

New *Helminthosporium* (Massarinaceae, Dothideomycetes) and *Nigrospora* (Incertae sedis, Sordariomycetes) species associated with walnut (*Juglans regia* L.) in China

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Abstract

Six collections of ascomycetes were obtained from samples collected from dead branches and leaves of *Juglans regia* in Guizhou and Yunnan provinces, China. By incorporating multigene phylogenetic analysis (ITS, LSU, *rpb2*, SSU, *tef1-a*, *tub2*) supplemented by morphological data, we establish two novel species, namely *Helminthosporium guizhouense* and *Nigrospora yunnanensis*. In morphology, *H. guizhouense* can be distinguished from *H. caespitosum* by its narrower conidia (13–16 µm vs. 27.3–35.5 µm), and *N. yunnanensis* is characterized by black, globose conidia (16.2 × 14.4 µm). The phylogenetic results further substantiated them as novel taxa. The present study contributes to our comprehension of the range of fungi found in *Juglans regia*, thereby expanding our knowledge of the diversity of fungi within this host.

Key words: Ascomycota, morphology, new taxa, phylogeny, taxonomy

Introduction

Dothideomycetes and Sordariomycetes comprise plant pathogens, endophytes, and saprobes, and they can be identified by their distinct fruiting bodies (Marek et al. 2009; Wijayawardene et al. 2016; Hongsanan et al. 2020; Healy et al. 2022). They are widespread inhabitants of plant tissues including walnut (*Juglans regia* L.). Walnuts are a nutritious and health-beneficial drupaceous nut, globally recognized for their valuable properties (Caglarirmak 2003).

Helminthosporium (Massarinaceae, Pleosporales, Dothideomycetes) is a group of asexual Ascomycota proposed by Link (1809) with the type species *H. velutinum*. Most *Helminthosporium* species are saprobes that primarily inhabit various natural substrates, such as plant tissues, wood, bark, dung, and insects, and are also human pathogens (Luttrell 1964, Alcorn 1988; Konta et al. 2023; Hyde et al. 2023, 2024). About 781 taxa have been placed in *Helminthosporium* (<http://www.indexfungorum.org>, June 2024), but most *Helminthosporium*

species differ from the generic type in the development of conidia and conidiophores and therefore are excluded from *Helminthosporium*. Furthermore, very few taxa have molecular data (Hyde et al. 2023). Very few instances of sexual morphs of *Helminthosporium* have been recorded, and the validity of most of these records is questionable as they have not been confirmed by sequence data (Voglmayr and Jaklitsch 2017).

Nigrospora (Apiosporaceae, Xylariales, and Sordariomycetes) was proposed by Zimmerman (1902) with the type species *N. panici* (Hyde et al. 2024). Initially, the characterization of *Nigrospora* species relied on morphological features, particularly large dark conidiospores. However, it was discovered that certain key morphological characteristics, such as the size of spores, were similar among species that are actually not closely phylogenetically related (Hao et al. 2020). To accurately identify different species, it is important to use a comprehensive approach that combines both morphological characteristics and phylogenetic analysis (Jayawardena et al. 2020, Maharachchikumbura et al. 2021). Wang et al. (2017) revised the classification of *Nigrospora*, increasing the known species from 15 to 27 by incorporating morphological and molecular data. Chethana et al. (2023) and Hyde et al. (2024) added records of further species. Wang et al. (2017) confirmed the placement of the genus in Apiosporaceae (Xylariales) based on multi-locus molecular phylogeny, including the internal transcribed spacer (ITS), translation elongation factor 1-alpha (*tef1-α*), and b-tubulin (*tub2*) gene regions. This was confirmed by Samarakoon et al. (2021).

Southwest China is a biodiverse region. In this study, six isolates were collected from walnut leaves and dead tissues from Qianxi County, Guizhou Province, and Lincang City, Yunnan Province. This study aimed to determine the taxonomic status of the pathogenic species of walnut in Guizhou and Yunnan provinces through an analysis of both morphological and molecular characteristics. After conducting a multi-locus phylogenetic analysis and morphological examination, two new species, *Helminthosporium guizhouense*, and *Nigrospora yunnanensis* are identified and introduced.

Materials and methods

Sample collection, fungal strain isolation, and morphology

Samples exhibiting signs of disease were collected from walnuts in Qianxi County, Guizhou Province, and Lincang City, Yunnan Province, from 2023 to 2024. To establish uncontaminated cultures, disinfection processes were implemented on the sample surfaces (Zhang et al. 2020). Conidia were identified on the surface under a dissecting microscope. These conidia were aseptically extracted from the leaves using a sterilized needle and relocated to a sterile, water-filled drip board. The spores were then dispersed in sterile water, and a small quantity of the resulting spore suspension was absorbed and uniformly dispersed onto a potato dextrose agar (PDA) incorporated with streptomycin. After a 12-hour incubation period at 25 °C, individual germinated spores were selected and transferred to fresh PDA. Additionally, we prepared Malt Extract Agar (MEA) and Oatmeal Agar (OA) media for fungal growth. The cultures were subsequently maintained at room temperature (28 °C) for a duration of 10 days.

VHX-7000 (Keyence, Osaka, Japan), Fully-Integrated Head VHX-7100 (Keyence, Osaka, Japan), and High-Performance Camera VHX-7020 (Keyence, Osaka, Japan) dissecting microscopes were used as vehicles for observing the fungal colonies and fruiting bodies. The morphological characteristics of the fungi were studied and documented using a compound light microscope (Zeiss Scope 5) equipped with an attached camera (AxioCam 208 color). Morphological measurements of the new species' features were taken using the ZEN 3.0 (blue edition) (Jena, Germany) software. All newly identified taxa have been registered in the Mycobank database (<https://www.mycobank.org>), accessed on 28 June 2024. For long-term conservation and research purposes, dried holotype specimens were preserved in the Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP). The ex-type cultures have been deposited in the Departmental Culture Collection (GUCC).

DNA extraction and sequencing

Upon reaching the border of a 90 mm diameter Petri dish, a sterile scalpel was used to transfer mycelium into a 1.5 mL centrifuge tube for the extraction of genomic DNA. This extraction was performed using PrepMan Ultra Reagent (Applied Biosystems, CA, USA) in line with the manufacturer's guidelines. The polymerase chain reaction (PCR) amplification was undertaken with a reaction volume of 25 µL. Primer pairs ITS5/ITS4 (White et al. 1990), LR0R/LR5 (Vilgalys and Hester 1990), dRPB2-5F/dRPB2-7cR (Voglmayr et al. 2016), NS1/NS4 (White et al. 1990), Bt2a/Bt2b (Glass and Donaldson 1995), EF1-728F/EF-2 (O'Donnell et al. 1998, Carbone and Kohn 1999) were used to amplify the internal transcribed spacer regions (ITS), partial large subunit nrRNA (LSU) gene, partial DNA-directed RNA polymerase II second largest subunit (*rpb2*), 18S small subunit ribosomal RNA (SSU), partial beta-tubulin (*tub2*) gene, and translation elongation factor 1-alpha (*tef1-a*) gene sequence fragments, respectively.

