Bhunjun CS, Niskanen T, Suwannarach N, Wannathes N, Chen YJ, McKenzie EH, Maharachchikumbura SS, Buyck B, Zhao CL, Fan YG, Zhang JY, Dissanayake AJ, Marasinghe DS, Jayawardena RS, Kumla J, Padamsee M, Chen YY, Liimatainen K, Ammirati JF, Phukhamsakda C, Liu JK, Phonrob W, Randrianjohany E, Hongsanan S, Cheewangkoon R, Bundhun D, Khuna S, Yu WJ, Deng LS, Lu YZ, Hyde KD, Lumyong S (2022) The numbers of fungi: Are the most speciose genera truly diverse? Fungal Diversity 27: 1–76. https://doi.org/10.1007/s13225-022-00501-4

Boonmee S, Wanasinghe DN, Calabon MS, Huanraluek N, Chandrasiri SK, Jones GE, Rossi W, Leonardi M, Singh SK, Rana S, Singh PN, Maurya DK, Lagashetti AC, Choudhary D, Dai YC, Zhao CL, Mu YH, Yuan HS, He SH, Phookamsak R, Jiang HB, Martín MP, Dueñas M, Telleria MT, Kałucka LK, Jagodziński AM, Liimatainen K, Pereira DS, Phillips AJL, Suwannarach N, Kumla J, Khuna S, Lumyong S, Potter TB, Shivas RG, Sparks AH, Vaghefi N, Abdel-Wahab MA, Abdel-Aziz FA, Li GJ, Lin WF, Singh U, Bhatt RP, Lee HB, Nguyen TTT, Kirk PM, Dutta AK, Acharya K, Sarma VV, Niranjan M, Rajeshkumar KC, Ashtekar N, Lad S, Wijayawardene NN, Bhat DJ, Xu RJ, Wijesinghe SN, Shen HW, Luo ZL, Zhang JY, Sysouphanthong P, Thongklang N, Bao DF, Aluthmuhandiram JVS, Abdollahzadeh J, Javadi A, Dovana F, Usman M, Khalid AN, Dissanayake AJ, Telagathoti A, Probst M, Peintner U, Garrido-Benavent I, Bóna L, Merényi Z, Boros L, Zoltán B, Stielow JB, Jiang N, Tian CM, Shams E, Dehghanizadeh F, Pordel A, Javan-Nikkhah M,

Denchev TT, Denchev CM, Kemler M, Begerow D, Deng CY, Harrower E, Bozorov T, Kholmuradova T, Gafforov Y, Abdurazakov A, Xu JC, Mortimer PE, Ren GC, Jeewon R, Maharachchikumbura SSN, Phukhamsakda C, Mapook A, Hyde KD (2021) Fungal diversity notes 1387–1511: Taxonomic and phylogenetic contributions on genera and species of fungal taxa. Fungal Diversity 111: 1–335. https://doi.org/10.1007/s13225-021-00489-3

Borelli D (1976) *Pyrenochaeta mackinnonii* nova species agente de micetoma. Castellania 4: 227–234.

Dayarathne MC, Jones E, Maharachchikumbura S, Devadatha B, Sarma V, Khongphinitbunjong K, Chomnunti P, Hyde KD (2020) Morpho-molecular characterization of microfungi associated with marine based habitats. Mycosphere 11: 1–188. https://doi.org/10.5943/mycosphere/11/1/1

de Gruyter JH (2012) Revised taxonomy of *Phoma* and allied genera. Wageningen University and Research, 1–181.

de Gruyter J, Woudenberg JH, Aveskamp MM, Verkley GJ, Groenewald JZ, Crous PW (2010) Systematic reappraisal of species in *Phoma* section *Paraphoma*, *Pyrenochaeta* and *Pleurophoma*. Mycologia 102: 1066–1081. https://doi.org/10.3852/09-240

de Gruyter J, Woudenberg JH, Aveskamp MM, Verkley GJ, Groenewald JZ, Crous PW (2013) Redisposition of Phoma-like anamorphs in Pleosporales. Studies in Mycology 75: 1–36. https://doi.org/10.3114/sim0004

Dighton J (2003) Fungi in ecosystem processes. Marcel Dekker, New York, 434 pp. https://doi.org/10.1201/9781315371528

Dissanayake AJ, Bhunjun CS, Maharachchikumbura SSN, Liu JK (2020) Applied aspects of methods to infer phylogenetic relationships amongst fungi. Mycosphere 11: 2652–2676. https://doi.org/10.5943/mycosphere/11/1/18

Hawksworth DL, Lücking R (2017) Fungal diversity revisited: 2.2 to 3.8 million species. Microbiology Spectrum 5(4). https://doi.org/10.1128/microbiolspec

Hongsanan S, Hyde KD, Phookamsak R, Wanasinghe DN, McKenzie EHC, Sarma VV, Boonmee S, Lücking R, Bhat DJ, Liu NG, Tennakoon DS, Pem D, Karunarathna A, Jiang SH, Jones EBG, Phillips AJL, Manawasinghe IS, Tibpromma S, Jayasiri SC, Sandamali DS, Jayawardena RS, Wijayawardene NN, Ekanayaka AH, Jeewon R, Lu Y, Dissanayake AJ, Zeng XY, Luo ZL, Tian Q, Phukhamsakda C, Thambugala KM, Dai DQ, Samarakoon MC, Chethana KWT, Ertz D, Doilom M, Liu JK, Pérez-Ortega S, Suija A, Senwanna C, Wijesinghe SN, Niranjan M, Zhang SN, Ariyawansa HA, Jiang HB, Zhang JF, Norphanphoun C, de Silva NI, Thiyagaraja V, Zhang H, Bezerra JDP, Miranda-González R, Aptroot A, Kashiwadani H, Harishchandra D, Sérusiaux E, Abeywickrama PD, Bao DF, Devadatha B, Wu HX, Moon KH, Gueidan C, Schumm F, Bundhun D, Mapook A, Monkai J, Bhunjun CS, Chomnunti P, Suetrong S, Chaiwan N, Dayarathne MC, Yang J, Rathnayaka AR, Xu JC, Zheng JS, Liu G, Feng Y, Xie N (2020a) Refined families of Dothideomycetes: Dothideomycetidae and Pleosporomycetidae. Mycosphere 11: 1553–2107. https://doi.org/10.5943/mycosphere/11/1/13

Hongsanan S, Hyde KD, Phookamsak R, Wanasinghe DN, McKenzie EHC, Sarma VV, Lücking R, Boonmee S, Bhat DJ, Liu NG, Tennakoon DS, Pem D, Karunarathna A, Jiang SH, Jones EBG, Phillips AJL, Manawasinghe IS, Tibpromma S, Jayasiri SC, Sandamali DS, Jayawardena RS, Wijayawardene NN, Ekanayaka AH, Jeewon R, Lu YZ, Phukhamsakda C, Dissanayake

AJ, Zeng XY, Luo ZL, Tian Q, Thambugala KM, Dai DQ, Samarakoon MC, Chethana KWT, Ertz D, Doilom M, Liu JK, Pérez-Ortega S, Suija A, Senwanna C, Wijesinghe SN, Niranjan M, Zhang SN, Ariyawansa HA, Jiang HB, Zhang JF, Norphanphoun C, de Silva NI, Thiyagaraja V, Zhang H, Bezerra JDP, Miranda-González R, Aptroot A, Kashiwadani H, Harishchandra D, Sérusiaux E, Abeywickrama PD, Bao DF, Devadatha B, Wu HX, Moon KH, Gueidan C, Schumm F, Bundhun D, Mapook A, Monkai J, Bhunjun CS, Chomnunti P, Suetrong S, Chaiwan N, Dayarathne MC, Yang J, Rathnayaka AR, Xu JC, Zheng JS, Liu G, Feng Y, Xie N (2020b) Refined families of Dothideomycetes: Orders and families incertae sedis in Dothideomycetes. Fungal Diversity 105(1): 17–318. https://doi.org/10.1007/s13225-020-00462-6

Hyde KD, Norphanphoun C, Abreu VP, Bazzicalupo A, Chethana KWT, Clericuzio M, Dayarathne MC, Dissanayake AJ, Ekanayaka AH, He MQ, Hongsanan S, Huang SK, Jayasiri SC, Jayawardena RS, Karunarathna A, Konta S, Kušan I, Lee H, Li J, Lin CG, Liu NG, Lu YZ, Luo ZL, Manawasinghe IS, Mapook A, Perera RH, Phookamsak R, Phukhamsakda C, Siedlecki I, Soares AM, Tennakoon DS, Tian Q, Tibpromma S, Wanasinghe DN, Xiao YP, Yang J, Zeng XY, Abdel-Aziz FA, Li WJ, Senanayake IC, Shang QJ, Daranagama DA, De Silva NI, Thambugala KM, Abdel-Wahab MA, Bahkali AH, Berbee ML, Boonmee S, Bhat DJ, Bulgakov TS, Buyck B, Camporesi E, Castañeda-Ruiz RF, Chomnunti P, Doilom M, Dovana F, Gibertoni TB, Jadan M, Jeewon R, Jones GEB, Kang JC, Karunarathna SC, Lim YW, Liu JK, Liu ZY, Plautz Jr HL, Lumyong S, Maharachchikumbura SSN, Matočec N, Mckenzie EHC, Mešič A, Miller D, Pawłowska J, Pereira OL, Promputtha I, Romero AI, Ryvarden L, Su HY, Suetrong S, Tkalčec Z, Vizzini A, Wen TC, Wisitrassameewong K, Wrzosek M, Xu JC, Zhao Q, Zhao RL, Mortimer PE (2017) Fungal diversity notes 603–708: Taxonomic and phylogenetic notes on genera and species. Fungal Diversity 87: 1–235. https://doi.org/10.1007/s13225-017-0391-3

Hyde KD, Jeewon R, Chen YJ, Bhunjun CS, Calabon MS, Jiang HB, Lin CG, Norphanphoun C, Sysouphanthong P, Pem D, Tibpromma S, Zhang Q, Doilom M, Jayawardena RS, Liu JK, Maharachchikumbura SSN, Phukhamsakda C, Phookamsak R, Al-Sadi AM, Thongklang N, Wang Y, Gaforov Y, Jones EBG, Lumyong S (2020) The numbers of fungi: Is the descriptive curve flattening? Fungal Diversity 103(1): 219–271. https://doi.org/10.1007/s13225-020-00458-2

Index Fungorum (2022) Index Fungorum. http://www.indexfungorum.org/Names/Names.asp [Retrieved 20 September 2022]

Jaklitsch WM, Voglmayr H (2016) Hidden diversity in *Thyridaria* and a new circumscription of the Thyridariaceae. Studies in Mycology 85(1): 35–64. https://doi.org/10.1016/j.simyco.2016.09.002

Jayasiri SC, Hyde KD, Ariyawansa HA, Bhat J, Buyck B, Cai L, Dai YC, Abd-Elsalam KA, Ertz D, Hidayat I, Jeewon R, Jones EBG, Bahkali AH, Karunarathna SC, Liu JK, Luangsa-ard JJ, Lumbsch HT, Maharachchikumbura SSN, McKenzie EHC, Moncalvo JM, Ghobad-Nejhad M, Nilsson H, Pang KA, Pereira OL, Phillips AJL, Raspé O, Rollins AW, Romero AI, Etayo J, Selçuk F, Stephenson SL, Suetrong S, Taylor JE, Tsui CKM, Vizzini A, Abdel-Wahab MA, Wen TC, Boonmee S, Dai DQ, Daranagama DA, Dissanayake AJ, Ekanayaka AH, Fryar SC, Hongsanan S, Jayawardena RS, Li WJ, Perera RH, Phookamsak R, de Silva NI, Thambugala KM, Tian Q, Wijayawarden NN, Zhao RL, Zhao Q, Kang JC, Promputtha I (2015) The Faces of Fungi database: Fungal names linked with morphol-

ogy, phylogeny and human impacts. Fungal Diversity 74: 3–18. https://doi.org/10.1007/s13225-015-0351-8

Jiang HB, Phookamsak R, Hyde KD, Mortimer PE, Xu JC, Kakumyan P, Karunarathna SC, Kumla JA (2021) A taxonomic appraisal of bambusicolous fungi in Occultibambusaceae (Pleosporales, Dothideomycetes) with new collections from Yunnan Province, China. Life 11(9): 932. https://doi.org/10.3390/life11090932

Kolařík M (2018) New taxonomic combinations in endophytic representatives of the genus *Nigrograna*. Czech Mycology 70: 123–126.

Kolařík M, Spakowicz DJ, Gazis R, Shaw J, Kubátová A, Nováková A, Chudíčková M, Forcina GC, Kang KW, Kelnarová I, Skaltsas D (2017) *Biatriospora* (Ascomycota: Pleosporales) is an ecologically diverse genus including facultative marine fungi and endophytes with biotechnological potential. Plant Systematics and Evolution 303(1): 35–50. https://doi.org/10.1007/s00606-016-1350-2

Li ZW (2014) Issues and suggestions on building an international brand of "Pu'er Coffee". Selected papers on rural agricultural reform, innovation and agricultural modernization 2: 719–721.

Liu YJ, Whelen S, Hall BD (1999) Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerse II subunit. Molecular Biology and Evolution 16: 1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092

Liu JK, Hyde KD, Jeewon R, Phillips AJ, Maharachchikumbura SS, Ryberg M, Liu ZY, Zhao Q (2017) Ranking higher taxa using divergence times: A case study in Dothideomycetes. Fungal Diversity 84(1): 75–99. https://doi.org/10.1007/s13225-017-0385-1

Lu L, Tibpromma S, Karunarathna SC, Thiyagaraja V, Xu JC, Jayawardena RS, Lumyong S, Hyde KD (2021) Taxonomic and phylogenic appraisal of a novel species and a new record of Stictidaceae from coffee in Yunnan Province, China. Phytotaxa 528(2): 111–124. https://doi.org/10.11646/phytotaxa.528.2.4

Lu L, Tibpromma S, Karunarathna SC, Jayawardena RS, Lumyong S, Xu JC, Hyde KD (2022a) Comprehensive review of fungi on coffee. pathogens 11: 411. https://doi.org/10.3390/pathogens11040411

Lu L, Karunarthna SC, Dai DQ, Xiong YR, Suwannarach N, Stephenson SL, Elgorban AM, Al-Rejaie S, Jayawardena RS, Tibpromma S (2022b) Description of four novel species in Pleosporales associated with coffee in Yunnan, China. Journal of Fungi 8(10): 1113. https://doi.org/10.3390/jof8101113

Luttrell ES (1955) The ascostromatic Ascomycetes. Mycologia 47: 511–532. https://doi.org/10.1080/00275514.1955.12024473

Mapook A, Hyde KD, McKenzie EH, Jones EB, Bhat DJ, Jeewon R, Stadler M, Samarakoon MC, Malaithong M, Tanunchai B, Buscot F (2020) Taxonomic and phylogenetic contributions to fungi associated with the invasive weed *Chromolaena odorata* (Siam weed). Fungal Diversity 101(1): 1–175. https://doi.org/10.1007/s13225-020-00444-8

Miller MA, Pfeiffer W, Schwartz T (2012) The CIPRES science gateway: Enabling high-impact science for phylogenetics researchers with limited resources. Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the Extreme to the Campus and Beyond, 1–8. https://doi.org/10.1145/2335755.2335836

Neilson J, Wang JHZ (2019) China and the changing economic geography of coffee value chains. Singapore Journal of Tropical Geography 40(3): 429–451. https://doi.org/10.1111/sjtg.12279

Nylander JAA (2004) MrModeltest v2. Program distributed by the author Evolutionary Biology Centre, Uppsala University, Uppsala.

Pandey A, Das N, Kumar B, Rinu K, Trivedi P (2008) Phosphate solubilization by *Penicillium* spp. isolated from soil samples of Indian Himalayan region. World Journal of Microbiology & Biotechnology 24(1): 97–102. https://doi.org/10.1007/s11274-007-9444-1

Phukhamsakda C, Nilsson RH, Bhunjun CS, de Farias AR, Sun YR, Wijesinghe SN, Raza M, Bao DF, Lu L, Tibpromma S, Dong W, Tennakoon DS, Tian XG, Xiong YR, Karunarathna SC, Cai L, Luo ZL, Wang Y, Manawasinghe IS, Camporesi E, Kirk PM, Promputtha I, Kuo CH, Su HY, Doilom M, Li Y, Fu YP, Hyde KD (2022) The numbers of fungi: Contributions from traditional taxonomic studies and challenges of metabarcoding. Fungal Diversity 28: 1–60. https://doi.org/10.1007/s13225-022-00502-3

Puing AG, Couture-Cossette A, Wang AX, Zygourakis CC, Cheng X, Stevens BA, Banaei N, Novoa RA, Ho DY, Subramanian AK (2020) Simultaneous coccidioidomycosis and phaeohyphomycosis in a kidney transplant recipient: A case report and literature review. Transplant Infectious Disease 22(6): e13365. https://doi.org/10.1111/tid.13365

Rambaut A (2014) FigTree v1. 4.2, a graphical viewer of phylogenetic trees.

Rehner SA, Buckley EA (2005) *Beauveria* phylogeny inferred from nuclear ITS and *EF*1-α sequences: Evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97: 84–98. https://doi.org/10.1080/15572536.2006.11832842

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Senanayake IC, Rathnayaka AR, Marasinghe DS, Calabon MS, Gentekaki E, Lee HB, Hurdeal VG, Pem D, Dissanayake LS, Wijesinghe SN, Bundhun D, Nguyen TT, Goonasekara ID, Abeywickrama PD, Bhunjun CS, Jayawardena RS, Wanasinghe DN, Jeewon R, Bhat DJ, Xiang MM (2020) Morphological approaches in studying fungi: Collection, examination, isolation, sporulation and preservation. Mycosphere 11: 2678–2754. https://doi.org/10.5943/mycosphere/11/1/20

Sharma H (2020) A detail chemistry of coffee and its analysis. In Coffee-Production and Research, 79 pp. https://doi.org/10.5772/intechopen.91725

Stamatakis A (2014) RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Tennakoon DS, Kuo CH, Maharachchikumbura SS, Thambugala KM, Gentekaki E, Phillips AJ, Bhat DJ, Wanasinghe DN, de Silva NI, Promputtha I, Hyde KD (2021) Taxonomic and phylogenetic contributions to *Celtis formosana*, *Ficus ampelas*, *F. septica*, *Macaranga tanarius* and *Morus australis* leaf litter inhabiting microfungi. Fungal Diversity 108: 1–215. https://doi.org/10.1007/s13225-021-00474-w

Tibpromma S, Hyde KD, Jeewon R, Maharachchikumbura SSN, Liu JK, Bhat DJ (2017) Fungal diversity notes 491–602: Taxonomic and phylogenetic contributions to fungal taxa. Fungal Diversity 83: 1–261. https://doi.org/10.1007/s13225-017-0378-0

Vaidya G, Lohman DJ, Meier R (2011) SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27(2): 171–180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

Wanasinghe DN, Wijayawardene NN, Xu J, Cheewangkoon R, Mortimer PE (2020) Taxonomic novelties in Magnolia-associated pleosporalean fungi in the Kunming Botanical Gardens (Yunnan, China). PLoS ONE 15(7): e0235855. https://doi.org/10.1371/journal.pone.0235855

White TJ, Bruns T, Lee SJWT, Taylor JL (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18: 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wijayawardene NN, Hyde KD, Dai DQ, Sánchez-García M, Goto BT, Saxena RK, Erdoğdu M, Selçuk F, Rajeshkumar KC, Aptroot A, Błaszkowski J, Boonyuen N, da Silva GA, de Souza FA, Dong W, Ertz D, Haelewaters D, Jones EBG, Karunarathna SC, Kirk PM, Kukwa M, Kumla J, Leontyev DV, Lumbsch HT, Maharachchikumbura SSN, Marguno F, Martínez-Rodríguez P, Mešić A, Monteiro JS, Oehl F, Pawłowska J, Pem D, Pfliegler WP, Phillips AJL, Pošta A, He MQ, Li JX, Raza M, Sruthi OP, Suetrong S, Suwannarach N, Tedersoo L, Thiyagaraja V, Tibpromma S, Tkalčec Z, Tokarev YS, Wanasinghe DN, Wijesundara DSA, Wimalaseana SDMK, Madrid H, Zhang GQ, Gao Y, Sánchez-Castro I, Tang LZ, Stadler M, Yurkov A, Thines M (2022) Outline of fungi and fungus-like taxa–2021. Mycosphere 13: 53–453. https://doi.org/10.5943/mycosphere/13/1/2

Yang EF, Tibpromma S, Karunarathna SC, Phookamsak R, Xu JC, Zhao ZX, Karunanayake C, Promputtha I (2022) Taxonomy and phylogeny of novel and extant taxa in Pleosporales associated with *Mangifera indica* from Yunnan, China (Series I). Journal of Fungi 8(2): 152. https://doi.org/10.3390/jof8020152

Zhang Y, Schoch CL, Fournier J, Crous PW, De Gruyter J, Woudenberg JH, Hirayama K, Tanaka K, Pointing SB, Spatafora JW, Hyde KD (2008) Multi-locus phylogeny of Pleosporales: A taxonomic, ecological and evolutionary re-evaluation. Studies in Mycology 61(1): 111–119. https://doi.org/10.3114/sim.2009.64.04

Zhang Y, Crous PW, Schoch CL, Hyde KD (2012) Pleosporales. Fungal Diversity 53(1): 1–221. https://doi.org/10.1007/s13225-011-0117-x

Zhang H, Li J, Zhou H, Chen Z, Song G, Peng Z, Pereira AP, Silva MC, Varzea VM (2014) Arabica coffee production in the Yunnan Province of China. Proceedings of the 24th International Conference on Coffee Science (ASIC).

Zhang JF, Liu JK, Thambugala KM, Yang J, Meng ZH, Liu ZY (2020) Two new species and a new record of *Nigrograna* (Nigrogranaceae, Pleosporales) from China and Thailand. Mycological Progress 19: 1365–1375. https://doi.org/10.1007/s11557-020-01633-0

Zhao YZ, Zhang ZF, Cai L, Peng JW, Liu F (2018) Four new filamentous fungal species from newly-collected and hive stored bee pollen. Mycosphere 9: 1089–1116. https://doi.org/10.5943/mycosphere/9/6/3

Zhou LW, Wang XW, Liu SL, Li GJ, Deng CY, Rossi W, Leonardi M, Liimatainen K, Kekki T, Niskanen T, Smith ME, Ammirati J, Bojantchev D, Abdel-Wahab MA, Zhang M, Tian E, Lu YZ, Zhang JY, Ma J, Dutta AK, Acharya K, Du TY, Xu JZ, Kim JS, Lim YW, Gerlach A, Zeng NK, Han YX, Razaghi P, Raza M, Cai L, Calabon MS, Jones EBG, Saha R, Kumar TKA, Krishnapriya K, Thomas A, Kaliyaperumal M, Kezo K, Gunaseelan S, Singh SK, Singh PN, Lagashetti AC, Pawar KS, Jiang SH, Zhang C, Zhang H, Qing Y, Bau T, Ramirez NA, Niveiro N, Li MX, Yang ZL, Wu G, Tarafder E, Tennakoon DS, Kuo CH, da Silva TM, Souza-Motta CM, Bezerra JDP, He G, Ji XH, Suwannarach N, Kumla J, Lumyong S, Wannathes N, Rana S, Hyde KD (2022) Fungal diversity notes: taxonomic and phylogenetic contributions on genera and species of fungal taxa. Fungal Diversity (in press).

MycoKeys 94: 73–89 (2022)
doi: 10.3897/mycokeys.94.95888
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Three novel species of *Helminthosporium* (Massarinaceae, Pleosporales) from China

Jingwen Liu[1], Yafen Hu[1], Xingxing Luo[1], Rafael F. Castañeda-Ruíz[2], Jian Ma[1]

1 *College of Agronomy, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China* **2** *Instituto de Investigaciones Fundamentales en Agricultura Tropical "Alejandro de Humboldt" (INIFAT), calle 1, esq. 2, Santiago de Las Vegas, Ciudad de La Habana, C. P. 17200, Cuba*

Corresponding author: Jian Ma (jxaumj@126.com)

Academic editor: Pedro Crous | Received 2 October 2022 | Accepted 12 December 2022 | Published 21 December 2022

Citation: Liu J, Hu Y, Luo X, Castañeda-Ruiz RF, Ma J (2022) Three novel species of *Helminthosporium* (Massarinaceae, Pleosporales) from China. MycoKeys 94: 73–89. https://doi.org/10.3897/mycokeys.94.95888

Abstract

Three new species of *Helminthosporium*, *H. nabanhensis*, *H. sinensis* and *H. yunnanensis* collected on dead branches of unidentified plants in Xishuangbanna, China, were proposed by morphological and molecular phylogenetic analysis. Phylogenetic analysis of the combined data of ITS-SSU-LSU-*TEF1-RPB2* sequences was performed using Maximum-Likelihood and Bayesian Inference, although *H. nabanhensis* and *H. sinensis* lack the *RPB2* sequences. Both molecular analyses and morphological data supported *H. nabanhensis*, *H. sinensis* and *H. yunnanensis* as three independent taxa within the Massarinaceae.

