MycoKeys 67: 1–18 (2020)
doi: 10.3897/mycokeys.67.49483
http://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
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Three new *Diaporthe* species from Shaanxi Province, China

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Academic editor: D. Haelewaters | Received 17 December 2019 | Accepted 5 April 2020 | Published 4 May 2020

Citation: Yang Q, Jiang N, Tian C-M (2020) Three new *Diaporthe* species from Shaanxi Province, China. MycoKeys 67: 1–18. https://doi.org/10.3897/mycokeys.67.49483

Abstract

Diaporthe species (Sordariomycetes, Diaporthales) are often reported as important plant pathogens, saprobes and endophytes on a wide range of plant hosts. In this study, *Diaporthe* specimens were collected from symptomatic twigs and branches at the Huoditang Forest Farm in Shaanxi Province, China. Identification was done using a combination of morphology and comparison of DNA sequence data of the nuclear ribosomal internal transcribed spacer (ITS), calmodulin (*cal*), histone H3 (*his3*), partial translation elongation factor-1α (*tef1*) and β-tubulin (*tub2*) gene regions. Three new *Diaporthe* species are proposed: *D. albosinensis*, *D. coryli* and *D. shaanxiensis*. All species are illustrated and their morphology and phylogenetic relationships with other *Diaporthe* species are discussed.

Keywords

Diaporthaceae, Dieback, DNA phylogeny, Systematics, Taxonomy

Introduction

Diaporthe species (Sordariomycetes, Diaporthales) are associated with a wide range of plant hosts as pathogens, endophytes or saprobes of crops, ornamentals and forest trees (Murali et al. 2006, Rossman et al. 2007, Garcia-Reyne et al. 2011, Gomes et al. 2013, Udayanga et al. 2015, Dissanayake et al. 2017, Guarnaccia and Crous 2017, 2018,

Wijayawardene et al. 2017, Yang et al. 2017a, b, 2018, Fan et al. 2018, Guarnaccia et al. 2018). The sexual morph of *Diaporthe* is characterised by immersed ascomata and an erumpent pseudostroma with elongated perithecial necks. Asci are unitunicate, clavate to cylindrical. Ascospores are fusoid, ellipsoid to cylindrical, hyaline, biseriate to uniseriate in the ascus, sometimes with appendages (Udayanga et al. 2011). The asexual morph is characterised by ostiolate conidiomata, with cylindrical phialides producing three types of hyaline, aseptate conidia (Udayanga et al. 2011, Gomes et al. 2013).

Species identification in *Diaporthe* has traditionally been based on host association, morphology and culture characteristics (Mostert et al. 2001, Santos and Phillips 2009, Udayanga et al. 2011), resulting in the description of over 200 species (Hyde et al. 2020). Multiple species of *Diaporthe* can colonise a single host and one species can be associated with different hosts (Santos and Phillips 2009, Diogo et al. 2010, Santos et al. 2011, Gomes et al. 2013). In addition, considerable within-species variability of phenotypic characters has been reported (Rehner and Uecker 1994, Mostert et al. 2001, Udayanga et al. 2011). Thus, a polyphasic taxonomic approach, based on multilocus DNA data, morphology and ecology, has been increasingly employed for species boundaries in the genus *Diaporthe* (Gomes et al. 2013, Huang et al. 2013, 2015, Udayanga et al. 2014a, b, 2015, Fan et al. 2015, Du et al. 2016, Gao et al. 2016, 2017, Guarnaccia and Crous 2017, Guarnaccia et al. 2018, Long et al. 2019).

Huoditang is located in the middle part of the southern slope of the Qinling Mountains at 33°18'~33°28'N, 108°21'~108°29'E. It belongs to the transitional zone of the northern subtropical and warm temperate zone in China. The terrain is complex and the climate is changeable (Zhang and Cao 2007). The plant communities are complex and, as a result, species diversity of fungi in the forest area is high (Zhang and Cao 2007). During trips to collect forest pathogens causing dieback in Shaanxi Province, cankered branches with typical *Diaporthe* fruiting bodies were investigated and sampled. The aim of the present study was to identify these fungi, based on modern polyphasic taxonomic concepts.

Materials and methods

Isolates

Fresh specimens of *Diaporthe* were collected from symptomatic twigs or branches in Shaanxi Province (Table 1). Isolates were obtained by removing a mucoid spore mass from conidiomata and spreading the suspension on the surface of 1.8% potato dextrose agar (PDA) in a 9 cm diam. Petri dish. Petri dishes were incubated at 25 °C until spores germinated. Single germinating conidia were transferred on to new PDA plates, which were kept at 25 °C in the dark. Specimens are deposited in the Museum of the Beijing Forestry University (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Centre (CFCC).

Table 1. Isolates and GenBank accession numbers used in the phylogenetic analyses of *Diaporthe*.

Species	Isolate	Host	Location	GenBank accession numbers				
				ITS	*cal*	*his3*	*tef1*	*tub2*
D. acericola	MFLUCC 17-0956	*Acer negundo*	Italy	KY964224	KY964137	NA	KY964180	KY964074
D. acerigena	CFCC 52554	*Acer tataricum*	China	MH121489	MH121413	MH121449	MH121531	NA
D. albosinensis	**CFCC 53066**	***Betula albosinensis***	**China**	**MK432659**	**MK442979**	**MK443004**	**MK578133**	**MK578059**
	CFCC 53067	***Betula albosinensis***	**China**	**MK432660**	**MK442980**	**MK443005**	**MK578134**	**MK578060**
D. alnea	CBS 146.46	*Alnus* sp.	Netherlands	KC343008	KC343250	KC343492	KC343734	KC343976
D. ambigua	CBS 114015	*Pyrus communis*	South Africa	KC343010	KC343252	KC343494	KC343736	KC343978
D. anacardii	CBS 720.97	*Anacardium occidentale*	East Africa	KC343024	KC343266	KC343508	KC343750	KC343992
D. angelicae	CBS 111592	*Heracleum sphondylium*	Austria	KC343027	KC343269	KC343511	KC343753	KC343995
D. apiculatum	CGMCC 3.17533	*Camellia sinensis*	China	KP267896	NA	NA	KP267970	KP293476
D. aquatica	IFRDCC 3051	*Aquatic habitat*	China	JQ797437	NA	NA	NA	NA
D. arctii	CBS 139280	*Arctium lappa*	Austria	KJ590736	KJ612133	KJ659218	KJ590776	KJ610891
D. aseana	MFLUCC 12-0299a	Unknown dead leaf	Thailand	KT459414	KT459464	NA	KT459448	KT459432
D. asheicola	CBS 136967	*Vaccinium ashei*	Chile	KJ160562	KJ160542	NA	KJ160594	KJ160518
D. baccae	CBS 136972	*Vaccinium corymbosum*	Italy	KJ160565	NA	MF418264	KJ160597	NA
D. beilharziae	BRIP 54792	*Indigofera australis*	Australia	JX862529	NA	NA	JX862535	KF170921
D. benedicti	BPI 893190	*Salix* sp.	USA	KM669929	KM669862	NA	KM669785	NA
D. betulae	CFCC 50469	*Betula platyphylla*	China	KT732950	KT732997	KT732999	KT733016	KT733020
D. betulina	CFCC 52560	*Betula albosinensis*	China	MH121495	MH121419	MH121455	MH121537	MH121577
D. bicincta	CBS 121004	*Juglans* sp.	USA	KC343134	KC343376	KC343618	KC343860	KC344102
D. caryae	CFCC 52563	*Carya illinoensis*	China	MH121498	MH121422	MH121458	MH121540	MH121580
D. cassines	CPC 21916	*Cassine peragua*	South Africa	KF777155	NA	NA	KF777244	NA
D. celeris	CPC 28262	*Vitis vinifera*	Czech Republic	MG281017	MG281712	MG281363	MG281538	MG281190
D. cercidis	CFCC 52565	*Cercis chinensis*	China	MH121500	MH121424	MH121460	MH121542	MH121582
D. chamaeropis	CBS 454.81	*Chamaerops humilis*	Greece	KC343048	KC343290	KC343532	KC343774	KC344016
D. charlesworthii	BRIP 54884m	*Rapistrum rugostrum*	Australia	KJ197288	NA	NA	KJ197250	KJ197268
D. chensiensis	CFCC 52567	*Abies chensiensis*	China	MH121502	MH121426	MH121462	MH121544	MH121584
D. cichorii	MFLUCC 17-1023	*Cichorium intybus*	Italy	KY964220	KY964133	NA	KY964176	KY964104
D. cinnamomi	CFCC 52569	*Cinnamomum* sp.	China	MH121504	NA	MH121464	MH121546	MH121586
D. citriasiana	CGMCC 3.15224	*Citrus unshiu*	China	JQ954645	KC357491	KJ490515	JQ954663	KC357459
D. citrichinensis	CGMCC 3.15225	*Citrus* sp.	China	JQ954648	KC357494	NA	JQ954666	NA
D. compactum	CGMCC 3.17536	*Camellia sinensis*	China	KP267854	NA	KP293508	KP267928	KP293434
D. conica	CFCC 52571	*Alangium chinense*	China	MH121506	MH121428	MH121466	MH121548	MH121588
D. coryli	**CFCC 53083**	***Corylus mandshurica***	**China**	**MK432661**	**MK442981**	**MK443006**	**MK578135**	**MK578061**
	CFCC 53084	***Corylus mandshurica***	**China**	**MK432662**	**MK442982**	**MK443007**	**MK578136**	**MK578062**
D. cucurbitae	CBS 136.25	*Arctium* sp.	Unknown	KC343031	KC343273	KC343515	KC343757	KC343999
D. cuppatea	CBS 117499	*Aspalathus linearis*	South Africa	KC343057	KC343299	KC343541	KC343783	KC344025

Species	Isolate	Host	Location	GenBank accession numbers				
				ITS	*cal*	*his3*	*tef1*	*tub2*
D. cynaroidis	CBS 122676	*Protea cynaroides*	South Africa	KC343058	KC343300	KC343542	KC343784	KC344026
D. cytosporella	FAU461	*Citrus limon*	Italy	KC843307	KC843141	NA	KC843116	KC843221
D. discoidispora	ZJUD89	*Citrus unshiu*	China	KJ490624	NA	KJ490566	KJ490503	KJ490445
D. dorycnii	MFLUCC 17-1015	*Dorycnium hirsutum*	Italy	KY964215	NA	NA	KY964171	KY964099
D. elaeagni-glabrae	CGMCC 3.18287	*Elaeagnus glabra*	China	KX986779	KX999281	KX999251	KX999171	KX999212
D. endophytica	CBS 133811	*Schinus terebinthifolius*	Brazil	KC343065	KC343307	KC343549	KC343791	KC343065
D. eres	AR5193	*Ulmus* sp.	Germany	KJ210529	KJ434999	KJ420850	KJ210550	KJ420799
D. eucalyptorum	CBS 132525	*Eucalyptus* sp.	Australia	NR120157	NA	NA	NA	NA
D. foeniculacea	CBS 123208	*Foeniculum vulgare*	Portugal	KC343104	KC343346	KC343588	KC343830	KC344072
D. fraxini-angustifoliae	BRIP 54781	*Fraxinus angustifolia*	Australia	JX862528	NA	NA	JX862534	KF170920
D. fraxinicola	CFCC 52582	*Fraxinus chinensis*	China	MH121517	MH121435	NA	MH121559	NA
D. fructicola	MAFF 246408	*Passiflora edulis* × *P. edulis* f. *flavicarpa*	Japan	LC342734	LC342738	LC342737	LC342735	LC342736
D. fusicola	CGMCC 3.17087	*Lithocarpus glabra*	China	KF576281	KF576233	NA	KF576256	KF576305
D. garethjonesii	MFLUCC 12-0542a	*Unknown dead leaf*	Thailand	KT459423	KT459470	NA	KT459457	KT459441
D. guangxiensis	JZB320094	*Vitis vinifera*	China	MK335772	MK736727	NA	MK523566	MK500168
D. helicis	AR5211	*Hedera helix*	France	KJ210538	KJ435043	KJ420875	KJ210559	KJ420828
D. heterophyllae	CBS 143769	*Acacia heterohpylla*	France	MG600222	MG600218	MG600220	MG600224	MG600226
D. hubeiensis	JZB320123	*Vitis vinifera*	China	MK335809	MK500235	NA	MK523570	MK500148
D. incompleta	CGMCC 3.18288	*Camellia sinensis*	China	KX986794	KX999289	KX999265	KX999186	KX999226
D. inconspicua	CBS 133813	*Maytenus ilicifolia*	Brazil	KC343123	KC343365	KC343607	KC343849	KC344091
D. infecunda	CBS 133812	*Schinus terebinthifolius*	Brazil	KC343126	KC343368	KC343610	KC343852	KC344094
D. juglandicola	CFCC 51134	*Juglans mandshurica*	China	KU985101	KX024616	KX024622	KX024628	KX024634
D. kadsurae	CFCC 52586	*Kadsura longipedunculata*	China	MH121521	MH121439	MH121479	MH121563	MH121600
D. litchicola	BRIP 54900	*Litchi chinensis*	Australia	JX862533	NA	NA	JX862539	KF170925
D. lusitanicae	CBS 123212	*Foeniculum vulgare*	Portugal	KC343136	KC343378	KC343620	KC343862	KC344104
D. masirevicii	BRIP 57892a	*Helianthus annuus*	Australia	KJ197277	NA	NA	KJ197239	KJ197257
D. middletonii	BRIP 54884e	*Rapistrum rugostrum*	Australia	KJ197286	NA	NA	KJ197248	KJ197266
D. millettiae	GUCC9167	*Millettia reticulata*	China	MK398674	MK502086	NA	MK480609	MK502089
D. miriciae	BRIP 54736j	*Helianthus annuus*	Australia	KJ197282	NA	NA	KJ197244	KJ197262
D. musigena	CBS 129519	*Musa* sp.	Australia	KC343143	KC343385	KC343627	KC343869	KC344111
D. neilliae	CBS 144.27	*Spiraea* sp.	USA	KC343144	KC343386	KC343628	KC343870	KC344112
D. neoarctii	CBS 109490	*Ambrosia trifida*	USA	KC343145	KC343387	KC343629	KC343871	KC344113
D. nothofagi	BRIP 54801	*Nothofagus cunninghamii*	Australia	JX862530	NA	NA	JX862536	KF170922
D. novem	CBS 127270	*Glycine max*	Croatia	KC343155	KC343397	KC343640	KC343881	KC344123
D. oraccinii	CGMCC 3.17531	*Camellia sinensis*	China	KP267863	NA	KP293517	KP267937	KP293443
D. ovalispora	ICMP20659	*Citrus limon*	China	KJ490628	NA	KJ490570	KJ490507	KJ490449
D. ovoicicola	CGMCC 3.17093	*Citrus* sp.	China	KF576265	KF576223	NA	KF576240	KF576289

Species	Isolate	Host	Location	GenBank accession numbers				
				ITS	*cal*	*his3*	*tef1*	*tub2*
D. osmanthi	GUCC9165	*Osmanthus fragrans*	China	MK398675	MK502087	NA	MK480610	MK502090
D. padina	CFCC 52590	*Padus racemosa*	China	MH121525	MH121443	MH121483	MH121567	MH121604
D. pandanicola	MFLU 18-0006	*Pandanus* sp.	Thailand	MG646974	NA	NA	NA	MG646930
D. pascoei	BRIP 54847	*Persea americana*	Australia	JX862532	NA	NA	JX862538	KF170924
D. passifloricola	CBS 141329	*Passiflora foetida*	Malaysia	KX228292	NA	KX228367	NA	KX228387
D. perseae	CBS 151.73	*Persea gratissima*	Netherlands	KC343173	KC343415	KC343657	KC343899	KC344141
D. pescicola	MFLUCC 16-0105	*Prunus persica*	China	KU557555	KU557603	NA	KU557623	KU557579
D. phaseolorum	AR4203	*Phaseolus vulgaris*	USA	KJ590738	NA	KJ659220	NA	KP004507
D. podocarpi-macrophylli	CGMCC 3.18281	*Podocarpus macrophyllus*	China	KX986774	KX999278	KX999246	KX999167	KX999207
D. pseudomangiferae	CBS 101339	*Mangifera indica*	Dominican Republic	KC343181	KC343423	KC343665	KC343907	KC344149
D. pseudophoenicicola	CBS 462.69	*Phoenix dactylifera*	Spain	KC343184	KC343426	KC343668	KC343910	KC344152
D. psoraleae-pinnatae	CBS 136413	*Psoralea pinnata*	South Africa	KF777159	NA	NA	NA	KF777252
D. pulla	CBS 338.89	*Hedera helix*	Yugoslavia	KC343152	KC343394	KC343636	KC343878	KC344120
D. racemosae	CBS 143770	*Euclea racemosa*	South Africa	MG600223	MG600219	MG600221	MG600225	MG600227
D. ravennica	MFLUCC 15-0479	*Tamarix* sp.	Italy	KU900335	NA	NA	KX365197	KX432254
D. rhusicola	CBS 129528	*Rhus pendulina*	South Africa	JF951146	KC843124	NA	KC843100	KC843205
D. rosae	MFLU 17-1550	*Rosa* sp.	Thailand	MG828894	NA	NA	NA	MG843878
D. rosicola	MFLU 17-0646	*Rosa* sp.	UK	MG828895	NA	NA	MG829270	MG843877
D. rudis	AR3422	*Laburnum anagyroides*	Austria	KC843331	KC843146	NA	KC843090	KC843177
D. sackstonii	BRIP 54669b	*Helianthus annuus*	Australia	KJ197287	NA	NA	KJ197249	KJ197267
D. salicicola	BRIP 54825	*Salix purpurea*	Australia	JX862531	NA	NA	JX862537	JX862531
D. sambucusii	CFCC 51986	*Sambucus williamsii*	China	KY852495	KY852499	KY852503	KY852507	KY852511
D. schini	CBS 133181	*Schinus terebinthifolius*	Brazil	KC343191	KC343433	KC343675	KC343917	KC344159
D. schoeni	MFLU 15-1279	*Schoenus nigricans*	Italy	KY964226	KY964139	NA	KY964182	KY964109
D. sennicola	CFCC 51634	*Senna bicapsularis*	China	KY203722	KY228873	KY228879	KY228883	KY228889
D. serafiniae	BRIP 55665a	*Helianthus annuus*	Australia	KJ197274	NA	NA	KJ197236	KJ197254
D. shaanxiensis	**CFCC 53106**	**on branches of liana**	**China**	**MK432654**	**MK442976**	**MK443001**	**MK578130**	**NA**
	CFCC 53107	**on branches of liana**	**China**	**MK432655**	**MK442977**	**MK443002**	**MK578131**	**NA**
D. siamensis	MFLUCC 10-573a	*Dasymaschalon* sp.	Thailand	JQ619879	NA	NA	JX275393	JX275429
D. sojae	FAU635	*Glycine max*	USA	KJ590719	KJ612116	KJ659208	KJ590762	KJ610875
D. sterilis	CBS 136969	*Vaccinium corymbosum*	Italy	KJ160579	KJ160548	MF418350	KJ160611	KJ160528
D. stictica	CBS 370.54	*Buxus sampervirens*	Italy	KC343212	KC343454	KC343696	KC343938	KC344180
D. subclavata	ICMP20663	*Citrus unshiu*	China	KJ490587	NA	KJ490529	KJ490466	KJ490408
D. subcylindrospora	MFLU 17-1195	*Salix* sp.	China	MG746629	NA	NA	MG746630	MG746631
D. subellipicola	MFLU 17-1197	on dead wood	China	MG746632	NA	NA	MG746633	MG746634