The PCR thermal cycle program used for amplifying of ITS, LSU, *rpb2*, SSU, *tub2*, and *tef1-a* started with an initial denaturation at 95 °C for 5 minutes. This was followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, elongation at 72 °C for one minute each, and a final extension step at 72 °C lasting 10 minutes. Sangon Biotech (Chengdu, China) handled the purification and sequencing of PCR amplicons. Sequences meeting the quality criteria were submitted to GenBank, and their corresponding accession numbers are listed in Table 1, which also contains a complete list of all the strains utilized in this research.

Phylogenetic analyses

Reference sequences obtained from GenBank (Table 1) were utilized to assist with the phylogenetic analyses. Multiple sequence alignments were created using the online platform of MAFFT v.7.307 (<http://mafft.cbrc.jp/alignment/server/>) (Katoh and Standley 2016). AliView (Larsson 2014) was utilized for manual refinement, with terminal ends and ambiguous regions of the alignment being manually excised. Phylogenetic analyses were performed using concatenated sequences from the six (ITS, LSU, *rpb2*, SSU, *tub2*, and *tef1-a*) through Maximum Likelihood (ML), and Bayesian Inference (BI) methodologies.

Table 1. Species and GenBank accession numbers of DNA sequences used in the phylogenetic analysis.

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	<i>rpb2</i>	SSU	<i>tef1-a</i>	<i>tub2</i>
<i>Byssothecium circinans</i>	CBS675.92	OM337536	GU205217	DQ767646	GU205235	–	–
<i>Haplohelminthosporium calami</i>	MFLUCC18-0074*	MT928158	MT928156	–	MT928160	–	–
<i>Helminthosporiella astilbacea</i>	COAD2126	MG668862	–	–	–	–	–
<i>Helminthosporiella stilbacea</i>	MFLUCC15-0813*	MT928159	MT928157	–	MT928161	–	–
<i>Helminthosporiella stilbacea</i>	CPHmZC-01	KX228298	KX228355	–	–	–	–
<i>Helminthosporium maquaticum</i>	MFLUCC15-0357 = S-096*	KU697302	KU697306	–	KU697310	–	–
<i>Helminthosporium austriacum</i>	CBS139924 = L132*	KY984301	KY984301	KY984365	KY984420	–	–
<i>Helminthosporium austriacum</i>	CBS14238 = L169	KY984303	KY984303	KY984367	–	–	–
<i>Helminthosporium austriacum</i>	L137	KY984302	KY984302	KY984366	–	–	–
<i>Helminthosporium caespitosum</i>	CBS484.77 = L99*	JQ044429	JQ044448	KY984370	KY984421	–	–
<i>Helminthosporium caespitosum</i>	L141	KY984305	KY984305	KY984368	–	–	–
<i>Helminthosporium caespitosum</i>	L151	KY984306	KY984306	KY984369	–	–	–
<i>Helminthosporium chengduense</i>	UESTC22.0024 = YQ071048 = CGMCC	ON557751	ON557745	ON563073	ON557757	–	–
<i>Helminthosporium chengduense</i>	UESTC22.0025 = YQ071047	ON557750	ON557744	ON563072	ON557756	–	–
<i>Helminthosporium chiangraiense</i>	MFLUCC21-0087*	MZ538504	MZ538538	–	–	–	–
<i>Helminthosporium chlorophorae</i>	BRIP14521	AF120259	–	–	–	–	–
<i>Helminthosporium dalbergiae</i>	MAFF243853 = H4628 = TS36	LC014555	AB807521	–	AB797231	–	–
<i>Helminthosporium endiandrae</i>	CBS138902 = CPC22194*	KP004450	KP004478	–	–	–	–
<i>Helminthosporium erythrinicola</i>	CPC35291 = CBS145569*	NR_165563	MK876432	MK876486	–	–	–
<i>Helminthosporium genistae</i>	CBS142597 = L142*	KY984310	KY984310	KY984374	–	–	–
<i>Helminthosporium genistae</i>	CBS139922 = L129	KY984309	KY984309	KY984373	KY984423	–	–
<i>Helminthosporium genistae</i>	CBS139921 = L128	KY984308	KY984308	KY984372	KY984422	–	–
<i>Helminthosporium guizhouense</i>	GUCC24-0011*	PP915799	PP949847	PP947940	PP949912	–	–
<i>Helminthosporium guizhouense</i>	GUCC24-0012	PP915800	PP949848	PP947941	PP949913	–	–
<i>Helminthosporium guizhouense</i>	GUCC24-0013	PP915801	PP949849	PP947942	PP949914	–	–
<i>Helminthosporium hispanicum</i>	CBS136917 = L109*	KY984318	KY984318	KY984381	KY984424	–	–
<i>Helminthosporium juglandinum</i>	CBS136922 = L118*	KY984321	KY984321	KY984384	–	–	–
<i>Helminthosporium juglandinum</i>	CBS136911 = L97	KY984322	KY984322	KY984385	KY984425	–	–
<i>Helminthosporium juglandinum</i>	CBS136912 = L101	KY984319	KY984319	KY984382	–	–	–
<i>Helminthosporium juglandinum</i>	CBS136913 = L102	KY984320	KY984320	KY984383	–	–	–
<i>Helminthosporium leucadendri</i>	CBS135133 = CPC19345*	KF251150	KF251654	KF252159	–	–	–
<i>Helminthosporium livistonae</i>	CPC32158 = CBS144413*	NR_160348	NG_064539	–	–	–	–