Keywords

asexual Ascomycota, hyphomycetes, lignicolous fungi, phylogenetic analysis, taxonomy

Introduction

Helminthosporium Link was originally erected by Link (1809) with *H. velutinum* as the type species, and was mainly characterized by macronematous, cylindrical, septate conidiophores with polytretic conidiogenous cells that producing solitary (rarely in short chains), acropleurogenous, clavate or obclavate, distoseptate conidia with a flat, ringed pore at the base (Ellis 1961, 1971; Luttrell 1964; Seifert

et al. 2011). The genus became a repository for a large amount of species due to a lack of understanding of the generic concepts. To date, about 770 epithets for *Helminthosporium* are listed in Index Fungorum (2022), but most of these were not congeneric with the generic type in development of conidia and conidiophores. Ellis (1961) provided a review on *Helminthosporium*, and accepted ten species. Luttrell (1963, 1964) examined the type species and defined the generic concept, and Sivanesan (1987) transferred several unrelated pathogens of the *Poaceae* from *Helminthosporium* to the genera *Cochliobolus* (anamorph *Bipolaris*), *Setosphaeria* (anamorph *Exserohilum*) and *Pyrenophora* (anamorph *Drechslera*). Siboe et al. (1999) subsequently provided a synoptic table of the main morphological features that distinguish 27 accepted *Helminthosporium* species. Since then, 27 additional species have been described in the genus (Zhang et al. 2004, 2007, 2010; Shirouzu and Harada 2008; Zhang and Zhang 2009; Zhang and Sun 2010; Zhao and Zhao 2012; Wang et al. 2014; Tanaka et al. 2015; Zhu et al. 2016; Alves-Barbosa et al. 2017; Tian et al. 2017; Crous et al. 2018, 2019; Zhao et al. 2018; Boonmee et al. 2021; Chen et al. 2022). Voglmayr and Jaklitsch (2017) revealed the phylogenetic relationships of *Corynespora*, *Exosporium* and *Helminthosporium* species, synonymized *Exosporium* with *Helminthosporium*, and confirmed 17 species in *Helminthosporium* by morphological and molecular systematic analysis, but the generic concept has been widened by adding four *Corynespora* species that produce terminal, monotretic conidiogenous cells. So it is challenging to classify *Corynespora* and *Helminthosporium* species based on morphology alone because the distinction between monotretic vs. polytretic conidiogenous cells is the only character for separating *Corynespora* and *Helminthosporium*. Based on the records of Species Fungorum 2021, Konta et al. (2021) summarized the morphology, host information, locality, sequence data and related references of 216 *Helminthosporium* species reported worldwide. Unfortunately, sequence data for most species are unavailable, and only 27 species are represented by the DNA sequence in GenBank (Chen et al. 2022).

Helminthosporium is worldwide in distribution, usually found as a common saprobe on leaf or twig litter, but one specie, *H. solani*, is an economically important pathogen causing silver scurf disease in potatoes worldwide (Alcorn 1983; Voglmayr and Jaklitsch 2017; Boonmee et al. 2021). To date, only 28 species have been recorded in China, viz. *H. aquaticum*, *H. bambusicola*, *H. cantonense*, *H. chengduense*, *H. chinense*, *H. citri*, *H. conidiophorellum*, *H. constrictum*, *H. corchori*, *H. dongxingense*, *H. guangxiense*, *H. hunanense*, *H. ipomoeae*, *H. juglandis*, *H. lablab*, *H. ligustri*, *H. marantae*, *H. multiseptatum*, *H. nanjingense*, *H. obpyriforme*, *H. oplismeni*, *H. ovoideum*, *H. piperis*, *H. pseudomicrosorum*, *H. rhodomyrti*, *H. sichuanense*, *H. subhyalinum* and *H. submersum* (Zhang et al. 2004; Zhang and Zhang 2009; Zhang et al. 2010; Zhang and Sun 2010; Zhao and Zhao 2012; Wang et al. 2014; Zhu et al. 2016; Zhao et al. 2018; Chen et al. 2022).

Xishuangbanna lies on the northern edge of tropical Southeast Asia. It is located in the southwestern part of Yunnan Province, China. It covers 19,125 km^2 and has a mountainous topography and humid tropical monsoon climate, with an av-

erage annual temperature of 19.3–23.9 °C, and an average annual precipitation of 1200–1800 mm. The primary forest vegetation types are tropical seasonal rain forest, tropical montane rain forest, evergreen broad-leaved forest, monsoon forest over limestone, and monsoon forest on river banks (Cao and Zhang 1997). Such conditions create a very wide range of habitats favoring the growth of various microbial species. During our continuing mycological surveys of saprobic microfungi from plant debris in this region, three interesting hyphomycetes with morphological features typical of *Helminthosporium* were collected on dead branches. Based on morphological data and multi-locus phylogenetic analysis, they were described as new to science in the present study.

Materials and methods

Sample collection, isolation and morphological studies

Samples of dead branches were collected from humid environments and river banks in the forest ecosystems of Xishuangbanna, Yunnan Province, China, and returned to the laboratory in Ziploc bags. Samples were processed and examined following the methods described in Ma et al. (2011). Fungi were mounted in a drop of lactic acid on microscope slides, and examined and photographed with an Olympus microscope (model BX 53), with a 100 × (oil immersion) objective at the same background color and scale. Adobe Photoshop 7.0 was used for image processing to assemble photographs into images. Single-spore isolations were made on potato dextrose agar (PDA) following Goh (1999). Colony colors were assessed according to the charts of Rayner (1970). All fungal strains were stored in 10% sterilized glycerin at 4 °C for further studies. The studied specimens and cultures were deposited in the Herbarium of Jiangxi Agricultural University, Plant Pathology, Nanchang, China (**HJAUP**).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fungal mycelia grown on PDA, using the Solarbio Fungi Genomic DNA Extraction Kit following the manufacturer's protocol (Solarbio, China). The DNA amplification was performed by polymerase chain reaction (PCR) using the respective loci (ITS, SSU, LSU, *TEF1*, *RPB2*). Primer sets used for these genes were as follows: ITS: ITS5/ITS4 (White et al. 1990), SSU: 18S-F/18S-R, LSU: 28S1-F/28S3-R (Xia et al. 2017), *TEF1*: EF1-983F/EF1-2218R (Rehner 2001; Zhao et al. 2018) and *RPB2*: dRPB2-5f/dRPB2-7r (Voglmayr et al. 2016). The final volume of the PCR reaction was 25 µl, containing 1 µl of DNA template, 1 µl each of the forward and reverse primer, 12.5 µl of 2 × Power Taq PCR MasterMix and 9.5 µl of double-distilled water (ddH_2O). The PCR thermal cycling conditions of ITS, SSU and LSU were initialized at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, elongation at 72 °C

for 1 min, a final extension at 72 °C for 10 min, and finally kept at 4 °C, the *TEF1* and *RPB2* were initialized at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at a suitable temperature for 30 s, elongation at 72 °C for 1 min, a final extension at 72 °C for 10 min, and finally kept at 4 °C. Annealing temperature was 60 °C for *TEF1*, 56 °C for *RPB2*. The PCR products were checked on 1% agarose gel electrophoresis stained with ethidium bromide. Purification and DNA sequencing were carried out at Beijing Tsingke Biotechnology Co., Ltd. China.

Sequence alignment and phylogenetic analysis

The newly generated sequences together with other sequences obtained from GenBank (Table 1) were initially aligned using MAFFTv.7 (Katoh and Standley 2013) on the online server (http://maffTh.cbrc.jp/alignment/server/), and optimized manually when needed. To establish the identity of the isolates at species level, phylogenetic analyses were conducted first individually for each locus and then as combined analyses of five gene loci (ITS, LSU, SSU, *TEF1* and *RPB2*). Five aligned data sets of ITS, LSU, SSU, *TEF1* and *RPB2* are concatenated using the concatenated sequence function of Phylosuite software v1.2.1 (Zhang et al. 2020a), and absent sequence data (i.e., ITS, LSU, SSU, *TEF1* and *RPB2* sequence data) in the alignments were treated with the question mark as missing data. Phylosuite software v1.2.1 (Zhang et al. 2020a) was used to construct the phylogenetic tree based on ITS, SSU, LSU, *TEF1* and *RPB2* sequence data. The concatenated aligned dataset was analyzed separately using Maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood phylogenies were inferred using IQ-TREE (Nguyen et al. 2015) under Edge-linked partition model for 10000 ultrafast bootstraps (Hoang et al. 2017). The final tree was selected among suboptimal trees from each run by comparing the likelihood scores using the TIM2e+I+G4 for ITS+*RBP2*, TVMe+I+G4 for LSU+SSU, and TNe+R2 for *TEF1* substitution model. Bayesian Inference phylogenies were inferred using MrBayes 3.2.6 (Ronquist et al. 2012) under partition model (2 parallel runs, 2000000 generations), in which the initial 25% of sampled data were discarded as burn-in. The best-fit model was SYM+I+G4 for ITS+*RBP2*, LSU+SSU; SYM+G4 for *TEF1*. ModelFinder (Kalyaanamoorthy et al. 2017) was used to select the best-fit partition model (Edge-linked) using BIC criterion. The trees were viewed in FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and further edited in Adobe Illustrator 2021.

Results

Molecular phylogeny

Three new strains of *Helminthosporium* isolated from dead branches in Xishuangbanna, Yunnan Province, China, were grown in culture and used for analyses of molecular

Table 1. Species and GenBank accession numbers of DNA sequences used in this study. New sequences are in bold.

Taxon	Strain	Genbank accession numbers				
		SSU	LSU	ITS	*RPB2*	*TEF1*
Byssothecium circinans	CBS 675.92	GU205235	GU205217	OM337536	DQ767646	GU349061
Corynespora cassiicola	CBS 100822	GU296144	GU301808	–	GU371742	GU349052
Corynespora smithii	L120	–	KY984297	KY984297	KY984361	KY984435
Corynespora smithii	L130	KY984419	KY984298	KY984298	KY984362	KY984436
Cyclothyriella rubronotata	TR, CBS 121892	–	KX650541	KX650541	KX650571	KX650516
Cyclothyriella rubronotata	TR9, CBS 141486	KX650507	KX650544	KX650544	KX650574	KX650519
Helminthosporium aquaticum	MFLUCC 15-0357, S-096[HT]	KU697310	KU697306	KU697302	–	–
Helminthosporium austriacum	L132[HT], CBS 139924	KY984420	KY984301	KY984301	KY984365	KY984437
Helminthosporium austriacum	L137	–	KY984302	KY984302	KY984366	KY984438
Helminthosporium austriacum	L169, CBS 142388	–	KY984303	KY984303	KY984367	KY984439
Helminthosporium caespitosum	L141	–	KY984305	KY984305	KY984368	–
Helminthosporium caespitosum	L151	–	KY984306	KY984306	KY984369	–
Helminthosporium caespitosum	L99[HT], CBS 484.77	KY984421	JQ044448	JQ044429	KY984370	KY984440
Helminthosporium chengduense	UESTC 22.0024, CGMCC 3.23575[HT]	ON557757	ON557745	ON557751	ON563073	ON600598
Helminthosporium chengduense	UESTC 22.0025	ON557756	ON557744	ON557750	ON563072	ON600597
Helminthosporium chiangraiense	MFLUCC 21-0087[HT]	–	MZ538538	MZ538504	–	–
Helminthosporium chinense	UESTCC 22.0026, CGMCC 3.23570[HT]	ON557760	ON557748	ON557754	–	ON600601
Helminthosporium chlorophorae	BRIP 14521	–	–	AF120259	–	–
Helminthosporium dalbergiae	H 4628, MAFF 243853	AB797231	AB807521	LC014555	–	AB808497
Helminthosporium endiandrae	CBS 138902, CPC 22194[HT]	–	KP004478	KP004450	–	–
Helminthosporium erythrinicola	CBS 145569[HT]	–	MK876432	NR_165563	MK876486	–
Helminthosporium genistae	L128, CBS 139921	KY984422	KY984308	KY984308	KY984372	–
Helminthosporium genistae	L129, CBS 139922	KY984423	KY984309	KY984309	KY984373	–
Helminthosporium genistae	L142[ET], CBS 142597	–	KY984310	KY984310	KY984374	–
Helminthosporium hispanicum	L109[HT], CBS 136917	KY984424	KY984318	KY984318	KY984381	KY984441
Helminthosporium italicum	MFLUCC 17-0241	–	KY815015	KY797638	–	KY815021
Helminthosporium juglandinum	L118[HT], CBS 136922	–	KY984321	KY984321	KY984384	KY984444
Helminthosporium juglandinum	L97, CBS 136911	KY984425	KY984322	KY984322	KY984385	KY984445
Helminthosporium leucadendri	CBS 135133, CPC 19345[HT]	–	KF251654	KF251150	KF252159	KF253110
Helminthosporium livistonae	CPC 32158, CBS 144413[HT]	–	NG_064539	NR_160348	–	–
Helminthosporium magnisporum	H 4627, MAFF 239278, TS 33[HT]	AB797232	AB807522	AB811452	–	AB808498
Helminthosporium massarinum	KT 1564[HT], CBS 139690	AB797234	AB807524	AB809629	–	AB808500
Helminthosporium massarinum	KT 838[EP], MAFF 239604	AB797233	AB807523	AB809628	–	AB808499
Helminthosporium microsorum	L94	KY984426	KY984327	KY984327	KY984388	KY984446
Helminthosporium microsorum	L95	–	KY984328	KY984328	KY984389	KY984447
Helminthosporium microsorum	L96[ET], CBS 136910	KY984427	KY984329	KY984329	KY984390	KY984448
Helminthosporium nabanhensis	**HJAUP C2054[ET]**	**OP555400**	**OP555398**	**OP555394**	–	**OP961931**
Helminthosporium nanjingensis	ZM020380	–	–	KF192322	–	–
Helminthosporium oligosporum	L106	–	KY984330	KY984330	KY984391	KY984449
Helminthosporium oligosporum	L92, CBS 136908	KY984428	KY984332	KY984332	KY984393	KY984450
Helminthosporium oligosporum	L93[ET], CBS 136909	–	KY984333	KY984333	KY984394	KY984451
Helminthosporium quercinum	L90[HT], CBS 136921	KY984429	KY984339	KY984339	KY984400	KY984453
Helminthosporium quercinum	L91	–	KY984340	KY984340	KY984401	KY984454
Helminthosporium sinensis	**HJAUP C2121[ET]**	**OP555399**	**OP555397**	**OP555393**	–	**OP961932**
Helminthosporium solani	CBS 365.75	KY984430	KY984341	KY984341	KY984402	KY984455
Helminthosporium solani	CBS 640.85	–	KY984342	KY984342	KY984403	–
Helminthosporium submersum	UESTCC 22.0021	ON557759	ON557747	ON557753	ON563075	ON600600
Helminthosporium submersum	MFLUCC 16-1360[HT]	MG098796	MG098787	–	–	MG098586
Helminthosporium submersum	MFLUCC 16-1290[PT]	MG098797	MG098788	MG098780	MG098592	MG098587

Taxon	Strain	Genbank accession numbers				
		SSU	LSU	ITS	*RPB2*	*TEF1*
Helminthosporium syzygii	CBS 145570 HT	–	MK876433	NR_165564	MK876487	–
Helminthosporium tiliae	L171	–	KY984343	KY984343	KY984404	KY984456
Helminthosporium tiliae	L88 ET, CBS 136907	KY984431	KY984345	KY984345	KY984406	KY984457
Helminthosporium tiliae	L89	–	KY984346	KY984346	KY984407	–
Helminthosporium velutinum	H 4626, MAFF 243854	AB797240	AB807530	LC014556	–	AB808505
Helminthosporium velutinum	H 4739, MAFF 243855	AB797235	AB807525	LC014557	–	AB808501
Helminthosporium velutinum	L115, CBS 136924	–	KY984347	KY984347	KY984408	KY984458
Helminthosporium velutinum	L116	–	KY984348	KY984348	KY984409	KY984459
Helminthosporium velutinum	L117	–	KY984349	KY984349	KY984410	KY984460
Helminthosporium velutinum	L126	–	KY984350	KY984350	KY984411	KY984461
*Helminthosporium velutin*um	L127	–	KY984351	KY984351	KY984412	KY984462
Helminthosporium velutinum	L131 ET, CBS 139923	KY984432	KY984352	KY984352	KY984413	KY984463
Helminthosporium velutinum	L98	KY984433	KY984359	KY984359	KY984417	KY984466
Helminthosporium velutinum	yone 96, MAFF 243859	AB797239	AB807529	LC014558	–	AB808504
Helminthosporium yunnanensis	**HJAUP C2071 ET**	**OP555392**	**OP555396**	**OP555395**	**OP961934**	**OP961933**
Massarina cisti	CBS 266.62, JCM 14140 HT	AB797249	AB807539	LC014568	FJ795464	AB808514
Massarina eburnea	CBS 473.64	AF164367	GU301840	AF383959	GU371732	GU349040
Massarina eburnea	H 3953, CBS 139697	AB521718	AB521735	LC014569	–	AB808517
Periconia byssoides	H 4600, MAFF 243872	AB797280	AB807570	LC014581	–	AB808546
Periconia digitata	CBS 510.77	AB797271	AB807561	LC014584	–	AB808537
Periconia pseudodigitata	KT 1395, CBS 139699, MAFF 239676 HT	NG_064850	NG_059396	NR_153490	–	AB808540
Pseudosplanchnonema phorcioides	L16, CBS 122935	KY984434	KY984360	KY984360	KY984418	KY984467
Stagonospora paludosa	CBS 135088, S601 NT	–	KF251760	KF251257	KF252262	KF253207
Stagonospora perfecta	KT 1726A, MAFF 239609	AB797289	AB807579	AB809642	–	AB808555
Stagonospora pseudoperfecta	KT 889, CBS 120236, MAFF 239607 HT	AB797287	AB807577	AB809641	–	AB808553
Stagonospora tainanensis	KT 1866, MAFF 243860	AB797290	AB807580	AB809643	–	AB808556

[1]"–", sequence is unavailable.

[2]Strain with ET (epitype), HT (holotype), NT (neotype), and PT (paratype).

[3]Abbreviations: **CBS:** Central Bureau voor Schimmel cultures, Utrecht, The Netherlands; **CGMCC:** China General Microbiological Culture Collection Center; **CPC:** Collection of Pedro Crous housed at CBS; **HJAUP:** Herbarium of Jiangxi Agricultural University, Plant Pathology; **MAFF:** the National Institute of Agrobiological Sciences, Japan; **MFLUCC:** Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; **UESTCC:** The University of Electronic Science and Technology Culture Collection, Chengdu, China; **ITS:** Internal Transcribed Spacer; **SSU:** Small Subunit Ribosomal; **LSU:** Large Subunit Ribosomal; ***TEF1*:** Transcriptional Enhancer Factor 1-alpha; ***RPB2*:** The Second Largest Subunit of RNA Polymerase II; others are not registered abbreviations.

sequence data. Unfortunately, our two species, *H. nabanhensis* and *H. sinensis* lack the *RPB2* sequences. Newly generated sequences were deposited in GenBank. Alignment has 75 sequences with 1511 total characters (The combined dataset, ITS:1–457, LSU:458–993, *RBP2*:994–1110, SSU:1111–1363, *TEF1*:1364–1511), 555 distinct patterns, 487 parsimony-informative, 89 singleton sites, 935 constant sites, and *Cyclothyriella rubronotata* (TR) and *C. rubronotata* (TR9) were regarded as an outgroup. Maximum likelihood and Bayesian Inference analyses of the combined dataset resulted in phylogenetic reconstructions with largely similar topologies, and bootstrap support values for Maximum likelihood higher than 90% and Bayesian posterior probabilities greater than 0.90 are given above the nodes. The best-scoring ML consensus tree (lnL = –10,686.191) with ultrafast bootstrap values from ML analyses and posterior probabilities from MrBayes analysis at the nodes are shown in Fig. 1. *Helminthosporium nabanhensis* form a distinct clade sister to *H. chlorophorae* with

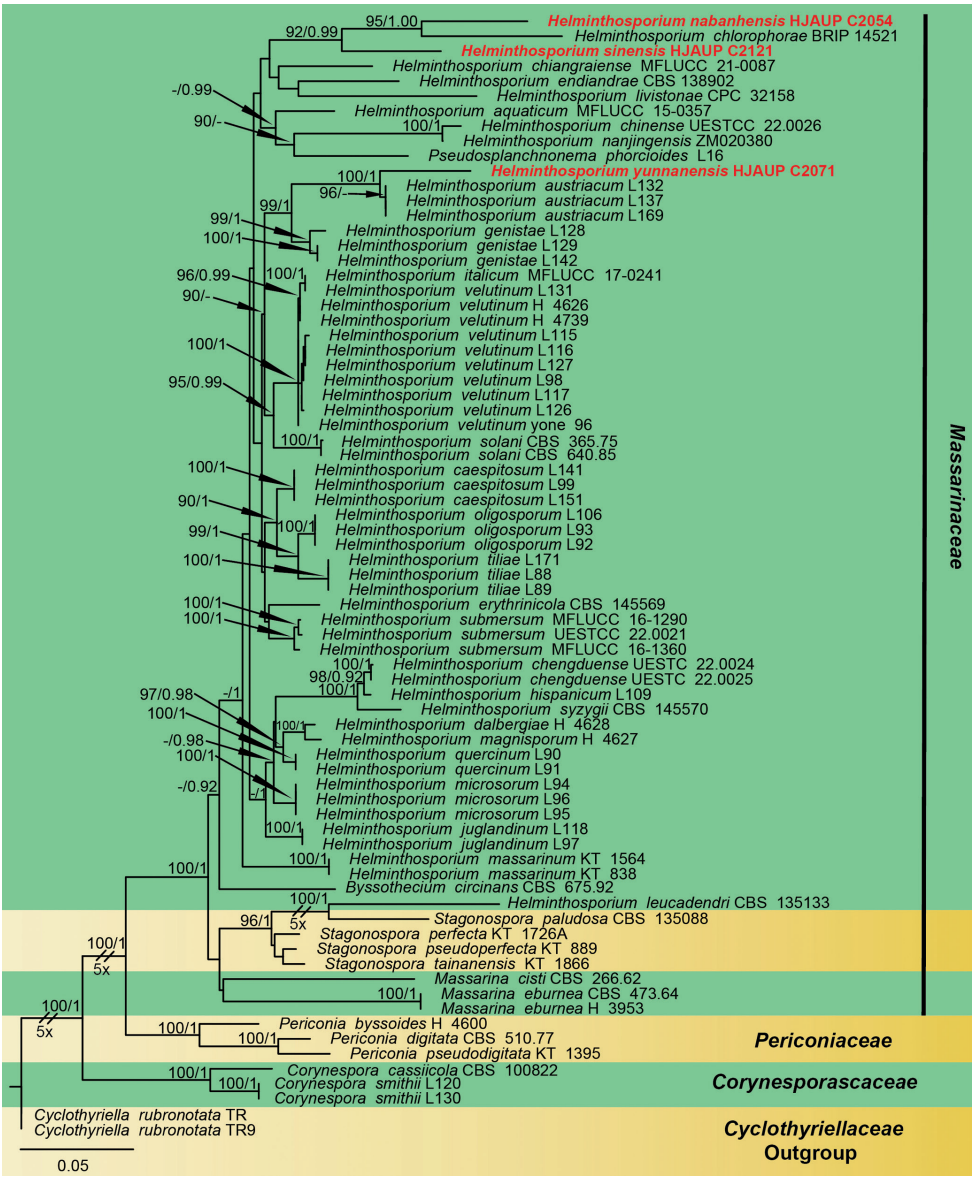

Figure 1. Phylogram of Massarinaceae based on combined ITS, SSU, LSU, *RPB*2 and *TEF*1 sequences. The ML and BI bootstrap support values above 90% and 0.90 are shown at the first and second position, respectively. The tree is rooted to *Cyclothyriella rubronotata* (TR) and *C. rubronotata* (TR9). Strains from the current study are in red. Some branches were shortened according to the indicated multipliers.

strong statistical support (ML/BI = 95/1.00); *H. sinensis* forms a high-support clade (ML/BI = 92/0.99) with the lineage consisting of *H. nabanhensis* and *H. chlorophorae*; *H. yunnanensis* is a sister to three different strains of *H. austriacum* with strong statistical support (ML/BI = 100/1.00).