Species	Isolate	Host	Location	GenBank accession numbers				
				ITS	*cal*	*his3*	*tef1*	*tub2*
D. subordinaria	CBS 464.90	*Plantago lanceolata*	New Zealand	KC343214	KC343456	KC343698	KC343940	KC344182
D. tectonendophytica	MFLUCC 13-0471	*Tectona grandis*	China	KU712439	KU749354	NA	KU749367	KU749354
D. tectonigena	MFLUCC 12-0767	*Tectona grandis*	China	KU712429	KU749358	NA	KU749371	KU743976
D. terebinthifolii	CBS 133180	*Schinus terebinthifolius*	Brazil	KC343216	KC343458	KC343700	KC343942	KC344184
D. ternstroemia	CGMCC 3.15183	*Ternstroemia gymnanthera*	China	KC153098	NA	NA	KC153089	NA
D. thunbergii	MFLUCC 10-576a	*Thunbergia laurifolia*	Thailand	JQ619893	JX197440	NA	JX275409	JX275449
D. tibetensis	CFCC 51999	*Juglandis regia*	China	MF279843	MF279888	MF279828	MF279858	MF279873
D. ueckerae	FAU656	*Cucumis melo*	USA	KJ590726	KJ612122	KJ659215	KJ590747	KJ610881
D. ukurunduensis	CFCC 52592	*Acer ukurunduense*	China	MH121527	MH121445	MH121485	MH121569	NA
D. unshiuensis	CFCC 52594	*Carya illinoensis*	China	MH121529	MH121447	MH121487	MH121571	MH121606
D. vaccinii	CBS 160.32	*Oxycoccus macrocarpos*	USA	KC343228	KC343470	KC343712	KC343954	KC344196
D. velutina	CGMCC 3.18286	*Neolitsea* sp.	China	KX986790	NA	KX999261	KX999182	KX999223
D. viniferae	JZB320071	*Vitis vinifera*	China	MK341551	MK500107	NA	MK500119	MK500112
D. xishuangbanica	CGMCC 3.18282	*Camellia sinensis*	China	KX986783	NA	KX999255	KX999175	KX999216
D. yunnanensis	CGMCC 3.18289	*Coffea* sp.	China	KX986796	KX999290	KX999267	KX999188	KX999228
Diaporthella corylina	CBS 121124	*Corylus* sp.	China	KC343004	KC343246	KC343488	KC343730	KC343972

Newly sequenced material is indicated in bold type. NA, not applicable.

Morphological analysis

Morphological observations of the asexual morph in the natural environment were based on features of the fruiting bodies produced on infected plant tissues and micromorphology, supplemented by cultural characteristics. Conidiomata from tree barks were sectioned by hand, using a double-edged blade and structures were observed under a dissecting microscope. The gross morphology of fruiting bodies was recorded using a Leica stereomicroscope (M205 FA). Fungal structures were mounted in clear lactic acid and micromorphological characteristics were examined at 1000× magnification using a Leica compound microscope (DM 2500) with differential interference contrast (DIC) optics. Thirty measurements of each structure were determined for each collection. Colony characters and pigment production on PDA were noted after 10 d. Colony colours were described according to Rayner (1970).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from colonies grown on cellophane-covered PDA, using the CTAB [cetyltrimethylammonium bromide] method (Doyle and Doyle 1990). PCR amplifications of phylogenetic markers were done using the same primer pairs

and conditions as in Yang et al. (2018). PCR products were assayed via electrophoresis in 2% agarose gels. DNA sequencing was performed using an ABI PRISM 3730XL DNA Analyzer with a BigDye Terminater Kit v.3.1 (Invitrogen, USA) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

Phylogenetic analyses

The quality of our amplified nucleotide sequences was checked and combined by SeqMan v.7.1.0 and reference sequences were retrieved from the National Center for Biotechnology Information (NCBI), based on recent publications on the genus *Diaporthe* (Guarnaccia et al. 2018, Yang et al. 2018, Long et al. 2019). Sequences were aligned using MAFFT v. 7.310 (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2016) and manually corrected using Bioedit 7.0.9.0 (Hall 1999). The best-fit nucleotide substitution models for each gene were selected using jModelTest v. 2.1.7 (Darriba et al. 2012) under the Akaike Information Criterion.

Phylogenetic analyses of the combined gene regions were performed using Maximum-Likelihood (ML) and Bayesian Inference (BI) methods. ML was conducted using PhyML v. 3.0 (Guindon et al. 2010), with 1000 bootstrap replicates. BI was performed using a Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003). Two MCMC chains, started from random trees for 1,000,000 generations and trees, were sampled every 100th generation, resulting in a total of 10,000 trees. The first 25% of trees were discarded as burn-in of each analysis. Branches with significant Bayesian Posterior Probabilities (BPP) were estimated in the remaining 7500 trees. Phylogenetic trees were viewed with FigTree v.1.3.1 (Rambaut and Drummond 2010) and processed by Adobe Illustrator CS5. Alignment and trees were deposited in TreeBASE (submission ID: S25522). The nucleotide sequence data of the new taxa have been deposited in GenBank (Table 1).

Results

Phylogenetic analyses

The five-gene sequence dataset (ITS, *cal*, *his3*, *tef1* and *tub2*) was analysed to infer the interspecific relationships within *Diaporthe*. The dataset consisted of 124 sequences including the outgroup, *Diaporthella corylina* (culture CBS 121124). A total of 2555 characters including gaps (505 for ITS, 513 for *cal*, 528 for *his3*, 475 for *tef1* and 522 for *tub2*) were included in the phylogenetic analysis. The best nucleotide substitution model for ITS, *his3* and *tub2* was TrN+I+G, while HKY+I+G was selected for both *cal* and *tef1*. The topologies resulting from ML and BI analyses of the concatenated dataset were congruent (Fig. 1). Isolates from Shaanxi Province formed three individual clades representing three undescribed species.

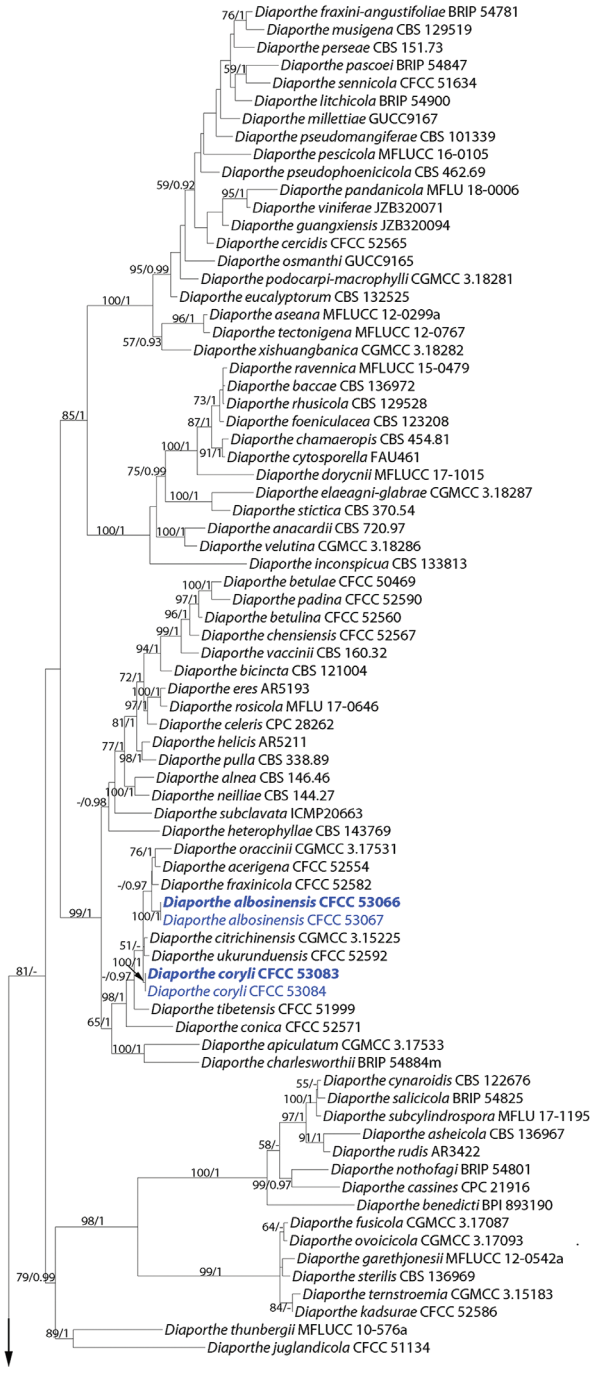

Figure 1. Phylogram of *Diaporthe* resulting from a maximum likelihood analysis based on combined ITS, *cal*, *his3*, *tef1* and *tub2*. Numbers above the branches indicate ML bootstraps (left, ML BS ≥ 50%) and Bayesian Posterior Probabilities (right, BPP ≥ 0.90). The tree is rooted with *Diaporthella corylina*. Isolates in current study are in blue. "-" indicates ML BS < 50% or BI PP < 0.90.

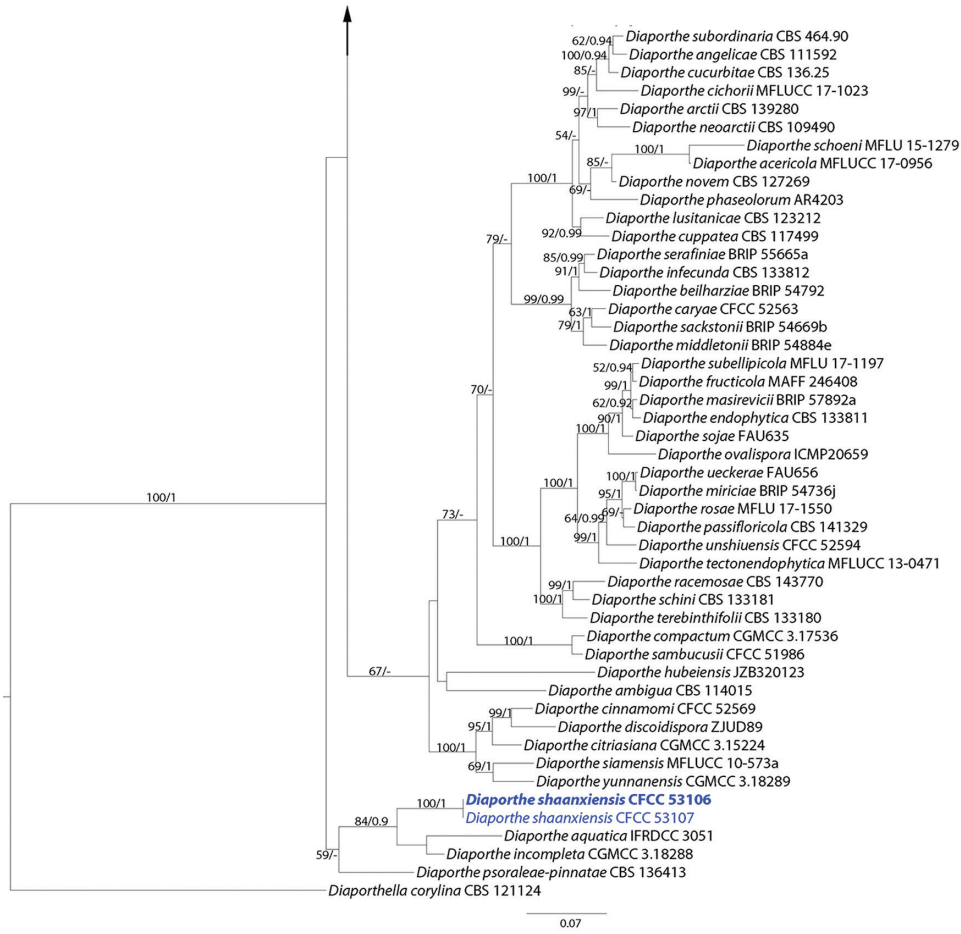

Figure 1. Continued.

Taxonomy

Diaporthe albosinensis **C.M. Tian & Q. Yang, sp. nov.**
MycoBank No: 829518
Fig. 2

Diagnosis. Distinguished from *D. fraxinicola* in having shorter conidiophores and longer beta conidia.

Etymology. Named after the host plant, *Betula albosinensis*, from which the holotype was collected.

Description. *Conidiomata* pycnidial, conical, immersed in bark, solitary to aggregated, erumpent through the bark surface, with a solitary undivided locule. *Ectostromatic disc* yellowish to brown, one ostiole per disc. *Ostiole* medium black,

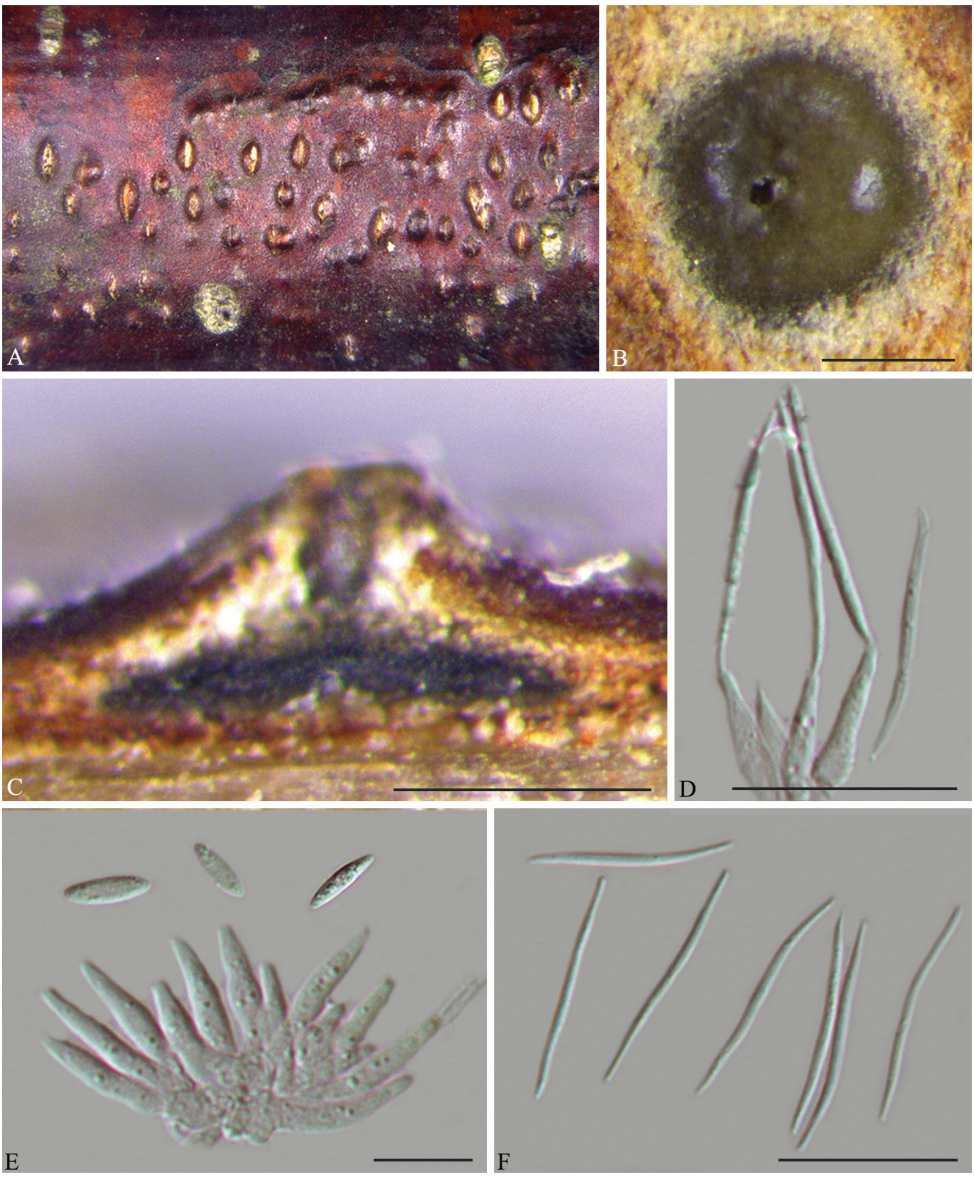

Figure 2. *Diaporthe albosinensis* on *Betula albosinensis* (BJFC-S1670). **A** Habit of conidiomata in wood **B** transverse section of conidiomata **C** longitudinal section through conidiomata **D** conidiogenous cells attached with beta conidia **E** conidiogenous cells attached with alpha conidia **F** beta conidia. Scale bars: 200 μm (**B–C**); 20 μm (**D, F**); 10 μm (**E**).

up to the level of disc. *Locule* undivided, (280–)290–375(–380) μm diam. *Conidiophores* (6–)8.5–13(–14.5) × (1.5–)2–2.5 μm, hyaline, cylindrical, smooth, phialidic, unbranched, straight or slightly curved. *Alpha conidia* hyaline, aseptate, fusiform, 0–1-guttulate, (7–)8–10(–11) × 2.5–3 μm. *Beta conidia* hyaline, aseptate, filiform,

straight or slightly curved, eguttulate, base subtruncate, tapering towards one apex, (24–)25.5–30(–32) × 1–1.5 μm.

Culture characters. Cultures incubated on PDA at 25 °C in the dark. Colony originally flat with white felted aerial mycelium, becoming light brown due to pigment formation, conidiomata irregularly distributed over agar surface, with yellowish conidial drops exuding from the ostioles.

Specimens examined. China. Shaanxi Province: Ningshan County, Huoditang Forest Farm, 33°28'25"N, 108°29'39"E, on branches of *Betula albosinensis*, 10 July 2018, *N. Jiang* (holotype BJFC-S1670; ex-type living culture: CFCC 53066; living culture: CFCC 53067).

Notes. Two isolates, representing *D. albosinensis,* are retrieved in a well-supported clade (ML BS/BPP=100/1) and appear most closely related to *D. fraxinicola* (Fig. 1). *Diaporthe albosinensis* can be distinguished from *D. fraxinicola*, based on *tef1* and *tub2* loci (3/335 in *tef1* and 19/429 in *tub2*). Morphologically, *D. albosinensis* differs from *D. fraxinicola* in having shorter conidiophores (8.5–13 vs. 10.5–17.5 μm) and longer beta conidia (25.5–30 vs. 19–29.5 μm) (Yang et al. 2018).

***Diaporthe coryli* C.M. Tian & Q. Yang, sp. nov.**
MycoBank No: 829520
Fig. 3

Diagnosis. Distinguished from *D. ukurunduensis* and *D. citrichinensis* in having larger alpha conidia.

Etymology. Named after the genus of the host plant from which the holotype was collected, *Corylus*.