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	<i>rpb2</i>	SSU	<i>tef1-a</i>	<i>tub2</i>
<i>Helminthosporium magnisporum</i>	MAFF239278 = H4627 = TS33*	AB811452	AB807522	–	AB797232	–	–
<i>Helminthosporium massarinum</i>	CBS139690 = JCM13095 = MAFF239605 = KT1564*	AB809629	AB807524	–	AB797234	–	–
<i>Helminthosporium massarinum</i>	JCM13094 = MAFF239604 = KT838*	AB809628	AB807523	–	AB797233	–	–
<i>Helminthosporium microsorum</i>	CBS136910 = L96*	KY984329	KY984329	KY984390	KY984427	–	–
<i>Helminthosporium microsorum</i>	L94	KY984327	KY984327	KY984388	KY984426	–	–
<i>Helminthosporium microsorum</i>	CBS136916 = L108	KY984323	KY984323	KY984386	–	–	–
<i>Helminthosporium microsorum</i>	L95	KY984328	KY984328	KY984389	–	–	–
<i>Helminthosporium nanjingensis</i>	HHAUF020380 = ZM020380	KF192322	–	–	–	–	–
<i>Helminthosporium oligosporum</i>	CBS136909 = L93*	KY984333	KY984333	KY984394	–	–	–
<i>Helminthosporium oligosporum</i>	CBS136908 = L92	KY984332	KY984332	KY984393	KY984428	–	–
<i>Helminthosporium oligosporum</i>	L106	KY984330	KY984330	KY984391	–	–	–
<i>Helminthosporium quercinum</i>	CBS136921 = L90*	KY984339	KY984339	KY984400	KY984429	–	–
<i>Helminthosporium quercinum</i>	CBS112393	KY984334	KY984334	KY984395	–	–	–
<i>Helminthosporium quercinum</i>	CBS136915 = L107	KY984336	KY984336	KY984397	–	–	–
<i>Helminthosporium solani</i>	CBS365.75	KY984341	KY984341	KY984402	KY984430	–	–
<i>Helminthosporium solani</i>	CBS640.85	KY984342	KY984342	KY984403	–	–	–
<i>Helminthosporium submersum</i>	MFLUCC16-1360*	–	MG098787	–	MG098796	–	–
<i>Helminthosporium submersum</i>	MFLUCC16-1290PT	MG098780	MG098788	MG098592	MG098797	–	–
<i>Helminthosporium submersum</i>	UESTCC22.0021 = Sara08_3 = CGMCC	ON557753	ON557747	ON563075	ON557759	–	–
<i>Helminthosporium syzygii</i>	CPC35312 = CBS145570*	NR_165564	MK876433	MK876487	–	–	–
<i>Helminthosporium tiliae</i>	CBS136907 = L88*	KY984345	KY984345	KY984406	KY984431	–	–
<i>Helminthosporium tiliae</i>	CBS136906 = L87	KY984344	KY984344	KY984405	–	–	–
<i>Helminthosporium tiliae</i>	L171	KY984343	KY984343	KY984404	–	–	–
<i>Helminthosporium velutinum</i>	CBS139923 = L131*	KY984352	KY984352	KY984413	KY984432	–	–
<i>Helminthosporium velutinum</i>	L98	KY984359	KY984359	KY984417	KY984433	–	–
<i>Helminthosporium velutinum</i>	CBS136924 = L115	KY984347	KY984347	KY984408	–	–	–
<i>Helminthosporium velutinum</i>	L116	KY984348	KY984348	KY984409	–	–	–
<i>Helminthosporium velutinum</i>	L117	KY984349	KY984349	KY984410	–	–	–
<i>Helminthosporium velutinum</i>	UESTCC22.0022 = BY14_2 = CGMCC3.23572	ON557755	ON557749	–	ON557761	–	–
<i>Helminthosporium chinense</i>	UESTCC22.0026 = YQ071.005 = CGMCC3.23570*	ON557754	ON557748	–	ON557760	–	–
<i>Massarina cisti</i>	CBS266.62 = JCM14140*	LC014568	AB807539	FJ795464	AB797249	–	–

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	<i>rpb2</i>	SSU	<i>tef1-a</i>	<i>tub2</i>
<i>Massarina eburnea</i>	CBS473.64	OM337528	GU301840	GU371732	GU296170	–	–
<i>Massarina eburnea</i>	CBS139697 = JCM14422 = H3953	LC014569	AB521735	–	AB521718	–	–
<i>Massarina pandanicola</i>	MFLUCC17-0596 = KUMCC17-0293*	MG646958	MG646947	–	MG646979	–	–
<i>Periconia pseudodigitata</i>	KT1395 = HHUF29370 = CBS139699 = JCM13166 = MAFF239676*	NR_153490	NG_059396	–	NG_064850	–	–
<i>Pseudodidymosphaeria spartii</i>	MFLUCC13-0273	KP325434	KP325436	–	KP325438	–	–
<i>Pseudodidymosphaeria spartii</i>	MFLUCC14-1212	KP325435	KP325437	–	KP325439	–	–
<i>Pseudosplanchnonema phorcioides</i>	L16 = CBS122935	KY984360	KY984360	KY984418	KY984434	–	–
<i>Pseudosplanchnonema phorcioides</i>	MFLUCC13-0533 = CGMCC3.17583	–	KM875454	–	KM875455	–	–
<i>Pseudosplanchnonema phorcioides</i>	MFLUCC13-0611	KP683375	KP683376	–	KP683377	–	–
<i>Pseudosplanchnonema phorcioides</i>	MFLUCC14-0618	KP683372	KP683373	–	KP683374	–	–
<i>Semifissispora natalis</i>	CPC25383 = CBS140659*	KT950846	KT950858	–	–	–	–
<i>Semifissispora rotundata</i>	CBS172.93 = CPC549	KT950847	KT950859	–	–	–	–
<i>Semifissispora tooloomensis</i>	CBS143431 = CPC31680*	NR_156674	NG_058526	–	–	–	–
<i>Stagonospora duoseptata</i>	CBS135093 = S618*	KF251255	KF251758	KF252260	–	–	–
<i>Stagonospora imperaticola</i>	MFLUCC15-0026 = ICMP21563*	KY706143	KY706133	KY706149	KY706138	–	–
<i>Stagonospora multiseptata</i>	MFLUCC15-0449 = ICMP21562*	NR_165854	NG_068239	–	–	–	–
<i>Stagonospora paludosa</i>	CBS135088*	KF251257	KF251760	KF252262	–	–	–
<i>Stagonospora perfecta</i>	KT1726A = JCM13099 = MAFF239609	AB809642	AB807579	–	AB797289	–	–
<i>Stagonospora perfecta</i>	CBS135099 = S656*	KF251258	KF251761	KF252263	–	–	–
<i>Stagonospora pseudocaricis</i>	CBS135132 = S610*	KF251259	KF251763	KF252265	–	–	–
<i>Stagonospora pseudopaludosa</i>	CPC22654 = CBS136424*	NR_137840	NG_058052	–	–	–	–
<i>Stagonospora pseudoperfecta</i>	CBS120236 = JCM13097 = MAFF239607*	AB809641	AB807577	–	AB797287	–	–
<i>Stagonospora tainanensis</i>	KT1866 = MAFF243860	AB809643	AB807580	–	AB797290	–	–
<i>Stagonospora trichophoricola</i>	CBS136764 = D652*	NR_156586	NG_058081	KJ869232	–	–	–
<i>Stagonospora uniseptata</i>	CBS135090 = S611*	KF251264	KF251767	KF252269	–	–	–
<i>Stagonospora uniseptata</i>	S607 = CPC22151	KF251265	KF251768	KF252270	–	–	–
<i>Stagonospora uniseptata</i>	S608 = CPC22150	KF251266	KF251769	KF252271	–	–	–