Taxonomy

Helminthosporium nabanhensis **Jing W. Liu & Jian Ma, sp. nov.**
IndexFungorum No: 559980
Fig. 2

Etymology. Referring to the collecting site of Nabanhe Nature Reserve in Yunnan Province, China.

Holotypus. HJAUP M2054.

Description. Saprobic on dead branches. ***Sexual morph*****:** Undetermined. ***Asexual morph*****:** Hyphomycetous. ***Colonies*** on natural substrate effuse, scattered, hairy, brown to black. ***Mycelium*** partly superficial, partly immersed in the substratum, composed of branched, septate, pale brown to brown, smooth hyphae. ***Conidiophores*** macronematous, mononematous, solitary or in groups of 2–4, simple, occasionally branched, erect, straight or flexuous, cylindrical, smooth, 8–21-septate, brown to dark brown, paler towards the apex, with well-defined small pores at the apex and rarely laterally beneath the upper 1–3 septa, 365–557 × 6.5–13.5 μm. ***Conidiogenous cells*** polytretic, integrated, terminal and intercalary, cylindrical, brown, smooth, with noncicatrized, distinct pores. Conidial secession schizolytic. ***Conidia*** acropleurogenous, solitary, dry, obclavate, pale brown to brown, 3–6-distoseptate, smooth, straight or curved, wider below than apex, truncate and dark at base, apically rostrate and pale, guttulate when young, non-guttulate at maturity, 26.5–46.5 μm long, 6.5–10 μm wide, tapering to 3–3.5 μm wide near the apex, 3–6 μm wide at the basal scar.

Cultural characteristics. Colony on PDA reaching 50–55 mm diam. after 2 weeks in an incubator under dark conditions at 25 °C, irregular circular, surface velvety, with white and denser mycelium at the center, becoming olivaceous and sparser towards the edge; reverse pale brown at the center, dark brown at the periphery.

Material examined. China, Yunnan Province: Xishuangbanna Dai Autonomous Prefecture, Nabanhe National Nature Reserve, on dead branches of an unidentified broadleaf tree, 12 July 2021, J.W. Liu, HJAUP M2054 (Holotype), ex-type living culture HJAUP C2054.

Notes. The phylogenetic tree shows that the strain of *H. nabanhensis* (HJAUP C2054) clusters with the ex-type strain of *H. chlorophorae* (BRIP 14521). The BLASTn analysis of ITS of our ex-type strain HJAUP C2054 showed 90% identity (425/471 bp, 10/471 gaps) with ex-type strain BRIP 14521 of *H. chlorophorae*. Moreover, *H. nabanhensis* morphologically differs from *H. chlorophorae* in bigger conidiophores (365–557 × 6.5–13.5 μm vs. 120–270 × 7–10 μm) occasionally branched, and smaller conidia (26.5–46.5 × 6.5–10 μm vs. 52–102 × 8–11 μm) with fewer septa (3–6 vs. 6–9), and from *H. sichuanense* (Zhang et al. 2004) in narrower conidiophores (6.5–13.5 μm vs. 14–25 μm) and smaller conidia (26.5–46.5 × 6.5–10 μm vs. 41–86 × 10–14 μm) with fewer septa (3–6 vs. 5–11).

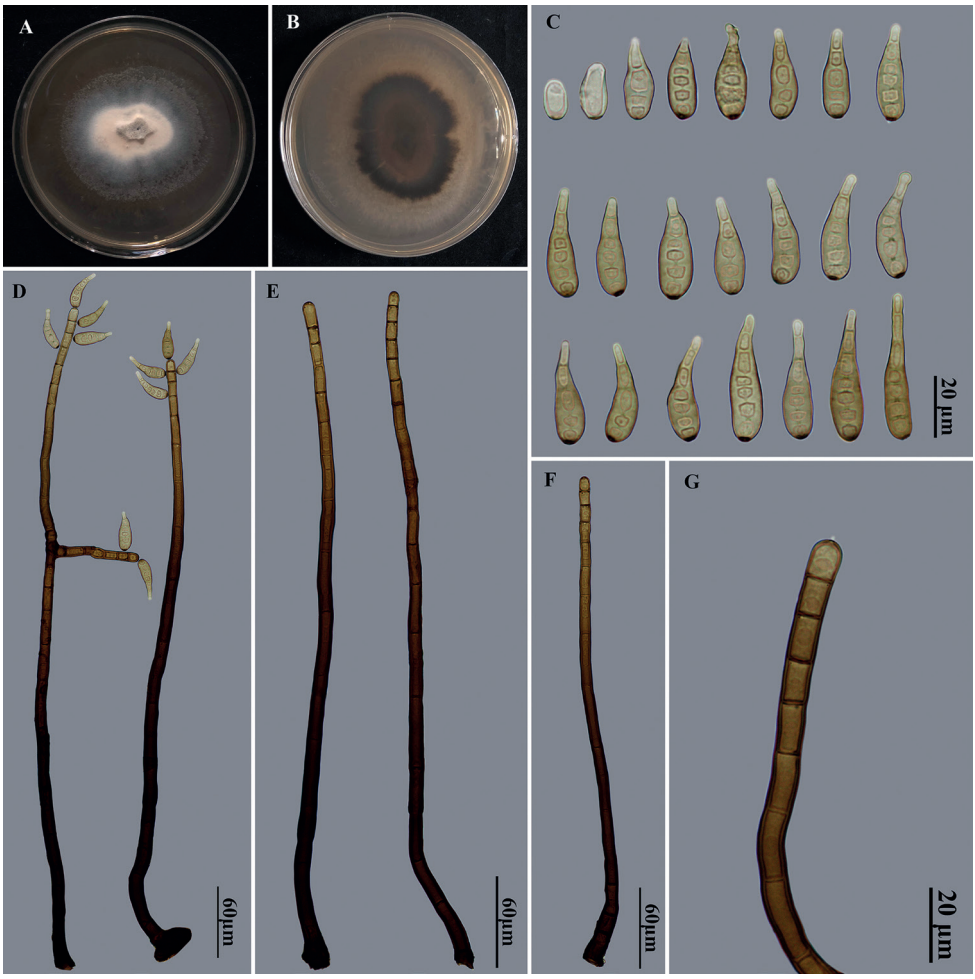

Figure 2. *Helminthosporium nabanhensis* (HJAUPM2054, holotype) **A, B** culture on PDA from above and reverse **C** conidia **D** conidiophores with conidia **E–G** conidiophores with conidiogenous cells.

Helminthosporium sinensis **Jing W. Liu & Jian Ma, sp. nov.**
IndexFungorum No: 559981
Fig. 3

Etymology. Referring to the country in which the fungus was collected.

Holotypus. HJAUP M2121.

Description. Saprobic on dead branches. ***Sexual morph*****:** Undetermined. ***Asexual morph*****:** Hyphomycetous. ***Colonies*** on natural substrate effuse, scattered, hairy, brown to black. ***Mycelium*** partly superficial, partly immersed in the substratum, composed of branched, septate, pale brown to brown, smooth hyphae.

Figure 3. *Helminthosporium sinensis* (HJAUPM2121, holotype) **A, B** culture on PDA from above and reverse **C** conidia **D, E** conidiophores, conidiogenous cells and conidia **F** conidiophores.

Conidiophores macronematous, mononematous, solitary or in groups of 2–4, simple, straight or flexuous, thick-walled, cylindrical, smooth, brown to dark brown, paler towards the apex, with well-defined small pores at the apex and rarely laterally beneath the upper 1–4 septa, 220–370 × 6–8.5 μm. ***Conidiogenous cells*** polytretic, integrated, terminal and intercalary, cylindrical, brown, smooth, with noncicatrized, distinct pores. Conidial secession schizolytic. ***Conidia*** acropleurogenous, solitary, rarely catenate, dry, obclavate, pale brown, 2–7-distoseptate, smooth, straight or curved, wider below than apex, truncate and dark at base, apically rostrate and pale, 37–60 μm long, 5.5–8.5 μm wide, tapering to 3–3.5 μm wide near the apex, 3–6 μm wide at the basal scar.

Cultural characteristics. Colony on PDA reaching 30–37 mm diam. after 2 weeks in an incubator under dark conditions at 25 °C, pale brown, irregular

circular, surface velvety, outermost layer gray; reverse dark brown, produces pale green pigment.

Material examined. China, Yunnan Province: Xishuangbanna Dai Autonomous Prefecture, Menghai County, Mengsong Township, on dead branches of an unidentified broadleaf tree, 13 July 2021, J.W. Liu, HJAUP M2121 (Holotype), ex-type living culture HJAUP C2121.

Notes. Phylogenetic analysis shows that the strain of *H. sinensis* (HJAUP C2121) forms an independent clade, and clusters with the strains of *H. nabanhensis* (HJAUP C2054) and *H. chlorophorae* (BRIP 14521). The BLASTn analysis of ITS of our ex-type strain HJAUP C2121 showed 89% identity (536/602 bp, 17/602 gaps) with ex-type strain HJAUP C2054 of *H. nabanhensis*, and showed 91% identity (430/471 bp, 13/471 gaps) with ex-type strain BRIP 14521 of *H. chlorophorae*. Moreover, *H. sinensis* differs from *H. nabanhensis* by its longer and narrower conidia (37–60 × 5.5–8.5 μm vs. 26.5–46.5 × 6.5–10 μm), and smaller conidiophores (220–370 × 6–8.5 μm vs. 365–557 × 6.5–13.5 μm), and from *H. chlorophorae* by its smaller conidia (37–60 × 5.5–8.5 μm vs. 52–102 × 8–11 μm) and longer and narrower conidiophores (220–370 × 6–8.5 μm vs. 120–270 × 7–10 μm), and from *H. guangxiense* (Zhang and Zhang 2009) in smaller conidiophores (220–370 × 6–8.5 μm vs. 330–850 × 14–25 μm) and smaller conidia (37–60 × 5.5–8.5 μm vs. 76–110 × 16–22 μm) with fewer septa (2–7 vs. 9–17). In addition, the conidia of *H. sinensis* are solitary or rarely catenate, whereas those of *H. guangxiense*, *H. nabanhensis* and *H. chlorophorae* are solitary.

Helminthosporium yunnanensis **Jing W. Liu & Jian Ma, sp. nov.**
IndexFungorum No: 559982
Fig. 4

Etymology. Referring to Yunnan province, where the type specimen was collected.

Holotypus. HJAUP M2071.

Description. Saprobic on dead branches. ***Sexual morph*****:** Undetermined. ***Asexual morph*****:** Hyphomycetous. ***Colonies*** on natural substrate effuse, scattered, hairy, brown to dark brown. Mycelium partly superficial, partly immersed in the substratum, composed of branched, septate, pale brown to brown, smooth hyphae. ***Conidiophores*** macronematous, mononematous, solitary or in groups of 2–4, simple, straight or flexuous, thick-walled, cylindrical, smooth, brown to dark brown, paler towards the apex, with one cylindrical, enteroblastic percurrent extension, and with well-defined small pores at the apex and rarely laterally beneath the upper 1–5 septa, 560–680 × 12.5–15.5 μm. ***Conidiogenous cells*** polytretic, integrated, terminal and intercalary, cylindrical, pale brown to brown, smooth, with noncicatrized, distinct pores. Conidial secession schizolytic. ***Conidia*** acropleurogenous, solitary, dry, obclavate, sigmoid, lunate or uncinate, pale brown, 4–7-distoseptate, smooth, straight or flexuous, wider below than apex, truncate and dark at base, apically rostrate and pale, 30.5–55.5 μm long, 9–11 μm wide, tapering to 2.5–3 μm near the apex, 3–7.5 μm wide at the basal scar.

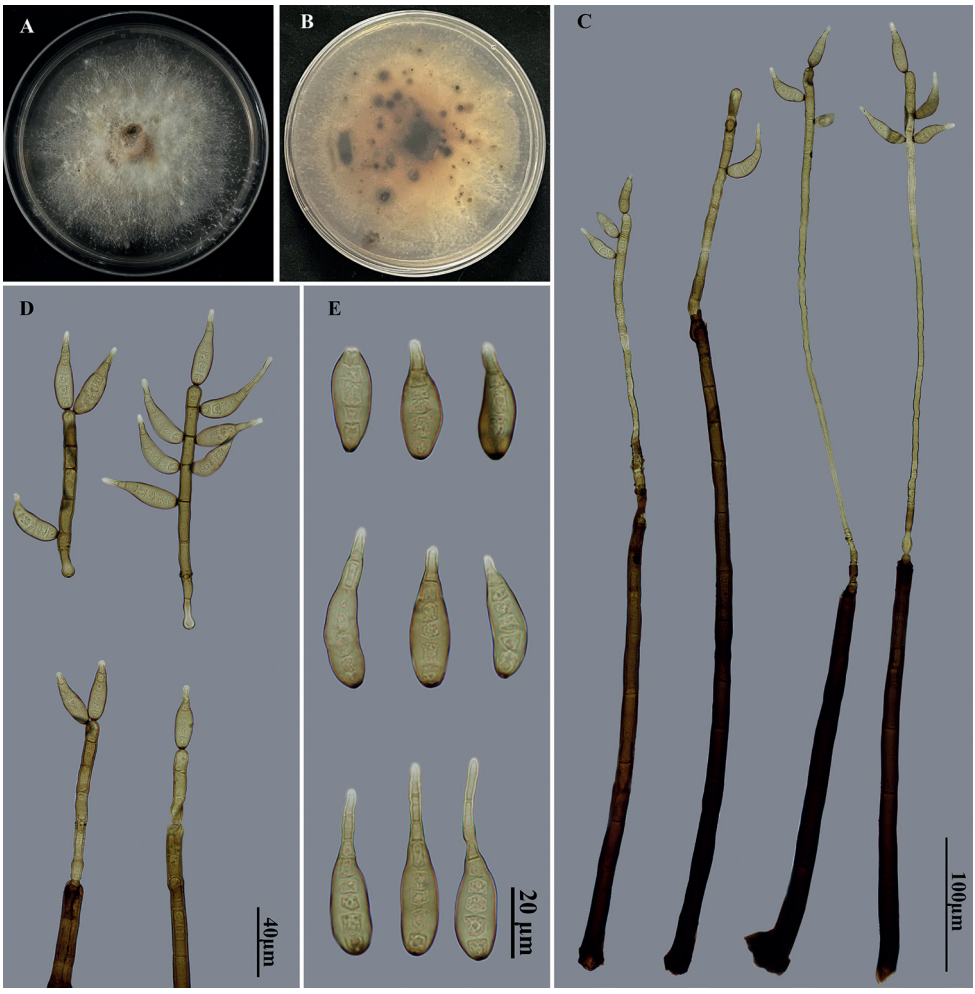

Figure 4. *Helminthosporium yunnanensis* (HJAUPM2071, holotype) **A, B** culture on PDA from above and reverse **C** conidiophores with conidia **D** conidiogenous cells and conidia **E** conidia.

Cultural characteristics. Colony on PDA reaching 75–82 mm diam. after 2 weeks in an incubator under dark conditions at 25 °C, irregular circular, surface velvety, with brown and denser mycelium at the center, becoming white and sparser towards the edge; reverse pale brown at the center, with little black dots.

Material examined. China, Yunnan Province: Xishuangbanna Dai Autonomous Prefecture, Nabanhe National Nature Reserve, on dead branches of an unidentified broadleaf tree, 12 July 2021, J.W. Liu, HJAUP M2071 (Holotype), ex-type living culture HJAUP C2071.

Notes. Phylogenetic analysis shows that the strain of *H. yunnanensis* (HJAUP C2071) clustered together and formed a sister clade with three different strains of *H. austriacum* (L132, L137, L169) (Voglmayr and Jaklitsch 2017). The BLASTn analysis of *H. yunnanensis* (HJAUP C2071) and *H. austriacum* (L132^{HT}) shows 97% identity

(524/541, 4 gaps) using ITS, 99% identity (550/553, 2 gaps) using LSU, 99% identity (872/873, 1 gap) using SSU, 98% identity (738/752, no gap) using *TEF1*, and 98% identity (1077/1095, no gap) using *RPB2*. *Helminthosporium yunnanensis* morphologically differs from *H. austriacum* in wider conidiophores (560–680 × 12.5–15.5 μm vs. 275–700 × 7–11 μm) with one cylindrical, enteroblastic percurrent extension, and narrower conidia (30.5–55.5 × 9–11 μm vs. 35–48 × 13.7–16.5 μm), and from *H. obpyriforme* (Zhang and Zhang 2009) in bigger conidiophores (560–680 × 12.5–15.5 μm vs. 225–460 × 9.5–13 μm) and smaller conidia (30.5–55.5 × 9–11 μm vs. 47–74 × 14–19 μm) with fewer septa (4–7 vs. 5–9).

Discussion

The taxonomic history of the genus *Helminthosporium* is complex. To date, about 770 epithets for *Helminthosporium* are listed in Index Fungorum (2022), but most of these were not congeneric with the generic type. Konta et al. (2021) listed 216 *Helminthosporium* species based on records from Species Fungorum, but most species are identified based on morphological studies, and so far only 27 species are represented by a DNA sequence in GenBank (Voglmayr and Jaklitsch 2017; Boonmee et al. 2021; Chen et al. 2022). Morphological comparison is important for fungal identification, but species identification only based on morphological studies is not comprehensive. With the availability of supplementary sequence data for *Helminthosporium* species, the molecular phylogenetic analysis is being used to evaluate previously described *Helminthosporium*-like species by molecular methods. The introduction of a phylogenetic analysis of *Helminthosporium* led to a better improvement of the heterogeneity of the genus and further clarified the taxonomic status of *Helminthosporium*. Voglmayr and Jaklitsch (2017) revisited *Corynespora*, *Exosporium* and *Helminthosporium*, with phylogenetic and morphological analyses. Zhang et al. (2020b) transferred *H. bigenum* into a new genus *Mirohelminthosporium* K. Zhang, D.W. Li & R.F. Castañeda and replaced the illegitimate *H. cylindrosporum* Matsush. with *H. matsushimae*. Chen et al. (2022) suggested four *Helminthosporium* species, *H. anomalum*, *H. asterinum*, *H. decacuminatum* and *H. gibberosporum* to *Bipolaris*, *Kirschsteiniothelia* or *Curvularia* by performing blastn analysis. Furthermore, seven new species were described under the genus *Helminthosporium* by molecular methods (Crous et al. 2018, 2019; Zhao et al. 2018; Boonmee et al. 2021; Chen et al. 2022). Based on previous studies, we proposed three new species by morphological and molecular phylogenetic analysis.

Chen et al. (2022) described two new species, *H. chengduense* and *H. chinense*, based on combined ITS, LSU, SSU, *TEF1* and *RPB2* sequence data and morphological characters. Accordingly, we also used ITS, LSU, SSU, *TEF1* and *RPB2* for phylogenetic analysis and obtained high phylogenetic support, although our two species, *H. nabanhensis* and *H. sinensis*, lack the *RPB2* sequences. They are considerably distinct from all other described *Helminthosporium* species by morphological characters and multi-locus phylogenetic analysis, so we are convinced that the newly introduced species are new to science.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (Nos. 31970018, 32160006, 31360011).

References

Alcorn JL (1983) Generic concepts in *Drechslera*, *Bipolaris* and *Exserohilum*. Mycotaxon 17: 1–86.

Alves-Barbosa M, Costa PMO, Malosso E, Castañeda-Ruíz RF (2017) Two new species of *Dictyosporium* and *Helminthosporium* from the Brazilian Atlantic Forest. Nova Hedwigia 105(1–2): 65–73. https://doi.org/10.1127/nova_hedwigia/2017/0401

Boonmee S, Wanasinghe DN, Calabon MS, Huanraluek N, Chandrasiri SKU, Jones GEB, Rossi W, Leonardi M, Singh SK, Rana S, Singh PN, Maurya DK, Lagashetti AC, Choudhary D, Dai YC, Zhao CL, Mu YH, Yuan HS, He SH, Phookamsak R, Jiang HB, Martín P, Dueñas M, Telleria MT, Kałucka IL, Jagodziński AM, Liimatainen K, Pereira DS, Phillips AJL, Suwannarach N, Kumla J, Khuna S, Lumyong S, Potter TB, Shivas RG, Sparks AH, Vaghef N, Abdel-Wahab MA, Abdel-Aziz FA, Li GJ, Lin WF, Singh U, Bhatt RP, Lee HB, Nguyen TTT, Kirk PM, Dutta AK, Acharya K, Sarma VV, Niranjan M, Rajeshkumar KC, Ashtekar N, Lad S, Wijayawardene NN, Bhat DJ, Xu RJ, Wijesinghe SN, Shen HW, Luo ZL, Zhang JY, Sysouphanthong P, Thongklang N, Bao DF, Aluthmuhandiram JVS, Abdollahzadeh J, Javadi A, Dovana F, Usman M, Khalid AN, Dissanayake AJ, Telagathoti A, Probst M, Peintner U, Garrido-Benavent I, Bóna L, Merényi Z, Boros L, Zoltán B, Stielow JB, Jiang N, Tian CM, Shams E, Dehghanizadeh F, Pordel A, Javan-Nikkhah M, Denchev TT, Denchev CM, Kemler M, Begerow D, Deng CY, Harrower E, Bozorov T, Kholmuradova T, Gaforov Y, Abdurazakov A, Xu JC, Mortimer PE, Ren GC, Jeewon R, Maharachchikumbura SSN, Phukhamsakda C, Mapook A, Hyde KD (2021) Fungal diversity notes 1387–1511: Taxonomic and phylogenetic contributions on genera and species of fungal taxa. Fungal Diversity 111(1): 1–335. https://doi.org/10.1007/s13225-021-00489-3

Cao M, Zhang JH (1997) Tree species diversity of tropical forest vegetation in Xishuangbanna, SW China. Biodiversity and Conservation 6(7): 995–1006. https://doi.org/10.1023/A:1018367630923

Chen YP, Tian WH, Guo YB, Madrid H, Maharachchikumbura SSN (2022) *Synhelminthosporium* gen. et sp. nov. and two new species of *Helminthosporium* (Massarinaceae, Pleosporales) from Sichuan Province, China. Journal of Fungi 8(7): 712. https://doi.org/10.3390/jof8070712

Crous PW, Wingfield MJ, Burgess TI, Hardy GESJ, Gené J, Guarro J, Baseia IG, García D, Gusmão LFP, Souza-Motta CM, Thangavel R, Adamčík S, Barili A, Barnes CW, Bezerra JDP, Bordallo JJ, Cano-Lira JF, de Oliveira RJV, Ercole E, Hubka V, Iturrieta-González I, Kubátová A, Martín MP, Moreau P-A, Morte A, Ordoñez ME, Rodríguez A, Stchigel AM, Vizzini A, Abdollahzadeh J, Abreu VP, Adamčíková K, Albuquerque GMR, Alexandrova AV, Álvarez Duarte E, Armstrong-Cho C, Banniza S, Barbosa RN, Bellanger J-M, Bezerra JL, Cabral TS, Caboň M, Caicedo E, Cantillo T, Carnegie AJ, Carmo LT, Castañeda-Ruiz RF, Clement CR, Čmoková A, Conceição LB, Cruz RHSF, Damm U, da Silva BDB, da Silva GA, da Silva RMF, Santiago ALCMA, de Oliveira LF, de Souza CAF, Déniel F, Dima B, Dong G, Edwards J, Félix CR, Fournier J, Gibertoni TB, Hosaka K, Iturriaga T, Jadan M, Jany J-L, Jurjević Ž, Kolařík

M, Kušan I, Landell MF, Leite Cordeiro TR, Lima DX, Loizides M, Luo S, Machado AR, Madrid H, Magalhães OMC, Marinho P, Matočec N, Mešić A, Miller AN, Morozova OV, Neves RP, Nonaka K, Nováková A, Oberlies NH, Oliveira-Filho JRC, Oliveira TGL, Papp V, Pereira OL, Perrone G, Peterson SW, Pham THG, Raja HA (2018) Fungal Planet description sheets: 716–784. Persoonia 40(1): 240–393. https://doi.org/10.3767/persoonia.2018.40.10

Crous PW, Carnegie AJ, Wingfield MJ, Sharma R, Mughini G, Noordeloos ME, Santini A, Shouche YS, Bezerra JDP, Dima B, Guarnaccia V, Imrefi I, Jurjević Ž, Knapp DG, Kovács GM, Magistà D, Perrone G, Rämä T, Rebriev YA, Shivas RG, Singh SM, Souza-Motta CM, Thangavel R, Adhapure NN, Alexandrova AV, Alfenas AC, Alfenas RF, Alvarado P, Alves AL, Andrade DA, Andrade JP, Barbosa RN, Barili A, Barnes CW, Baseia IG, Bellanger J-M, Berlanas C, Bessette AE, Bessette AR, Biketova AYu, Bomfim FS, Brandrud TE, Bransgrove K, Brito ACQ, Cano-Lira JF, Cantillo T, Cavalcanti AD, Cheewangkoon R, Chikowski RS, Conforto C, Cordeiro TRL, Craine JD, Cruz R, Damm U, de Oliveira RJV, de Souza JT, de Souza HG, Dearnaley JDW, Dimitrov RA, Dovana F, Erhard A, Esteve-Raventós F, Félix CR, Ferisin G, Fernandes RA, Ferreira RJ, Ferro LO, Figueiredo CN, Frank JL, Freire KTLS, García D, Gené J, Gęsiorska A, Gibertoni TB, Gondra RAG, Gouliamova DE, Gramaje D, Guard F, Gusmão LFP, Haitook S, Hirooka Y, Houbraken J, Hubka V, Inamdar A, Iturriaga T, Iturrieta-González I, Jadan M, Jiang N, Justo A, Kachalkin AV, Kapitonov VI, Karadelev M, Karakehian J, Kasuya T, Kautmanová I, Kruse J, Kušan I, Kuznetsova TA, Landell MF, Larsson K-H, Lee HB, Lima DX, Lira CRS, Machado AR, Madrid H, Magalhães OMC, Majerova H, Malysheva EF, Mapperson RR, Marbach PAS, Martín MP, Martín-Sanz A, Matočec N, McTaggart AR, Mello JF, Melo RFR, Mešić A, Michereff SJ, Miller AN, Minoshima A, Molinero-Ruiz L, Morozova OV, Mosoh D, Nabe M, Naik R, Nara K, Nascimento SS, Neves RP, Olariaga I, Oliveira RL, Oliveira TGL, Ono T, Ordoñez ME de M, Ottoni A, Paiva LM, Pancorbo F, Pant B, Pawłowska J, Peterson SW, Raudabaugh DB, Rodríguez-Andrade E, Rubio E, Rusevska K, Santiago ALCMA, Santos ACS, Santos C, Sazanova NA, Shah S, Sharma J, Silva BDB, Siquier JL, Sonawane MS, Stchigel AM, Svetasheva T, Tamakeaw N, Telleria MT, Tiago PV, Tian CM, Tkalčec Z, Tomashevskaya MA, Truong HH, Vecherskii MV, Visagie CM, Vizzini A, Yilmaz N, Zmitrovich IV, Zvyagina EA, Boekhout T, Kehlet T, Læssøe T, Groenewald JZ (2019) Fungal Planet description sheets: 868–950. Persoonia 42: 291–473. https://doi.org/10.3767/persoonia.2019.42.11

Ellis MB (1961) Dematiaceous hyphomycetes. III. Mycological Papers 82: 1–55.