Description. *Conidiomata* pycnidial, conical to spherical, immersed in the host bark, erumpent from surface of host branches, scattered, 950–1200 × 420–650 μm diam., covered by orange discharged conidial masses at maturity, usually conspicuous. *Ectostromatic disc* inconspicuous. *Central column* beneath the disc more or less conical, bright yellow. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* cylindrical, hyaline, smooth, unbranched, tapering towards the apex, (8.5–)10–12(–13) × (2–)2.5–3 μm. *Alpha conidia* hyaline, aseptate, fusiform, multiguttulate, rarely 2-guttulate, (10.5–)11.5–13(–13.5) × 3–3.5 μm. *Beta conidia* not observed.

Culture characters. Cultures incubated on PDA at 25 °C in the dark. Colony flat, felty with thick texture at the marginal area, with thin texture in the centre, producing beige pigment after 7–10 d. Aerial mycelium white, dense, conidiomata distributed in the centre, with translucent conidial drops exuding from the ostioles.

Specimens examined. CHINA. Shaanxi Province: Ningshan County, Huoditang Forest Farm, 33°28'26"N, 108°29'40"E, on branches of *Corylus mandshurica*, 10 July 2018, *N. Jiang* (holotype BJFC-S1671; ex-type living culture: CFCC 53083); 33°28'26"N, 108°29'38"E, on branches of *Corylus mandshurica*, 10 July 2018, *N. Jiang* (paratype BJFC-S1672; living culture: CFCC 53084).

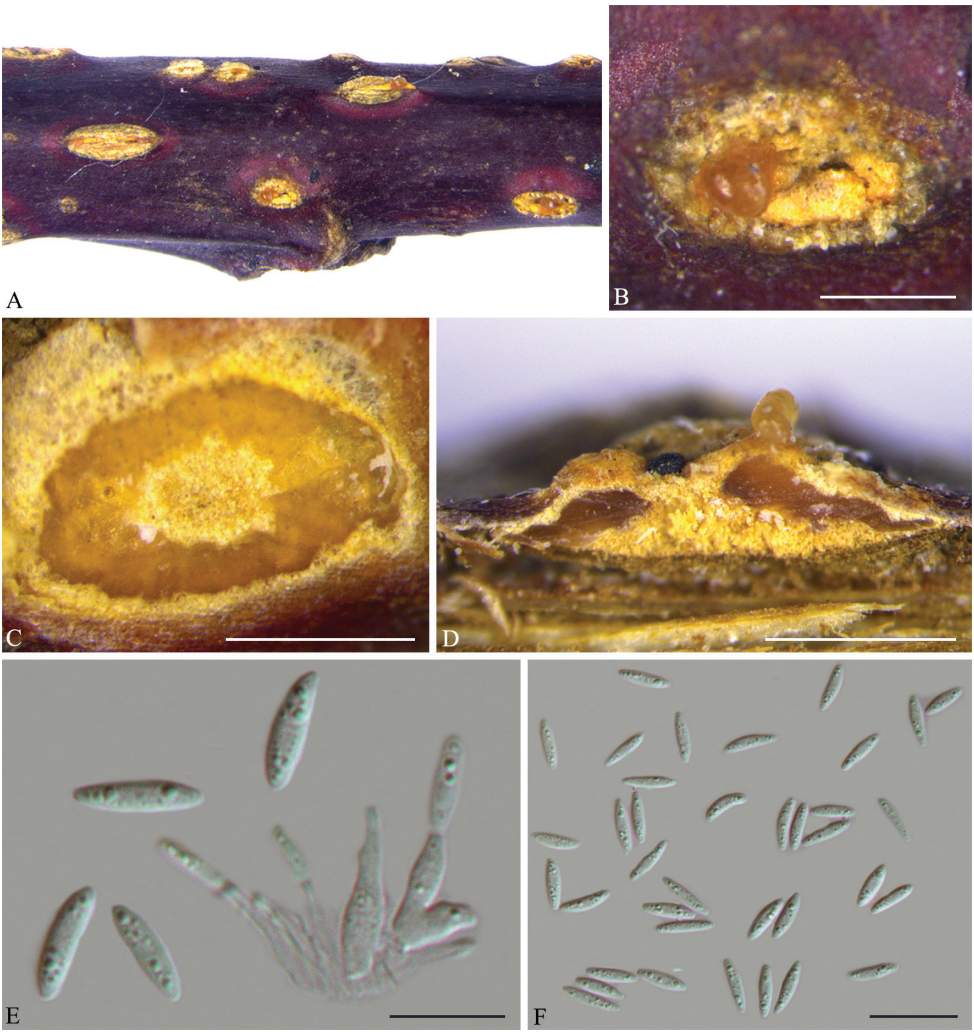

Figure 3. *Diaporthe coryli* on *Corylus mandshurica* (BJFC-S1671). **A, B** Habit of conidiomata in wood **C** transverse section of conidiomata **D** longitudinal section through conidiomata **E** conidiogenous cells attached with alpha conidia **F** alpha conidia. Scale bars: 500 μm (**B–D**); 10 μm (**E**); 20 μm (**F**).

Notes. We generated sequences for two isolates of *D. coryli*, CFCC 53083 and CFCC 53084. This new species is phylogenetically most closely related to *D. ukurunduensis* and *D. citrichinensis* (Fig. 1). *Diaporthe coryli* can be distinguished from *D. ukurunduensis*, based on ITS, *his3* and *tef1* loci (8/467 in ITS, 1/460 in *his3* and 1/336 in *tef1*); and from *D. citrichinensis* based on *tef1* and *tub2* loci (4/335 in *tef1* and 25/428 in *tub2*). Morphologically, *D. coryli* can be distinguished from both *D. ukurunduensis* (11.5–13 × 3–3.5 vs. 5–6 × 2–3 μm) and *D. citrichinensis* (11.5–13 × 3–3.5 vs. 5.5–9 × 1.5–2.5 μm) in having larger alpha conidia (Huang et al. 2013, Gao et al. 2016).

Diaporthe shaanxiensis **C.M. Tian & Q. Yang, sp. nov.**
MycoBank No: 829527
Fig. 4

Diagnosis. Distinguished from *D. aquatica* and *D. incompleta* in having longer beta conidia.

Etymology. Named after Province Shaanxi, where the holotype was collected.

Description. *Conidiomata* pycnidial, immersed in bark, scattered, erumpent through the bark surface, discoid, with a solitary undivided locule. *Ectostromatic disc* yellowish to pale brown, one ostiole per disc, usually conspicuous, (485–)500–687(–

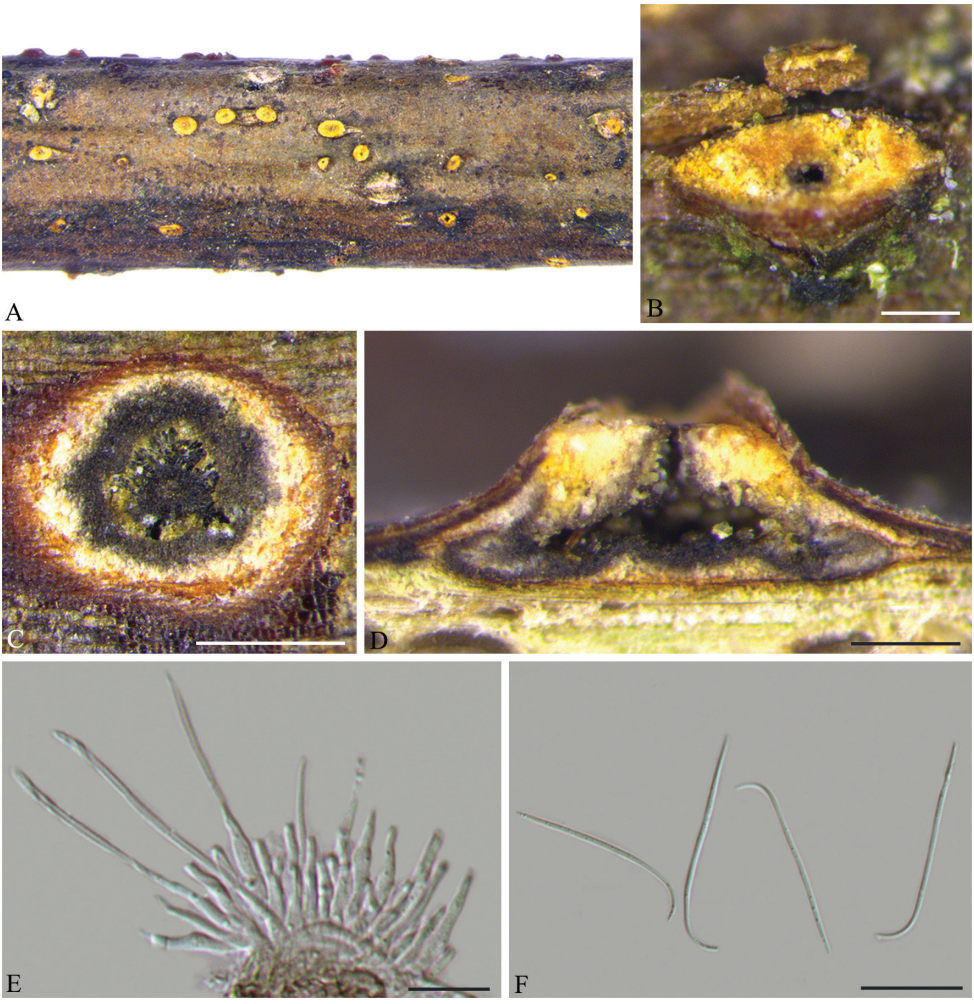

Figure 4. *Diaporthe shaanxiensis* on liana (BJFC-S1674). **A, B** Habit of conidiomata on twig **C** transverse section through conidiomata **D** longitudinal section through conidiomata **E** conidiogenous cells attached with beta conidia **F** beta conidia. Scale bars: 200 μm (**B–D**); 10 μm (**E, F**).

695) µm diam. *Locule* circular, undivided, (500–)526–765(–792) µm diam. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline, cylindrical, unbranched, slightly curved, tapering towards the apex, (12.5–)14.5–17(–18) × 1–1.5(–2) µm. *Alpha conidia* not observed. *Beta conidia* hyaline, aseptate, filiform, straight to curved, eguttulate, (35.5–)37–47.5(–50) × 1 µm.

Culture characters. Cultures incubated on PDA at 25 °C in the dark. Colony originally flat with white fluffy aerial mycelium, becoming pale brown with pigment, with visible solitary conidiomata at maturity.

Specimens examined. CHINA. Shaanxi Province: Ningshan County, Huoditang Forest Farm, 33°28'25"N, 108°29'39"E, on branch of liana, 10 July 2018, *N. Jiang* (holotype BJFC-S1674; ex-type living culture: CFCC 53106); 33°28'24"N, 108°29'38"E, on branch of liana, 10 July 2018, *N. Jiang* (Paratype BJFC-S1675; living culture: CFCC 53107).

Notes. In the combined tree, *D. shaanxiensis* is a distinct clade with maximum support and it appears to be most closely related to *D. aquatica* and *D. incompleta* (Fig. 1). *Diaporthe shaanxiensis* can be distinguished from *D. aquatica* by a 17 nt difference in the ITS region. For *D. aquatica*, only ITS sequences are available in NCBI GenBank (Hu et al. 2012). The new species can be distinguished from *D. incompleta*, based on ITS, *cal*, *his3* and *tef1* (24/454 in ITS, 14/443 in *cal*, 66/468 in *his3* and 24/311 in *tef1*). Morphologically, *D. shaanxiensis* differs from both *D. aquatica* (37–47.5 vs. 9–12.5 µm) and *D. incompleta* (37–47.5 vs. 19–44 µm) in having longer beta conidia (Gao et al. 2016, 2017).

Discussion

In this study, an investigation of forest pathogens from Huoditang in Shaanxi Province was carried out and Diaporthe canker was observed as a common disease. Identification of our collections was conducted, based on isolates from fruiting bodies using five combined loci (ITS, *cal*, *his3*, *tef1* and *tub2*), as well as morphological characters. Three new *Diaporthe* species were described. These are *D. albosinensis* sp. nov., *D. coryli* sp. nov. and *D. shaanxiensis* sp. nov.

Diaporthe albosinensis is associated with *Betula albosinensis*. Thus far, six *Diaporthe* species have been reported from *Betula*. These are *D. alleghaniensis*, *D. betulae*, *D. betulicola*, *D. betulina*, *D. eres* and *D. melanocarpa* (Kobayashi 1970, Gomes et al. 2013, Du et al. 2016, Yang et al. 2018). Morphologically, *D. albosinensis* differs from *D. betulae* (600–1250 µm), *D. betulicola* (700–1300 µm) and *D. betulina* (670–905 µm) in having smaller locules (Du et al. 2016, Yang et al. 2018); and from *D. alleghaniensis* (5–8 × 1.5–2 µm) and *D. eres* (6.5–8.5 × 3–4 µm) in having larger alpha conidia (Arnold 1967, Anagnostakis 2007, Gomes et al. 2013). In addition, our phylogenetic reconstruction of a five-locus dataset adds support for the new species, although no sequence data are currently available for *D. alleghaniensis*, *D. betulicola* and *D. melanocarpa* (Fig. 1). Interestingly, *D. melanocarpa* is found on different plant hosts; it was described from

Pyrus melanocarpa in London and then recorded from *Amelanchier*, *Betula* and *Cornus* (Dearness 1926, Wehmeyer 1933, Kobayashi 1970). *Diaporthe coryli* is characterised by the ostiole with orange discharged conidial masses and a yellow central column (Fig. 3). *Diaporthe shaanxiensis* was found on branches of liana with an obvious ostiole per disc and characterised by hyaline, filiform beta conidia. Alpha conidia were found neither in the natural environment nor in culture for this species.

Species delimitation of *Diaporthe* has improved considerably by using a combination of morphological, cultural, phytopathological and molecular phylogenetic analyses (Udayanga et al. 2014a, b, 2015, Fan et al. 2015, Gao et al. 2017, Guarnaccia and Crous 2017, Hyde et al. 2017, 2020, Guarnaccia et al. 2018, Yang et al. 2018, Long et al. 2019). As a result, many Diaporthe canker diseases and new species have been discovered and reported from all over the world and also in China. The descriptions and molecular data of *Diaporthe* species represent an important resource for plant pathologists, plant quarantine officials and taxonomists.

Acknowledgements

This study is financed by the Research Foundation of Education Bureau of Hunan Province, China (Project No.: 19B608) and the introduction of talent research start-up fund project of CSUFT (Project No.: 2019YJ025). We are grateful to Chungen Piao, Minwei Guo (China Forestry Culture Collection Center, Chinese Academy of Forestry, Beijing) and reviewers Lu Quan and Jadson Bezerra.

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MycoKeys 67: 19–32 (2020)
doi: 10.3897/mycokeys.67.51133
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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Gnomoniopsis chinensis (Gnomoniaceae, Diaporthales), a new fungus causing canker of Chinese chestnut in Hebei Province, China

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Academic editor: Huzefa Raja | Received 14 February 2020 | Accepted 17 April 2020 | Published 14 May 2020

Citation: Jiang N, Liang L-Y, Tian C-M (2020) *Gnomoniopsis chinensis* (Gnomoniaceae, Diaporthales), a new fungus causing canker of Chinese chestnut in Hebei Province, China. MycoKeys 67: 19–32. https://doi.org/10.3897/mycokeys.67.51133

Abstract

Chinese chestnut (*Castanea mollissima*) is an important crop tree species in China. However, branch canker and fruit rot are two kinds of severe diseases, which weaken the host and decrease chestnut production. During our investigations into chestnut diseases in China, several fungi have been confirmed as casual agents in previous studies, namely *Aurantiosacculus castaneae*, *Cryphonectria neoparasitica*, *Cry. parasitica*, *Endothia chinensis* and *Gnomoniopsis daii*. In this study, a new canker pathogen is introduced based on morphology, phylogeny and pathogenicity. Typical *Gnomoniopsis* canker sign of wide, orange tendrils emerging from hosts' glaucous lenticels were obvious on the diseased trees in the field. Symptomatic branches or bark on stems from different chestnut plantations were sampled and isolated, then strains were identified by comparisons of DNA sequence data for the nuclear ribosomal internal transcribed spacer (ITS), partial translation elongation factor-1α (*tef1*) and β-tubulin (*tub2*) gene regions as well as morphological features. As a result, these strains appeared different from any known *Gnomoniopsis* species. Hence, we propose a novel species named *Gnomoniopsis chinensis*. Pathogenicity was further tested using the ex-type strain (CFCC 52286) and another strain (CFCC 52288) on both detached branches and 3-year-old chestnut seedlings. The inoculation results showed that *Gnomoniopsis chinensis* is mildly pathogenic to Chinese chestnut. However, further studies are required to confirm its pathogenicity to the other cultivated *Castanea* species in America, Europe and Japan.

Keywords

Castanea mollissima, chestnut disease, taxonomy

Introduction

The Chinese chestnut (*Castanea mollissima*), as well as the American chestnut (*C. dentata*), the European chestnut (*C. sativa*) and the Japanese chestnut (*C. crenata*), are known as the four main cultivated sweet chestnut species in the world (Conedera et al. 2004; Yi 2017). In recent studies, several important fungal pathogens have been reported from chestnut trees, including *Aurantiosacculus castaneae*, *Cryphonectria neoparasitica*, *Cry. parasitica*, *Endothia chinensis* and *Gnomoniopsis daii* from *C. mollissima* (Jiang et al. 2018a, 2019b; Jiang and Tian 2019); *Cry. parasitica*, *G. smithogilvyi* (syn. *G. castaneae*), *Phytophthora cinnamomi* and *Sirococcus castaneae* from *C. sativa* (Anagnostakis 1987; Visentin et al. 2012; Shuttleworth et al. 2013; Meyer et al. 2017; Shuttleworth and Guest 2017; Rigling and Prospero 2018; Akilli Şimşek et al. 2019; Lione et al. 2019). In China, *Castanea mollissima* is widely cultivated for its gluten-free, low fat, and cholesterol-free chestnuts (Lu and Guo 2017), but suffering from several fungal diseases (Li et al. 2006; Zhang et al. 2009).

The fungal genus *Gnomoniopsis* (Gnomoniaceae, Diaporthales) includes species all occurring in plant tissues as pathogens, endophytes or saprobes (Danti et al. 2002; Rossman et al. 2007; Walker et al. 2010; Sogonov et al. 2008). Until now, *Gnomoniopsis* species have been found on hosts from three plant families, Fagaceae, Onagraceae and Rosaceae (Sogonov et al. 2008; Walker et al. 2010). Two species occur as pathogens on *Castanea* species (family Fagaceae), i.e. *Gnomoniopsis smithogilvyi* (syn. *G. castaneae*) and *G. daii* (Crous et al. 2012; Jiang and Tian 2019). *Gnomoniopsis smithogilvyi* and *G. castaneae* were proposed by two independent studies, from rotten fruits of *Castanea sativa* (Crous et al. 2012; Visentin et al. 2012). However, Shuttleworth et al. (2015) proved that *Gnomoniopsis smithogilvyi* and *G. castaneae* are conspecific based on a comparative morphological analysis and five-marker phylogenetic analysis. The fungal name *Gnomoniopsis smithogilvyi* was published earlier than *G. castaneae*, hence *G. smithogilvyi* has priority over *G. castaneae*.