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	<i>rpb2</i>	SSU	<i>tef1-a</i>	<i>tub2</i>
<i>Suttonomyces clematidis</i>	MFLUCC14-0240 = GUCC18	–	KP842917	–	KP842920	–	–
<i>Suttonomyces rosae</i>	MFLUCC15-0051*	MG828973	MG829085	–	MG829185	–	–
<i>Synhelminthosporium synnematiferum</i>	UESTCC22.0023 = HLG072894 = CGMCC3.23574*	ON557752	ON557746	ON563074	ON557758	–	–
<i>Apiospora malaysiana</i>	CBS 102053	KF144896	–	–	–	KF145030	KF144988
<i>Apiospora pseudoparenchymatica</i>	LC7234*	KY494743	–	–	–	KY705139	KY705211
<i>Nigrospora aurantiaca</i>	CGMCC 3.18130*	KX986064	–	–	–	KY019295	KY019465
<i>Nigrospora aurantiaca</i>	LC7034	KX986093	–	–	–	KY019394	KY019598
<i>Nigrospora bambusae</i>	CGMCC 3.18327*	KY385307	–	–	–	KY385313	KY385319
<i>Nigrospora bambusae</i>	LC7245	KY385305	–	–	–	KY385315	KY385321
<i>Nigrospora brasiliensis</i>	CMM 1214*	KY569629	–	–	–	MK753271	MK720816
<i>Nigrospora brasiliensis</i>	CMM 1217	KY569630	–	–	–	MK753272	MK720817
<i>Nigrospora camelliae-sinensis</i>	CGMCC 3.18125*	KX985986	–	–	–	KY019293	KY019460
<i>Nigrospora chinensis</i>	LC6851	KX986049	–	–	–	KY019450	KY019579
<i>Nigrospora chinensis</i>	CGMCC 3.18127*	KX986023	–	–	–	KY019422	KY019462
<i>Nigrospora covidalis</i>	CGMCC 3.20538*	OK335209	–	–	–	OK431485	OK431479
<i>Nigrospora covidalis</i>	LC158337	OK335210	–	–	–	OK431486	OK431480
<i>Nigrospora endophytica</i>	URM8712 = A.R.M. 687	OM265226	–	–	–	OP572415	OP572418
<i>Nigrospora endophytica</i>	URM8462 = A.R.M. 973*	OM265233	–	–	–	OP572416	OP572420
<i>Nigrospora falsivesicularis</i>	CGMCC 3.19678*	MN215778	–	–	–	MN264017	MN329942
<i>Nigrospora falsivesicularis</i>	LC13553	MN215779	–	–	–	MN264018	MN329943
<i>Nigrospora globospora</i>	CGMCC 3.20539*	OK335211	–	–	–	OK431487	OK431481
<i>Nigrospora globospora</i>	LC15839	OK335212	–	–	–	OK431488	OK431482
<i>Nigrospora gorlenkoana</i>	CBS 480.73*	KX986048	–	–	–	KY019420	KY019456
<i>Nigrospora guangdongensis</i>	CFCC:53917*	MT017509	–	–	–	MT024493	MT024495
<i>Nigrospora guilinensis</i>	LC7301	KX986063	–	–	–	KY019404	KY019608
<i>Nigrospora guilinensis</i>	CGMCC 3.18124*	KX985983	–	–	–	KY019292	KY019459
<i>Nigrospora hainanensis</i>	CGMCC 3.18129*	KX986091	–	–	–	KY019415	KY019464
<i>Nigrospora hainanensis</i>	URM8714 = A.R.M.967	OM265228	–	–	–	OM642834	OM793057
<i>Nigrospora hainanensis</i>	URM8715 = A.R.M.968	OM265229	–	–	–	OM642835	OM793058
<i>Nigrospora lacticolonia</i>	CGMCC 3.18123*	KX985978	–	–	–	KY019291	KY019458
<i>Nigrospora lacticolonia</i>	URM8713 = A.R.M. 921	OM265227	–	–	–	OM642833	OM642838
<i>Nigrospora magnoliae</i>	MFLUCC 19–0112*	MW285092	–	–	–	–	MW438334
<i>Nigrospora manihoticola</i>	URM8461 = A.R.M. 645*	OM265224	–	–	–	OM914791	OM869479
<i>Nigrospora musae</i>	CBS 319.34*	KX986076	–	–	–	KY019419	KY019455

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	<i>rpb2</i>	SSU	<i>tef1-a</i>	<i>tub2</i>
<i>Nigrospora musae</i>	LC6385	KX986042	–	–	–	KY019371	KY019567
<i>Nigrospora oryzae</i>	LC2724	KX985959	–	–	–	KY019312	KY019486
<i>Nigrospora oryzae</i>	LC4265	KX985994	–	–	–	KY019335	KY019518
<i>Nigrospora osmanthi</i>	CGMCC 3.18126*	KX986010	–	–	–	KY019421	KY019461
<i>Nigrospora osmanthi</i>	LC4487	KX986017	–	–	–	KY019438	KY019540
<i>Nigrospora pernambucoensis</i>	URM8711 = A.R.M.651	OM265225	–	–	–	OM914792	OM869480
<i>Nigrospora pernambucoensis</i>	URM8463 = A.R.M.974*	OM265234	–	–	–	OM914793	OM869481
<i>Nigrospora philosophiae-doctoris</i>	CGMCC 3.20540*	OK335214	–	–	–	OK431490	OK431484
<i>Nigrospora pyriformis</i>	CGMCC 3.18122*	KX985940	–	–	–	KY019290	KY019457
<i>Nigrospora pyriformis</i>	URM8716 = A.R.M.970	OM265231	–	–	–	OM513904	OM642839
<i>Nigrospora rubi</i>	LC2698*	KX985948	–	–	–	KY019302	KY019475
<i>Nigrospora saccharicola</i>	LC12057	MN215789	–	–	–	MN264028	MN329952
<i>Nigrospora saccharicola</i>	CGMCC 3.19362*	MN215788	–	–	–	MN264027	MN329951
<i>Nigrospora sacchari-ofcinarum</i>	CGMCC 3.19335*	MN215791	–	–	–	MN264030	MN329954
<i>Nigrospora sacchari-ofcinarum</i>	LC13531	MN215792	–	–	–	MN264031	MN329955
<i>Nigrospora singularis</i>	CGMCC 3.19334*	MN215793	–	–	–	MN264032	MN329956
<i>Nigrospora singularis</i>	LC12068	MN215794	–	–	–	MN264033	MN329957
<i>Nigrospora sphaerica</i>	LC2839	KX985964	–	–	–	KY019317	KY019491
<i>Nigrospora sphaerica</i>	LC2840	KX985965	–	–	–	KY019318	KY019492
<i>Nigrospora</i> sp. 1	LC2725	KX985960	–	–	–	KY019313	KY019487
<i>Nigrospora</i> sp. 1	LC4566	KX986022	–	–	–	KY019354	KY019545
<i>Nigrospora</i> sp. 2	LC6704	KX986047	–	–	–	KY019373	KY019571
<i>Nigrospora stoneae</i>	BRIP 75022a	OR608744	–	–	–	OR604065	OR604067
<i>Nigrospora vesicularis</i>	LC0322	KX985939	–	–	–	KY019296	KY019467
<i>Nigrospora vesicularis</i>	CGMCC 3.18128*	KX986088	–	–	–	KY019294	KY019463
<i>Nigrospora vesicularifera</i>	CGMCC 3.19333*	MN215812	–	–	–	MN264051	MN329975
<i>Nigrospora vesicularifera</i>	URM8718 = A.R.M.975	OM265235	–	–	–	OM513905	OM642840
<i>Nigrospora yunnanensis</i>	GUCC24-0008*	PP915796	–	–	–	PP947933	PP947937
<i>Nigrospora yunnanensis</i>	GUCC24-0009	PP915797	–	–	–	PP947934	PP947938
<i>Nigrospora yunnanensis</i>	GUCC24-0010	PP915798	–	–	–	PP947935	PP947939
<i>Nigrospora zimmermanii</i>	CBS 290.62*	KY385309	–	–	–	KY385311	KY385317
<i>Nigrospora zimmermanii</i>	CBS 984.69	KY385310	–	–	–	KY385316	KY385322

* = Type specimens. Our strains in this study were in bold.