Ellis MB (1971) Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England, 608 pp. https://doi.org/10.1079/9780851986180.0000

Goh TK (1999) Single-spore isolation using a hand-made glass needle. Fungal Diversity 2: 47–63.

Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS (2017) UFBoot2: Improving the ultrafast bootstrap approximation. Molecular Biology and Evolution 35(2): 518–522. https://doi.org/10.1093/molbev/msx281

Index Fungorum (2022) Fungal names search. http://www.indexfungorum.org/names/Names.asp

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017) ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods 14(6): 587–589. https://doi.org/10.1038/nmeth.4285

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Konta S, Hyde KD, Karunarathna SC, Mapook A, Senwanna C, Dauner LAP, Nanayakkara CM, Xu JC, Tibpromma S, Lumyong S (2021) Multi-gene phylogeny and morphology reveal *Haplohelminthosporium* gen. nov. and *Helminthosporiella* gen. nov. associated with palms in Thailand and a checklist for *Helminthosporium* reported worldwide. Life 11(5): 454. https://doi.org/10.3390/life11050454

Link JHF (1809) Observationes in ordines plantarum naturales. Dissertatio 1. Magazin der Gesellschaft Naturforschender Freunde zu Berlin 3: 3–42.

Luttrell ES (1963) Taxonomic criteria in *Helminthosporium* 11. Mycologia 55(5): 643–674. https://doi.org/10.1080/00275514.1963.12018057

Luttrell ES (1964) Systematics of *Helminthosporium* and related genera. Mycologia 56(1): 119–132. https://doi.org/10.1080/00275514.1964.12018090

Ma J, Wang Y, Ma LG, Zhang YD, Castañeda-Ruíz RF, Zhang XG (2011) Three new species of *Neosporidesmium* from Hainan, China. Mycological Progress 10(2): 157–162. https://doi.org/10.1007/s11557-010-0685-2

Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution 32(1): 268–274. https://doi.org/10.1093/molbev/msu300

Rayner RW (1970) A mycological colour chart. British Mycological Society. Commonwealth Mycological Institute.

Rehner S (2001) Primers for Elongation Factor 1-α (EF1-α). http://ocid.nacse.org/research/deephyphae/EF1primer.pdf

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Seifert K, Morgan-Jones G, Gams W, Kendrick B (2011) The genera of hyphomycetes. CBS Biodiversity Series 9: 1–997.

Shirouzu T, Harada Y (2008) Lignicolous dematiaceous hyphomycetes in Japan: Five new records for Japanese mycoflora, and proposals of a new name, *Helminthosporium magnisporum*, and a new combination, *Solicorynespora foveolata*. Mycoscience 49(2): 126–131. https://doi.org/10.1007/S10267-007-0399-8

Siboe GM, Kirk PM, Cannon PF (1999) New dematiaceous hyphomycetes from Kenyan rare plants. Mycotaxon 73: 283–302.

Sivanesan A (1987) Graminicolous species of *Bipolaris*, *Curvularia*, *Exserohilum* and their teleomorphs. Mycological Papers 158: 1–261. https://doi.org/10.2307/3759472

Tanaka K, Hirayama K, Yonezawa H, Sato G, Toriyabe A, Kudo H, Hashimoto A, Matsumura M, Harada Y, Kurihara Y, Shirouzu T, Hosoya T (2015) Revision of the Massarineae (Pleosporales, Dothideomycetes). Studies in Mycology 82(1): 75–136. https://doi.org/10.1016/j.simyco.2015.10.002

Tian Q, Li WJ, Hyde KD, Camporesi E, Bhat DJ, Chomnunti P, Xu JC (2017) Molecular taxonomy of five species of microfungi on *Alnus* spp. from Italy. Mycological Progress 17(1–2): 255–274. https://doi.org/10.1007/s11557-017-1336-7

Voglmayr H, Jaklitsch WM (2017) *Corynespora*, *Exosporium* and *Helminthosporium* revisited – New species and generic reclassification. Studies in Mycology 87(1): 43–76. https://doi.org/10.1016/j.simyco.2017.05.001

Voglmayr H, Akulov OY, Jaklitsch WM (2016) Reassessment of *Allantonectria*, phylogenetic position of *Thyronectroidea*, and *Thyronectria caraganae* sp. nov. Mycological Progress 15(9): 921–937. https://doi.org/10.1007/s11557-016-1218-4

Wang XJ, Wu HY, Zhang M (2014) A new species of *Helminthosporium* from Jiangsu, China. Mycotaxon 127(1): 1–4. https://doi.org/10.5248/127.1

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR protocols: a guide to methods and applications. Academic Press Inc, New York, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Xia JW, Ma YR, Li Z, Zhang XG (2017) *Acrodictys*-like wood decay fungi from southern China, with two new families Acrodictyaceae and Junewangiaceae. Scientific Reports 7(1): 7888. https://doi.org/10.1038/s41598-017-08318-x

Zhang TY, Sun GY (2010) Flora fungorum sinicorum. Vol. 30. Helminthosporioid hyphomycetes. Science Press, Beijing, 1–272. https://doi.org/10.3724/SP.J.1142.2010.20137

Zhang M, Zhang TY (2009) Taxonomic studies of *Helminthosporium* from China 4. Six new species and a key to *Helminthosporium* from China. Mycotaxon 109(1): 399–413. https://doi.org/10.5248/109.399

Zhang M, Zhang TY, Wu WP (2004) Taxonomic studies of *Helminthosporium* from China II. Two new species in Sichuan Province. Junwu Xuebao 23(2): 179–182.

Zhang M, Zhang TY, Wu WP (2007) Taxonomic studies of *Helminthosporium* from China III. Three new species in Guangdong Province. Mycotaxon 99: 137–142.

Zhang M, Wu HY, Wang ZY (2010) Taxonomic studies of *Helminthosporium* from China 5. Two new species in Hunan and Sichuan Province. Mycotaxon 113(1): 95–99. https://doi.org/10.5248/113.95

Zhang D, Gao FL, Jakovlić I, Zou H, Zhang J, Li WX, Wang GT (2020a) PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Molecular Ecology Resources 20(1): 348–355. https://doi.org/10.1111/1755-0998.13096

Zhang K, Zhang H, Li DW, Castañeda-Ruíz RF (2020b) *Mirohelminthosporium* gen. nov. for an atypical *Helminthosporium* species and *H. matsushimae* nom. nov. Mycotaxon 135(4): 777–783. https://doi.org/10.5248/135.777

Zhao GC, Zhao RL (2012) The Higher Microfungi from Forests of Yunnan Province. Yunnan Science and Technology Press, Kunming, 572 pp.

Zhao N, Luo ZL, Hyde KD, Su HY, Bhat DJ, Liu JK, Bao DF, Hao YE (2018) *Helminthosporium submersum* sp. nov. (Massarinaceae) from submerged wood in north-western Yunnan Province. China. Phytotaxa 348(4): 269–278. https://doi.org/10.11646/phytotaxa.348.4.3

Zhu D, Luo ZL, Baht DJ, McKenzie EC, Bahkali AH, Zhou DQ, Su HY, Hyde KD (2016) *Helminthosporium velutinum* and *H. aquaticum* sp. nov. from aquatic habitats in Yunnan Province, China. Phytotaxa 253(3): 179–190. https://doi.org/10.11646/phytotaxa.253.3.1

MycoKeys 94: 91–108 (2022)
doi: 10.3897/mycokeys.94.89425
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Morphology, phylogeny, mitogenomics and metagenomics reveal a new entomopathogenic fungus *Ophiocordyceps nujiangensis* (Hypocreales, Ophiocordycipitaceae) from Southwestern China

Tao Sun[1,2*], Weiqiu Zou[1*], Quanying Dong[1,2], Ou Huang[1], Dexiang Tang[1,2], Hong Yu[1]

1 *Yunnan Herbal Laboratory, College of Ecology and Environmental Sciences, Yunnan University, Kunming 650504, Yunnan, China* **2** *School of Life Sciences, Yunnan University, Kunming 650504, Yunan, China*

Corresponding author: Hong Yu (hongyu@ynu.edu.cn, herbfish@163.com)

Academic editor: Francesco Dal Grande | Received 23 June 2022 | Accepted 4 December 2022 | Published 21 December 2022

Citation: Sun T, Zou W, Dong Q, Huang O, Tang D, Yu H (2022) Morphology, phylogeny, mitogenomics and metagenomics reveal a new entomopathogenic fungus *Ophiocordyceps nujiangensis* (Hypocreales, Ophiocordycipitaceae) from Southwestern China. MycoKeys 94: 91–108. https://doi.org/10.3897/mycokeys.94.89425

Abstract

Ophiocordyceps contains the largest number of *Cordyceps sensu lato*, various species of which are of great medicinal value. In this study, a new entomopathogenic fungus, *Ophiocordyceps nujiangensis*, from Yunnan in southwestern China, was described using morphological, phylogenetic, and mitogenomic evidence, and its fungal community composition was identified. It was morphologically characterized by a solitary, woody, and dark brown stromata, smooth-walled and septate hyphae, solitary and gradually tapering conidiogenous cells with plenty of warty protrusions, and oval or fusiform conidia (6.4–11.2 × 3.7–6.4 μm) with mucinous sheath. The phylogenetic location of *O. nujiangensis* was determined based on the Bayesian inference (BI) and the maximum likelihood (ML) analyses by concatenating nrSSU, nrLSU, *tef-1a*, *rpb1*, and *rpb2* datasets, and ten mitochondrial protein-coding genes (PCGs) datasets (*atp6*, *atp9*, *cob*, *cox2*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, and *nad5*). Phylogenetic analyses revealed that *O. nujiangensis* belonged to the *Hirsutella sinensis* subclade within the *Hirsutella* clade of *Ophiocordyceps*. And *O. nujiangensis* was phylogenetically clustered with *O. karstii*, *O. liangshanensis*, and *O. sinensis*. Simultaneously, five fungal phyla and 151 fungal genera were recognized in the analysis of the fungal community of *O. nujiangensis*. The fungal community composition differed from that of *O. sinensis*, and differences in the microbial community composition of closely related species might be appropriate as further evidence for taxonomy.

* These authors contributed equally to this work.

Keywords
microbial community, mitochondrial genome, new species, *Ophiocordyceps nujiangensis*, phylogenetic analyses

Introduction

The genus *Ophiocordyceps* was introduced by Petch (1931), with *O. blattae* Petch as the type. This genus accommodated species with features of head-cover asci, septate and non-disarticulating ascospores (Petch 1931). Then, the genus was regarded as a subgenus of *Cordyceps* (Kobayasi 1941, 1982; Mains 1958). Until 2007, Sung et al. (2007) erected a new family Ophiocordycipitaceae based on phylogenetic analysis and the characteristics of darkly pigmented stromata, which were pliant to wiry or fibrous to tough in texture. And they revised the classification of *Ophiocordyceps*, treating it as the type genus of Ophiocordycipitaceae. *Ophiocordyceps* has the largest number of species in Ophiocordycipitaceae, with 307 species named in Ophiocordyceps to date. (http://www.indexfungorum.org/, retrieval on November 3, 2022).

The methods of morphology and phylogeny were utilized for species identification, and the phylogenetic analyses based on concatenating nrSSU, nrLSU, *tef-1α*, *rpb1*, and *rpb2* datasets became the popular means (Sung et al. 2007; Quandt et al. 2014; Sanjuan et al. 2015; Wang et al. 2020a). Moreover, the mitochondrial genome had been an effective instrument for studying species' origin, classification, and evolution due to its advantages of high copy number, low mutation rate, and fast evolution rate (Alexeyev et al. 2013; Aguileta et al. 2014; Williams et al. 2014). The significant difference in the mitochondrial genome of fungi could be distinguished (Nie et al. 2019). The biogenetic analyses of the fungal mitochondrial genome could verify the genetically related species. NCBI has published the mitochondrial genomes of more than 680 fungi, including approximately 60 species of Hypocreales (Chen et al. 2021; Zhao et al. 2021).

Some species in *Ophiocordyceps* have enormous medicinal and commercial value, such as *O. sinensis*, traditional in Chinese medicine. Owing to their extraordinary efficacy, wild sources were widely sold as commodities and gradually became scarce. (Han et al. 2019; Dai et al. 2020). Therefore, seeking additional new resources would defuse the tense situation. For example, *O. lanpingensis* and *O. xuefengensis* had been authenticated as possessing ingredients that were beneficial for health and considered to be desirable alternatives for *O. sinensis* (Zou et al. 2017; Zhang et al. 2017). *Ophiocordyceps* is widely distributed in China, and of particular note are some recent reports of new species from southwestern China (Wang et al. 2018; Wang et al. 2020b; Chen et al. 2021).

The companion fungi were essential for the growth and development of the host. For example, *Tuber*-associated microbial communities played a potentially important role in mycelial growth, ascocarp development, and mycorrhizal synthesis of *Tuber* (Li et al. 2018). And adding *Grifola* sp. in the cultivation process of *G. umbellate* could promote sclerotia formation (Guo et al. 2002). Thus, the composition and diversity of companion fungi should be analyzed to gain insight into new species and their microbial resources.

In this study, a new species of *Ophiocordyceps*, which parasitized on the larvae of Hepialidae, was collected from Yunnan in southwestern China. The phylogenetic location was elucidated based on the Bayesian inference (BI) and the maximum likelihood (ML) analyses by concatenating nrSSU, nrLSU, *tef-1a*, *rpb1*, and *rpb2* datasets, and mitochondrial protein-coding genes (PCGs) datasets. Morphological characteristics were observed and recorded. The composition and diversity of the fungal communities hosting the new species were identified.

Methods

Sample collection and isolation

Samples were collected on Hepialidae larvae in the soil in Yajiaoluo (27°07'48"N, 98°52'12"E), Fugong County, Nujiang Prefecture, Yunnan Province, China. Specimens were photographed in the fields with a Canon 750D digital camera. The fresh specimens were placed into the sterile culture dish, then transferred to the laboratory and deposited in the Yunnan Herbal Herbarium (**YHH**), Yunnan University.

Specimens were isolated and cultured using the tissue isolating method (Yin and Zhang 2015; Wang et al. 2020b) as follows. Specimens were dipped into 75% alcohol for 2 min to sterilize the surface and then washed with sterile water. The 2–3 mm sclerotium was ripped by tweezers and put on the culture medium (200 g potato, 20 g dextrose 20, 15–20 g agar, 10 g yeast extract, 5 g peptone in 1 L sterile water) (Xu et al. 2019), with three replications. Then they were transferred to the room at 25 °C for culturing. The cultures were deposited in the Yunnan Fungal Culture Collection (**YFCC**), at Yunnan University.

Morphological observations

A moderate quantity of pure cultures was picked by an inoculating needle onto the center of the culture medium and maintained at 25 °C. After 6–10 weeks, shape, size, texture, and color were photographed with a Canon 750D camera. The superficial pure cultures were lightly stuck on transparent adhesive tapes, then the tapes were patched on slides, and the slides were placed on the Olympus BX53 microscope for micro-morphological observations and measurements (Wang et al. 2020a; Wang et al. 2020b).

DNA extraction, PCR amplification, and sequencing of nuclear genes

The genomic DNA of the samples (containing specimens and pure cultures) was isolated using the ZR Fungal DNA kit (Zymo, California, USA), then the DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, USA). The nrSSU and nrLSU (nuclear ribosomal small and large subunits), *rpb1* and *rpb2* (the largest and second-largest subunit sequences of RNA polymerase II), and *tef-1α* (the

translation elongation factor 1α) regions were amplified with the primer pairs used by Wang et al. (2020b). The PCR mixtures contained 2 × Taq PCR Master Mix (Tiangen, Beijing, China) 25 μL, forward primer (10 μM) 0.5 μL, reverse primer (10 μM) 0.5 μL, template DNA (1 ng/μL) 1 μL, and finally added sterile ddH_2O up to 50 μL. Finally, the PCR amplification and sequencing were performed as described by Wang et al. (2015).

Sequencing, assembly, and annotation of mitogenome

The genomic DNA of the pure cultures was isolated through the above-mentioned method, the extracted DNA was transported to BGI genomics Co., Ltd (Wuhan, China) for sequencing. The sequencing library was built by the IlluminaTruseq DNA Sample Preparation Kit (BGI, Shenzhen, China), and the Illumina HiSeq 4000 Platform was applied to the PE2 × 150 bp sequencing. After data quality control, the unpaired, short, and low-quality reads were removed, and the clean reads were obtained (Zhao et al. 2021). Next, the reads of the mitogenome were collected from the clean data employing GetOrganelle v.1.6.2e, and the mitogenome was assembled using BLAST 2.2.30 and SPAdes. V.3.13.0. The mitogenome was initially annotated by MFannot (https://megasun.bch.umontreal.ca/RNAweasel/, accessed on 10 December 2020) and MITOS (http://mitos2.bioinf.uni-leipzig.de/index.py, accessed on 10 December 2020) (Valach et al. 2014; Jin et al. 2020; Chen et al. 2021).

Phylogenetic analyses

For determining the phylogenetic location of the species, phylogenetic analyses were conducted with the combined sequence data of nrSSU, nrLSU, *rpb1*, *rpb2*, and *tef-1α* (Wang et al. 2015; Wang et al. 2020a; Wang et al. 2020b), and ten protein-coding genes (PCGs, *atp6*, *atp9*, *cob*, *cox2*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*) of mitogenomes, respectively (Chen et al. 2021; Zhao et al. 2021). The Bayesian inference (BI) and the maximum likelihood (ML) methods were performed for the phylogenetic analyses by MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and RaxML 7.0.3 (Stamatakis et al. 2008). The GTR + G + I model was determined by jModelTest version 2.1.4 (Darriba et al. 2012) with 10 million generations for the BI analysis. And the ML analysis was run with the GTR + I model on 10,000 rapid bootstrap replicates. *Tolypocladium inflatum* W. Gams and *T. ophioglossoides* (J.F. Gmel.) C.A. Quandt, Kepler & Spatafora were designated as the outgroup taxa for the analysis of nrSSU, nrLSU, *rpb1*, *rpb2*, and *tef-1α* datasets. And *Penicillium citrinum* Thom and *Neurospora crassa* Shear & B.O. Dodge were designated as the outgroup taxa for the analysis of 10 PCGs. The GenBank accession numbers of the 10 PCGs (*atp6*, *atp9*, *cob*, *cox2*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, and *nad5*) annotated from the specimen YFCC8894 were ON868828–ON868837.

Isolation of total DNA, PCR amplification, and high-throughput sequencing

The microbial genomic DNA of the fruiting body from four different specimens (S1–S4) was isolated through the method mentioned above. The ITS (internal transcribed

spacer) regions were amplified with primer pairs ITS5 (5'-GGAAGTAAAAGTCG-TAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), by an ABI GeneAmp 9700 PCR thermocycler (ABI, CA, USA). The PCR amplifications were performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, and single extension at 72 °C for 10 min. The PCR mixtures contained 5 × Fast Pfu buffer 4 μL, 0.4 μL Fast Pfu polymerase, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, template DNA (1ng/μL) 10 μL, and finally added sterile ddH_2O up to 20 μL. The PCR products were extracted from 2% agarose gel and purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, USA) and quantified by Quantus Fluorometer (Promega, Madison, USA).

Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA), following the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Sequence Read Archive (SRA) Accession Number: SAMN28950406–SAMN28950409).

Raw FASTQ files were de-multiplexed using an in-house Perl script, and then quality-filtered by fastp version 0.19.6 (Chen et al. 2018) and merged by FLASH version 1.2.7 (Magoč and Salzberg 2011). Then the optimized sequences were clustered into operational taxonomic units (OTUs) employing UPARSE 7.1 (Edgar 2013) with the 97% sequence similarity level. Chimeric sequences, chloroplast sequences, mitochondrial sequences, and the OTUs identified as Plantae, Rhizaria, Chromista, and those with no rank and unclassified kingdom were removed from samples.

Composition and phylogenetic analysis of microbial communities

Bioinformatic analysis was carried out by the Majorbio Cloud platform (https://cloud.majorbio.com). The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 (Wang et al. 2007) against the ITS gene database (Unite V7.2) through a confidence threshold of 0.7. A phylogenetic tree was constructed to illustrate the relationships between the fungi at the family level, employing FastTree version 2.1.3 (http://www.microbesonline.org/fasttree/) and the ML algorithm (Zhang et al. 2015).