Gnomoniopsis smithogilvyi is an important nut rot agent on chestnut nuts, an endophyte in asymptomatic flowers, leaves and stems, and a saprobe on dead burrs and branches (Crous et al. 2012; Visentin et al. 2012). Moreover, this species has been reported as a severe bark pathogen on *Castanea* in several countries (Dar and Rai 2013, 2015; Pasche et al. 2016; Lewis et al. 2017; Trapiello et al. 2018; Lione et al. 2019). In China, *Gnomoniopsis* from rotten Chinese chestnut has proved to be a different species, namely *Gnomoniopsis daii* (Jiang and Tian 2019). In this study, we focused on the symptom, taxonomy and pathogenicity aspects of *Gnomoniopsis* species from cankered tissues on Chinese chestnut trees.

Materials and methods

Sample collection and isolation

During 2016 to 2019, investigations were conducted in chestnut plantations of nine provinces/municipalities in China, including Beijing, Fujian, Hebei, Hubei, Hunan,

Liaoning, Shandong, Shaanxi and Tianjin. Typical *Gnomoniopsis* canker symptoms were only observed in Hebei Province (Fig. 1). Symptomatic barks from stems and cankered branches were collected in brown paper bags and transported to the laboratory for fungal isolations and further study. Single conidial isolates were acquired from asexual fruiting structures by removing a mucoid conidial mass from pycnidial ostioles, and spreading the suspension on the surface of potato dextrose agar (PDA; 200 g potatoes, 20 g dextrose, 20 g agar per L). Agar plates were incubated at 25 °C to induce germination of conidia. After inoculation for up to 36 h, single germinating conidia were then transferred to clean plates under a dissecting stereomicroscope with a sterile needle. Specimens and cultures were deposited and maintained in the Museum of Beijing Forestry University (BJFC) and China Forestry Culture Collection Center (CFCC), Beijing, China, respectively.

DNA extraction and phylogenetic analysis

Genomic DNA was extracted from mycelium grown on PDA using a CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1990). Three partial loci, including the 5.8S nuclear ribosomal DNA gene with the two flanking internally transcribed spacer (ITS) regions, the translation elongation factor 1a (*tef1*), and the β-tubulin gene 2 (*tub2*), were amplified using the following primer pairs: ITS1 and ITS4 for ITS (White et al. 1990), EF1-728F and EF1-1567R for *tef1* (Carbone and Kohn 1999), and Bt2a and Bt2b for *tub2* (Glass and Donaldson 1995). The PCR conditions were: initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 50 s at 48 °C (ITS) or 54 °C (tef1) or 52 °C (tub2), and 1 min at 72 °C, and a final elongation step of 7 min at 72 °C. The PCR amplification products were scored visually by electrophoresis in 2 % agarose gels. The DNA sequencing was performed using an ABI PRISM 3730XL DNA Analyzer with BigDye Terminater Kit v.3.1 (Invitrogen) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China). To assess the phylogenetic position of our isolates within the genus *Gnomoniopsis*, phylogenetic analyses were performed based on combined ITS, *tef1* and *tub2* sequence data, with *Sirococcus castaneae* (CBS 142041) and *Apiognomonia errabunda* (CBS 342.86) selected as outgroup taxa. The GenBank accession numbers of sequences used in the analysis are given in Table 1, which were aligned and edited manually in MEGA6 (Tamura et al. 2013). Maximum likelihood (ML) analysis was used for phylogenetic inferences of the concatenated alignments. ML analysis was implemented on the CIPRES Science Gateway portal using RAxML-HPC BlackBox v. 8.2.10 (Stamatakis 2014).

Morphological identification and characterization

Species identification was based on morphological features of the asexual fruiting bodies produced on infected plant tissues, supplemented by cultural characteristics.

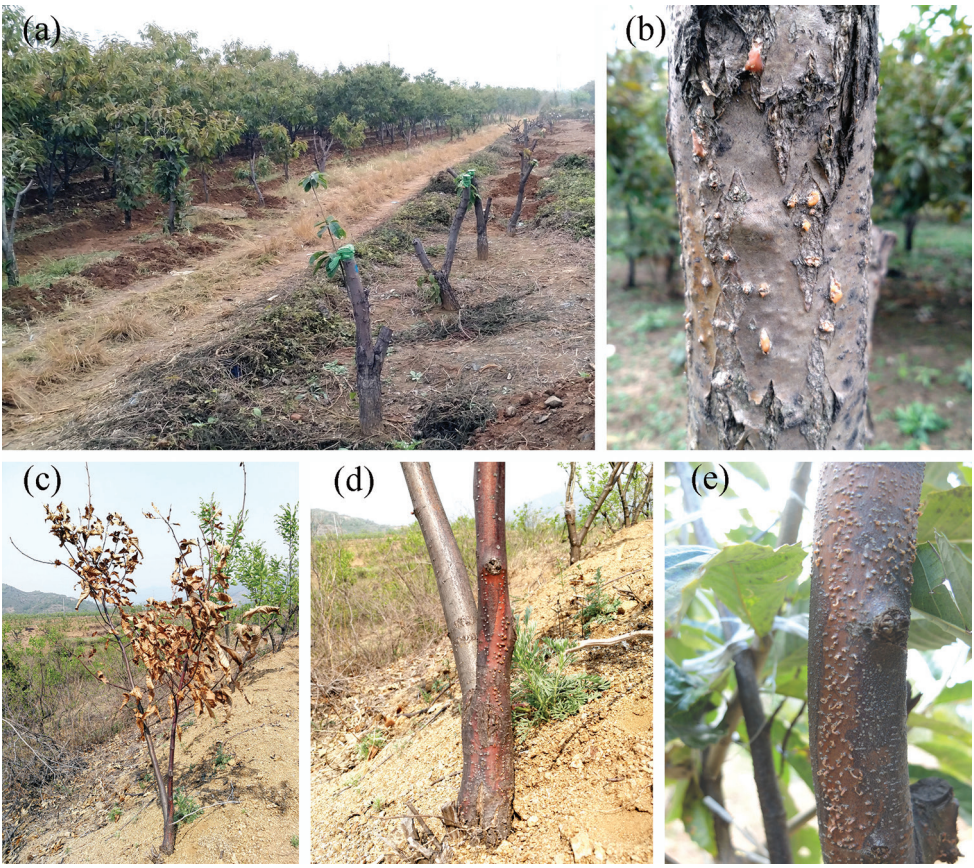

Figure 1. Symptoms caused by *Gnomoniopsis chinensis* on Chinese chestnut (*Castanea mollissima*) **a, b** severe cankers on adult trees **c** a dead young tree **d** lesion with conidiomata on the bark near the root **e** lesion with conidiomata on the stem.

Hence, cross-sections were prepared by hand using a double-edge blade. Morphological characteristics of the fruiting bodies including: size of conidiomata and locules; size and shape of conidiophores and conidia were determined under a Nikon AZ100 dissecting stereomicroscope. More than 20 fruiting bodies were sectioned, and 50 conidia were selected randomly for measurement using a Leica compound microscope (LM, DM 2500). Cultural characteristics of isolates incubated on PDA in the dark at 25 °C were recorded, including the colony color and pycnidium structures (Rayner 1970).

Pathogenicity trials

Two isolates of *Gnomoniopsis chinensis* (ex-type strain: CFCC 52286; CFCC 52288) were used for inoculations, and agar plugs were used as the negative control. Isolates

Table 1. Isolates and GenBank accession numbers used in this study.

Species	Country	Host	Strain	GenBank Accession Number		
				ITS	*tub2*	*tef1*
Apiognomonia veneta	France	*Platanus occidentalis*	CBS 342.86	DQ313531	EU219235	DQ318036
Gnomoniopsis alderdunensis	USA	*Rubus pedatus*	CBS 125679	GU320826	GU320788	GU320813
Gnomoniopsis alderdunensis	USA	*Rubus parviflorus*	CBS 125680	GU320825	GU320787	GU320801
Gnomoniopsis alderdunensis	USA	*Rubus parviflorus*	CBS 125681	GU320827	GU320789	GU320802
Gnomoniopsis chamaemori	Finland	*Rubus chamaemorus*	CBS 804.79	GU320817	GU320777	GU320809
Gnomoniopsis chinensis	**China**	***Castanea mollissima***	**CFCC 52286**	**MG866032**	**MH545366**	**MH545370**
Gnomoniopsis chinensis	**China**	***Castanea mollissima***	**CFCC 52287**	**MG866033**	**MH545367**	**MH545371**
Gnomoniopsis chinensis	**China**	***Castanea mollissima***	**CFCC 52288**	**MG866034**	**MH545368**	**MH545372**
Gnomoniopsis chinensis	**China**	***Castanea mollissima***	**CFCC 52289**	**MG866035**	**MH545369**	**MH545373**
Gnomoniopsis clavulata	USA	*Quercus falcata*	CBS 121255	EU254818	EU219211	GU320807
Gnomoniopsis comari	Finland	*Comarum palustre*	CBS 806.79	EU254821	EU219156	GU320810
Gnomoniopsis comari	Finland	*Comarum palustre*	CBS 807.79	EU254822	GU320779	GU320814
Gnomoniopsis comari	Switzerland	*Comarum palustre*	CBS 809.79	EU254823	GU320778	GU320794
Gnomoniopsis daii	China	*Castanea mollissima*	CFCC 54043	MN598671	MN605517	MN605519
Gnomoniopsis daii	China	*Castanea mollissima*	CMF002B	MN598672	MN605518	MN605520
Gnomoniopsis fructicola	USA	*Fragaria vesca*	CBS 121226	EU254824	EU219144	GU320792
Gnomoniopsis fructicola	France	*Fragaria* sp.	CBS 208.34	EU254826	EU219149	GU320808
Gnomoniopsis fructicola	USA	*Fragaria* sp.	CBS 125671	GU320816	GU320776	GU320793
Gnomoniopsis guttulata	Bulgaria	*Agrimonia eupatoria*	MS 0312	EU254812	NA	NA
Gnomoniopsis idaeicola	USA	*Rubus* sp.	CBS 125672	GU320823	GU320781	GU320797
Gnomoniopsis idaeicola	USA	*Rubus pedatus*	CBS 125673	GU320824	GU320782	GU320798
Gnomoniopsis idaeicola	France	*Rubus* sp.	CBS 125674	GU320820	GU320780	GU320796
Gnomoniopsis idaeicola	USA	*Rubus procerus*	CBS 125675	GU320822	GU320783	GU320799
Gnomoniopsis idaeicola	USA	*Rubus procerus*	CBS 125676	GU320821	GU320784	GU320811
Gnomoniopsis macounii	USA	*Spiraea* sp.	CBS 121468	EU254762	EU219126	GU320804
Gnomoniopsis occulta	USA	*Potentilla* sp.	CBS 125677	GU320828	GU320785	GU320812
Gnomoniopsis occulta	USA	*Potentilla* sp.	CBS 125678	GU320829	GU320786	GU320800
Gnomoniopsis paraclavulata	USA	*Quercus alba*	CBS 123202	GU320830	GU320775	GU320815
Gnomoniopsis racemula	USA	*Chamerion angustifolium*	CBS 121469	EU254841	EU219125	GU320803
Gnomoniopsis sanguisorbae	Switzerland	*Sanguisorba minor*	CBS 858.79	GU320818	GU320790	GU320805
Gnomoniopsis smithogilvyi	Australia	*Castanea* sp.	CBS 130190	JQ910642	JQ910639	KR072534
Gnomoniopsis smithogilvyi	Australia	*Castanea* sp.	CBS 130189	JQ910644	JQ910641	KR072535
Gnomoniopsis smithogilvyi	Australia	*Castanea* sp.	CBS 130188	JQ910643	JQ910640	KR072536
Gnomoniopsis smithogilvyi	Italy	*Castanea sativa*	MUT 401	HM142946	KR072532	KR072537
Gnomoniopsis smithogilvyi	New Zealand	*Castanea sativa*	MUT 411	HM142948	KR072533	KR072538
Gnomoniopsis tormentillae	Switzerland	*Potentilla* sp.	CBS 904.79	EU254856	EU219165	GU320795
Sirococcus castaneae	Switzerland	*Castanea sativa*	CBS 142041	KX929744	KX958443	KX929710

Note: NA, not applicable. Strains in this study are identified in bold.

were grown on PDA for five days at 25 °C before the tests. Inoculations were performed on detached branches and 3-year-old seedlings of *Castanea mollissima*, respectively. The detached branches and young seedling were collected from Hebei Province where the disease is emerging. The healthy chestnut branches (2 cm in diameter) were sampled from an adult chestnut tree and cut into pieces of 20 cm length. A total of 30 fresh and healthy branches and 15 seedlings were used for the pathogenicity tests. Ten branches and five seedlings were inoculated with each isolate and the negative control. For incubations, incisions were made on the middle of the detached branches and 1 cm above the midpoint of the seedling stem to expose the cambium using a 5-mm-diameter cork borer. Discs of agar were cut from the actively growing margins of the

cultures and these were placed into wounds of the same size on the chestnut barks. Inoculated wounds and ends of inoculated branches were sealed with parafilm to reduce desiccation and the chance of contamination. The tested seedlings and branch segments were maintained in the greenhouse randomly at 25 °C under natural light conditions. Detached branches were inoculated in November 2017, and the young seedlings were tested in July 2019. The results from detached branches were evaluated after one month, and seedlings after three months, by measuring the lengths of the lesions on the cambium. The re-isolations were made from the resultant lesions from all tested branches and seedlings by cutting small pieces of discolored xylem and placing them onto the PDA plates. Re-isolations were identified based on morphology on PDA and ITS sequences. Differences among isolates in lesion length were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) tests. Statistical analysis was carried out by R software (version 3.4.3.) and considered as significant at $p < 0.05$.

Results

Phylogenetic analyses

The final combined ITS-*tef1-tub2* matrix of *Gnomoniopsis* included 35 ingroup and two outgroup taxa, comprising 1364 alignment characters. Of these, 783 characters were constant, 117 variable characters were parsimony-uninformative and 464 characters were parsimony informative. The phylogenetic tree obtained from ML analysis is shown in Figure 2, indicating that all isolates from the present study are phylogenetically different from other known species with 100% ML bootstrap support.

Taxonomy

Gnomoniopsis chinensis **C.M. Tian & N. Jiang, sp. nov.**
MycoBank No: 823868
Figures 3, 4

Etymology. Named after the country where it was first collected.

Description. Pathogenic on stems and branches of *Castanea mollissima*. Conidiomata pseudostromatic, globose to pulvinate, occurring separately, yellow to orange, semi-immersed in bark, 400–1000 µm high, 500–1500 µm diam, unilocular, single ostiolate, forming long, wide orange tendrils, 1500–2000 µm × 400–500 µm. Conidiophores indistinct, often reduced to conidiogenous cells. Conidiogenous cells oval, hyaline, 1-celled, 6–12 µm. Conidia oval, oblate, fusiform, straight to curved, hyaline, 2–3 guttules, (6.0–)6.5–8.5(–9.0) × (2.2–)2.7–3(–3.5) µm (mean = 7.5 × 2.7 µm).

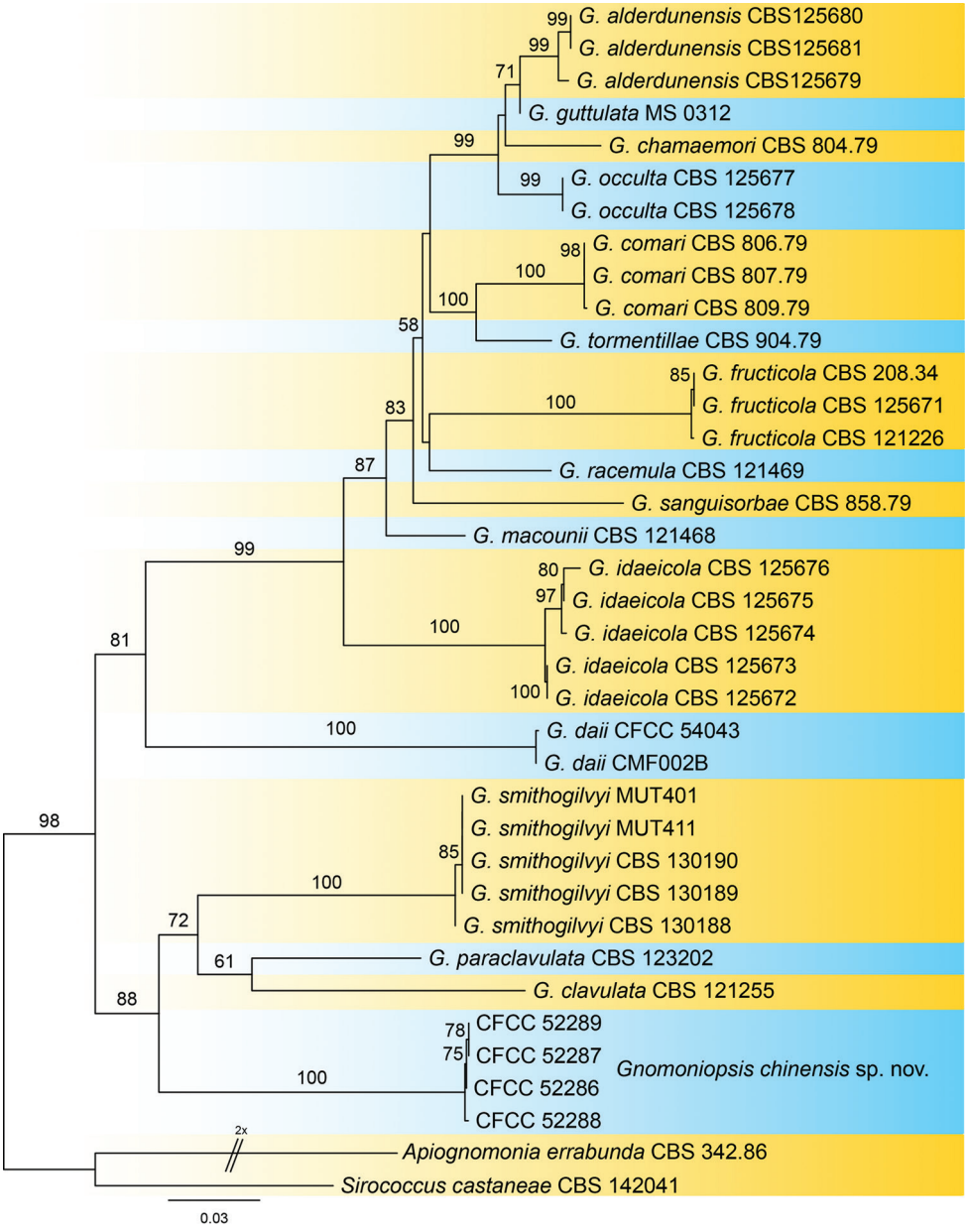

Figure 2. Consensus tree resulting from a RAxML analysis of combined ITS, *tef1* and *tub2* sequence alignment for species of *Gnomoniopsis*. The scale bar represents the expected number of changes per site.