The Maximum Likelihood analysis was executed on the IQ-TREE web server (<http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos et al. 2016). The models is: In Fig. 1, TIM2e+I+G4 for ITS, TIM2e+I+G4 for LSU, TN+F+I+G4 for *rpb2* and K2P+G4 for SSU; In Fig. 2, TNe+I+G4 for ITS, TN+F+I+G4 for *tef1-a*, HKY+F+I+G4

for *tub2*. The number of bootstrap alignments is 1000 (Nguyen et al. 2015). Bayesian analysis (BI) was carried out using PhyloSuite v.1.2.2 as a tool (Zhang et al. 2019). In Fig. 1, SYM+I+G4 as the optimal model for ITS and LSU, HKY+F+I+G4 as the best-fit model for *rpb2*, K2P G4 as the optimal model for SSU; In Fig. 2, SYM+I+G4 as the optimal model for ITS, HKY+F+I+G4 as the best-fit model for *tef1-α* and *tub2*. Four chains were run for 10,000,000 generations and sampled every 500 generations. The initial 25% of the resulting trees were discarded as burn-in, and the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree. The final phylogenetic topology was visualized with FigTree v.1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>) and was modified in Microsoft Office PowerPoint 2019.

Results

Phylogenetic analyses

For the *Helminthosporium* and related genera (Fig. 1), the phylogenetic trees accommodated 96 sequences listed in Table 1. The strains GUCC24-0011, GUCC24-0012, and GUCC24-0013 were characterized based on their molecular properties, specifically sequencing of the ITS, LSU, *rpb2*, and SSU genes regions. An outgroup consisting of the type strain KT1395 of *Periconia pseudodigitata* was also included in the study based on concatenated datasets, as shown in Table 1. The combined alignment consists of 5596 characters, including ITS (1766 characters), LSU (1648 characters), *rpb2* (1114 characters), and SSU (1065 characters) regions. We constructed two phylogenetic trees: an ML tree and a BI tree. The ML tree was selected to represent the phylogenetic relationship of different *Helminthosporium* taxa (Fig. 1). *Helminthosporium guizhouense* (GUCC24-0011, GUCC24-0012, and GUCC24-0013) was found to be a sister taxon to *H. caespitosum* (CBS484.77, L141, and L151) with high support values from both ML and BI analyses (ML/BI: 100/1).

For the *Nigrospora* and related genera (Fig. 2), the phylogenetic trees accommodated 68 sequences listed in Table 1. The strains GUCC24-0008, GUCC24-0009, and GUCC24-0010 were characterized based on their molecular properties, and accurate sequencing of the ITS, *tef1-α*, and *tub2* gene regions. *Apiospora malaysiana* (CBS 102053) and *A. pseudoparenchymatica* (LC7234) were selected as outgroups. The combined alignment consists of 1523 characters, including ITS (571 characters), *tef1-α* (562 characters), *tub2* (385 characters) regions. We constructed two phylogenetic trees: an ML tree and a BI tree. The ML tree was selected to represent the phylogenetic relationship of different *Nigrospora* taxa (Fig. 2). *Nigrospora yunnanensis* (GUCC24-0011, GUCC24-0012, and GUCC24-0013) formed an independent branch without the DNA base differences in three loci supported by strong statistic data (ML/BI: 100/1) and were adjacent to the branch of *N. falsivesicularis* (CGMCC 3.19678 and LC13553), *N. vesicularis* (LC0322 and CGMCC 3.18128), *N. aurantiaca* (CGMCC 3.18130 and LC7034), *N. stoneae* (BRIP 75022a), *N. lacticolonia* (CGMCC 3.18123 and URM8713), *N. osmanthi* (CGMCC 3.18126 and LC4487), *N. endophytica* (URM8712 and URM8462), *N. pernambucoensis* (URM8711 and URM8463), and *N. guilinensis* (LC730 and CGMCC 3.18124) (ML/BI: 97/0.99).