Results

Phylogenetic analyses of nuclear genes

The phylogenetic tree was built with the 72 taxa by the Bayesian inference (BI) and the maximum likelihood (ML) methods. *Tolypocladium inflatum* OSC 71235 and *Tolypocladium ophioglossoides* CBS 100239 were designated as the outgroup taxa (Fig. 1; Suppl. material 1). The five-gene phylogenetic trees based on the BI and the ML analyses had similar topologies. The reconstructed phylogenetic tree of *Ophiocordyceps*

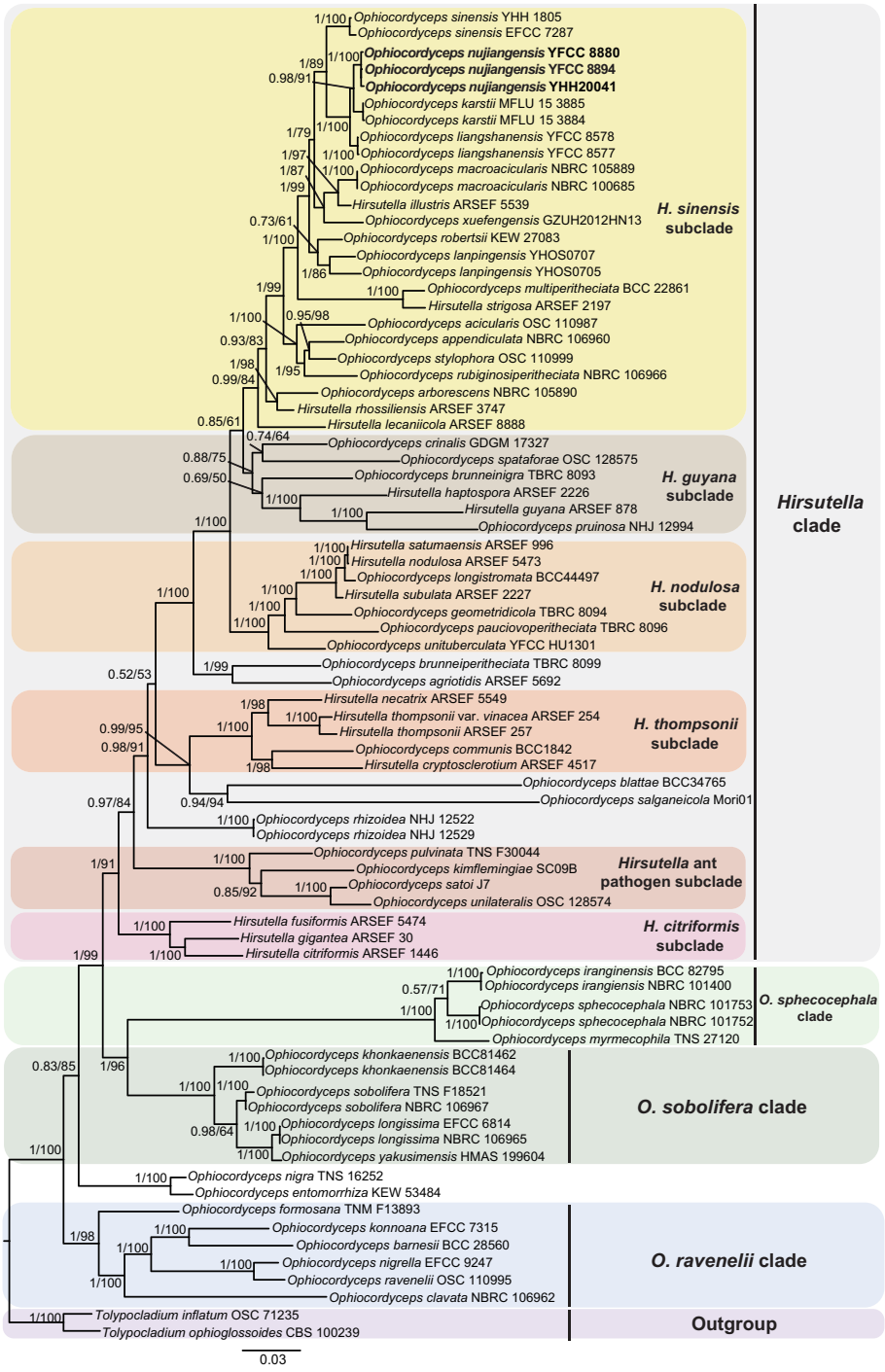

Figure 1. Phylogenetic placement of *Ophiocordyceps nujiangensis* inferred from the Bayesian inference (BI) and the maximum likelihood (ML) analyses by concatenating nrSSU, nrLSU, *tef-1a*, *rpb1*, and *rpb2* datasets. The BI posterior probabilities (≥ 0.5) and the ML bootstrap values (≥ 50%) were indicated at the nodes. The specimens analyzed in this study were shown in bold type.

contained four statistically well-supported clades. And the *Hirsutella* clade had six statistically well-supported subclades. It was similar to the analyses by Sanjuan et al. (2015), Simmons et al. (2015), and Wang et al. (2018). The three specimens of *Ophiocordyceps nujiangensis* (Wild sample YHH20041, pure cultures YFCC 8880, and YFCC 8894) were clustered together and formed a separate clade (the BI posterior probabilities = 1, the ML bootstrap = 98%). *O. nujiangensis* was closely related to *O. karstii*, *O. liangshanensis* and *O. sinensis* with strong support (Fig. 1). The similarities between the YFCC 8880 strain of *O. nujiangensis* and the most relevant were 99.66% (nrSSU), 99.87% (nrLSU), 98.53% (*tef-1*α), 98.53% (*rpb1*) and 98.80% (*rpb2*) in the BALST (The basic local alignment search tool) results of NCBI database. The BALST results of the YFCC 8894 strain were 99.87% (nrLSU), 98.52% (*tef-1*α), and 98.69% (*rpb1*). And the BALST results of sample YHH20041 were 100% (nrSSU), 99.87% (nrLSU), 98.64% (*tef-1*α), 98.36% (*rpb1*) and 98.66% (*rpb2*).

Phylogenetic analyses of mitochondrial genes

The mitogenome of *O. nujiangensis* was assembled and annotated. And 10 PCGs (protein-coding genes) were chosen for the phylogenetic analyses, including 2 subunits of ATP synthase (*atp6* and *atp9*), 1 cytochrome b gene (*cob*), 1 subunit of cytochrome c oxidase (*cox2*), and 6 subunits of NADH dehydrogenase complex (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, and *nad5*). The BI and the ML trees were estimated for phylogenetic analyses of Hypocreales based on the mitochondrial PCG dataset of 55 species from GenBank. *Penicillium citrinum* and *Neurospora crassa* were designated as the outgroup taxa (Suppl. material 2). As shown in Figure 2, six well-supported clades were recognized in Hypocreales, namely Bionectriaceae, Clavicipitaceae, Cordycipitaceae, Hypocreaceae, Nectriaceae, and Ophiocordycipitaceae. And *Ophiocordyceps nujiangensis* was clustered collectively with *O. sinensis*, *H. rhossiliensis*, *H. vermicola*, *O. pingbianensis*, *H. minnesotensis*, and *H. thompsonii* in *Ophiocordyceps*. *O. nujiangensis* formed a separate clade (the BI posterior probabilities = 1, the ML bootstrap = 100%), and was also closely grouped with *O. sinensis* (Fig. 2).

Taxonomy

Ophiocordyceps nujiangensis **H. Yu, T. Sun & W.Q. Zou, sp. nov.**
MycoBank No: MB 844428
Fig. 3

Etymology. Nujiangensis, referring to the collection site of this species, Nujiang.

Holotype. Yajiaoluo, Fugong County, Nujiang Prefecture, Yunnan Province, China. 98°52.20'N, 27°07.80'E, alt 1980 m, on the larvae of Hepialidae in soil, 6 June 2021, Hong Yu (YHH 20039, holotype; YFCC 8880, ex-holotype culture).

Sexual stage. Stromata grew from the head of Hepialidae larva, solitary, certain branches at middle, gradually tapering from base to tip, woody, hard, dark brown (1545C, the number of PANTONE color, https://www.pantone.com), 14.8–18.2 cm long. Microscopic morphology to be determined.

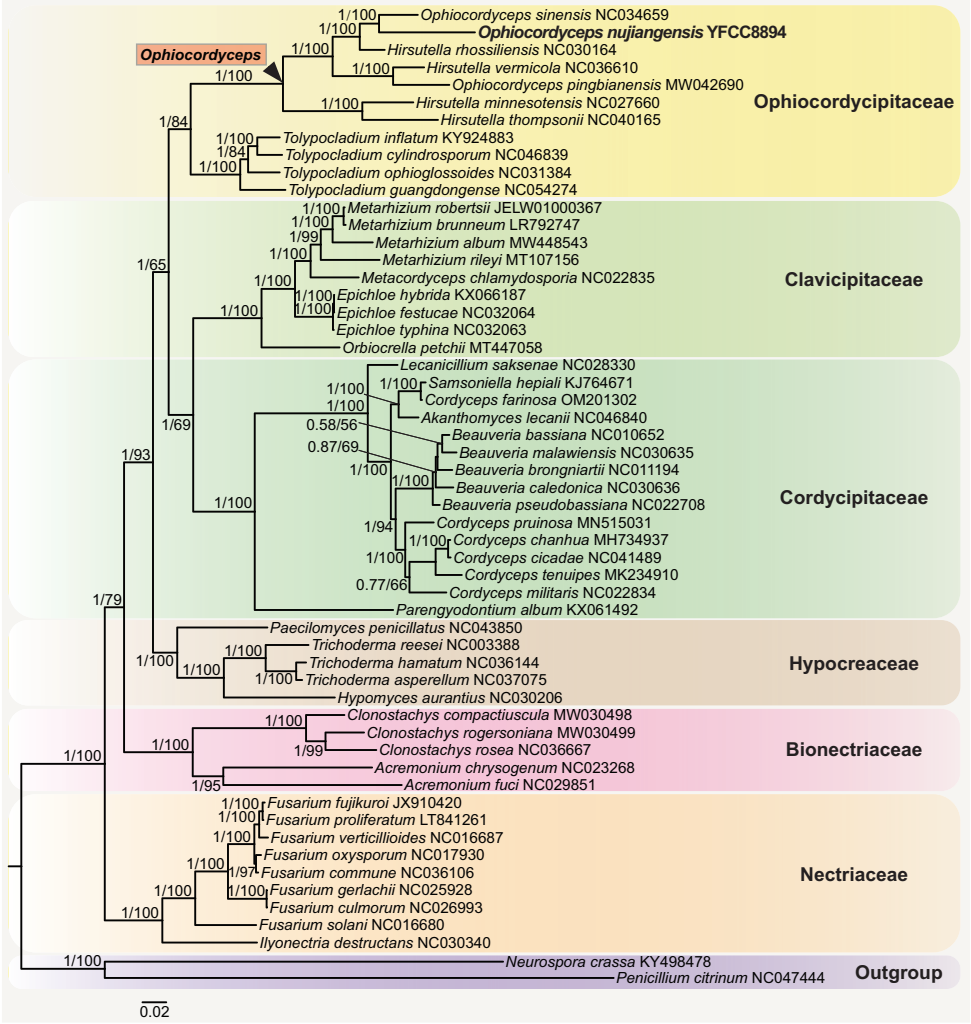

Figure 2. Phylogenetic tree of Hypocreales based on the Bayesian inference (BI) and the maximum likelihood (ML) analyses of 10 PCGs. The 10 PCG genes included *atp6*, *atp9*, *cob*, *cox2*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L* and *nad5*. The values at the nodes were the BI posterior probabilities and the ML bootstrap proportions, respectively. The specimen analyzed in this study was given in bold type.

Asexual stage. *Hirsutella*. The colonies grew slowly on PDA, adding peptone (5 g/L) and yeast extract powder (10g/L) to PDA could accelerate the growth. Culturing at room temperature (16–20 °C) after 14 weeks, the colonies increased to 20–21 mm, hard, slight protuberance in the middle, pale gray (Cool gray 1 C), reverse black brown (Black 4 XGC). Hyphae hyaline, septate, smooth-walled. Conidiogenous cells hyaline, solitary, 54.9–76.5 (AVE = 50.50 ± 0.24) μm long, gradually tapering, base width 3.6–4.9 (AVE = 4.32 ± 0.11) μm, tip width 1.0–1.5 (AVE = 1.30 ± 0.11) μm, with warty protrusions from the middle to the top and more on the top, smooth-walled.

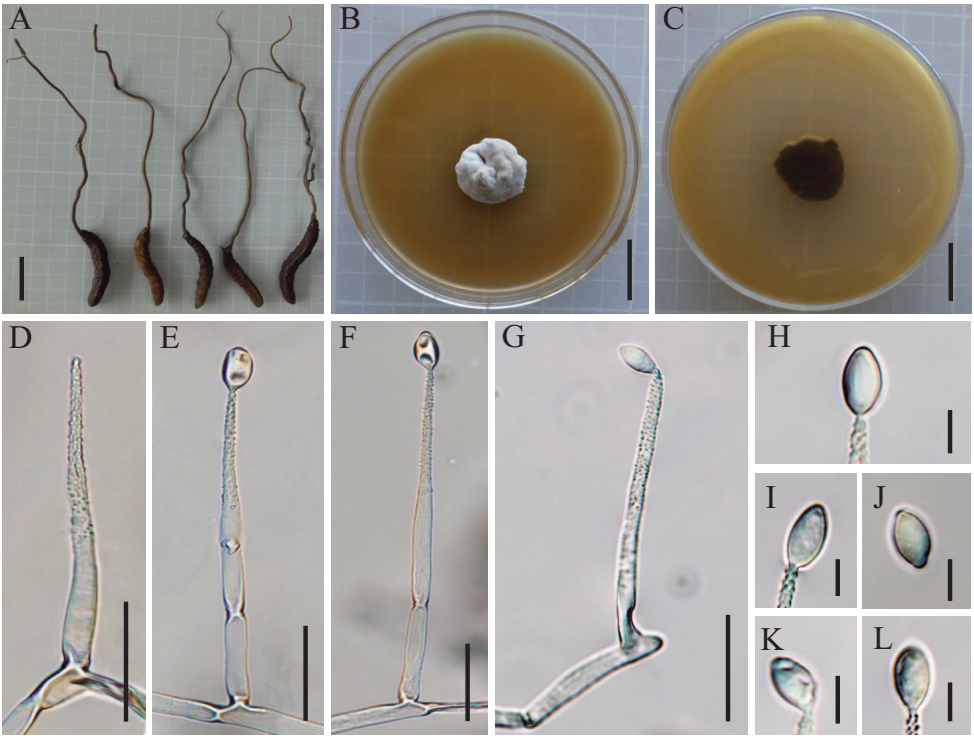

Figure 3. *Ophiocordyceps nujiangensis* **A** intact wild material **B** colony obverse on PDA with peptone and yeast extract powder **C** colony reverse on PDA with peptone and yeast extract powder **D** conidiogenous cells **E–G** conidiogenous cells and conidia **H–L** conidia. Scale bars: 3 cm (**A**); 2 cm (**B, C**); 20 μm (**D–G**); 5 μm (**H–L**).

Conidia hyaline, oval or fusiform, with smooth walls and mucinous sheath, 6.4–11.2 (AVE = 7.95 ± 0.15) × 3.7–6.4 (AVE = 4.73 ± 0.16) μm.

Host. Larvae of Hepialidae.

Habitat. Parasitized on Hepialidae larvae in the soil.

Distribution. Yajiaoluo, Fugong County, Nujiang Prefecture, Yunnan Province, China.

Other material examined. Yajiaoluo, Fugong County, Nujiang Prefecture, Yunnan Province, China. 98°52.20'N, 27°07.80'E, alt 1980 m, on the larvae of Hepialidae in soil, 6 June 2021, Hong Yu (YHH20040, YFCC 8894; YHH 20041).

Notes. *Ophiocordyceps nujiangensis* was closely phylogenetically related to *O. karstii* and *O. liangshanensis*. The formation of stromata on the head of the host was a feature common to all three species. However, the length of the stromata varies between the three species. *O. nujiangensis* had a stromata length longer than *O. karstii*, but shorter than *O. liangshanensis* (Table 1). *O. nujiangensis*, on the other hand, had slightly longer conidiophores and slightly smaller conidia than *O. liangshanensis* (Table 1).

Table 1. A morphological comparison of *Ophiocordyceps nujiangensis* and its allies.

Species	Host	Stromata	Ascomata	Asci	Ascospores	Phialides	Conidia	Reference
O. nujiangensis	Hepialidae larvae	Solitary, 148–182 mm long	–	–	–	54.9–76.5 μm long, base width 3.6–4.9 μm, tip width 1.0–1.5 μm	Oval or fusiform, 6.4–11.2 × 3.7–6.4 μm	This study
O. karstii	On dead larva of *Hepialus jianchuanensis*	Mostly single, 140–145 × 2–4 mm	Superficial, flask-shaped, 600–765 × 247–323 μm	Narrow cylindrical, 186–228 × 8–12 μm	Fusiform, 173–202 × 3–5 μm, not breaking into secondly spores	–	–	Li et al. (2016)
O. liangshanensis	Hepialidae larvae	Single or occasionally, 200–300 × 1.5–2.5 mm	Superficial, long ovoid, 450–740 × 300–450 μm	Cylindrical, 260–480 × 8–12 μm	Fasciculate, thread-like, slender, and long, 170–240 × 2.5–4.1 μm	Monophialidic, 46.9–75.6 μm long, subcylindrical, 3.8–4.7 μm basal wide	Ellipsoid, citriform or shape of an orange segment, 8.0–12.6 × 3.6–5.0 μm	Wang et al. (2021)
O. sinensis	Hepialidae larva	Single, occasionally 2–3, 40–110 mm long	Nearly superficial, ellipsoidal to ovate, 380–550 × 140–240 μm	Slender, long, 240–485 × 12–16 μm	Usually 2–4 mature ascospores, multiseptate, not breaking into secondary ascospores, 160–470 × 5–6 μm	–	–	Liang et al. (2007)

Fungal community composition

In total, 135,048 effective sequences were obtained. Based on the minimum number of reads in the sample, 33,762 reads were randomly selected for each sample to avoid bias in the sequencing depth. The rarefaction curve (the Shannon-Wiener curve) showed that the sequencing depth was very reasonable for representing the diversity of the fungal community (Suppl. material 3).

At the phylum level, a total of five phyla were identified, including Ascomycota, Basidiomycota, Mortierellomycota, Rozellomycota, and Glomeromycota. Of these, Ascomycota was dominant, with an average of 99.66%. The rest averaged no more than 1 percent. And the unclassified was dominant in the 151 identified genera, the average proportion was 29.56%, followed by *Trichothecium* (27.16%) and *Microdochium* (26.81%) (Fig. 4). Namely, numerous companion fungi were verified in the fruiting body of *O. nujiangensis*. The results also indirectly suggested that *O. nujiangensis* might be a new species as its ITS sequence could not be aligned in the database.

Phylogenetic analyses of the fungi at the family level

The top 50 families were classified into four phyla (Suppl. material 4), comprising Ascomycota, Basidiomycota, Mortierellomycota, and Rozellomycota; however, none in Glomeromycota. There were 41 families subordinated to Ascomycota, including the three families (Clavicipitaceae, Ophiocordycipitaceae, and Cordycipitaceae), which distributed *Cordyceps sensu lato*. And the phylogenetic locations of the three families

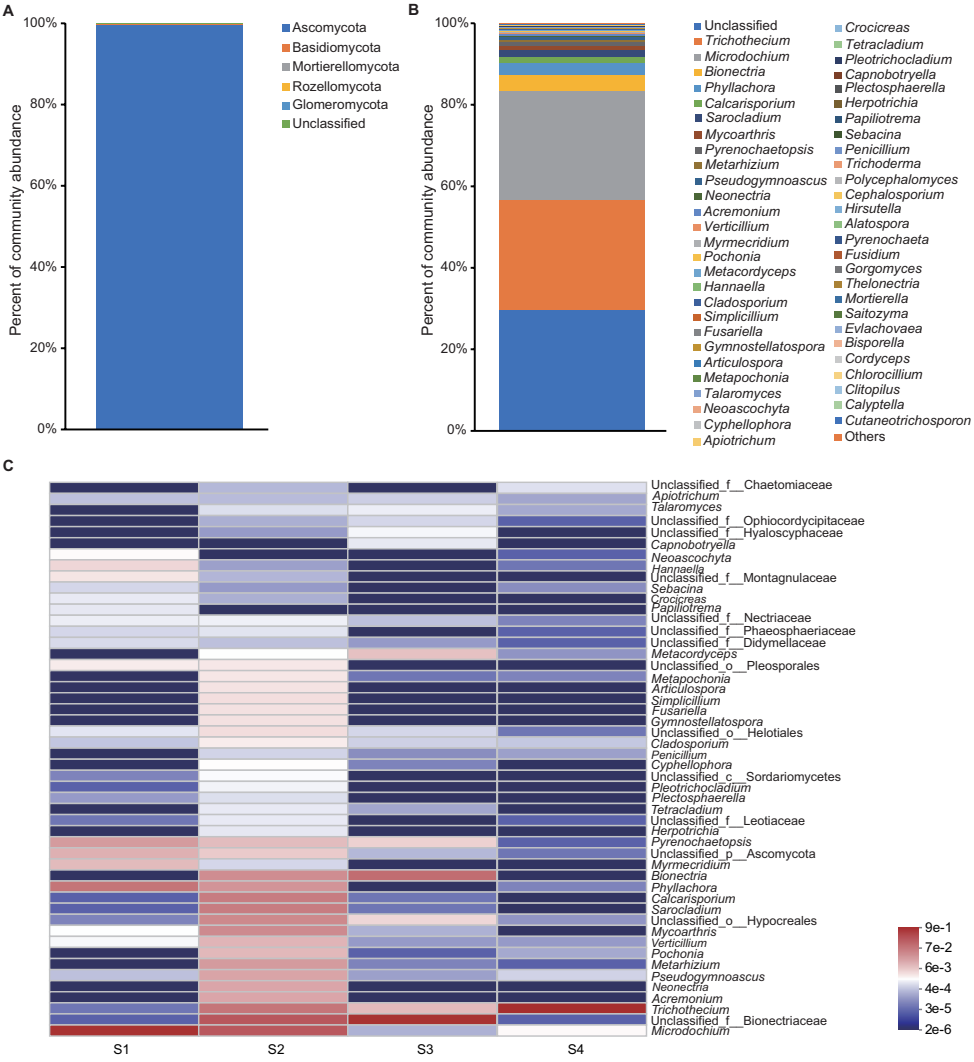

Figure 4. Composition of fungal community inhabiting *Ophiocordyceps nujiangensis*. A Composition of fungal community on phylum level. B Composition of fungal community on genus level. C Community heatmap analysis of the four specimens on genus level.

were essentially the same as previously reported in the study by Sung et al. (2007) and Wang et al. (2020a). The results implied that *O. nujiangensis* might have many companion fungi, which belongs to *Cordyceps sensu lato*.

Discussion

Ophiocordyceps nujiangensis was morphologically characterized by solitary, woody, and dark brown stromata, smooth-walled and septate hyphae, solitary and gradually

tapering conidiogenous cells with plenty of warty protrusions, and oval or fusiform conidia with mucinous sheath. In this research, the five-gene phylogenetic tree was rebuilt with four clades of *Ophiocordyceps* (the clade of *Hirsutella*, the clade of *O. ravenelii*, the clade of *O. sobolifera*, and the clade of *O. sphecocephala*,) and six subclades of *Hirsutella* clade (the subclade of *H. citriformis*, the subclade of *H. guyana*, the subclade of *H. nodulosa*, the subclade of *H. sinensis*, the subclade of *H. thompsonii*, and the subclade of *Hirsutella* ant pathogen), and the results were similar to the analyses by Sanjuan et al. (2015), Simmons et al. (2015), and Wang et al. (2018). *O. nujiangensis* was grouped phylogenetically with *O. karstii*, *O. liangshanensis*, and *O. sinensis*. Nevertheless, there was an obvious distinction between them in their morphological characteristics, especially in the length of the stromata. However, further comparisons were difficult due to the lack of anamorph observation of *O. karstii*. In the phylogenetic analyses of nuclear genes, the three specimens of *O. nujiangensis* united to form a single clade, and the result of phylogenetic analysis was consistent with that based on mitochondrial genes. Not only that, metagenomic data of *O. karstii* and *O. liangshanensis* had not been reported, and the differences between the allied species could not be discriminated.

A total of five fungal phyla and 151 fungal genera were identified in this study. Among them, Ascomycota and the unclassified were the dominant phylum and genus. Except for the dominant, *Trichothecium* and *Microdochium* also had high proportions at the genus level. The genus, *Trichothecium*, was a heterogonous group of filamentous fungi; some species were pathogenic fungi (Summerbell et al. 2011; Han et al. 2021). *Microdochium* was a common cereal pathogen fungus that adapted nicely to the cool (Parry et al. 1995; Gagkaeva et al. 2020). Some companion fungi had been confirmed that had vital functions (Guo et al. 2002; Li et al. 2018). The growth and development of the host were mostly due to the combined effect of the microbial adding peptone and yeast community (Han et al. 2019; Xie et al. 2021). Thus, the genera might have had an essential influence on the growth and development of *O. nujiangensis*. Furthermore, a comparison of the fungal communities of *O. sinensis* and *O. nujiangensis* showed that they had different community compositions. However, *Trichothecium* and *Microdochium* could not be found among the top 19 genera in fungal communities of *O. sinensis* reported (Xia et al. 2016). Consequently, the differences in the microbial community composition of closely related species might be suitable as further evidence for identifying species.