Culture characters. Colonies on PDA attaining 90 mm after 20 days at 25 °C, flat, velutinous to shortly woolly, dark brown in center, gradually lightening to pale grey at margin; margin diffuse; reverse of almost same colors as surface.

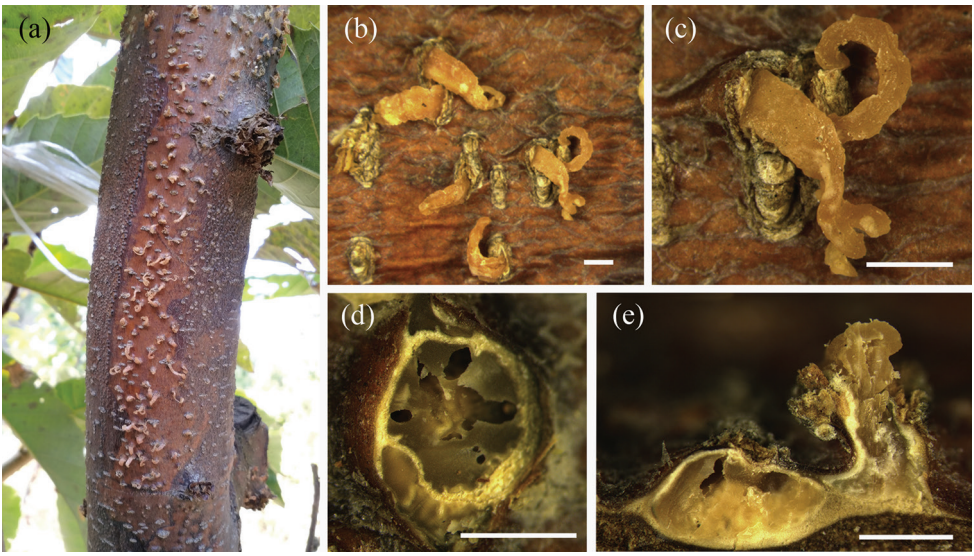

Figure 3. Conidiomata of *Gnomoniopsis chinensis* from *Castanea mollissima* (BJFC-S1380, holotype) **a–c** habit of conidiomata on the chestnut stem **d** transverse sections through conidiomata **e** longitudinal sections through conidiomata. Scale bars: 1 mm (**b–e**).

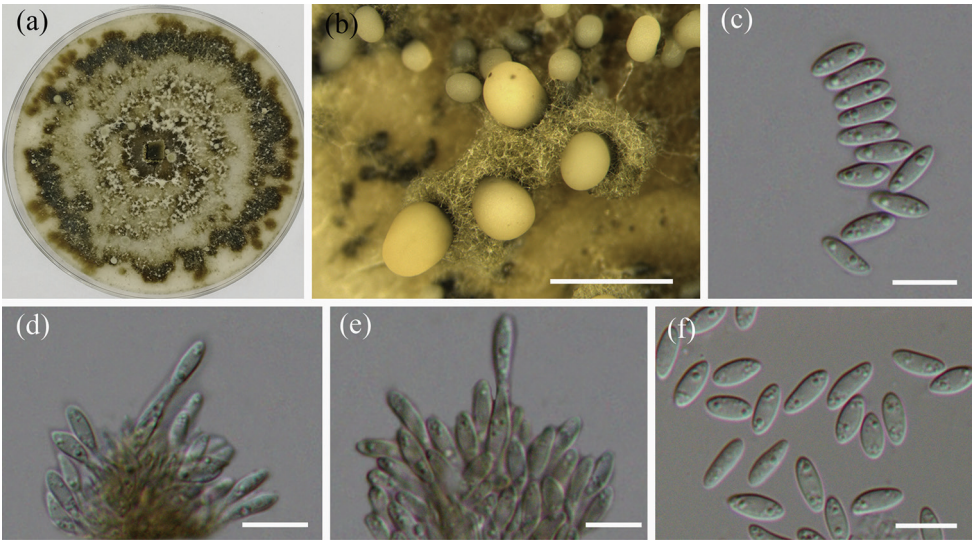

Figure 4. Morphology of *Gnomoniopsis chinensis* from PDA (CFCC 52286, ex-type culture) **a** colonies on PDA **b** conidiomata formed on PDA **c, f** conidia **d, e** conidiogenous cells. Scale bars: 1 mm (**b**); 10 μm (**c–f**).

Specimens examined. China, Hebei Province, Chengde City, chestnut plantation, 40°24'32.16"N, 117°28'56.24"E, 262 m asl, on stems and branches of *Castanea mollissima*, Ning Jiang, 11 October 2017 (BJFC-S1380, holotype; ex-type culture, CFCC 52286). Hebei Province, Qinhuangdao City, chestnut plantation, 40°22'52.32"N,

119°11'52.18"E, 246 m asl, on branches and twigs of *Castanea mollissima*, Ning Jiang, 14 October 2017 (BJFC-S1382, paratype; living culture, CFCC 52288). Hebei Province, Tangshan City, chestnut plantation, 40°12'59.76"N, 117°59'7.24"E, 67 m asl, on stems and branches of *Castanea mollissima*, Ning Jiang, 18 October 2017 (BJFC-S1383; living culture, CFCC 52289).

Notes. Three *Gnomoniopsis* species have been discovered from the host genus *Castanea*. They share similar conidial dimension (6.0–9.0 × 2.2–3.5 µm in *Gnomoniopsis chinensis* vs. 5.0–8.0 × 2.0–3.5 µm in *G. daii* vs. 6.0–9.5 × 2.0–4.0 µm in *G. smithogilvyi*) (Crous et al. 2012; Jiang and Tian 2019). However, we can distinguish them easily by the phylogram of ITS, *tef1* and *tub2* (Fig. 2). In addition, *Gnomoniopsis chinensis* and *G. daii* inhabit the Chinese chestnut (*Castanea mollissima*), but *G. smithogilvyi* on the European chestnut (*C. sativa*) and *C. crenata* × *C. sativa* hybrids.

Pathogenicity trials

One month after inoculation on detached branches, the two *Gnomoniopsis chinensis* isolates produced lesions in the cambium of detached chestnut branches. In contrast, there was no lesion development in any of the negative control inoculations (Fig. 5). The lesion size of the two *Gnomoniopsis chinensis* isolates (CFCC 52286 and CFCC 52288) showed no significantly difference, while both of them were significantly longer than the negative control ($P < 0.05$). *Gnomoniopsis chinensis* was consistently re-isolated from lesions.

Three months after inoculation on young seedlings, two isolates *Gnomoniopsis chinensis* and the negative control, produced minor lesions (Fig. 6). Statistical analyses of data showed no significant difference among two isolates *Gnomoniopsis chinensis* and the negative control ($P < 0.05$). However, *Gnomoniopsis chinensis* was still re-isolated successfully from the minor lesions caused by CFCC 52286 and CFCC 52288 and not from the negative control inoculations.

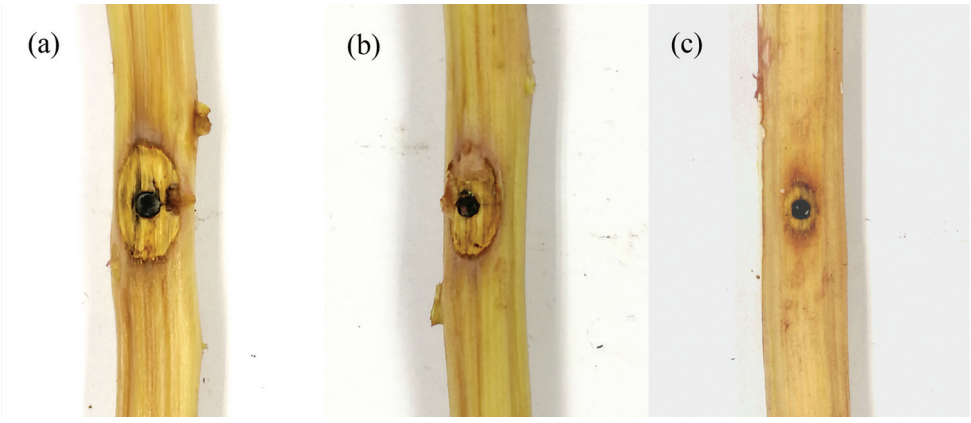

Figure 5. Lesions resulting from inoculation of *Gnomoniopsis chinensis* onto detached *Castanea mollissima* branches, and wound response on the negative control **a** CFCC 52288 **b** CFCC 52286 **c** negative control.

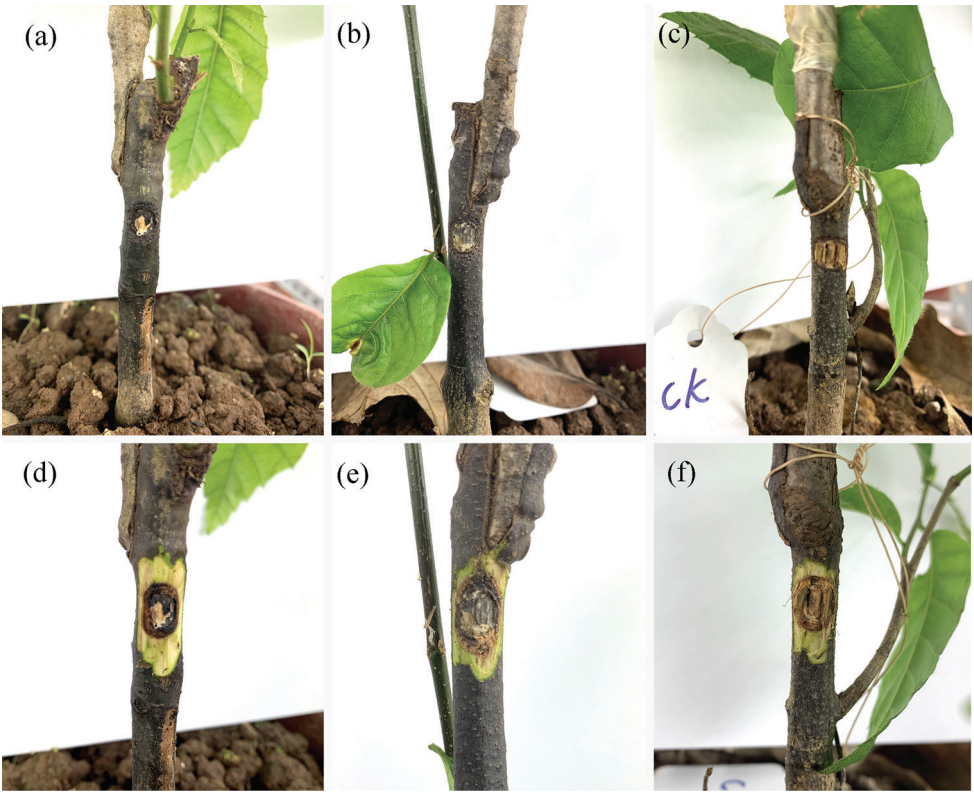

Figure 6. Lesions resulting from inoculation of *Gnomoniopsis chinensis* onto 3-year-old *Castanea mollissima* seedlings, and wound response on the negative control **a, d** CFCC 52288 **b, e** CFCC 52286 **c, f** negative control. Row 1: lesions on the bark; row 2: lesions beneath the bark.

Discussion

In the past years, our team focused on the fungi inhabiting Chinese chestnut (*Castanea mollissima*) trees from their taxonomy and pathogenicity aspects. Several fungi including *Aurantiosacculus castaneae*, *Cryphonectria neoparasitica*, *Cry. parasitica*, *Endothia chinensis* and *Gnomoniopsis daii* have been proven to cause branch canker or fruit rot (Jiang et al. 2019b; Jiang and Tian 2019). Other fungi were reported to be associated with branch canker, however, they were not confirmed by incubation tests, including *Aplosporella javeedii*, *Coryneum gigasporum*, *Co. sinense*, *Co. suttonii*, *Co. umbonatum*, *Cytospora ceratospermopsis*, *Cy. kuanchengensis*, *Cy. leucostoma*, *Cy. myrtagena*, *Cy. Schulzeri*, *Cy. xinglongensis*, *Dendrostoma aurorae*, *Den. castaneae*, *Den. castaneicola*, *Den. chinense*, *Den. parasiticum*, *Den. shaanxiense*, *Den. shandongense*, *Lopadostoma americanum*, *Melanops castaneicola*, *Myrmaecium fulvopruinatum*, *Neopseudomelanconis castaneae* (Jiang et al. 2018b, c, d, 2019a, 2020). Subsequently, *Dendrostoma atlanticum* and *Den. castaneum* were reported from European chestnut (*Castanea sativa*) trees (Jaklitsch and

Voglmayr 2019). Different *Dendrostoma* species were discovered from the Chinese and European chestnut stems, branches and twigs, which indicates similar plant and fungi interactions in different continents. Another example is that *Gnomoniopsis daii* causes Chinese chestnut rot and *Gnomoniopsis smithogilvyi* causes European chestnut rot (Crous et al. 2012; Jiang and Tian 2019). Interestingly, this study reveals a novel *Gnomoniopsis* species, *G. chinensis*, as an opportunistic pathogen causing bark cankers on Chinese chestnut, which is different from *Gnomoniopsis smithogilvyi* causing both nut rot and bark cankers (Crous et al. 2012; Visentin et al. 2012; Dar and Rai 2013, 2015; Pasche et al. 2016; Lewis et al. 2017; Trapiello et al. 2018).

Gnomoniopsis species appear host-specific, inhabiting hosts of three families, viz. Betulaceae, Fagaceae, Rosaceae and Onagraceae (Sogonov et al. 2008; Walker et al. 2010; Visentin et al. 2012; Linaldeddu et al. 2016). Five species have been discovered from fagaceous hosts, and they are similar in conidial size (Table 2). *Gnomoniopsis clavulata* and *G. paraclavulata* were recorded on *Fagus* or *Quercus* trees (Sogonov et al. 2008). *Gnomoniopsis chinensis* and *G. daii* were discovered only from *Castanea* trees. It is hard to distinguish them by the currently known conidial characteristics. However, all currently known *Gnomoniopsis* species can be successfully distinguished by phylogenetic analysis based on ITS, *tef1* and *tub2*.

Stevanović et al. (2019) reported *Gnomoniopsis idaeicola* to cause blackberry canker and wilting in Serbia. With the same signs on the host bark, especially the wide, orange tendrils emerging from hosts' glaucous lenticels, *Gnomoniopsis chinensis* appeared to be an emerging pathogen on *Castanea mollissima*. Chestnut blight, caused by *Cryphonectria parasitica*, a notorious bark disease on chestnut trees worldwide (Rigling and Prospero 2018), can be distinguished from chestnut *Gnomoniopsis* canker, and the presence of mycelial fans in the cambial region. Nowadays, we have characterized the two canker pathogens on Chinese and European chestnut trees, *Gnomoniopsis chinensis* and *G. smithogilvyi*. They appear not to be very pathogenic to their native hosts, but the pathogenicity to non-native hosts is still unknown. *Gnomoniopsis* and *Cryphonectria* belong to the same fungal order Diaporthales, and are similar in some aspects. Hence, more work on these two pathogens is necessary on both *Castanea mollissima* and *C. sativa*. In addition, considering the high value of the plant genus, *Castanea*, and the current situation of serious commercial loss caused by various fungi, more comprehensive and detailed investigations are necessary to understand the diversity of microbes on the hosts and their functions.

Table 2. Conidial size of *Gnomoniopsis* species from fagaceous hosts.

Species	Conidial length (µm)	Conidial width (µm)	Reference
Gnomoniopsis chinensis	(6.0–)6.5–8.5(–9.0)	(2.2–)2.7–3(–3.5)	This study
Gnomoniopsis clavulata	(5–)6–6.5(–8)	(2–)2.5–3(–4)	Sogonov et al. 2008
Gnomoniopsis daii	(5.0–)5.5–7.0(–8.0)	2.0–3.5	Jiang and Tian 2019
Gnomoniopsis paraclavulata	(6–)7.5–8(–9.5)	(2–)3–3(–3.5)	Sogonov et al. 2008
Gnomoniopsis smithogilvyi	(6.0–)8(–9.5)	(2.0–)2.5(–4.0)	Crous et al. 2012

Acknowledgements

This study is financed by National Natural Science Foundation of China (Project No.: 31670647). We are grateful to Chungen Piao, Minwei Guo (China Forestry Culture Collection Center (CFCC), Chinese Academy of Forestry, Beijing.

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MycoKeys 67: 33–44 (2020)
doi: 10.3897/mycokeys.67.51020
http://mycokeys.pensoft.net

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New species of *Retiboletus* (Boletales, Boletaceae) from China based on morphological and molecular data

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Academic editor: M. P. Martín | Received 11 February 2020 | Accepted 30 March 2020 | Published 14 May 2020

Citation: Liu H-Y, Li Y-C, Bau T (2020) New species of *Retiboletus* (Boletales, Boletaceae) from China based on morphological and molecular data. MycoKeys 67: 33–44. https://doi.org/10.3897/mycokeys.67.51020

Abstract

Species of the genus *Retiboletus* in China were investigated based on morphology and phylogenetic analysis of DNA sequences from the nuclear ribosomal large subunit (nrLSU) and the translation elongation factor 1-α gene (TEF1-α). Nine species were recovered from China, including two new and seven known species. The new species, namely *Retiboletus ater* and *R. sinogriseus*, from southwestern and northeastern China respectively, are documented and illustrated in this paper. *Retiboletus ater* is morphologically characterized by its black to grayish black pileus, white to grayish hymenophore, black to blackish stipe and white to grayish white context. *Retiboletus sinogriseus* is morphologically characterized by its brown to grayish-brown pileus, yellow to grayish-yellow hymenophore, pale yellow to brownish stipe and yellow to brownish-yellow context. Descriptions and line drawings of these two novel species and their comparisons with allied taxa are presented.

Keywords

Boletes, morphology, new taxa, phylogeny, taxonomy

Introduction

The genus *Retiboletus* Manfr. Binder & Bresinsky was erected to accommodate *Boletus ornatipes* Peck and allied species (Binder and Bresinsky 2002). The genus is morphologically characterized by the combination of the following characters: pileus convex to plane, dry, subtomentose, black, dark gray, mustard yellow or olive-brown; hymenophore pallid, grayish or yellow, unchanging or staining brown or orange-brown when bruised; hymenial cystidia present; stipe reticulate; context pallid, yellow or vivid yellow, unchanging or bruising orange-brown; clamp connections absent; spore deposit olive-brown to yellow-brown, basidiospores smooth, ellipsoid to subfusoid, inamyloid and partly dextrinoid (Binder and Bresinsky 2002; Zeng et al. 2016). The separation of *Retiboletus* from *Boletus* s. str. and its establishment at the generic rank is strongly supported (Wu et al. 2016; Zeng et al. 2016, 2018; Badou et al. 2018). So far thirteen species of this genus have been described from North/Central America and East Asia, seven out of which have been reported from China (Binder and Bresinsky 2002; Wu et al. 2016; Zeng et al. 2016, 2018).