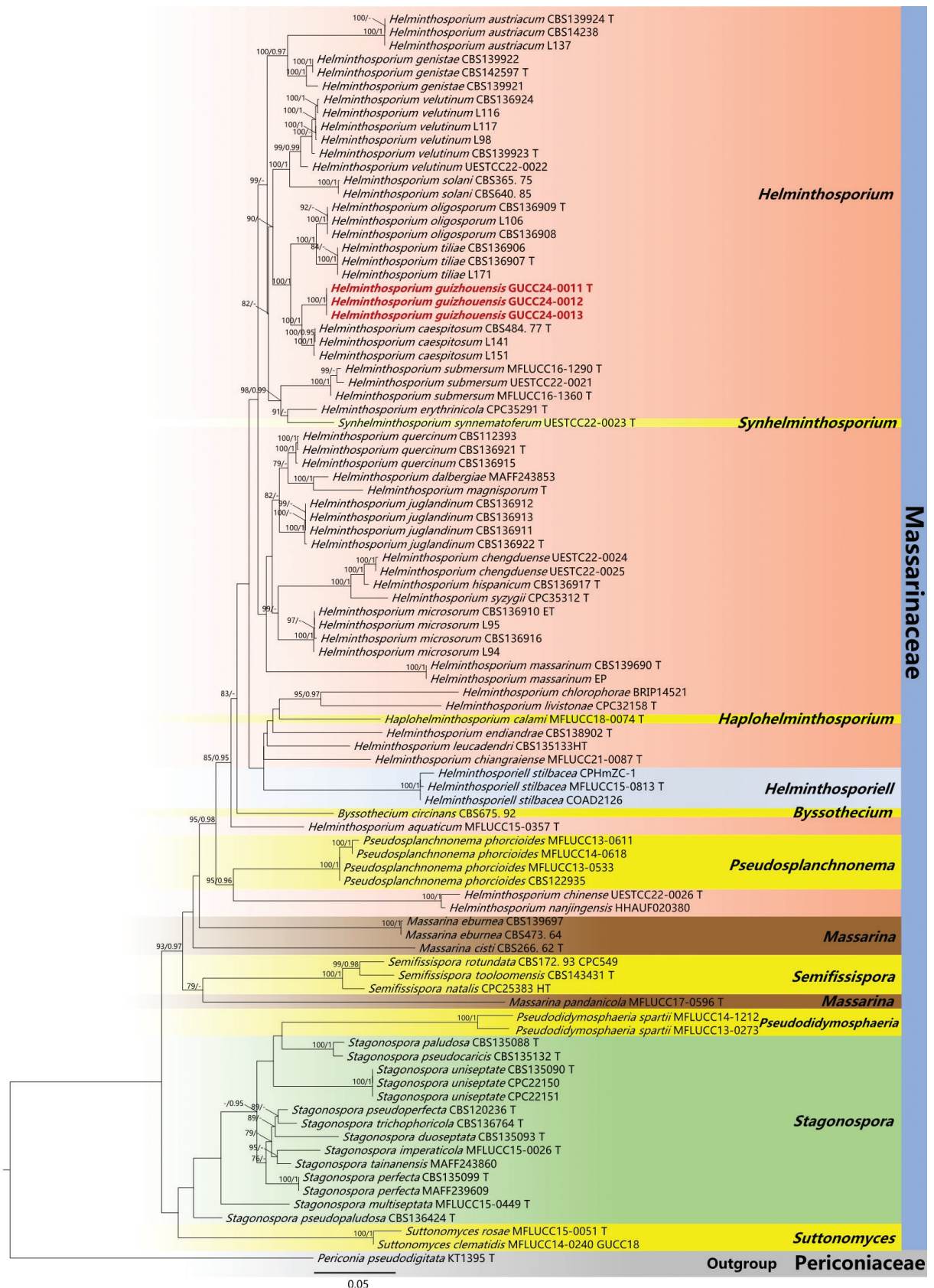


Figure 1. The maximum parsimony tree of 96 *Helminthosporium* taxa is based on ITS, LSU, *rpb2*, and SSU genes. The tree was rooted with *Periconia pseudodigitata* (KT1395). Bootstrap support values for ML greater than 75% and Bayesian posterior probabilities greater than 0.95 are given near nodes, respectively. The new isolates were in red. Ex-type strains were marked by T. The scale bar indicates 0.05 expected changes per site.

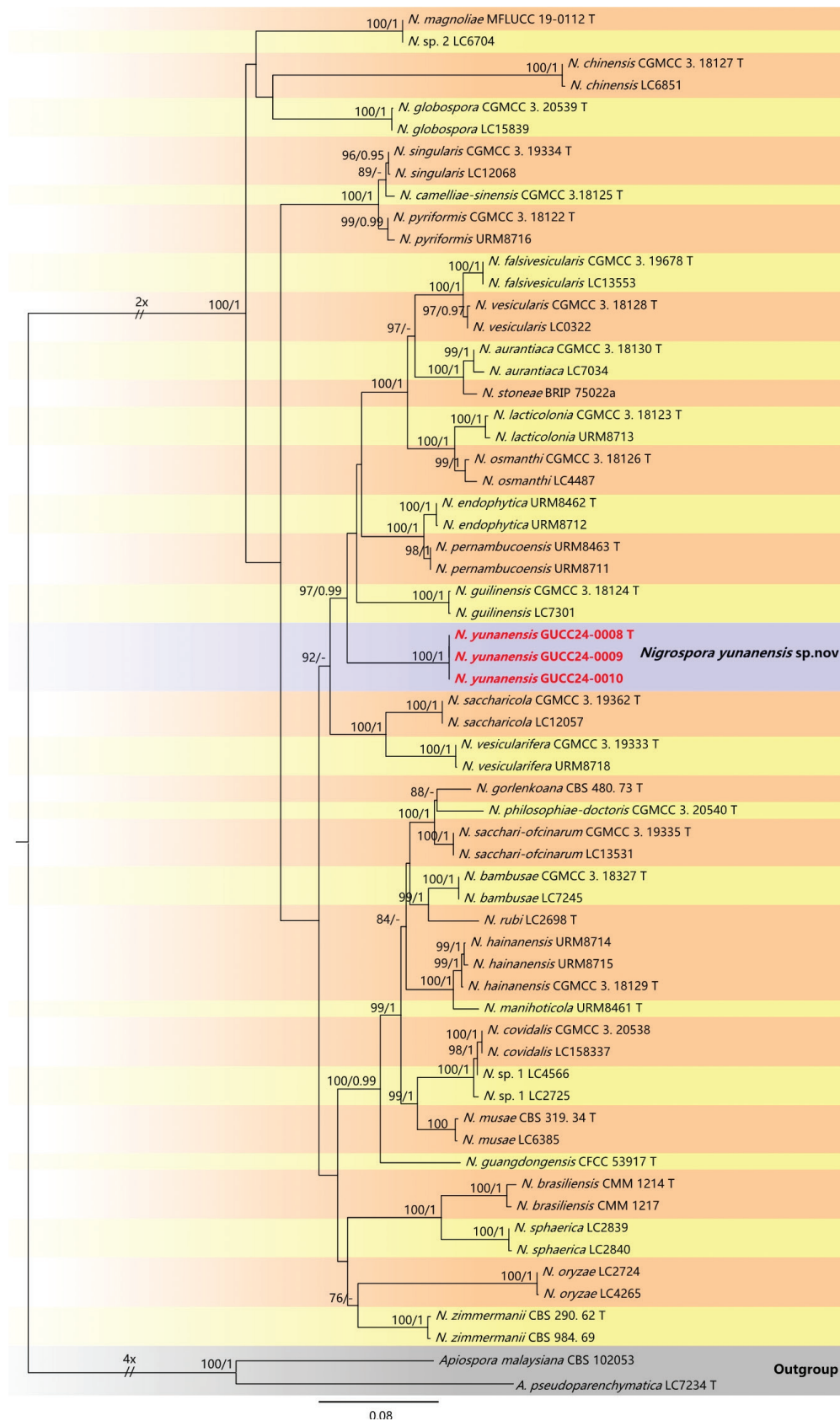


Figure 2. The maximum parsimony tree of 68 *Nigrospora* taxa is based on ITS, *tef1-α*, and *tub2* genes. The tree was rooted with *Apiospora malaysiana* (CBS 102053) and *A. pseudoparenchymatica* (LC7234). Bootstrap support values for ML greater than 75% and Bayesian posterior probabilities greater than 0.95 are given near nodes, respectively. The new isolates were in red. Ex-type strains were marked by T. The scale bar indicates 0.08 expected changes per site.

Taxonomy

Helminthosporium guizhouense M.T. Zou & Yong Wang bis, sp. nov.

MycoBank No: 854537

Fig. 3a–r

Etymology. The name refers to Guizhou, the province where the fungus was collected.

Diagnosis. *Helminthosporium guizhouense* can easily be distinguished from *H. caespitosum* by its narrower conidia (13–16 µm vs. 27.3–35.5 µm).

Type. CHINA • Guizhou Province, Qianxi City; 26°56'11.58"N, 105°55'15.46"E; 1235 m; 24 January 2023; from rotten dead branch of *Juglans regia*, coll. M.T. Zou; HGUP24-0007 (holotype); ex-type culture GUCC23-0011 (ITS: PP915799, LSU: PP949847, *rpb2*: PP947940; SSU: PP949912).

Description on the natural substrate. Colonies hairy, brown, or blackish-brown, in groups. Mycelium partly immersed in the substratum, towards the surface forming stroma-like aggregations of light to brown pseudoparenchymatous cells.