The phylogenetic analysis of mitochondrial genes became an adequate means to delimit fungal species, except for morphological observation and the five-gene phylogenetic tree (Nie et al. 2019; Meng et al. 2020). Similar topologies were obtained by utilizing 14 PCGs, PCGs + rRNA, or mitochondrial whole genomes (Hu et al. 2021). It was illustrated that the stable phylogenetic trees could be reconstructed using the phylogenetic analysis of mitochondrial genes. In the present research, the phylogenetic tree of Hypocreales was rebuilt, which was similar to the report by Chen et al. (2021). It had been shown that the phylogenetic trees with mitochondrial genes were reliable.

The characteristic differences between the new species and other species could be distinguished through the morphology data, and the phylogenetic location of the new species could be determined by the phylogeny and mitogenomics data. It was attempted to further

study the companion fungi of the new species, but the available data on the species and their phylogenetic relationship were considerably lacking. Metagenomics provided more comprehensive genetic information about microorganisms and the microorganisms with which they associated (Venter et al. 2004; Truong et al. 2017; Huang and Wang 2020). Therefore, the method might be an efficient avenue for reconstructing the "Tree of Life".

Acknowledgements

This work was funded by the National Natural Science Foundation of China (31870017), the Ministry of Ecology and Environment of China (2019HJ2096001006).

References

Aguileta G, de Vienne DM, Ross ON, Hood ME, Giraud T, Petit E, Gabaldón T (2014) High variability of mitochondrial gene order among fungi. Genome Biology and Evolution 6(2): 451–465. https://doi.org/10.1093/gbe/evu028

Alexeyev M, Shokolenko I, Wilson G, LeDoux S (2013) The maintenance of mitochondrial DNA integrity critical analysis and update. Cold Spring Harbor Perspectives in Biology 5(5): a012641. https://doi.org/10.1101/cshperspect.a012641

Chen S, Zhou Y, Chen Y, Gu J (2018) fastp: An ultra-fast all-in-one FASTQ preprocessor. Bioinformatics (Oxford, England) 34(17): i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Chen SQ, Wang YB, Zhu KF, Yu H (2021) Phylogeny and morphology reveal *Ophiocordyceps pingbianensis* sp. nov., an entomopathogenic fungus from China. Life (Basel, Switzerland) 11(7): 686. https://doi.org/10.3390/life11070686

Dai YD, Wu CK, Yuan F, Wang YB, Huang LD, Chen ZH, Zeng WB, Wang Y, Yang ZL, Zeng PS, Lemetti P, Mo XX, Yu H (2020) Evolutionary biogeography on *Ophiocordyceps sinensis*: An indicator of molecular phylogeny to geochronological and ecological exchanges. Geoscience Frontiers 11(3): 807–820. https://doi.org/10.1016/j.gsf.2019.09.001

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: Moremodels, new heuristics and parallel computing. Nature Methods 9(8): 772–772. https://doi.org/10.1038/nmeth.2109

Edgar RC (2013) UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10(10): 996–998. https://doi.org/10.1038/nmeth.2604

Gagkaeva TY, Orina AS, Gavrilova OP, Gogina NN (2020) Evidence of *Microdochium* fungi associated with cereal grains in Russia. Microorganisms 8(3): 340. https://doi.org/10.3390/microorganisms8030340

Guo SX, Wang QY, Zhuang WY, Zhang JH, Xing XK (2002) Discovery and application of the companion fungus related to sclerotial formation from hyphae of *Grifola umbellata*. Journal of Integrative Plant Biology 44: 1151–1154. https://www.jipb.net/EN/Y2002/V44/I10/1239

Han RC, Wu H, Tao HP, Qiu XH, Liu GQ, Rao ZC, Cao L (2019) Research on Chinese cordyceps during the past 70 years in China. Yingyong Kunchong Xuebao 56: 849–883. https://doi.org/10.7679/j.issn.2095-1353.2019.099

Han Z, Wang Z, Bi Y, Zong Y, Gong D, Wang B, Li B, Sionov E, Prusky D (2021) The Effect of Environmental pH during *Trichothecium roseum* (Pers.:Fr.) Link Inoculation of Apple Fruits on the Host Differential Reactive Oxygen Species Metabolism. Antioxidants 10(5): 692. https://doi.org/10.3390/antiox10050692

Hu ZY, Liu SC, Xu ZG (2021) Sequencing and phylogenetic analysis of the mitochondrial genome of the "jinhua-jun" isolated from fermented dark tea. Junwu Xuebao 40: 135–151. https://doi.org/10.13346/j.mycosystema.200153

Huang JM, Wang Y (2020) Genomic differences within the phylum Marinimicrobia: From waters to sediments in the Mariana Trench. Marine Genomics 50: 100699. https://doi.org/10.1016/j.margen.2019.100699

Jin JJ, Yu WB, Yang JB, Song Y, dePamphilis CW, Yi TS, Li DZ (2020) GetOrganelle: A fast and versatile toolkit for accurate de novo assembly of organelle genomes. Genome Biology 21(1): 241. https://doi.org/10.1186/s13059-020-02154-5

Kobayasi Y (1941) The genus *Cordyceps* and its allies. Science Reports of the Tokyo Bunrika Daigaku. Section B 5: 53–260. https://www.science-open.com/document?vid=0fa805f8-29da-4082-b30f-f1d4708e6dff

Kobayasi Y (1982) Keys to the taxa of the genera *Cordyceps* and *Torrubiella*. Nippon Kingakkai Kaiho 23: 329–364. https://www.scienceopen.com/document?vid=74f7ff8b-cd2d-4882-a902-d87963e26322

Li GJ, Hyde KD, Zhao RL, Hongsanan S, Abdel-Aziz FA, Abdel-Wahab MA, Alvarado P, Alves-Silva G, Ammirati JF, Ariyawansa HA, Baghela A, Bahkali AH, Beug M, Bhat DJ, Bojantchev D, Boonpratuang T, Bulgakov TS, Camporesi E, Boro MC, Ceska O, Chakraborty D, Chen JJ, Chethana KWT, Chomnunti P, Consiglio G, Cui BK, Dai DQ, Dai YC, Daranagama DA, Das K, Dayarathne MC, De Crop E, De Oliveira RJV, de Souza CAF, de Souza JI, Dentinger BTM, Dissanayake AJ, Doilom M, Drechsler-Santos ER, Ghobad-Nejhad M, Gilmore SP, Góes-Neto A, Gorczak M, Haitjema CH, Hapuarachchi KK, Hashimoto A, He MQ, Henske JK, Hirayama K, Iribarren MJ, Jayasiri SC, Jayawardena RS, Jeon SJ, Jerônimo GH, Jesus AL, Jones EBG, Kang JC, Karunarathna SC, Kirk PM, Konta S, Kuhnert E, Langer E, Lee HS, Lee HB, Li WJ, Li XH, Liimatainen K, Lima DX, Lin CG, Liu JK, Liu XZ, Liu ZY, Luangsa-ard JJ, Lücking R, Lumbsch HT, Lumyong S, Leaño EM, Marano AV, Matsumura M, McKenzie EHC, Mongkolsamrit S, Mortimer PE, Nguyen TTT, Niskanen T, Norphanphoun C, O'Malley MA, Parnmen S, Pawłowska J, Perera RH, Phookamsak R, Phukhamsakda C, Pires-Zottarelli CLA, Raspé O, Reck MA, Rocha SCO, de Santiago ALCMA, Senanayake IC, Setti L, Shang QJ, Singh SK, Sir EB, Solomon KV, Song J, Srikitikulchai P, Stadler M, Suetrong S, Takahashi H, Takahashi T, Tanaka K, Tang LP, Thambugala KM, Thanakitpipattana D, Theodorou MK, Thongbai B, Thummarukcharoen T, Tian Q, Tibpromma S, Verbeken A, Vizzini A, Vlasák J, Voigt K, Wanasinghe DN, Wang Y, Weerakoon G, Wen HA, Wen TC, Wijayawardene NN, Wongkanoun S, Wrzosek M, Xiao YP, Xu JC, Yan JY, Yang J, Da Yang S, Hu Y, Zhang JF, Zhao J, Zhou LW, Peršoh D, Phillips AJL, Maharachchikumbura SSN (2016) Fungal

diversity notes 253–366: Taxonomic and phylogenetic contributions to fungal taxa. Fungal Diversity 78(1): 1–237. https://doi.org/10.1007/s13225-016-0366-9

Li JM, Chen J, Guo SX (2018) Diversity and ecological functions of *Tuber*-associated microbial communities during *Tuber* ascocarp development. Junwu Xuebao 37: 971–987. https://doi.org/10.13346/j.mycosystema.170269

Liang ZQ, Liu AY, Liu ZY (2007) *Cordyceps*. Flora Fungorum sinicorum, vol. 32. Science Press, Beijing, China.

Magoč J, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27: 2957–2963. https://doi.org/10.1093/bioinformatics/btr507

Mains EB (1958) North American entomogenous species of *Cordyceps*. Mycologia 50(2): 169–222. https://doi.org/10.1080/00275514.1958.12024722

Meng GL, Zeng PM, Wang ZY, Cheng B, Ye LY, Wu XP (2020) Application of the *Ganoderma multipileum* mitogenome to identification of *Ganoderma* species. Junwu Xuebao 39: 23–33. https://doi.org/10.13346/j.mycosystema.190313

Nie Y, Wang L, Cai Y, Tao W, Zhang YJ, Huang B (2019) Mitochondrial genome of the entomophthoroid fungus *Conidiobolus heterosporus* provides insights into evolution of basal fungi. Applied Microbiology and Biotechnology 103(3): 1379–1391. https://doi.org/10.1007/s00253-018-9549-5

Parry DW, Rezanoor HN, Pettitt TR, Hare MC, Nicholson P (1995) Analysis of *Microdochium nivale* isolates from wheat in the UK during 1993. Annals of Applied Biology 126(3): 449–455. https://doi.org/10.1111/j.1744-7348.1995.tb05379.x

Petch T (1931) Notes on entomogenous fungi. Transactions of the British Mycological Society 16(1): 55–75. https://doi.org/10.1016/S0007-1536(31)80006-3

Quandt CA, Kepler RM, Gams W, Araújo JPM, Ban S, Evans HC, Hughes D, Humber R, Hywel-Jones N, Li ZZ, Luangsa-Ard JJ, Rehner SA, Sanjuan T, Sato H, Shrestha B, Sung GH, Yao YJ, Zare R, Spatafora GW (2014) Phylogenetic-based nomenclatural proposals for Ophiocordycipitaceae (Hypocreales) with new combinations in *Tolypocladium*. IMA Fungus 5(1): 121–134. https://doi.org/10.5598/imafungus.2014.05.01.12

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxford, England) 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Sanjuan TI, Franco-Molano AE, Kepler RM, Spatafora JW, Tabima J, Vasco-Palacios AM, Restrepo S (2015) Five new species of entomopathogenic fungi from the Amazon and evolution of Neotropical *Ophiocordyceps*. Fungal Biology 119(10): 901–916. https://doi.org/10.1016/j.funbio.2015.06.010

Simmons DR, Kepler RM, Rehner SA, Groden E (2015) Phylogeny of *Hirsutella* species (Ophiocordycipitaceae) from the UsA: Remedying the paucity of *Hirsutella* sequence data. IMA Fungus 6(2): 345–356. https://doi.org/10.5598/imafungus.2015.06.02.06

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web servers. Systematic Biology 57(5): 758–771. https://doi.org/10.1080/10635150802429642

Summerbell RC, Gueidan C, Schroers HJ, de Hoog GS, Starink M, Rosete YA, Guarro J, Scott JA (2011) Acremonium phylogenetic overview and revision of *Gliomastix*, *Sarocladium*, and *Trichothecium*. Studies in Mycology 68: 139–162. https://doi.org/10.3114/sim.2011.68.06

Sung GH, Hywel-Jones NL, Sung JM, Luangsa-ard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Studies in Mycology 57: 5–59. https://doi.org/10.3114/sim.2007.57.01

Truong DT, Tett A, Pasolli E, Huttenhower C, Segata N (2017) Microbial strain-level population structure and genetic diversity from metagenomes. Genome Research 27(4): 626–638. https://doi.org/10.1101/gr.216242.116

Valach M, Burger G, Gray MW, Lang BF (2014) Widespread occurrence of organelle genome-encoded 5S rRNAs including permuted molecules. Nucleic Acids Research 42(22): 13764–13777. https://doi.org/10.1093/nar/gku1266

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304(5667): 66–74. https://doi.org/10.1126/science.1093857

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73(16): 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wang YB, Yu H, Dai YD, Wu CK, Zeng WB, Yuan F, Liang ZQ (2015) *Polycephalomyces agaricus*, a new hyperparasite of *Ophiocordyceps* sp. infecting melolonthid larvae in southwestern China. Mycological Progress 14(9): 70. https://doi.org/10.1007/s11557-015-1090-7

Wang YB, Nguyen TT, Dai YD, Yu H, Zeng WB, Wu CK (2018) Molecular phylogeny and morphology of *Ophiocordyceps unituberculata* sp. nov. (Ophiocordycipitaceae), a pathogen of caterpillars (Noctuidae, Lepidoptera) from Yunnan, China. Mycological Progress 17(6): 1–9. https://doi.org/10.1007/s11557-017-1370-5

Wang YB, Wang Y, Fan Q, Duan DE, Zhang GD, Dai RQ, Dai YD, Zeng WB, Chen ZH, Li DD, Tang DX, Xu ZH, Sun T, Nguyen TT, Tran NL, Dao VM, Zhang CM, Huang LD, Liu YJ, Zhang XM, Yang DR, Sanjuan T, Liu XZ, Yang ZL, Yu H (2020a) Multigene phylogeny of the family Cordycipitaceae (Hypocreales): New taxa and the new systematic position of the Chinese cordycipitoid fungus *Paecilomyces hepiali*. Fungal Diversity 103(1): 1–46. https://doi.org/10.1007/s13225-020-00457-3

Wang Y, Wu HJ, Tran NL, Zhang GD, Souvanhnachit S, Wang YB, Yu H (2020b) *Ophiocordyceps furcatosubulata*, a new entomopathogenic fungus parasitizing beetle larvae (Coleoptera: Elateridae). Phytotaxa 482(3): 268–278. https://doi.org/10.11646/phytotaxa.482.3.5

Wang Y, Dai YD, Yang ZL, Guo R, Wang YB, Yang ZL, Ding L, Yu H (2021) Morphological and molecular phylogenetic data of the chinese medicinal fungus *Cordyceps liangshanensis* reveal its new systematic position in the family Ophiocordycipitaceae. Mycobiology 49(4): 297–307. https://doi.org/10.1080/12298093.2021.1923388

White TJ, Bruns S, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR Protocols, A Guide to Methods and Application, Academic Press 1, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Williams S, Foster P, Littlewood D (2014) The complete mitochondrial genome of a turbinid vetigastropod from MiSeq Illumina sequencing of genomic DNA and steps towards a resolve gastropod phylogeny. Gene 533(1): 38–47. https://doi.org/10.1016/j.gene.2013.10.005

Xia F, Chen X, Guo MY, Bai XH, Liu Y, Shen GR, Li YL, Lin J, Zhou XW (2016) High-throughput sequencing-based analysis of endogenetic fungal communities inhabiting the Chinese Cordyceps reveals unexpectedly high fungal diversity. Scientific Reports 6(1): 33437. https://doi.org/10.1038/srep33437

Xie F, Chen ZH, Zhu YL, Su QJ, Chen C, Zhang Y (2021) Relative infectiveness of six *Cordyceps sinensis*-related fungal strains to *Hepialus armoricanus* larvae. Yingyong Kunchong Xuebao 58: 1312–1321. https://doi.org/10.7679/j.issn.2095-1353.2021.131

Xu TM, Chen B, Zheng YQ, Zhao YX, Su ZT, Zhang LY (2019) Artificial culture method of *Cordyceps myrmecophila*, 2019, CN110583360A. https://wenku.baidu.com/view/8d3ed6fa846fb84ae45c3b3567ec102de3bddf68?fr=xueshu

Yin XY, Zhang XM (2015) Improved tissue isolation method for *Auricularia auricula*. Heilongjiang Nongye Kexue 3: 132–134. http://en.cnki.com.cn/Article_en/CJFDTOTAL-HLJN201503043.htm

Zhang T, Wang NF, Zhang YQ, Liu YH, Yu LY (2015) Diversity and distribution of fungal communities in the marine sediments of Kongsfjorden, Svalbard (High Arctic). Scientific Reports 5(1): 14524. https://doi.org/10.1038/srep14524

Zhang Y, Du Y, Hong Y, Zhou YC, Ge F (2017) Protective Effects of *Ophiocordyceps lanpingensis* on Glycerol-Induced Acute Renal Failure in Mice. Journal of Immunology Research 2017: 1–8. https://doi.org/10.1155/2017/6915912

Zhao ZY, Zhu KF, Tang DX, Wang YB, Wang Y, Zhang GD, Geng YP, Yu H (2021) Comparative analysis of mitochondrial genome features among four *Clonostachys* species and insight into their systematic positions in the order Hypocreales. International Journal of Molecular Sciences 22(11): 5530. https://doi.org/10.3390/ijms22115530

Zou J, Wu L, He ZM, Zhang P, Chen ZH (2017) Determination of the main nucleosides and nucleobases in natural and cultured *Ophiocordyceps xuefengensis*. Molecules (Basel, Switzerland) 22(9): 1530. https://doi.org/10.3390/molecules22091530

Supplementary material 1

Relevant species information and GeneBank accession numbers for phylogenetic research in this study

Authors: Tao Sun, Weiqiu Zou, Quanying Dong, Ou Huang, Dexiang Tang, Hong Yu

Data type: table (word document)

Link: https://doi.org/10.3897/mycokeys.94.89425.suppl1

Supplementary material 2

The information of species and their mitochondrial genomes for constructing the mitochondrial-genome phylogenetic tree of Hypocreales

Authors: Tao Sun, Weiqiu Zou, Quanying Dong, Ou Huang, Dexiang Tang, Hong Yu

Data type: table (PDF file)

Link: https://doi.org/10.3897/mycokeys.94.89425.suppl2

Supplementary material 3

Rarefaction curves (Shannon-Wiener curve) of the fungal communities collected from the fruiting body from four different specimens of *Ophiocordyceps nujiangensis*

Authors: Tao Sun, Weiqiu Zou, Quanying Dong, Ou Huang, Dexiang Tang, Hong Yu

Data type: figure (eps. file)

Link: https://doi.org/10.3897/mycokeys.94.89425.suppl3

Supplementary material 4

Phylogenetic analyses of the ranked top 50 families identified from *Ophiocordyceps nujiangensis* based on maximum likelihood (ML). Values at the nodes are ML bootstrap proportions

Authors: Tao Sun, Weiqiu Zou, Quanying Dong, Ou Huang, Dexiang Tang, Hong Yu

Data type: figure (PDF file)

Link: https://doi.org/10.3897/mycokeys.94.89425.suppl4

MycoKeys 94: 109–124 (2022)
doi: 10.3897/mycokeys.94.96782
https://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
Launched to accelerate biodiversity research

Exophiala yunnanensis and *Exophiala yuxiensis* (Chaetothyriales, Herpotrichiellaceae), two new species of soil-inhabiting *Exophiala* from Yunnan Province, China

Ruili Lv[1], Xiaoqian Yang[1], Min Qiao[1], Linlin Fang[1], Jianying Li[2], Zefen Yu[1]

1 *Laboratory for Conservation and Utilization of Bio-resources, Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, Yunnan, 650091, China* **2** *Kunming Edible Fungi Institute of All China Federation of Supply and Marketing Cooperatives, Kunming 650221, China*

Corresponding author: Zefen Yu (zfyu2021@163.com)

Academic editor: Cecile Gueidan | Received 28 October 2022 | Accepted 12 December 2022 | Published 21 December 2022

Citation: Lv R, Yang X, Qiao M, Fang L, Li J, Yu Z (2022) *Exophiala yunnanensis* and *Exophiala yuxiensis* (Chaetothyriales, Herpotrichiellaceae), two new species of soil-inhabiting *Exophiala* from Yunnan Province, China. MycoKeys 94: 109–124. https://doi.org/10.3897/mycokeys.94.96782

Abstract

During a survey of soil fungi collected from Yunnan Province, China, two new species of *Exophiala*, *E. yunnanensis* and *E. yuxiensis*, were isolated from the soil of karst rocky desertification (KRD). The DNA sequences of these respective strains, including internal transcribed spacers (ITS), large subunit nuclear ribosomal RNA (LSU rRNA), partial small subunit (SSU) and β-tubulin (*tub2*) were sequenced and compared with those from species closely-related to *Exophiala*. *Exophiala yunnanensis* differs from the phylogenetically closely related *E. nagquensis* and *E. brunnea* by its smaller aseptate conidia. *Exophiala yuxiensis* is phylogenetically related to *E. lecanii-corni*, *E. lavatrina* and *E. mali*, but can be distinguished from them by its larger conidia. Full descriptions, illustrations and phylogenetic positions of *E. yunnanensis* and *E. yuxiensis* were provided.

Keywords

Exophiala, multi-locus phylogeny, morphology, new species, taxonomy

Introduction

Exophiala J.W. Carmich. (Chaetothyriales, Herpotrichiellaceae) was established with *E. salmonis* J.W. Carmich. as type species (Carmichael 1966) in Alberta, Canada. Due to their yeast-like melanised colonies, these fungi are often also referred to as "black yeasts" (Matsumoto et al. 1987). The genus is characterised by annellidic conidiogenous cells producing slimy heads of conidia, conidiophores upright or bent, not or irregularly branched, smooth, light olive to brown. However, there are several synanamorphs recorded in this genus (Thitla et al. 2022). Nearly all species are recognisable within the order by the way they produce cells by budding (De Hoog et al. 2011).

Exophiala spp. are widely distributed and can be isolated from bulk soil, biological crusts, rock surfaces, air, natural water masses, rhizosphere, plant tissues, and infected animals and human tissue (Addy et al. 2005; Bates et al. 2006; Neubert et al. 2006; Bukovská et al. 2010; Julou et al. 2010; De Hoog et al. 2011). Most studies on *Exophiala* species focused on their importance as etiologic agents of disease in animals and humans (Zeng and De Hoog 2008; Najafzadeh et al. 2013; Wen et al. 2016). Several *Exophiala* species are opportunistic pathogens of immunocompetent humans (Woo et al. 2013; Yong et al. 2015), in rare occasions causing nervous system phaeohyphomycosis (Chang et al. 2000) or causing cutaneous and subcutaneous skin infections, including *E. spinifera* (H.S. Nielsen & Conant) Mcginnis, which has the strongest pathogenicity to human skin (Vitale and De Hoog 2002). Furthermore, some *Exophiala* species, such as *E. salmonis*, *E. aquamarina* de Hoog et al. and *E. equina* (Pollacci) de Hoog et al. may cause cutaneous or disseminated infections of cold-blooded animals (De Hoog et al. 2011). Therefore, the classification and identification of this genus are significantly important for clinical diagnosis, treatment and prevention.

In the past, taxonomic and diagnostic schemes for *Exophiala* were morphological characteristics, but the anamorphic states of some species are highly pleomorphic (De Hoog et al. 1995; Haase et al. 1995; Thitla et al. 2022), which make them difficult to be recognised and circumscribed (Naveau 1999; Zeng and De Hoog 2008), so only a small number of *Exophiala* species are, in fact, recognisable using morphology. With the development of molecular systematics, more and more species were redefined, redesignated or described mainly depending on genetic, morphological, physiological and ecological features (Haase et al. 1999; De Hoog et al. 2003; Vitale et al. 2003; De Hoog et al. 2006). At present, 80 names in *Exophiala* were recorded in Index Fungorum, amongst them *E. nigra* (Issatsch.) Haase & de Hoog, *E. placitae* Crous & Summerell, *E. prototropha* (Bulanov & Malama) Haase et al. and *E. werneckii* (Horta) Arx, have been moved to *Nadsoniella* Issatsch., *Neophaeococcomyces* Crous & M.J. Wingf., *Pullularia* Berkhout and *Hortaea* Nishim. & Miyaji, respectively. Currently, 68 species have been accepted into this genus after a brief review of Thitla et al. (2022) and Crous et al. (2022), who described new species from Thailand and Australia.