During field investigation of Boletaceae across China, we encountered two impressive *Retiboletus* species from southwestern and northeastern China, respectively. These species can be easily recognized by their conspicuous colors in the field. Molecular phylogenetic analysis of this genus based on the nuclear ribosomal large subunit (nrLSU) and the translation elongation factor 1-α gene (TEF1-α) indicated that they represent two distinct species. Combined with morphological characters, *Retiboletus ater* and *R. sinogriseus*, are proposed and described herein. It is noteworthy that an additional collection from northeastern China, labeled *R.* aff. *kauffmanii* (HY56), was included in our molecular phylogenetic analysis. But its classification can't be clarified due to its paucity of mature material. Further collections are needed to better estimate its taxonomic status.

Materials and methods

Morphological studies

Specimens were described and photographed in the field and deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN) and Herbarium of Jilin Agriculture University (HMJAU). In the descriptions, macroscopic characters were based on field notes and color slides of the specimens. Color codes are from Kornerup and Wanscher (1981). Microscopic characters were from the observations of the specimens through light microscopy. For microscopic study, dried materials were sectioned and mounted in 5% KOH solution. Sections of the pileipellis were cut tangentially and halfway between the center and margin of the pileus. All measurements were made in KOH mounts and observed under the light phase. All line drawings of microstructures were made from rehydrated material. Melzer's reagent was used for testing color reactions of the tissue fragments to the solution. The notations "basidi-

ospores (n/m/p)" indicate that the measurements were made on n basidiospores from m basidiomata of p collections. The expressions (a)b–c(d) stand for the dimensions of basidiospores; the range b–c contains a minimum of 90% of the measured values, a and d in the brackets stand for the extreme values. The following abbreviations are used: Q (length/width ration of basidiospores) and Q_m (average Q ± standard deviation).

DNA extraction, PCR and DNA sequencing

Protocols for DNA extraction, PCR, sequencing and sequence alignment followed those in Vadthanarat et al. (2019), Gelardi et al. (2019), Zhang et al. (2019) and references therein. The primer pair used for amplifying the nrLSU region was LROR and LR5 (Vilgalys and Hester 1990). DNA sequences were compiled with SeqMan (DNASTAR Lasergene 9). Sequences were aligned with MUSCLE 3.6 (Edgar 2004) and manually adjusted where necessary. Edited sequences were deposited in GenBank (Table 1).

Phylogenetic analysis

19 sequences (10 of nrLSU and 9 of TEF1-α) from 10 collections were newly generated in this study and aligned with selected sequences from GenBank and previous studies (Binder and Bresinsky 2002; Ortiz-Santana et al. 2007; Zeng et al. 2016, 2018) (Table 1). *Boletus edulis* Bull. and *Boletus reticuloceps* (M. Zang, M.S. Yuan & M.Q. Gong) Q.B. Wang & Y.J. Yao were chosen as outgroup. The combined nuclear dataset (nrLSU + TEF1-α) was analyzed with maximum likelihood (ML). Maximum-likelihood tree generation and bootstrap analysis were performed with the program RAxML 7.2.6 (Stamatakis 2006) running 1000 bootstrap replicates combined with a ML search.

Result

Molecular analysis

The combined nuclear dataset (nrLSU + TEF1-α) consists of 64 sequences and is 1526 bp long. The alignment was submitted to TreeBASE (S25798). Phylograms with branch lengths inferred with RAxML, including the support values, are illustrated (Fig. 1). The monophyly of *Retiboletus* was moderately supported (bootstrap = 56%) in our analysis (Fig. 1). Two new species were recovered within *Retiboletus*, including two collections of *R. ater* from southwestern China and two collections of *R. sinogriseus* from northeastern China. Phylogenetically, sequences of *R. ater* form a unique lineage with 100% bootstrap support, while *R. sinogriseus* is closely related to *R. griseus* with high bootstrap support (99%). The collection labeled *R.* aff. *kauffmanii* (HY56) from northeastern China, clusters together with *R. kauffmanii* and another Indian species labeled *Retiboletus* sp. (CAL_F_1397).

Table 1. Specimens used in molecular phylogenetic study and their GenBank accession numbers.

Species	Voucher	Locality	Accession		Reference
			nrLSU	TEF1-α	
Retiboletus ater	Li1215	SW China	**MT010611**	**MT010621**	**This study**
R. ater	Li1224	SW China	**MT010612**	**MT010622**	**This study**
R. brunneolus	LC_LJW237	SW China	**MT010615**	**MT010625**	**This study**
R. brunneolus	Li993	SE China	KF112424	KF112179	Wu et al. 2016
R. flavoniger	RH7247	Costa Rica	AF456828	–	Binder and Bresinsky 2002
R. flavoniger	RH7189	Costa Rica	AF456829	–	Binder and Bresinsky 2002
R. fuscus	Wu445	SW China	KT990636	KT990830	Wu et al. 2016
R. fuscus	Cui47	SW China	**MT010614**	**MT010624**	**This study**
R. griseus	BD210	USA	HQ161858	–	Binder and Bresinsky 2002
R. griseus	snBoth	USA	KF030308	KF030414	Binder and Bresinsky 2002
R. griseus	Halling10162	USA	**MT010608**	**MT010618**	**This study**
R. kauffmanii	Wu317	SW China	KP739282	KP739301	Zeng et al. 2016
R. nigerrimus	Tyni1	Japan	AF456832	–	Binder and Bresinsky 2002
R. nigrogriseus	FHMU2045	Southern China	MH367475	MH367487	Zeng et al. 2018
R. nigrogriseus	FHMU2800	Southern China	MH367476	MH367488	Zeng et al. 2018
R. ornatipes	201/97	USA	AF456815	–	Binder and Bresinsky 2002
R. ornatipes	Halling10163	USA	**MT010617**	**MT010626**	**This study**
R. ornatipes	161/97	USA	AF456817	–	Binder and Bresinsky 2002
R. pseudogriseus	Zeng647	Southern China	**MT010613**	**MT010623**	**This study**
R. pseudogriseus	FHMU375	Southern China	MH367477	MH367489	Zeng et al. 2018
R. pseudogriseus	Zeng668	SE China	KP739285	–	Zeng et al. 2016
R. retipes	96/97	USA	AF456830	–	Binder and Bresinsky 2002
R. retipes	22/97	USA	AF456831	–	Binder and Bresinsky 2002
R. retipes	116/96	USA	AF456823	–	Binder and Bresinsky 2002
R. retipes	57/97	USA	AF456811	–	Binder and Bresinsky 2002
R. sinensis	Zeng1299	SE China	KP739291	KP739303	Zeng et al. 2016
R. sinensis	Zeng1278	SE China	KP739289	KP739302	Zeng et al. 2016
R. sinensis	Zeng569	Southern China	KP739286	–	Zeng et al. 2016
R. sinogriseus	LJ258	NE China	**MT010610**	**MT010620**	**This study**
R. sinogriseus	LJ260	NE China	**MT010609**	**MT010619**	**This study**
R. sp.	CAL_F_1397	India	KY290586	–	GenBank
R. aff. *kauffmanii*	HY56	NE China	**MT010616**	–	**This study**
R. vinaceipes	CFMR:DR-1035	Dominican Republic	MN250180	–	Ortiz-Santana et al. 2007
R. vinaceipes	CFMR:BZ-2386	Belize	MN250190	–	Ortiz-Santana et al. 2007
R. zhangfeii	Li1951	SE China	JQ928627	JQ928582	Wu et al. 2016
R. zhangfeii	Li1073	SE China	KT990630	KT990824	Wu et al. 2016
Boletus edulis	HMJAU4637	NE China	KF112455	KF112202	Wu et al. 2016
B. reticuloceps	Liang521	SW China	KT990537	KT990739	Wu et al. 2016
Pseudoaustroboletus valens	LF690	Southern China	KM274870	KM274878	Li et al. 2014
P. valens	Li915	SE China	KM274869	KM274877	Li et al. 2014

Sequences obtained in this study are shown in bold. SW = southwestern; NE = northeastern; SE = southeastern.

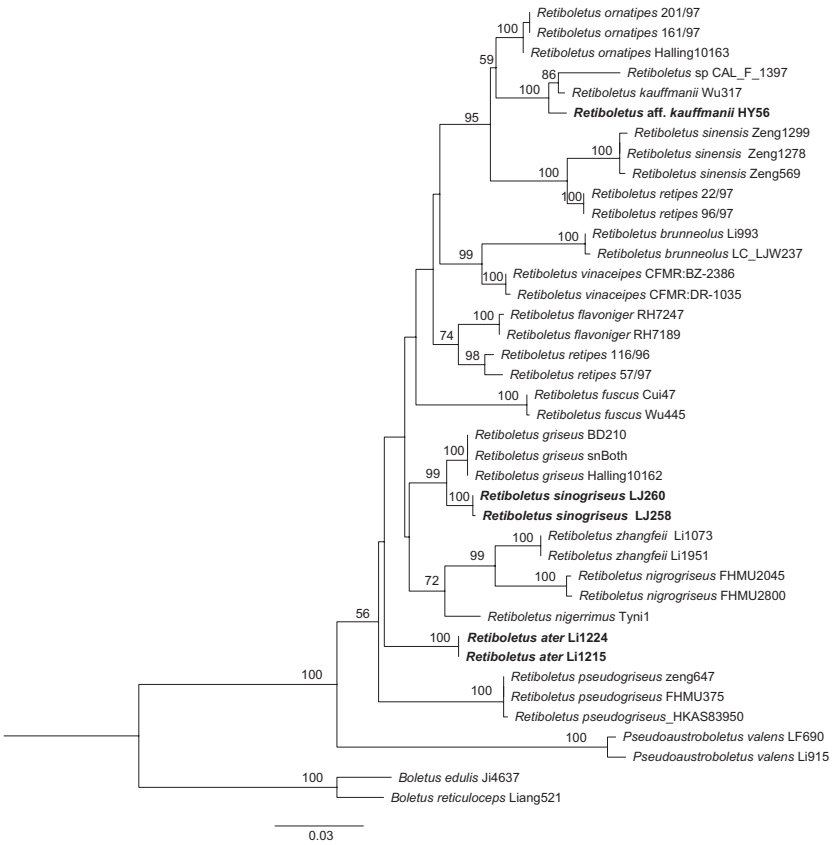

Figure 1. Maximum likelihood phylogenetic tree of *Retiboletus* inferred from the combined nuclear dataset (nrLSU + TEF1-α). Bootstrap frequencies (> 50%) are shown above supported branches. Newly sequenced collections are boldfaced in black. Species vouchers are provided after the species name.

Taxonomy

***Retiboletus ater* Yan C. Li & T. Bau, sp. nov.**
MycoBank No: 834293
Figures 2a–c, 3

Etymology. *ater* referring to the color of the basidiomata.

Type. China. Yunnan Province: Jingdong County, Ailaoshan National Nature Reserve, alt. 2500 m, 14 July 2008, Y.C. Li 1215 (holotype: KUN-HKAS 56069!).

Description. Basidiomata small to medium-sized. Pileus 3–5 cm in diameter, hemispherical to applanate, surface dry, densely subtomentose, black (4F3) to blackish (4E2) in the center and gray (3D1) or yellowish-gray (3C2-3) towards margin, context 2.5 cm thick in the center of the pileus, pallid gray (2D1) to cream (2C3-4), unchanging when bruised. Hymenophore adnate or slightly depressed around apex of stipe;

Figure 2. Habitat of the new *Retiboletus* species. **a–c** *R. ater* (from KUN-HKAS 56069) **d–f** *R. sinogriseus* (**d** from KUN-HKAS 91288 **e–f** from KUN-HKAS 91286).

pores angular, tubes up to 11 mm long, 0.3–1 mm wide, white (2B1) when young and yellowish (2A2) in age, becoming brownish-yellow (5C7-8) when injured. Stipe 4–6 × 0.8–1.2 cm, clavate to flexuous, solid; surface dry, blackish to gray, prominently and coarsely reticulate over the upper 1/3; context white (2A1) in the upper part and yellowish to cream yellow downwards, unchanging when injured; basal mycelium white (2A1). Taste and odor indistinct.

Basidia 26–38 × 6–10 μm, clavate, thin-walled, 4-spored, hyaline to yellowish in KOH. Basidiospores [60/3/2] (7)8–10.5(11) × 3–4.5(5) μm [Q = (1.89) 2–3.33 (3.67),

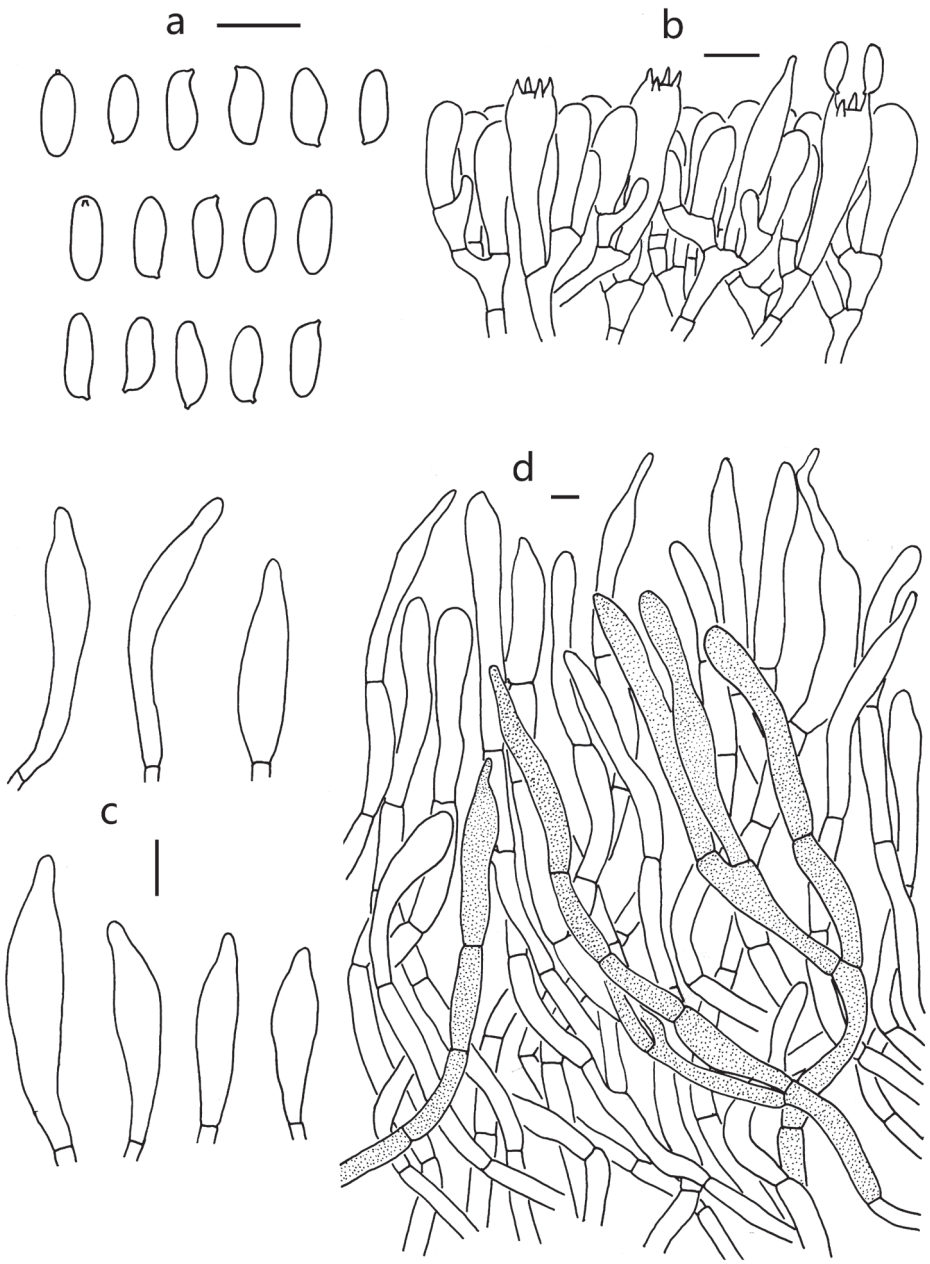

Figure 3. Microscopic features of *R. ater* (KUN-HKAS 56069). **a** Basidiospores **b** basidia and pleurocystidium **c** cheilo- and pleurocystidia **d** pileipellis. Scale bars: 10 μm.

Q_m = 2.52 ± 0.42], subfusiform and inequilateral in side view with shallow suprahilar depression, elongate fusoid or narrowly oblong in ventral view, slightly thick-walled (up to 0.5 μm), brownish to yellowish-brown in KOH, olive-brown to brown in Melzer's reagent, smooth. Hymenophoral trama boletoid; hyphae cylindrical, 3.5–9 μm wide,

hyaline to yellowish in KOH, yellowish to brownish-yellow in Melzer's reagent. Cheilo- and pleurocystidia 26–55 × 6–10 μm, abundant, subfusiform to fusiform, thin-walled, with yellowish-brown contents, surface without encrustations. Caulocystidia forming the reticulum over the stipe surface, similar to cheilo- and pleurocystidia. Pileipellis a trichoderm about 280 μm thick, composed of more or less vertically arranged, slightly interwoven, brown to dark brown hyphae, 5–15 μm wide; terminal cells 45–111 × 9–15 μm, narrowly clavate to subcylindrical or subfusiform, sometimes narrowly mucronate, rostrate, slightly thick-walled (up to 0.5 μm), hyaline to yellowish in KOH, yellowish to brownish-yellow in Melzer's reagent. Pileal trama composed of thin- to slightly thick-walled (up to 0.5 μm) hyphae, 5–11 μm wide, hyaline to yellowish in KOH, yellowish to brownish-yellow in Melzer's reagent. Clamp connections absent in all tissues.

Habitat, ecology and distribution. Solitary on the ground in forests dominated by plants in the family Fagaceae; currently known from southwestern China.

Additional specimens examined. China. Yunnan Province: Jingdong County, Ailaoshan National Nature Reserve, alt. 2500 m, 14 July 2008, Y.C. Li 1224 (KUNHKAS 56078).

Discussion. *Retiboletus ater* is characterized by the black to blackish or gray to yellowish-gray pileus, the white to yellowish hymenophore, the gray to brownish-gray stipe, the prominent and coarse reticulum over the upper 1/3 of the stipe and the trichoderm pileipellis with hyphae 9–15 μm wide. It generally shares the same colored pileus and hymenophore with *R. fuscus* (Hongo) N.K. Zeng & Zhu L. Yang, *R. griseus* (Frost) Manfr. Binder & Bresinsky, *R. nigrogriseus* N.K. Zeng, S. Jiang & Zhi Q. Liang, and *R. pseudogriseus* N.K. Zeng & Zhu L. Yang. However, *R. fuscus* is characterized by an overall reticulate stipe, slight longer basidiospores (9–12 × 3.5–4.5 μm) and narrower pileipellis hyphae (4–8 μm wide) (Zeng et al. 2016). *Retiboletus griseus* has a reticulum over the upper 2/3 of the stipe, a cream or grayish-brown stipe often with orange-yellow stains when hurt, and a distribution in North/Central America (Smith and Thiers 1971; Ortiz-Santana et al. 2007). *Retiboletus nigrogriseus* is characterized by the white to olivaceous contex in the stipe, the entirely reticulate stipe and the cutis pileipellis with hyphae 4–10 μm wide. *Retiboletus pseudogriseus* has a grayish white pileus which is covered with brown to blackish brown squamules, white context becoming brown when injured, and a slender and completely reticulate stipe.