Culture characteristics. Colony on PDA 25 mm diam after 2 weeks in an incubator under dark conditions at 28 °C, irregular circular, fat, raised, undulate, rough, with white and denser mycelium at the center, with white to deep-gray to creamy yellow, entire margin; reverse cream to yellow, with dark yellowish-brown spots. Teleomorph: Unknown. Anamorph: Conidiophores macronematous, erect, straight, or slightly curved, cylindrical, smooth, 171–718 µm long, 12–25 µm wide at the base, tapering to 7–13.5 µm near the apex, arising solitary or in fascicles from the stroma cells, erect, simple, straight or flexuous, thick-walled, brown to dark brown, with sympodial proliferation, 1–13-septate. Conidia 61–114 × 13–16 µm ($\bar{x}$ = 85 × 18, n = 45), gradually tapering to 3–7 µm ($\bar{x}$ = 5, n = 45) at the distal end, with a 4–10 µm ($\bar{x}$ = 6, n = 42) wide, blackish-brown to black scars at the base, straight or flexuous, solitary, obclavate to rostrate, smooth-walled, hyaline, pale golden brown to brown, 8–12-distoseptate, with angular lumina; wall up to 6 µm thick.

Habit. Saprobiic on decaying wood of *Juglans regia*.

Distribution. China, Guizhou Province, Qianxi City

Other material examined. CHINA • Guizhou Province, Qianxi City; 105°92'E, 26°93'N; 1235 m; 24 January 2023; from rotten dead branch of *Juglans regia*, coll. M.T. Zou, HGUP24-0008 (holotype); living culture GUCC24-0011, GUCC24-0012, and GUCC24-0013.

Notes. Based on the multi-gene phylogenetic tree (Fig. 1), our strains are clustered in a distinct branch adjacent to the strain of *Helminthosporium caespitosum* (CBS 484.77). Topologically, there is a clear genetic distance between these taxa with ML-BS = 100%, BYPP = 1 support. When comparing the ITS, LSU, *rpb2*, and SSU nucleotides of *H. guizhouense* with *H. caespitosum* in the clade, there are 22 bp (0 gap) differences of 569 bp in ITS, 2 bp (0 gap) differences of 904 bp in LSU, and 38 bp (0 gap) differences of 401 bp in *rpb2*, and 4 bp (0 gap) differences of 1098 bp in SSU. Our collection of *H. guizhouense* (HGUP24-0008) differs significantly from the holotype of *H. caespitosum* (WU 38825 and WU 38826) (Voglmayr and Jaklitsch 2017) in the length of conidiophores (171–718 × 9.5–23 µm vs. 27–37 × 12.2–14.5 µm), the size of conidia (61–114 × 13–16 µm vs. 82–109 × 27.3–35.5 µm), the number of septa (8–12



Figure 3. *Helminthosporium guizhouense* sp. nov. (HGUP24-0008, holotype) on rotten dead branch of *Juglans regia* **a–c** colonies on the natural substrat; **d, e** culture on PDA after 2 weeks (**d** above **e** reverse) **f** conidiophore bases, stroma cells, and conidia **l** conidiophore **m** colony, conidiophores, and stroma cells **n** conidiophore **g–k, o–r** conidia. Scale bars: 1000 µm (**a**); 500 µm (**b, c**); 50µm (**f–r**).

vs. 6–10) and the wall thickness of angular lumina (6 μm vs. 8 μm). In addition, the colonies on the natural substrate of *H. guizhouense* are hairy, brown, or blackish brown, in groups, whereas *H. caespitosum* is dark-red-brown, scattered, or crowded. Through our analysis and classification process, we have identified these three strains as a new species *Helminthosporium guizhouense*.

***Nigrospora yunnanensis* M.T. Zou & Yong Wang bis, sp. nov.**

MycoBank No: 854538

Fig. 4a–k

Etymology. The name refers to Yunnan, the province where the fungus was collected.

Diagnosis. *Nigrospora yunnanensis* is characterized by black, globose conidia (16.2 $\times$ 14.4 μm).

Type. CHINA • Yunnan Province: Lincang City; 23°40'26.08"N, 99°56'47.70"E; 1900 m; 22 Dec 2023; on *Juglans regia*, coll. M.T. Zou; HGUP24-0007 (holotype); ex-type culture GUCC24-0008 (ITS: PP915796, *tef1- α* : PP947933, *tub2*: PP947937).

Culture characteristics. Colonies on PDA reaching 90 mm diam after ten days at 25 °C. The anterior surface and posterior surface are white, while the mycelium is thick and fluffy. Colonies on OA reach 90 mm diam. after ten days at 25 °C. The mycelium is circular, filiform, fluffy, while the surface and reverse are initially white, becoming gray to black, or black and producing a few black areas with age. Colonies on MEA reaching a diameter of 90 mm after ten days at 25 °C. The mycelium circular, filiform, thick, and fluffy. The surface and reverse are initially white, but they become gray to dark black with abundant black areas spreading from the periphery to the center as they age. Sexual morph undetermined. Asexual morph on OA: Hyphae 2.5–8 μm diam, smooth, hyaline to pale brown, branched, septate. Conidiophores smooth, hyaline to brown, branched, septate, sometimes reduced to conidiogenous cells. Conidiogenous cells ($n = 30$) 8–14 $\times$ 6–10 μm (av. = 10.4 $\times$ 8.2 μm), aggregated in clusters on hyphae, pale brown, subglobose to ampulliform. Conidia ($n = 40$) 14.5–18.5 $\times$ 11–17.5 μm (av. = 16.2 $\times$ 14.4 μm) solitary, globose to subglobose, black, shiny, smooth, aseptate.

Habitat. On *Juglans regia*.

Known distribution. China, Yunnan Province, Lincang city.

Additional material examined. CHINA • Yunnan Province: Lincang city; 23°67'N, 99°94'E; 1900 m; 22 Dec 2023; on *Juglans regia*; coll. M.T. Zou; HGUP24-0007; living culture GUCC24-0008, GUCC24-0009, and GUCC24-0010.

Notes. Three isolates from walnut leaves were obtained in this study and clustered in a well-supported clade distinguished from other known species (Fig. 4). *Nigrospora yunnanensis* formed an independent branch. Morphological differences (Table 2) support that they belong to different taxa.

Discussion

In the family Massarinaceae, along with *Helminthosporium*, there are ten other accepted genera: *Bysothecium*, *Haplohelminthosporium*, *Helminthosporiella*, *Massarina*, *Mirohelminthosporium*, *Pseudodidymosphaeria*, *Pseudosplanchnonema*, *Semifissispora*, *Stagonospora*, and *Suttonomyces* (Wijayawardene et al. 2022).