During a survey of fungi from rocky desertification area, two unknown fungi were found. Based on morphology and phylogenetic analysis combined ITS, SSU, LSU and *tub2*, we proposed two new species, *E. yunnanensis* and *E. yuxiensis*.

Materials and methods

Isolation and morphological characterisation of strains

Soil samples were collected from Yiliang and Yuxi in Yunnan Province, southwest China. Samples were placed in plastic bags, labelled and transported to the laboratory. All the samples were stored at 4 °C before further processing. Fungal strains were obtained by serial dilutions (1,000 to 1,000,000 fold) and spread on to the surface of Rose Bengal agar with antibiotics (40 mg streptomycin, 30 mg ampicillin per litre) added in a 9 cm diam. Petri dish, followed by incubation at 25 °C for 5 days (Zheng et al. 2021a). Representative colonies were picked up with a sterilised needle and transferred to potato dextrose agar (PDA, 200 g potato, 20 g dextrose, 18 g agar, 1000 ml distilled water). After 7 days, colonies were transferred to cornmeal agar (CMA, 20 g cornmeal, 18 g agar, 1000 ml distilled water). Characteristics of colonies, growth rate and other morphological aspects from PDA were observed after 10 days. Microscopic characteristics including mycelium, 10 conidiophores and 30 conidia were examined and measured after 7 days on CMA using an Olympus BX51 microscope. Pure cultures were deposited in the Herbarium of the Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming, Yunnan, P.R. China (**YMF**, formerly Key Laboratory of Industrial Microbiology and Fermentation Technology of Yunnan), China General Microbiological Culture Collection Center (**CGMCC**), the Guangdong Microbial Culture Collection Center (**GDMCC**) and Japan Collection of Microorganisms (**JCM**).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted following the protocol of Zheng et al. (2021b). The internal transcribed spacer (ITS), the large subunit nuclear ribosomal RNA (LSU rRNA), the partial small subunit (SSU) and the β-tubulin (*tub2*) were amplified using the primer pairs ITS1/ITS4 (White 1990), LR0R/LR5 (Vilgalys and Hester 1990), NSSU131/NS24 (Kauff and Lutzoni 2002) and Bt2a/Bt2b (Glass and Donaldson 1995), respectively. The PCR amplifications were conducted in 25 µl final volumes which consisted of 1.0 µl DNA template, 1.0 µl of each forward and reverse primers, 12.5 µl 2 × Master Mix and 9.5 µl ddH_2O. The PCR reaction cycles were as follows: initial denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 40 s; the annealing extension dependent on the amplified loci (48 °C for LSU, 54 °C for ITS, 51 °C for SSU and 58 °C for *tub2*) for 1 min and extension at 72 °C for 2 min; a final extension at 72 °C for 10 min. PCR products were sequenced by TSINGKE Biological Technology in Kunming, China.

Sequence alignment and phylogenetic analysis

Preliminary BLAST searches with ITS, LSU, SSU and *tub2* gene sequences of the new isolates against NCBI databases had identified species closely related to our two isolates. Based on this information, ITS, LSU, SSU and *tub2* sequences of 62 strains were

downloaded and used in the phylogenetic analysis with *Cyphellophora oxyspora* (CBS 698.73) as outgroup. The GenBank accession numbers of sequences used in the phylogenetic analysis are shown in Table 1. DNA sequence data were aligned using ClustalX 1.83 (Thompson et al. 1997) with default parameters. Aligned sequences of multiple loci were concatenated and manually adjusted through BioEdit version v. 7.0.4.1 (Hall 1999) and ambiguously aligned regions were excluded. The combined sequence was converted to a NEXUS file using MEGA6 (Tamura et al. 2013) and it was uploaded to TreeBASE (www.treebase.org; accession number: S29757).

Phylogenetic analyses were conducted using both the Bayesian Inference (BI) and Maximum Likelihood (ML) methods. Bayesian Inference analysis was conducted using MrBayes v.3.2 (Ronquist et al. 2012) with the NEXUS file. The following parameters were used: ngen = 1,000,000; samplefr = 1,000; printfr = 1,000. The Akaike Information Criterion (AIC) implemented in jModelTest version 2.0 (Posada 2008) was used to select the best fit models after likelihood score calculations were done. TPM1uf + I + G was estimated as the best-fit model under the output strategy of AIC. Two independent analyses with four chains each (one cold and three heated) were run until stationary distribution was achieved. The initial 25% of the generations of MCMC sampling were excluded as burn-in. The refinement of the phylogenetic tree was used for estimating Bayesian Inference posterior probability (BIPP) values. The ML trees, based on four gene loci, were constructed with the GTR+GAMMA model using RAxML version 7.2.6 (Stamatakis 2006) and the robustness of branches was assessed by bootstrap analysis with 1000 replicates. The tree was viewed in TreeView 1.6.6 (Page 1996) with Maximum Likelihood bootstrap proportions (MLBP) greater than 50% and Bayesian Inference posterior probabilities (BIPP) greater than 70%, as shown at the nodes.

Results

Molecular phylogeny

The Bayesian tree, based on ITS sequence data, confirmed that two strains were distinct from known species of *Exophiala* (Fig. 1), *Exophiala yunnanensis* is phylogenetically close to *E. nagquensis* CGMCC 3.17284 and ITS similarity between *E. yunnanensis* and *E. nagquensis* is 92.21%. *Exophiala yuxiensis* is phylogenetically related to *E. lecaniicorni* CBS 123.33, *E. mali* CBS 146791 and *E. lavatrina* NCPF 7893 and the similarities between the holotype of *E. yuxiensis* and the representative strains of three species are 90.27%, 89.86% and 85.08%, respectively.

In the combined phylogenetic analyses (ITS, LSU, SSU and *tub2*), which contained 2218 characters, a similar topological structure was observed between the two phylogenetic trees constructed by BI and ML. The support values with BI analysis are relatively higher than the ML bootstrap support values (Fig. 2) In this tree, *E. yunnanensis*, *E. nagquensis* W. Sun et al., *E. brunnea* Papendorf and *E. frigidotolerans* Rodr.-Andr. et al. formed a clade with high statistical support (BIBP/MLBP = 100/97).

Table 1. Species, strains and their corresponding GenBank accession numbers of sequences used for phylogenetic analyses. *Exophiala* strains of the present study were marked in bold. [T]ex-type cultures; "-" The gene fragment representing this strain was not attainable.

Species	Strain no.	GenBank accession no.			
		ITS	LSU	SSU	*tub*2
Exophiala abietophila	CBS 145038[T]	MK442581	NG066323	–	–
Exophiala alcalophila	CBS 520.82[T]	JF747041	AF361051	JN856010	JN112423
Exophiala angulospora	CBS 482.92[T]	JF747046	KF155190	JN856011	JN112426
Exophiala aquamarina	CBS 119918[T]	JF747054	–	JN856012	JN112434
Exophiala asiatica	CBS 122847[T]	EU910265	–	–	–
Exophiala attenuata	F10685	KT013095	KT013094	–	–
Exophiala bergeri	CBS 353.52[T]	EF551462	FJ358240	FJ358308	EF551497
Exophiala bonariae	CBS 139957[T]	JX681046	KR781083	–	–
Exophiala brunnea	CBS 587.66[T]	JF747062	KX712342	JN856013	JN112442
Exophiala campbellii	NCPF 2274	LT594703	LT594760	–	–
Exophiala cancerae	CBS 120420[T]	JF747064	–	–	JN112444
Exophiala capensis	CBS 128771[T]	JF499841	MH876538	–	–
Exophiala castellanii	CBS 158.58[T]	JF747070	KF928522	JN856014	KF928586
Exophiala cinerea	CGMCC 3.18778[T]	MG012695	MG197820	MG012724	MG012745
Exophiala clavispora	CGMCC 3.17512	KP347940	MG197829	MG012733	KP347931
Exophiala crusticola	CBS 119970[T]	AM048755	KF155180	KF155199	–
Exophiala dermatitidis	CBS 207.35[T]	AF050269	KJ930160	–	KF928572
Exophiala ellipsoidea	CGMCC 3.17348[T]	KP347955	KP347956	KP347965	KP347921
Exophiala embothrii	CBS 146560	MW045819	MW045823	–	–
Exophiala equina	CBS 119.23[T]	JF747094	–	JN856017	JN112462
Exophiala eucalypti	CBS 142069	KY173411	KY173502	–	–
Exophiala eucalyptorum	CBS 121638[T]	NR132882	KC455258	KC455302	KC455228
Exophiala exophialae	CBS 668.76[T]	AY156973	KX822326	KX822287	EF551499
Exophiala frigidotolerans	CBS 146539[T]	LR699566	LR699567	–	–
Exophiala halophila	CBS 121512[T]	JF747108	–	JN856015	JN112473
Exophiala heteromorpha	CBS 232.33[T]	MH855419	MH866871	–	–
Exophiala hongkongensis	CBS 131511	JN625231	–	–	JN625236
Exophiala italica	MFLUCC 16-0245[T]	KY496744	KY496723	KY501114	–
Exophiala jeanselmei	CBS 507.90[T]	AY156963	FJ358242	FJ358310	EF551501
Exophiala lacus	FMR 3995	KU705830	KU705847	–	–
Exophiala lavatrina	NCPF 7893	LT594696	LT594755	–	–
Exophiala lecanii-corni	CBS 123.33[T]	AY857528	FJ358243	FJ358311	–
Exophiala lignicola	CBS 144622[T]	MK442582	MK442524	–	–
Exophiala macquariensis	CBS 144232[T]	MF619956	–	–	MH297438
Exophiala mali	CBS 146791[T]	MW175341	MW175381	–	–
Exophiala mansonii	CBS 101.67[T]	AF050247	AY004338	X79318	–
Exophiala mesophila	CBS 402.95[T]	JF747111	KX712349	JN856016	JN112476
Exophiala moniliae	CBS 520.76[T]	KF881967	KJ930162	–	–
Exophiala nagquensis	CGMCC 3.17284	KP347947	MG197838	MG012742	KP347922
Exophiala nidicola	FMR 3889	MG701055	MG701056	–	–
Exophiala nigra	CBS 535.94[T]	KY115191	KX712353	–	–
Exophiala nishimurae	CBS 101538[T]	AY163560	KX822327	KX822288	JX482552
Exophiala oligosperma	CBS 725.88[T]	AY163551	KF928486	FJ358313	EF551508
Exophiala opportunistica	CBS 109811[T]	JF747123	KF928501	–	JN112486
Exophiala palmae	CMRP 1196[T]	KY680434	KY570929	–	KY689829
Exophiala phaeomuriformis	CBS 131.88[T]	AJ244259	–	–	–
Exophiala pisciphila	CBS 537.73[T]	NR121269	AF361052	JN856018	JN112493
Exophiala placitae	CBS 121716[T]	MH863143	MH874694	–	–
Exophiala prostantherae	CBS 146794[T]	MW175344	MW175384	–	–

Species	Strain no.	GenBank accession no.			
		ITS	LSU	SSU	*tub2*
Exophiala polymorpha	CBS 138920^{T}	KP070763	KP070764	–	–
Exophiala pseudooligosperma	YMF 1.6741	MW616557	MW616559	MW616558	MZ127830
Exophiala psychrophila	CBS 191.87^{T}	JF747135	–	JN856019	JN112497
Exophiala quercina	CPC 33408	MT223797	MT223892	–	–
Exophiala radicis	P2772	KT099203	KT723447	KT723452	KT723462
Exophiala salmonis	CBS 157.67^{T}	AF050274	AY213702	JN856020	JN112499
Exophiala sideris	CBS 121818^{T}	HQ452311	–	HQ441174	HQ535833
Exophiala spinifera	CBS 899.68^{T}	AY156976	–	–	EF551516
Exophiala tremulae	CBS 129355^{T}	FJ665274	–	KT894147	KT894148
Exophiala xenobiotica	CBS 128104	MH864829	MH876272	–	–
Exophiala yunnanensis	**YMF1.06739**	**MZ779226**	**MZ779229**	**MZ781222**	**OM095379**
Exephiala yuxiensis	**YMF1.07354**	**OL863155**	**OL863154**	**OM149370**	**OL944581**
Cyphellophora oxyspora	CBS 698.73^{T}	KC455249	KC455262	KC455305	KC455232

Exophiala yuxiensis is phylogenetically close to *E. lecanii-corni* (Benedek & G. Specht) Haase & de Hoog and the clade formed by these species and six additional ones also has high statistical support (BIBP/MLBP = 100/89).

Taxonomy

***Exophiala yunnanensis* Z.F. Yu & R.L. Lv, sp. nov.**
MycoBank No: 842373
Fig. 3

Etymology. *yunnanensis*, pertaining to Yunnan, a province of southwest China from where the type was collected.

Description. Colonies on CMA medium after 7 days with hyphae olive green, smooth, septate, thin walled, branched, 1.6–3.0 μm wide. Conidiogenous cells slightly differentiated from simple or branched vegetative hyphae, terminal or intercalary, flask-shaped, ovoid to elongate, pale brown, loci at tips and lateral; annellated zones inconspicuous or occasionally finely fimbriate, often inserted on intercalary cells. Conidia aseptate, ellipsoidal, cylindrical or allantoid, 1–2 guttulate, smooth, brown, 2.9–4.8 × 1.8–3.3 μm, with a conspicuous scar of approx. 1 μm wide at the base, containing no evident or few small oil drops.

Culture characteristics. Colonies on PDA medium, at 25 °C, were slow-growing, mycelium immersed and partly superficial, irregular, umbonate, surface olivaceous-grey to black. Radial growth rates were 0.8–0.9 mm day^{-1}on PDA. Colonies on CMA medium were restricted, mycelium immersed and partly superficial, effuse, cottony, reverse olivaceous-buff to olivaceous, reaching 12 mm diam. in 15 days at 25 °C.

Type. China. Yiliang County, Yunnan Province, isolated from soil of rocky desertification area, 24°96'N, 102°66'E, ca. 1886 m elev., Oct 2020, Z.F.Yu, preserved by lyophilisation (a metabolically-inactive state) in State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan (holotype YMFT 1.06739), ex-holotype live culture: YMF 1.06739; CGMCC 3.16095; GDMCC 3.725; JCM 39339.

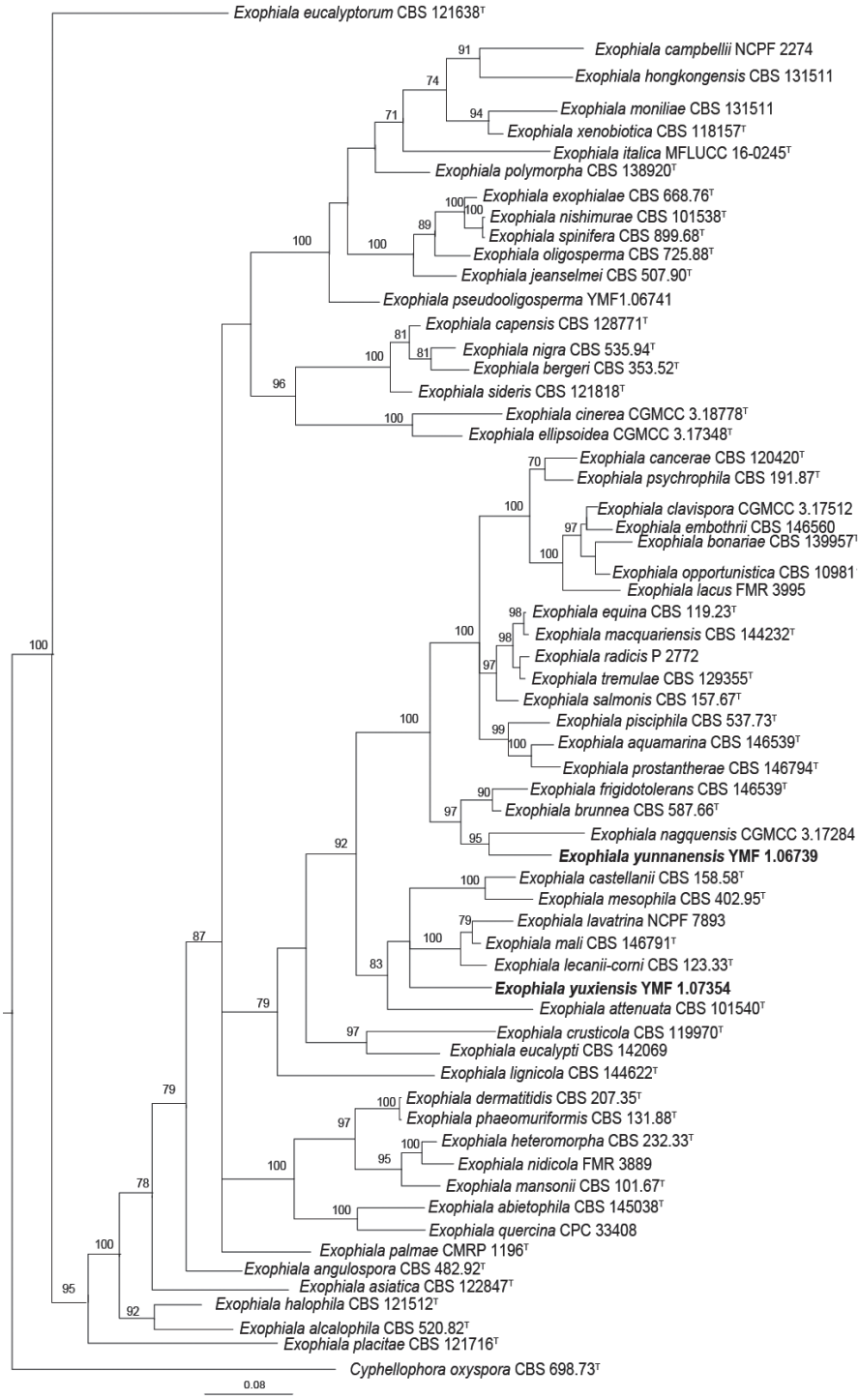

Figure 1. Phylogenetic tree generated by Bayesian Inference, based on sequences of the ITS. *Cyphellophora oxyspora* CBS 698.73 serves as outgroup. Bayesian posterior probability over 75 is shown at the nodes. Two new species were shown in bold.

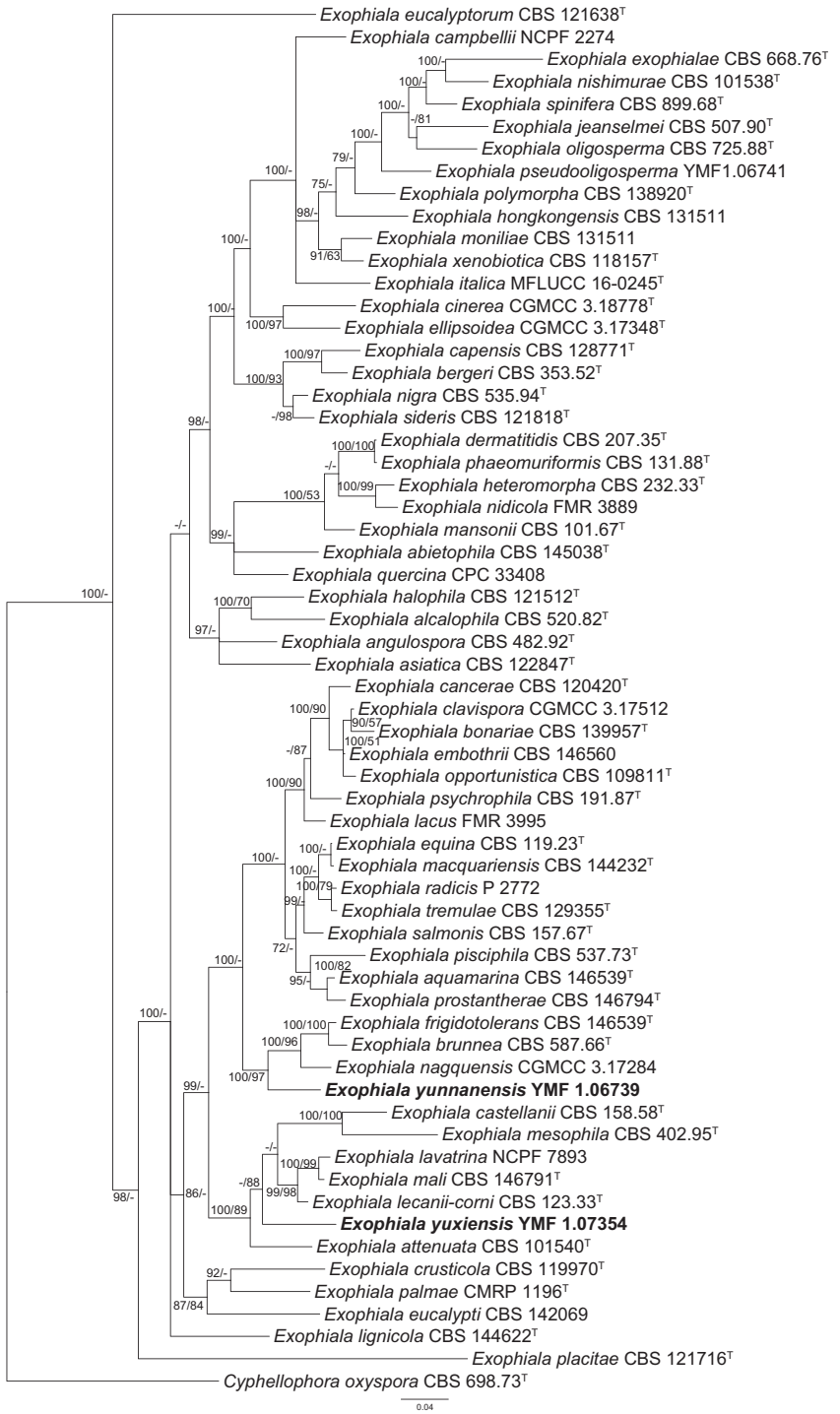

Figure 2. Phylogenetic tree generated by Bayesian analyses combined sequences of ITS, LSU, SSU and *tub2.* Bayesian posterior probability values > 70 (left) and Bootstrap values > 50 (right) are indicated at nodes (BIBP/MLBP). *Cyphellophora oxyspora* CBS 698.73 serves as outgroup.

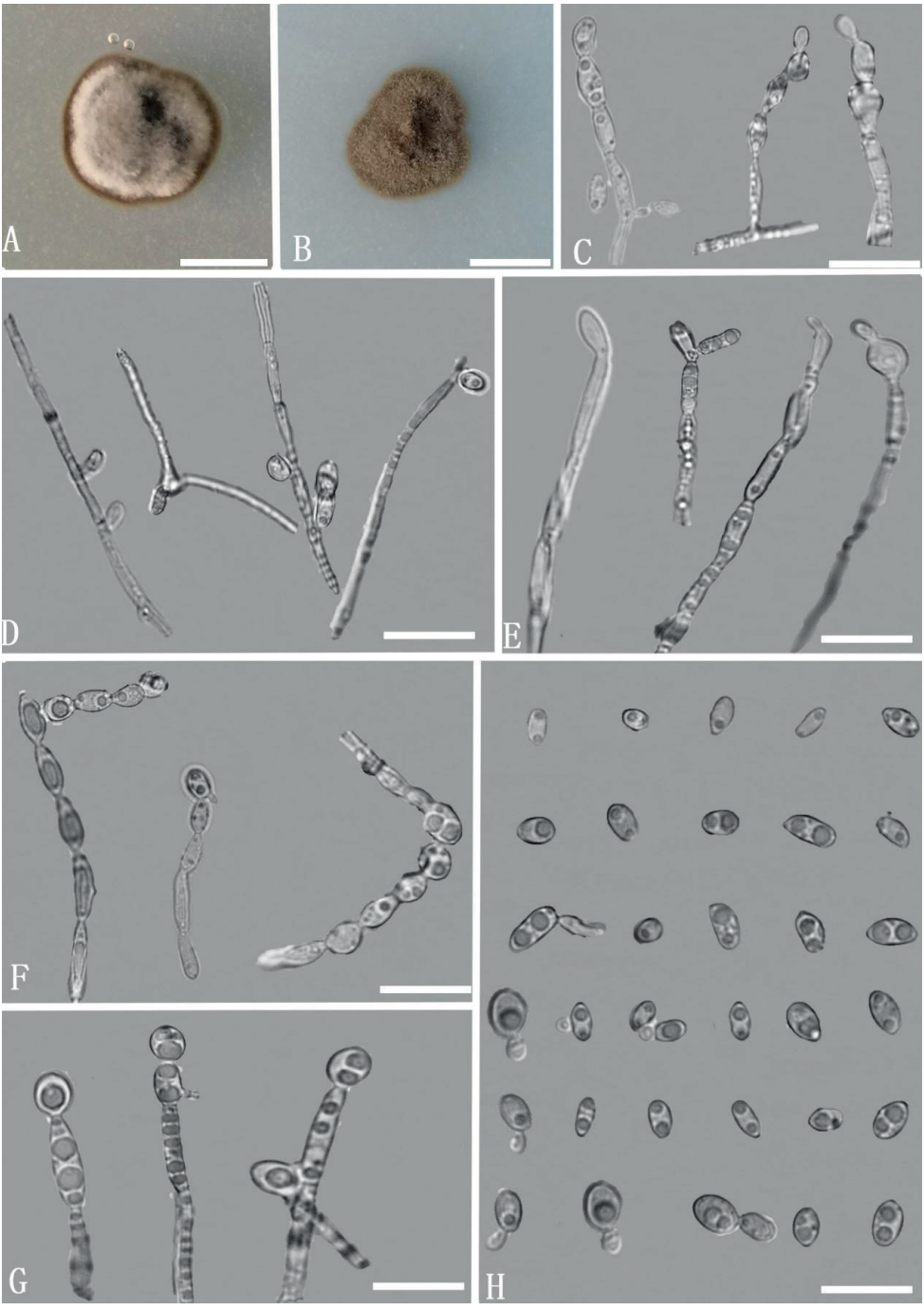

Figure 3. *Exophiala yunnanensis* (YMFT 1.06739, holotype) **A** colony on PDA after 14 days **B** colony on CMA after 14 days **C–G** conidiogenous cells **H** conidia and budding cells. Scale bars: 3.2 cm (**A, B**); 10 μm (**C–H**).