In the phylogenetic analysis (Fig. 1), *R. ater* forms an independent lineage within *Retiboletus*, future studies would require more molecular sequence data to help fully resolve its evolutionary relationships to the other species.

Retiboletus sinogriseus Yan C. Li & T. Bau, sp. nov.

MycoBank No: 834294
Figures 2d–f, 4

Etymology. *sino* (Latin) = China, reflecting that the basidiomata were collected in China + *griseus* for similarity of the basidiomata of this species to *Retiboletus griseus*.

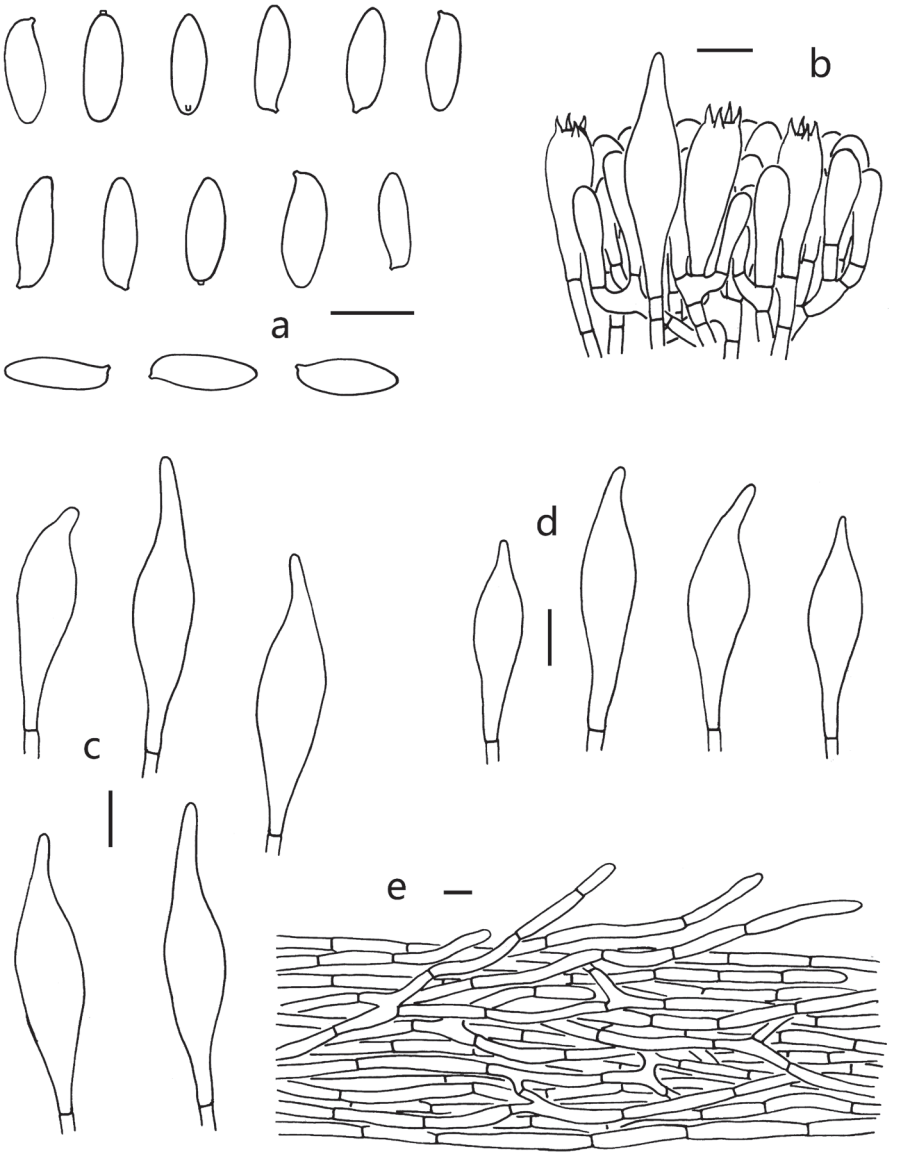

Figure 4. Microscopic features of *R. sinogriseus* (KUN-HKAS 912889). **a** Basidiospores **b** basidia and pleurocystidium **c** pleurocystidia **d** cheilocystidia **e** pileipellis. Scale bars: 10 μm.

Type. China. Liaoning Province: Anshan City, Qianshan, alt. 400 m, 25 Aug 2015, J. Li 260 (holotype: KUN-HKAS 91288!).

Description. Basidiomata medium-sized. Pileus 6–7.2 cm in diameter, subhemispherical to applanate, sometimes convex; surface tomentose, grayish-brown (5D2-3) to brown (5E4), rimose when dry, always cracked into small squamules on grayish (4B1) to whitish (2A1) background; context 1–2 cm thick in the center of pileus,

white (1A1), unchanging when injured. Hymenophore adnate, sometimes slightly depressed around apex of stipe; pores angular, 0.3–1 mm wide, tubes up to 14 mm long, yellow (4C3-4) to grayish-yellow (4B2-3), unchanging when injured. Stipe 6–8 × 1.1–1.5 cm, subcylindrical, solid; surface dry, pale yellow at apex, blackish-yellow towards the base, entirely covered with moderately developed reticulum; context white to cream in the upper part and yellowish to yellow downwards, unchanging when injured; basal mycelium yellow. Taste and odor indistinct.

Basidia 21–27 × 9–11 μm, clavate, thin-walled, four-spored; sterigmata 4–5 μm long. Basidiospores [40/2/2] (9) 10.0–13.5 (–14.0) × (3) 4.0–5.0 (–5.5) μm, Q = (2.25–) 2.5–3.25 (–3.42), Q_m = 2.88 ± 0.32, subfusiform to ellipsoid, slightly thick-walled (up to 0.5 μm), hyaline to yellowish in KOH, olive-brown to yellowish-brown in Melzer's reagent, smooth. Hymenophoral trama boletoid. Cheilo- and pleurocystidia 35–56 × 7–12 μm, abundant, subfusiform to fusiform, thin-walled, with yellowish-brown to brown contents, without encrustations. Pileipellis a subcutis, 100–120 μm thick, composed of thin-walled filamentous hyphae 4–7 μm wide, with subcylindrical to clavate terminal cells 33–72 × 4–6 μm, sometimes with subacute apex, colorless to pale yellowish-brown in KOH, yellow-brown to brownish in Melzer's reagent. Pileal trama composed of thin-walled hyphae 4–9 μm wide, colorless to pale yellowish-brown in KOH, yellow-brown to brownish in Melzer's reagent. Clamp connections absent in all tissues.

Habitat, ecology and distribution. Solitary on the ground in mixed forests dominated by plants in the families Fagaceae and Pinaceae; currently known from northeastern China.

Additional specimens examined. China. Liaoning Province: Anshan City, Qianshan, alt. 400 m, 25 Aug 2015, J. Li 258 (KUN-HKAS 91286).

Discussion. *Retiboletus sinogriseus* has a grayish-brown to brown pileus, a pale yellow to blackish-yellow stipe. Such traits are very similar to those of *R. griseus*. Interestingly, *R. sinogriseus* clusters with *R. griseus* with strong statistical support (Fig. 1). However, *R. griseus*, originally described from North America but not found in China yet, has a distinctly pallid hymenohore and broad pileipellis hyphae which are up to 17 μm wide (Singer 1947; Smith and Thiers 1971; Ortiz-Santana et al. 2007). Additionally, the *R. sinogriseus*/*R. griseus* clade is clustered with *R. zhangfeii* N.K. Zeng & Zhu L. Yang, *R. nigrogriseus* and *R. nigerrimus* (R. Heim) Manfr. Binder & Bresinsky (however without bootstrap support). In this assemblage, *R. zhangfeii* differs significantly from *R. sinogriseus* by its differently colored pileus, hymenophore, stipe and context (Zeng et al. 2016). *Retiboletus nigrogriseus* has a black to gray pileus, white to grayish white hymenophore, white to olivaceous contex in the stipe and much smaller basidiospores 8–10.5 × 3.5–4.5 μm. *Retiboletus nigerrimus*, originally described from Papua New Guinea, has a pileus with a distinctive blue tinge, a context lemon yellow in pileus and orange in the base of stipe and longer and narrower basidiospores 11.5–14.5 × 3.6–4.6 μm (Heim 1963).

Nine species of *Retiboletus* were recorded from China, including two new species described herein. For the convenience of identification, a key to the species in China is given below.

Key to *Retiboletus* species in China

1 Hymenophore bright yellow to brownish-yellow, stipe yellow to orange-yellow, mycelium on the base of stipe yellow to brownish-yellow **2**
– Hymenophore whitish to grayish white, stipe black to blackish or grayish-black, mycelium on the base of stipe whitish to grayish-white..... **4**
2 Pileus grayish-brown to brown without olivaceous tinge, context in pileus white to grayish-white..... ***R. sinogriseus***
– Pileus yellow-brown to olive-brown, context in pileus yellow to pale yellow... **3**
3 Basidiomata medium-sized to large, pileus up to 15 cm in diameter, basidiospores 9–13 × 4–5 μm, cheilo- and pleurocystidia 30–60 × 6–10 μm ..... ***R. kauffmanii***
– Basidiomata small to medium-sized, pileus up to 8 cm in diameter, basidiospores 8–11 × 3.5–4 μm, cheilo- and pleurocystidia 20–46 × 4.5–7 μm ..... ***R. sinensis***
4 Context in the stipe white to grayish-white with olivaceous tinge **5**
– Context in the stipe white to grayish-white with grayish-yellow tinge **6**
5 Hymenophore white when young, lilac to purplish when old, basidiospores 9–11 × 4–5 μm..... ***R. zhangfeii***
– Hymenophore white to grayish-white without lilac to purplish tinge, basidiospores relatively small 8–10.5 × 3.5–4 μm..... ***R. nigrogriseus***
6 Stipe entirely reticulate..... **7**
– Stipe without reticulum or with reticulum restricted to the upper part **8**
7 Pileus brown to blackish-brown, basidiospores 9.5–11 × 4–4.5 μm, pileipellis hyphae up to 8 μm wid..... ***R. pseudogriseus***
– Pileus grayish-brown to grayish-black, basidiospores slightly narrower 9–12 × 3.5–4 μm, pileipellis hyphae broad up to 13 μm wide ..... ***R. fuscus***
8 Pileus pale brown to grayish-brown, stipe without reticulum, basidiospores 10–12.5 × 4.5–5 μm, pleurocystidia 50–80 × 9–14.5 μm ..... ***R. brunneolus***
– Pileus black to blackish, stipe covered with reticulum over the upper 1/3, basidiospores much smaller 8–10.5 × 3–4.5 μm, pleurocystidia relatively small 26–55 × 6–10 μm..... ***R. ater***

Acknowledgements

The authors are indebted to Dr. Jing Li (Kunming Institute of Botany, Chinese Academy of Sciences) for her kind help in collecting the specimens. They are grateful to Prof. Roy E. Halling (the New York Botanical Garden) for providing American specimens. The anonymous reviewers are acknowledged for their valuable comments and suggestions. This study was supported by the Funds of the National Natural Science Foundation of China (31570025, 31750001, 31872618), the Youth Innovation Promotion Association, CAS (2016348), the Key Research Program of Frontier Sciences of CAS (QYZDY-SSW-SMC029-5) and the Ten Thousand Talents Program of Yunnan (YNWR-QNBJ-2018-125).

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MycoKeys 67: 45–53 (2020)
doi: 10.3897/mycokeys.67.51811
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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The first Laboulbeniales (Ascomycota, Laboulbeniomycetes) from an American millipede, discovered through social media

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Academic editor: D. Haelewaters | Received 10 March 2020 | Accepted 8 April 2020 | Published 14 May 2020

Citation: Santamaria S, Enghoff H, Reboleira AS (2020) The first Laboulbeniales (Ascomycota, Laboulbeniomycetes) from an American millipede, discovered through social media. MycoKeys 67: 45–53. https://doi.org/10.3897/mycokeys.67.51811

Abstract

Laboulbeniales are highly specialized arthropod-associated fungi. The majority of the almost 2200 known species live on insects, although they also occur on other arthropod hosts. Recently, the number of Laboulbeniales associated with millipedes has increased considerably. Here we describe the first species of a Laboulbeniales fungus, *Troglomyces twitteri* **sp. nov.**, from an American millipede. The new species was initially discovered on a photo of *Cambala annulata* (Say, 1821) from Ohio, USA, which had been shared on Twitter. A subsequent microscopic study of *Cambala* millipedes in museum collections in Denmark and France confirmed the discovery.

Keywords

animal-fungus interaction, collections-based research, Diplopoda, Laboulbeniaceae, social media

Introduction

Fungi of the order Laboulbeniales form a rather large group of ascomycetous fungi with around 2200 described species in 142 genera (Reboleira et al. 2018). They are obligatorily associated with living arthropods and spend their entire life cycle on their host (Blackwell et al. 2020). Traditionally they have been defined as parasites, with complex haustoria penetrating into the host (Jensen et al. 2019). However, the ab-

sence of haustoria in most Laboulbeniales questions their parasitic nature (Tragust et al. 2016). The majority of Laboulbeniales hosts are insects, mostly Coleoptera (80% of described species) and Diptera (10%) (Weir and Hammond 1997), but also other arthropods have been reported as hosts: mites, millipedes and harvestmen, the latter with a single species (Santamaria et al. 2017).

Laboulbeniales have been long neglected both by mycologists and entomologists. The reason may be that entomologists are often unaware of their presence in part due to their small size and the lack of collaboration between entomologists and mycologists that have less access to the hosts on which these fungi depend. In addition, the study of Laboulbeniales was hindered by technical issues due to their size and difficulty to isolate DNA until recently (Haelewaters et al. 2015; Sundberg et al. 2017).

Research on Laboulbeniales has traditionally been taxonomic, with a recent emergence of molecular phylogenetic studies both at species-level and higher taxonomic levels (e.g., Sundberg et al. 2018; Haelewaters et al. 2019a, b). A few studies have provided insights into the interaction of Laboulbeniales and their hosts, especially in those parasitizing insects (Báthori et al. 2015, 2017; Jensen et al. 2019), but very little is known about general Laboulbeniales biology (Tragust et al. 2016; Szentiványi et al. 2020).

During the last decade, the number of Laboulbeniales species associated with millipedes (Diplopoda) has grown significantly from eight prior to 2014 to a current count of 30 species (Santamaria et al. 2014, 2016, 2018; Enghoff and Santamaria 2015; Reboleira et al. 2018). These species have been collected in Europe, Macaronesia, the Middle East, Africa, SE Asia, Indonesia, Australia and New Zealand, but until now, no Laboulbeniales from American millipedes have been reported.

Millipede hosts of Laboulbeniales usually combine the following traits: i) successive generations of adults overlap in time; ii) their populations are large and stable, and iii) they inhabit moist environments (Santamaria et al. 2014). The transmission of the ascospores in millipede hosts most often occurs directly, by contacts of the hosts during copulation, hence this is why most thalli are found growing around the gonopode and gonopores (Reboleira et al. 2018).

After the observation of a shared photo of a North American *Cambala annulata* (Say, 1821) millipede on Twitter (Fig. 1), we identified the presence of Laboulbeniales on this specimen. Subsequently, we decided to screen *Cambala* millipedes in museum collections resulting in the discovery of an undescribed species in the laboulbenialean genus *Troglomyces*, which was found on several specimens. This new species is formally described here.

Methods

Specimens of *Cambala* spp. from the collections of the Natural History Museum of Denmark in Copenhagen (**NHMD**) and in the National Museum of Natural History in Paris (**MNHN**) were investigated for the presence of Laboulbeniales under a binocular stereomicroscope Leica M165C. The thalli of the fungus on the infected *Cambala* specimens were removed using an insect pin and mounted with lactophenol on a mi-

Figure 1. *Cambala annulata*, male. USA, Ohio, Adams County, West Union, Greene Township, Edge of Appalachia Preserve System, Abner Hollow Rd., on Bisher Dolostone Cliffs, 38.7139N, 83.4187W, 26 Jun 2014; M. Zloba leg. Original of image shared on Twitter on 31 Oct 2018 by Derek Hennen. Courtesy of D. Hennen. The red circles indicate two thalli of Laboulbeniales.

croscope slide following the methodology of Santamaría et al. (2018). Specimens were studied using a Leica DMR microscope equipped with differential interference contrast (DIC) optics and photographed with a Jenoptik ProgRes 10 Plus digital camera.

Taxonomy

Order Laboulbeniales Lindau
Suborder Laboulbeniineae Thaxt
Family Laboulbeniaceae Peyr
Subfamily Laboulbenioideae s. str.
Tribe Laboulbenieae Thaxt
Subtribe Stigmatomycetinae (Thaxt.) I.I. Tav.

Genus *Troglomyces* S. Colla, Nuovo Giornale Botanico Italiano 39: 450 (1932).

Type species. *T. manfrediae* S. Colla

Brief description. Receptacle three-celled. Cell III very narrow and adnate to the perithecium. Perithecium with 5-6 outer wall cells in each vertical row. Perithecial apex typically with four protruding lips. Nine species.

***Troglomyces twitteri* Santam., Enghoff & Reboleira, sp. nov.**
MycoBank No: 834938
Figure 2

Diagnosis. Septa II–III and II–VI approximately at the same level. Dorsal and ventral margin of cell II of equal to subequal height, in contrast to all other *Troglomyces*, such that cell II is not adnate to either cell VI or the perithecium. Primary appendage branched. Perithecial apex bearing four slightly protruding lips, one of them being longer.

Types. *Holotype*: USA, Georgia, Peach County, Fort Valley, 25 Feb 1984, Jerry A. Payne leg., "Leaf litter in hardwood forest", on *Cambala annulata*, RL Hoffman 1984 det. (host: MNHN GA-003-5, slide: C-F-95157, deposited at NHMD). ***Paratypes*:** Same data as the holotype (host: MNHN GA003, slides: GA003-1, GA003-2, GA003-3, GA003-4, deposited at MNHN); USA, North Carolina, Swain Co., Smokemont Campground in Great Smoky Mountains National Park, 10 Aug 1981, H. Enghoff & R.M. Shelley leg., on *Cambala hubrichti* Hoffman, H. Enghoff det. (host: NHMD 621689, slides: C-F-95156, C-F-95155, C-F-95154, C-F-95153, deposited at NHMD).