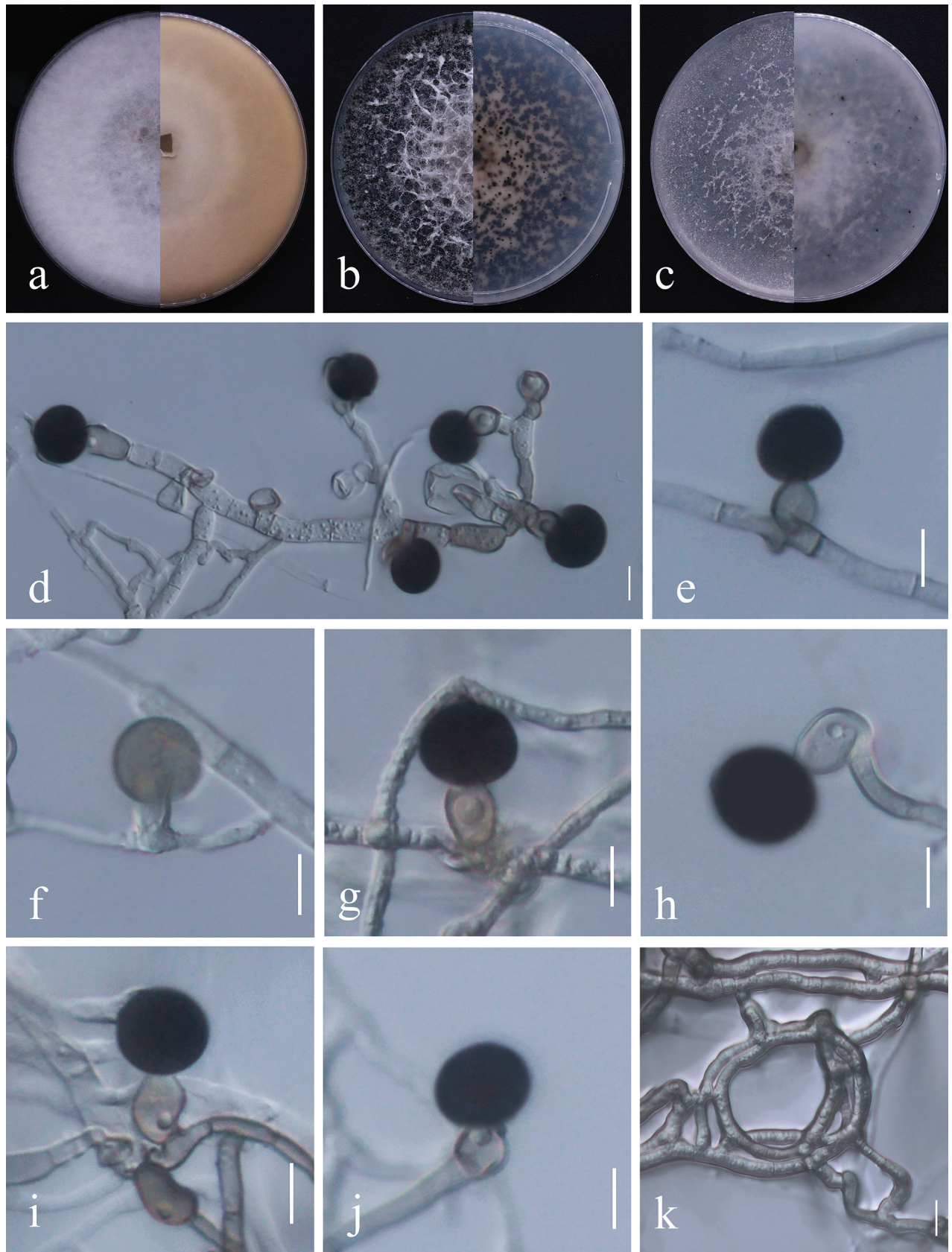


Figure 4. *Nigrospora yunnanensis* (GUCC23-0008) **a–c** culture characteristics on media after ten days (**a** on PDA **b** on CMA **c** on OA) **d–j** conidia attached to conidiogenous cells **k** coiled hyphae. Scale bars: 10 μm (**d–k**).

Table 2. Comparison of conidia and conidiogenous cells of *Nigrospora* species related to this study.

Species	Strain	Conidia (μm)	Conidiogenous cells (μm)	Reference
<i>Nigrospora endophytica</i>	URM8462	10–17.5	6.2–10	(Brito et al. 2023)
<i>N. guilinensis</i>	CGMCC 3.18124	11.5–15	6–11 × 4–7.5	(Wang et al. 2017)
<i>N. pernambucoensis</i>	URM8463	12.5–20	5–22.5 × 5–12.5	(Brito et al. 2023)
<i>N. saccharicola</i>	CGMCC 3.19362	13.5–16.5	7.5–10.5 × 5–7.5	(Raza et al. 2019)
<i>N. vesicularifera</i>	CGMCC 3.19333	11–19	7.5–10 × 12.5–15.5	(Raza et al. 2019)
<i>N. yunnanensis</i>	GUCC24-0008	14.4 × 16.2	6–10 × 8–14	This study

Helminthosporium is polyphyletic, as confirmed by Konta et al. (2021), and its members were found mixed with other taxa of *Byssothecium*, *Helminthosporiella*, and *Pseudosplanchnonema*. According to a study by Chen et al. (2022), a new genus (*Synhelminthosporium*) was identified through morphological examination and multi-locus phylogenetic analyses. Most *Helminthosporium* species are saprobic, primarily found on woody plant materials. However, some are plant pathogens, and others thrive on fungi, particularly in Diaporthales, but the role of these *Helminthosporium* species on their fungal hosts is still uncertain (Voglmayr and Jaklitsch 2017).

Nigrospora belongs to the Apiosporaceae, and are endophytes, saprobes, and plant pathogens, causing harm to economically important plant species within both forestry and agricultural domains. Examples include *N. oryzae* causing panicle branch rot disease on *Oryza sativa* in China (Liu et al. 2021), *N. sphaerica* causing Leaf blight disease of Cacao in the Philippines (Villanueva et al. 2023), *N. chinensis* causing stem spot on dragon fruit in China (Guo et al. 2024). *Nigrospora* is also a human pathogen. *N. oryzae* and *N. sphaerica* can cause human corneal keratitis (Ananya et al. 2014; Takayama et al. 2024). In addition, *N. yunnanensis* was isolated from *Juglans regia*, which could potentially be a pathogen for the walnuts.

The nutritional benefits of walnut kernels are substantial, as they are rich in fat, protein, vitamins, and minerals, while also containing essential compounds such as flavonoids and phenolic acids (Caglarirmak 2003). Moreover, walnuts are hosts to multiple forms of microfungi, including pathogens, endophytes, and saprobes (Zhang et al. 2024). Given this, it is crucial to undertake a comprehensive study of the microfungi present on walnuts in previously unexplored regions, for instance, the provinces of Yunnan and Guizhou in China, and to perform a thorough taxonomic classification of these microorganisms.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Data curation: MZ. Formal analysis: FAO, KDH. Funding acquisition: XJP, YW. Supervision: XJP, YW. Writing – original draft: MZ. Writing – review and editing: FAO, XJP, YW, KDH.

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Data availability

All of the data that support the findings of this study are available in the main text.

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