***Exophiala yuxiensis* Z.F. Yu & R.L. Lv, sp. nov.**
MycoBank No: MB842374
Fig. 4

Etymology. *yuxiensis*, pertaining to Yuxi, a city of Yunnan Province in China, from which the type was collected.

Description. Colonies on CMA medium after 7 days with hyphae pale olivaceous-green, smooth, irregularly septate, thin-walled, branched, 1.5–3 μm wide, with lateral branches originating close to septa. Conidiogenous cells slightly differentiated from hyphae, arising from hyphal tips or lateral, terminal or intercalary, variable in shape, flask-shaped, ovoid to elongate, clavate, obtuse at the base, tapering towards inconspicuous annellate loci, 5.5–10.5 × 3–5 μm; annellated zones inconspicuous or occasionally finely fimbriate, often inserted on intercalary cells of hyphae. Conidia aseptate, ellipsoidal to cylindrical, 1–2 (mostly 2) bi-guttulate, smooth, pale olivaceous-green, 4.5–8 × 3.5–5 μm, without conspicuous scar.

Culture characteristics. Colonies on PDA medium, at 25 °C, were slow-growing, mycelium immersed and partly superficial, umbonate, dense, powdery or velvety, dry, margin irregular, surface olivaceous-grey, reverse olivaceous-black, attaining 1 cm diam. in 4 days. Colonies on CMA medium were restricted, mycelium immersed and partly superficial, cottony, surface olivaceous-green, some floccose aerial hyphae in the centre, front distinct, reverse pale olivaceous-black, reaching 3 cm diam. in 5–7 days.

Type. China. Yuxi City, Yunnan Province, isolated from soil of rocky desertification area, 24°44'N, 102°55'E, 1660 m altitude, Jul 2021, Z.F. Yu, preserved by lyophilisation (a metabolically-inactive state) in State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan (holotype YMFT 1.07354), ex-holotype live culture: YMF 1.07354; CGMCC 3.16094; GDMCC 3.726; JCM 39376).

Discussion

In this study, we propose two new species of *Exophiala*, based on combined morphological characteristics and phylogenetic analyses. *Exophiala yunnanensis* and *E. yuxiensis* are different from their phylogenetically closely-related species. Amongst them, *E. nagquensis* (Sun et al. 2020) and *E. brunnea* (Papendorf 1969) are distinguished from *E. yunnanensis* by their larger conidia (*E. nagquensis*: 4.8–10.4 × 2.6–5.0 μm; *E. brunnea*: 4.5–10 μm in length; *E. yunnanensis* 2.9–4.8 × 1.8–3.3 μm), while *E. frigidotolerans* differs from *E. yunnanensis* by ellipsoidal to reniform and larger conidia (4.0–7.0 × 2.0–4 .0 μm) (Crous et al. 2020). Additionally, *E. yunnanensis* resembles *E. nagquensis* and *E. frigidotolerans* in the shape of budding cells, but *E. yunnanensis* has smaller budding cells (Maciá-Vicente et al. 2016; Sun et al. 2020).

Exophiala yuxiensis is phylogenetically related to *E. lecanii-corni*, *E. lavatrina* Borman et al. and *E. mali* Crous. Amongst these species, *E. mali* is the most similar to

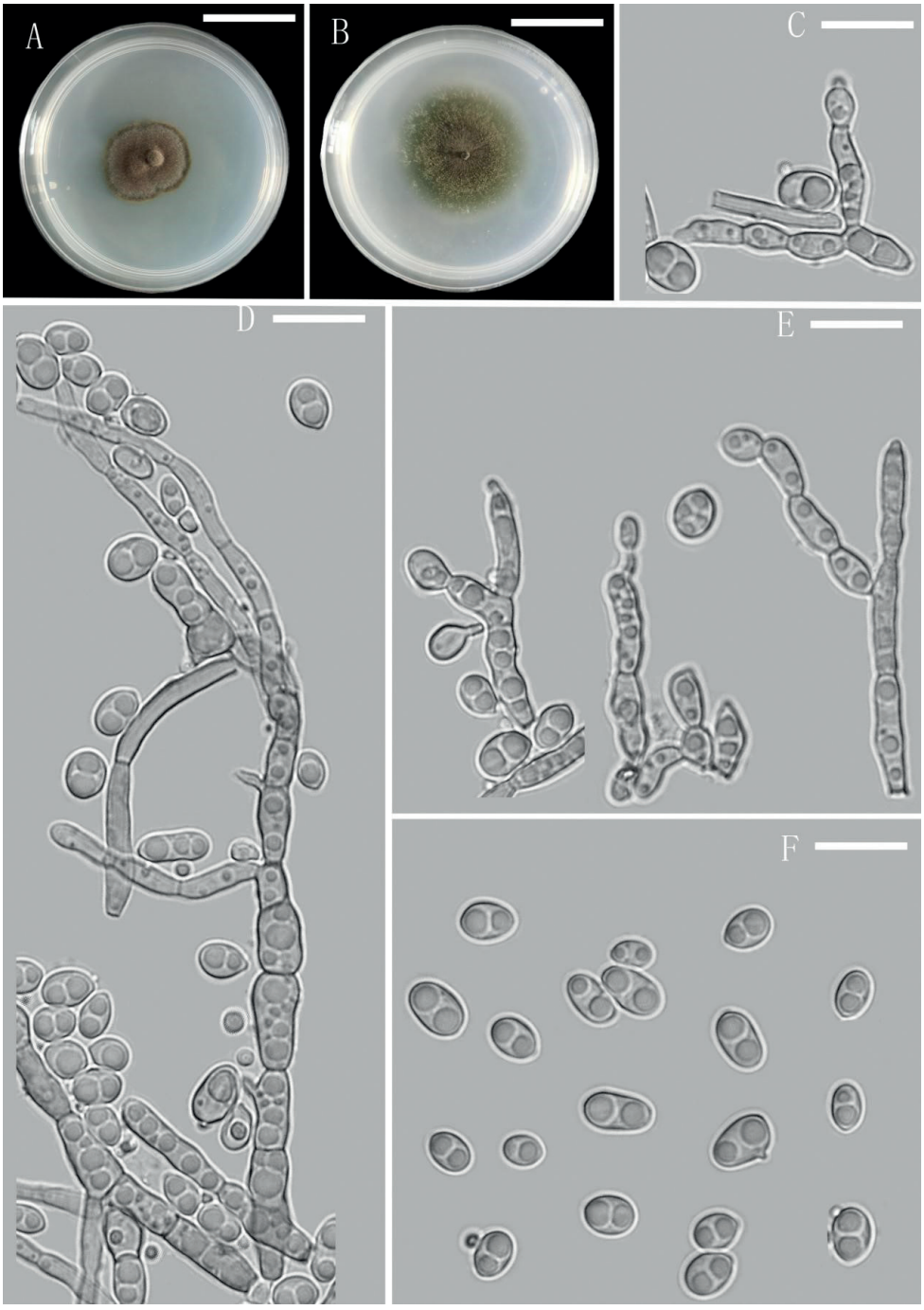

Figure 4. *Exophiala yuxiensis* (YMFT 1.07354, holotype) **A** colony on PDA after 30 days **B** colony on CMA after 30 days **C–E** conidiogenous cells **F** conidia and budding cells. Scale bars: 3.2 cm (**A, B**), 10 μm (**C–F**).

E. yuxiensis by ellipsoidal to cylindrical conidia, but the conidia of *E. mali* are larger (8.0–10.0 × 3.0–5.0 μm vs. 4.5–8.0 × 3.5–5.0 μm) and the hyphae of *E. mali* are constricted at the septa in the terminal region, forming chains of disarticulating conidia (Crous et al. 2020). *Exophiala lavatrina* can be distinguished from *E. yuxiensis* by smaller conidia (4.5–7 × 2.5–4 μm) (Borman et al. 2017).

The species of *Exophiala* have a wide distribution, with isolation from diverse substrates, such as plants, fruit juices, shower rooms, seawater, sports drinks, arable soil, wood pulp, oil sludge and the decaying shell of babassu coconut (De Hoog et al. 1994; De Hoog et al. 2006; De Hoog et al. 2011; Feng et al. 2014; Madrid et al. 2016). Some species were reported as opportunistic pathogens on the superficial skin or internal organs in humans and animals. For example, the type species *E. salmonis*, was isolated from cerebral mycetoma of *Salmo clarkii* Richardson, 1836 (Carmichael 1966), while isolates of *E. equina* (Pollacci) de Hoog et al. and *E. pisciphila* McGinnis & Ajello cause disease on cold-blooded animals such as fish, turtles, crabs, sea horses and frogs (De Hoog et al. 2011). In addition, some species were frequently isolated as endophytes (Addy et al. 2005), although they seldom represent important components of endophytic communities.

The present work increased the number of *Exophiala* species to 70 in the world (Crous et al. 2022; Thitla et al. 2022). In China, Yunnan Province has diverse climate and vegetation, which provides natural advantages for the study of environmental microbial diversity. However, further extensive samplings and investigation of fungi are necessary to generate a complete knowledge about the biodiversity, distribution, habitats and adaptation mechanisms from *Exophiala* to environmental stresses.

Acknowledgements

This work was financed by the National Natural Science Foundation Program of PR China (31970013, 32170017), Yunnan University Research and Innovation Fund for Postgraduates (2021Y294).

References

Addy HD, Piercey MM, Currah RS (2005) Microfungal endophytes in roots. Canadian Journal of Botany 83(1): 1–13. https://doi.org/10.1139/b04-171

Bates ST, Reddy G, Garcia-Pichel F (2006) *Exophiala crusticola* anam. nov. (affinity Herpotrichiellaceae), a novel black yeast from biological soil crusts in the Western United States. International Journal of Systematic and Evolutionary Microbiology 56(11): 2697–2702. https://doi.org/10.1099/ijs.0.64332-0

Borman AM, Fraser M, Szekely A, Larcombe DE, Johnson EM, Diekema DJ (2017) Rapid identification of clinically relevant members of the genus *Exophiala* by matrix-assisted laser desorption ionization-time of flight mass spectrometry and description of two novel

species, *Exophiala campbellii* and *Exophiala lavatrina*. Journal of Clinical Microbiology 55(4): 1162–1176. https://doi.org/10.1128/JCM.02459-16

Bukovská P, Jelínková M, Hrselová H, Sýkorová Z, Gryndler M (2010) Terminal restriction fragment length measurement errors are affected mainly by fragment length, G+C nucleotide content and secondary structure melting point. Journal of Microbiological Methods 82(3): 223–228. https://doi.org/10.1016/j.mimet.2010.06.007

Carmichael JW (1966) Cerebral mycetoma of trout due to a *phialophora*-like fungus. Medical Mycology 5(2): 120–123. https://doi.org/10.1080/00362176785190211

Chang CL, Kim DS, Dong JP, Kim HJ, Chang HL, Shin JH (2000) Acute cerebral phaeohyphomycosis due to *Wangiella dermatitidis* accompanied by cerebrospinal fluid eosinophilia. Journal of Clinical Microbiology 38(5): 1965–1966. https://doi.org/10.1128/JCM.38.5.1965-1966.2000

Crous PW, Cowan DA, Maggs-Kolling G, Yilmaz N, Larsson E, Angelini C, Brandrud TE, Dearnaley JDW, Dima B, Dovana F, Fechner N, García D, Gené J, Halling RE, Houbraken J, Leonard P, Luangsa-ard JJ, Noisripoom W, Rea-Ireland AE, Ševčíková H, Smyth CW, Vizzini A, Adam JD, Adams GC, Alexandrova AV, Alizadeh A, Álvarez Duarte E, Andjic V, Antonín V, Arenas F, Assabgui R, Ballarà J, Banwell A, Berraf-Tebbal A, Bhatt VK, Bonito G, Botha W, Burgess TI, Caboň M, Calvert J, Carvalhais LC, Courtecuisse R, Cullington P, Davoodian N, Decock CA, Dimitrov R, Di Piazza S, Drenth A, Dumez S, Eichmeier A, Etayo J, Fernández I, Fiard J-P, Fournier J, Fuentes-Aponte S, Ghanbary MAT, Ghorbani G, Giraldo A, Glushakova AM, Gouliamova DE, Guarro J, Halleen F, Hampe F, Hernández-Restrepo M, Iturrieta-González I, Jeppson M, Kachalkin AV, Karimi O, Khalid AN, Khonsanit A, Kim JI, Kim K, Kiran M, Krisai-Greilhuber I, Kučera V, Kušan I, Langenhoven SD, Lebel T, Lebeuf R, Liimatainen K, Linde C, Lindner DL, Lombard L, Mahamedi AE, Matočec N, Maxwell A, May TW, McTaggart AR, Meijer M, Mešić A, Mileto AJ, Miller AN, Molia A, Mongkolsamrit S, Muñoz Cortés C, Muñoz-Mohedano J, Morte A, Morozova OV, Mostert L, Mostowfizadeh-Ghalamfarsa R, Nagy LG, Navarro-Ródenas A, Örstadius L, Overton BE, Papp V, Para R, Peintner U, Pham THG, Pordel A, Pošta A, Rodríguez A, Romberg M, Sandoval-Denis M, Seifert KA, Semwal KC, Sewall BJ, Shivas RG, Slovák M, Smith K, Spetik M, Spies CFJ, Syme K, Tasanathai K, Thorn RG, Tkalčec Z, Tomashevskaya MA, Torres-Garcia D, Ullah Z, Visagie CM, Voitk A, Winton LM, Groenewald JZ (2020) Fungal Planet description sheets: 1112–1181. Persoonia 45(1): 251–409. https://doi.org/10.3767/persoonia.2020.45.10

Crous PW, Begoude BAD, Boers J, Braun U, Declercq B, Dijksterhuis J, Elliott TF, Garay-Rodriguez GA, Jurjević Ž, Kruse J, Linde CC, Loyd A, Mound L, Osieck ER, Rivera-Vargas LI, Quimbita AM, Rodas CA, Roux J, Schumacher RK, Starink-Willemse M, Thangavel R, Trappe JM, van Iperen AL, Van Steenwinkel C, Wells A, Wingfield MJ, Yilmaz N, Groenewald JZ (2022) New and interesting fungi. 5. Fungal Systematics and Evolution 10: 19–90. https://doi.org/10.3114/fuse.2022.10.02

De Hoog GS, Takeo K, Yoshida S, Gottlich E, Nishimura K, Miyaji M (1994) Pleoanamorphic life cycle of *Exophiala* (*Wangiella*) *dermatitidis*. Antonie van Leeuwenhoek 65(2): 143–153. https://doi.org/10.1007/BF00871755

De Hoog GS, Ende A, Uijthof J, Untereiner WA (1995) Nutritional physiology of type isolates of currently accepted species of *Exophiala* and *Phaeococcomyces*. Antonie van Leeuwenhoek 68(1): 43–49. https://doi.org/10.1007/BF00873291

De Hoog GS, Vicente V, Caligiorne RB, Kantarcioglu S, Tintelnot K, Gerrits van den Ende AHG, Haase G (2003) Species diversity and polymorphism in the *Exophiala spinifera* clade containing opportunistic black yeast-like fungi. Journal of Clinical Microbiology 41(10): 4767–4778. https://doi.org/10.1128/JCM.41.10.4767-4778.2003

De Hoog GS, Zeng JS, Harrak MJ, Sutton DA (2006) *Exophiala xenobiotica* sp. nov., an opportunistic black yeast inhabiting environments rich in hydrocarbons. Antonie van Leeuwenhoek 90(3): 257–268. https://doi.org/10.1007/s10482-006-9080-z

De Hoog GS, Vicente V, Najafzadeh MJ, Harrak MJ, Badali H, Seyedmousavi S (2011) Waterborne *Exophiala* species causing disease in cold-blooded animals. Persoonia 27(1): 46–72. https://doi.org/10.3767/003158511X614258

Feng PY, Hoog GS, Najafzadeh MJ, Gerrits van den Ende AHG, Stielow JB, Badali H, Boeger WA, Vicente VA (2014) *Cladophialophora abundans*, a novel species of Chaetothyriales isolated from the natural environment. Mycological Progress 13(2): 381–391. https://doi.org/10.1007/s11557-013-0924-4

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61(4): 1323–1330. https://doi.org/10.1128/aem.61.4.1323-1330.1995

Haase G, Sonntag L, Peer YVD, Uijthof J, Melzer-Krick B (1995) Phylogenetic analysis of ten black yeast species using nuclear small subunit rRNA gene sequences. Antonie van Leeuwenhoek 68(1): 19–33. https://doi.org/10.1007/BF00873289

Haase G, Sonntag L, Melzer-Krick B, De Hoog GS (1999) Phylogenetic inference by SSU-gene analysis of members of the Herpotrichiellaceae with special reference to human pathogenic species. Studies in Mycology 43: 80–97. https://doi.org/10.1017/S0953756298007552

Hall TA (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series 41(41): 95–98. https://doi.org/10.1021/bk-1999-0734.ch008

Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse M-A (2010) Mixotrophy in orchids: Insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. The New Phytologist 166(2): 639–653. https://doi.org/10.1111/j.1469-8137.2005.01364.x

Kauff F, Lutzoni F (2002) Phylogeny of the gyalectales and ostropales (Ascomycota, Fungi): Among and within order relationships based on nuclear ribosomal RNA small and large subunits. Molecular Phylogenetics and Evolution 25(1): 138–156. https://doi.org/10.1016/S1055-7903(02)00214-2

Maciá-Vicente JG, Glynou K, Piepenbring M (2016) A new species of *Exophiala* associated with roots. Mycological Progress 15(2): 1–12. https://doi.org/10.1007/s11557-016-1161-4

Madrid H, Hernández-Restrepo M, Gené J, Cano J, Guarro J, Silva V (2016) New and interesting chaetothyrialean fungi from Spain. Mycological Progress 15(10–11): 1179–1201. https://doi.org/10.1007/s11557-016-1239-z

Matsumoto T, Padhye AA, Ajello L (1987) Medical significance of the so-called black yeasts. European Journal of Epidemiology 3(2): 87. https://doi.org/10.1007/BF00239744

Najafzadeh MJ, Dolatabadi S, Keisari MS, Naseri A, Feng P, de Hoogbfghijk GS (2013) Detection and identification of opportunistic *Exophiala* species using the rolling circle amplification of ribosomal internal transcribed spacers. Journal of Microbiological Methods 94(3): 338–342. https://doi.org/10.1016/j.mimet.2013.06.026

Naveau U (1999) Molecular systematics of the Herpotrichiellaceae with an assessment of the phylogenetic positions of *Exophiala dermatitidis* and *Phialophora americana*. Mycologia 91(1): 67–83. https://doi.org/10.1080/00275514.1999.12060994

Neubert K, Mendgen K, Brinkmann H, Wirsel SGR (2006) Only a few fungal species dominate highly diverse mycofloras associated with the common reed. Applied and Environmental Microbiology 72(2): 1118–1128. https://doi.org/10.1128/AEM.72.2.1118-1128.2006

Page RD (1996) Tree View: An application to display phylogenetic trees on personal computers. Bioinformatics 12(4): 357–358. https://doi.org/10.1093/bioinformatics/12.4.357

Papendorf MC (1969) New South African soil fungi. Transactions of the British Mycological Society 52(3): 483–489. https://doi.org/10.1016/S0007-1536(69)80132-4

Posada D (2008) jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution 25(7): 1253–1256. https://doi.org/10.1093/molbev/msn083

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Stamatakis E (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22(21): 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Sun W, Su L, Yang S, Sun J, Liu B, Fu R, Wu B, Liu X, Cai L, Guo L, Xiang M (2020) Unveiling the hidden diversity of rock-Inhabiting Fungi: Chaetothyriales from China. Journal of Fungi 6(4): 1–37. https://doi.org/10.3390/jof6040187

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Thitla T, Kumla J, Khuna S, Lumyong S, Suwannarach N (2022) Species diversity, distribution, and phylogeny of *Exophiala* with the addition of four new species from Thailand. Journal of Fungi 8(8): 766. https://doi.org/10.3390/jof8080766

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25(24): 4876–4882. https://doi.org/10.1093/nar/25.24.4876

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172(8): 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

Vitale RG, De Hoog GS (2002) Molecular diversity, new species and antifungal susceptibilities in the *Exophiala spinifera* clade. Medical Mycology 6(6): 545–556. https://doi.org/10.1080/mmy.40.6.545.556

Vitale RG, Afeltra J, Hoog G, Rijs AJ, Verweij PE (2003) In vitro activity of amphotericin B and itraconazole in combination with flucytosine, sulfadiazine and quinolones against *Exophiala spinifera*. The Journal of Antimicrobial Chemotherapy 51(5): 1297–1300. https://doi.org/10.1093/jac/dkg218

Wen YM, Rajendran RK, Lin YF, Kirschner R, Hu S (2016) Onychomycosis Associated with *Exophiala oligosperma* in Taiwan. Mycopathologia 181(1–2): 83–88. https://doi.org/10.1007/s11046-015-9945-7

White TJ (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR protocols, a guide to methods applications 38, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Woo PC, Ngan AH, Tsang CC, Ling IW, Chan JF, Leung S-Y, Yuen K-Y, Lau SKP (2013) Clinical spectrum of *Exophiala* infections and a novel *Exophiala* species, *Exophiala hongkongensis*. Journal of Clinical Microbiology 51(1): 260–267. https://doi.org/10.1128/JCM.02336-12

Yong LK, Wiederhold NP, Sutton DA, Sandoval-Denis M, Lindner JR, Fan H, Sanders C, Guarro J (2015) Morphological and molecular characterization of *Exophiala polymorpha* sp. nov. isolated from sporotrichoid lymphocutaneous lesions in a patient with Myasthenia Gravis. Journal of Clinical Microbiology 53(9): 2816–2822. https://doi.org/10.1128/JCM.00622-15

Zeng JS, De Hoog GS (2008) *Exophiala spinifera* and its allies: Diagnostics from morphology to DNA barcoding. Medical Mycology 46(3): 193–208. https://doi.org/10.1080/13693780701799217

Zheng H, Li J, Guo JS, Qiao M, Yu ZF (2021a) *Anacraspedodidymum submersum*, a new species of aquatic hyphomycetes from southwest China. International Journal of Systematic and Evolutionary Microbiology 71: 004650. https://doi.org/10.1099/ijsem.0.004650

Zheng H, Qiao M, Lv YF, Du X, Zhang KQ, Yu ZF (2021b) New species of soil-inhabiting and aquatic endophytic *Trichoderma* from southwest China. Journal of Fungi 7: 467. https://doi.org/10.3390/jof7060467