Description. Thallus hyaline, except for the blackened foot. ***Basal cell of the receptacle*** (I) about twice as long as broad, enlarged distally. ***Suprabasal cell of the receptacle*** (II) pentagonal, isodiametric, up to 1.5 times as long as broad, margins parallel to somewhat broadened distally. Septa II-III and II-VI variably oblique, located approximately at the same level. Septum II-VI slightly longer than II-III. ***Cell III*** very narrow, up to 8 times longer than broad; adnate to the perithecium along half or three quarters of the latter's length. ***Primary appendage*** branched above the first or, more frequently, the second cell, into several simple or once ramified branches; surpassing the perithecial apex. Basal and suprabasal cells of appendage similar in size and shape; about two times as long as broad. Primary septum (Fig. 2C, "a") slightly constricted and strongly oblique. Only one antheridium has been seen in an immature thallus, as a simple phialid on a branch of the primary appendage (Fig. 2G, "an"). ***Perithecial stalk cell*** (VI) very inconspicuous, strongly flattened (Fig. 2B, 2F, "VI"). ***Perithecium*** ovoidal, broadest at the middle or third basal part, gradually tapering upwards. Apex bearing four not quite protruding lips, one of them slightly longer (Fig. 2E, arrow). A small tooth-like outgrowth on the outer side near the apex (Fig. 2F, arrow).

Measurements. Length from foot to apex of perithecium 81–129 μm. Perithecium (including basal cells) 45–66 × 14–23 μm. Appendage maximum length if undamaged, from primary septum 61–76 μm.

Etymology. Named after the social media platform Twitter, where it was observed for the first time.

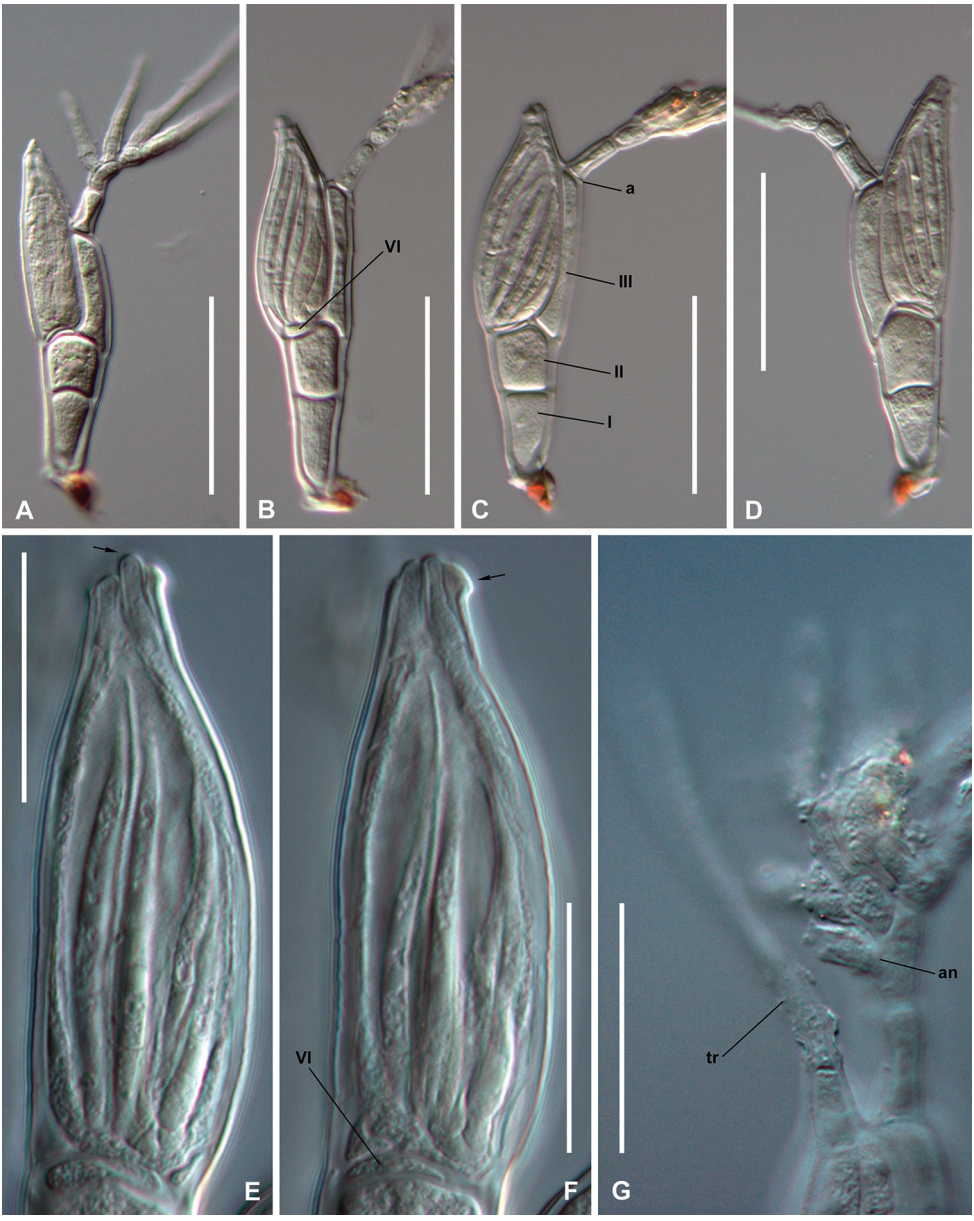

Figure 2. *Troglomyces twitteri* Santam., Enghoff & Reboleira, sp. nov. **A–D** mature thalli with labelling of cells and other elements in **B, C E, F** detail of perithecium at two focusing levels to show the slightly longer lip (**E**, arrow), and tooth-like outgrowth (**F**, arrow). In Fig. **F**, cell VI is labelled **G** detail of an immature thallus showing the trichogyne (tr) and the antheridium (an). Scale bars: 50 µm (**A–D**), 25 µm (**E–G**). Photographs from: slides GA003-1 (**A, D**), GA003-2 (**E–G**), and C-F-95157 (**B, C**).

Discussion

The most distinctive characteristic of *Troglomyces twitteri* vis-à-vis its congeners is found in the shape and location of cell II, which is bigger than in other species and does not extend laterally to cell VI or the perithecium. The strongly flattened and inconspicuous cell VI is shared with *T. tetralabiatus* Santam., Enghoff & Reboleira, probably the mostly similar species. *Troglomyces twitteri* differs from the other species as follows: *Troglomyces dioicus* Santam., Enghoff & Reboleira is dioecious, has a conspicuous spiny process and an unbranched appendage; *T. tetralabiatus* shows four very conspicuous and elongated perithecial lips; *T. bilabiatus* Santam., Enghoff & Reboleira has two elongated lips, an unbranched appendage, and the antheridia are placed directly on the lower cells of the appendage; *T. pusillus* Santam & Enghoff has an unbranched appendage and the second cell of this appendage functions as an intercalary antheridium; *T. triandrus* Santam & Enghoff has three superposed antheridia formed by the third, fourth and fifth cells of the appendage; *T. botryandrus* Santam., Enghoff & Reboleira has two groups of antheridia in bunches near the base of appendage; *T. manfrediae* S. Colla has an unbranched appendage and an antheridium on the corner of the appendage basal cell; *T. rossii* Santam., Enghoff & Reboleira has a bifurcate appendage with a characteristic trapezoidal, small cell in the bifurcation.

Arthropods of the class Diplopoda, commonly known as millipedes, play an important role in the decomposition of organic matter above and below the ground (Hopkin and Read 1992; David 2015; Reboleira and Enghoff 2015). Millipedes have poor dispersal abilities and consequently show high endemism patterns, converting them into excellent models for the study of Laboulbeniales biogeographical patterns (Santamaria et al. 2014, 2016, 2018; Reboleira et al. 2018). The gonopores of millipedes are situated on the third body ring from the front, and in the vast majority of millipedes, mating takes place by the introduction of modified appendages (gonopods) on the seventh body ring into the female gonopore; the distribution of laboulbenialean thalli on millipede hosts very often reflects this behavior (Enghoff and Santamaria 2015).

Species of *Troglomyces* have so far been found only on millipedes belonging to the orders Julida and Chordeumatida. The here reported find of *T. twitteri* on *Cambala* is not only a first record of Laboulbeniales from an American millipede, it also represents the first record of *Troglomyces* from the order Spirostreptida. Species of Spirostreptida are, on the other hand, hosts for many species of another Laboulbeniales genus: *Rickia* Cavara (Santamaria et al. 2016). Spirostreptidan hosts include one species of Cambalidae, *Chiraziulus kaiseri* (Mauriès, 1983), which is host to *Rickia appendicifera* Santam., Enghoff & Reboleira.

The genus *Cambala* Gray, 1832 is endemic to North America. *Cambala annulata* (Say, 1821) and *C. hubrichti* Hoffman, 1958 are dominant members of the litter fauna in the southern Appalachian Mountains (Shelley 1979). *Troglomyces twitteri* is probably overlooked but widespread in this area, i.e. the potential geographic distribution of the fungus is likely to match the distribution of its hosts. Like most other millipedes, *Cambala* species secrete strongly smelling defensive chemicals from glands along

their body. Eisner et al. (1965) identified 2-methyl-1,4-benzoquinone and 2-methyl-3-methoxy-1.4 benzoquinone in the secretion of *C. hubrichiti*.

The abundance of thalli on the host was reduced compared to some other species of millipedes that are known to have high load of Laboulbeniales. For example, thalli of *Rickia gigas* Santam., Enghoff & Reboleira on *Archispirostreptus* spp. were reported as "hairs" in internet fora by keepers of millipedes as pets (Santamaría et al. 2016). The distribution of *T. twitteri* thalli on the host body follows a transmission pattern that is associated with mating behavior, the fungi being mostly found around the gonopods/gonopores of the millipedes (Santamaria et al. 2014, 2016, 2018; Reboleira et al. 2015; Reboleira and Enghoff 2015). However, the thalli observed on Twitter were on the dorsal side of the first two body rings. This suggests that, under higher thallus densities, thalli can spread from the genital areas of the millipedes to the back, i.e. higher than in the specimens studied.

The use of social media is now a considerable part of how humans interact and perceive the news of a changing world. Photographs in online databases (e.g., Flickr and iNaturalist) and social media (e.g., Facebook and Instagram) have previously provided new species of insects and plants for science, and new hosts for parasites – after careful examination by taxonomists (Winterton et al. 2012; Gonella et al. 2015; Báthori et al. 2017; Jaume-Schinkel et al. 2020). There is an increasing interplay between research and social media platforms, and many scientists use Twitter to promote and share research, a phenomenon also promoted by scientific publisher companies (Bik and Goldstein 2013). To our knowledge, this is the first time that a new species for science has been discovered on Twitter, as a result of a casual observation of a photo shared by a colleague. This, again, emphasizes the importance of such platforms for sharing research and making new discoveries. The circumstances of this species' discovery should encourage data sharing among amateur naturalists and professional scientists.

Acknowledgements

Derek Hennen kindly provided a better resolution copy of his image from Twitter. ASR was supported by a research grant (15471) from the VILLUM FONDEN.

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MycoKeys 67: 55–80 (2020)
doi: 10.3897/mycokeys.67.52055
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First record of Harpellales, Orphellales (Kickxellomycotina) and Amoebidiales (Mesomycetozoea) from Bulgaria, including a new species of *Glotzia*

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Academic editor: T. Lumbsch | Received 13 March 2020 | Accepted 9 April 2020 | Published 27 May 2020

Citation: Valle LG, Stoianova D (2020) First record of Harpellales, Orphellales (Kickxellomycotina) and Amoebidiales (Mesomycetozoea) from Bulgaria, including a new species of *Glotzia*. MycoKeys 67: 55–80. https://doi.org/10.3897/mycokeys.67.52055

Abstract

This paper presents the results obtained from a short survey performed in Bulgaria, southeast Europe, where the trichomycetes (sensu lato), an ecological group of arthropod gut endosymbionts, were previously completely unknown. The present study initiates the comprehension of these cryptic organisms, members of the Kickxellomycotina (Harpellales, Orphellales) and the Mesomycetozoea (Amoebidiales), in this Balkan country. Eighteen new geographic records for Bulgaria are reported, including 10 species of Harpellales, three species of Orphellales and five species of Amoebidiales. Within the Harpellales, the species *Glotzia balkanensis* **sp. nov.** is described. This new species is most related to the rare species *G. centroptili* Gauthier ex Manier & Lichtw. and *G. stenospora* White & Lichtw., but is differentiated by spore and thallial characteristics. Photographs are provided and biogeographic implications of these records are discussed.

Keywords

aquatic insects, Balkans, gut fungi, symbiosis, trichomycetes, Zoopagomycotina

Introduction

The ecological group trichomycetes includes filamentous or sac-like protozoan organisms (Mesomycetozoea Order Eccrinales and Amoebidiales) (Benny and O'Donnell 2000; Ustinova et al. 2000; Lutzoni et al. 2004; Tanabe et al. 2004; Adl et al. 2005; Cafaro 2005) and filamentous Fungi (Zoopagomycota, Kickxellomycotina, O. Harpellales and Asellariales) (Hibbet et al. 2007), all of them sharing the same ecological behaviour and living symbiotically inside the gut of arthropod hosts. The intense conditions and constrictions of this peculiar environment have shaped the convergent evolution, resulting in parallel morphology for all members in this ecological assemblage of organisms, explaining the previous classification of these phylogenetically unrelated orders within the Class Trichomycetes (Lichtwardt 1986). The Order Harpellales is the most diverse and well-known within the trichomycetes, with nearly 250 species, living associated mainly with immature stages of aquatic insects (Lichtwardt et al. 2001). Recently, a new order has been raised from within the Harpellales: the Orphellales L.G. Valle, M.M. White, Strongman & Lichtw to include the Plecopteran-associated genus, *Orphella*, with unusual characteristics in its sexual spores, amongst other particularities that make this genus exceptional within the Kickxellomycotina (White et al. 2018). The relationship between trichomycetes and their hosts is considered commensalistic, since they feed on the digestive content transiting those portions of the insect gut where most of the nutrients have already been absorbed by the animal (Misra 2001). However, they can behave mutualistically in some developmental and environmental conditions (Horn and Lichtwardt 1981), while other species may be deleterious to their hosts, like a few dipteran-associated species of *Smittium* which can be lethal to mosquitoes (Dubitskii 1978; Sweeney 1981; López-Lastra 1990) and may have an added interest for mosquito-control research.

Bulgaria is a biogeographically attractive region and, like most other Oriental European countries, it has not, until now, been studied by trichomycetologists. Europe transitions to Asia through the Balkans, acting as a connecting corridor, with Siberian and central European fauna and flora, together with Mediterranean components. This, combined with other geological factors, makes the Balkan Peninsula one of the two – together with the Iberian Peninsula– most interesting biogeographic regions in Europe, both being considered hotspots of biodiversity (Griffiths et al. 2004; Popov and Fet 2007). Bulgaria is actually the best-studied of all Balkan countries concerning biodiversity, because of the great tradition of zoological and botanical research (Popov and Fet 2007). With all these concurrent factors, it is our wish to contribute to the biodiversity data of the country with this preliminary study of arthropod endosymbionts.

At present, there are 54 species of trichomycetes documented from the Iberian Peninsula (Casas et al. 2019, Valle 2004, 2007, 2014a, 2014b, Valle and Santamaria 2002, 2004); it can be used as an indicator of what the diversity might be like in Bulgaria if more collections of trichomycetes are made in the future.

Material and methods

All taxa reported here were collected from diverse localities (Table 1) in Bulgaria, most of them in the Provinces of Sofia and Pernik, since fresh material was processed in the laboratory of the Institute of Biodiversity and Ecosystem Research of the Bulgarian Academy of Sciences, in Sofia. Consequently, collecting trips where done not far away from the capital. Arthropod hosts, mainly aquatic insect larvae, nymphs and some aquatic isopods, were captured following the methods described by Lichtwardt et al. (2001), using aquatic dip nets and/or by hand-picking from stones, pebbles and vegetation. Hosts were transported to the lab in jars containing stream water on ice. Insect guts were dissected in water on microscope slides using a stereomicroscope and the gut symbionts transferred to a drop of water on another slide with the aid of ultrafine forceps and entomological needles. Lactophenol cotton-blue was used as the mounting medium for semi-permanent voucher slides, then these were sealed with clear finger-

Table 1. Collection site information.

Ref	Province	Locality	EUNIS habitat type: name and code	Water Temp °C / pH	Geographic coordinates	Alt. (m a.s.l.)	Date (in 2016)
1	Sofia	Rakita River near Pasarel Village	Temporary running waters; C2.5	14°/7	42.547797N, 23.497202E	748	19 Aug
2	Sofia	Iskar River near Pasarel Village	Permanent non-tidal, smooth-flowing watercourses; C2.3	11.5°/7.2	42.535885N, 23.508824E	712	19 Aug
3	Sofia	Small creek next to Iskar	Non-permanent temporary	12°/7	Close to the previous locality	710	19 Aug
4	Pernik	Small brook tributary of Struma River, Chuypetlovo Village	Permanent non-tidal, fast, turbulent watercourses; C2.2	12°/6.2	42.520650N, 23.245939E	1258	20 Aug
5	Pernik	Struma River, Chuypetlovo Village	Permanent non-tidal, fast, turbulent watercourses; C2.2	11°/6.2	42.520608N, 23.245650E	1255	20 Aug
6	Pernik	Small brook tributary of Struma River after Bosnek Village near the bridge	Permanent non-tidal, smooth-flowing watercourses; C2.3	18°/7	42.494986N, 23.171681E	909	20 Aug
7	Sofia	Tributary of Chureshka River, near Eleshnitsa Village	Permanent non-tidal, fast, turbulent watercourses; C2.2	14°/6.8	42.760899N, 23.648960E	707	21 Aug
8	Sofia	Chureshka River, bridge before Potop Village	Permanent non-tidal, fast, turbulent watercourses; C2.2	14.5°/6.8	42.752736N, 23.647861E	669	21 Aug
9	Sofia	Small pond (swamp) near highway Hemus	Permanent eutrophic lakes, ponds and pools; C1.4/ C1.3	18.7°/6.2	42.773999N, 23.774886E	903	21 Aug
10	Kyustendil	Manastirska River before Rilski Manastir	Permanent non-tidal, fast, turbulent watercourses; C2.2	9°/6.1	42.153581N, 23.389001E	1422	22 Aug
11	Kyustendil	Rilska River before Pastra Village	Permanent non-tidal, fast, turbulent watercourses; C2.2	10.5°/6.1	42.113840N, 23.318704E	1027	22 Aug
12	Sofia	Darvenishka River, Sofia city, Park Vertopo	Permanent non-tidal, smooth-flowing watercourses; C2.3	18°/7.2	42.645710N, 23.364568E	585	23 Aug

nail polish. Most of the photographs were taken later at the Autonomous University of Barcelona (Catalonia, Spain) with a Zeiss Axioscope compound microscope, equipped with a Jenoptik ProgResC3 digital camera. For each of the endosymbiont species, the corresponding hosts were preserved in 70% ethanol for identification. Microscope slides are deposited in BCB-Mycotheca (herbarium at the institutional address of the corresponding author), except for some duplicates of the slides that were deposited in the Institute of Biodiversity and Ecosystem Research (Sofia, Bulgaria).

To reference the microscopic slides (specimens), a reference number was selected for each locality, preceded with the geographic reference BUL (Bulgaria: BUL–1, BUL–2 etc…). A second number was assigned sequentially for each microscope slide within the corresponding site (i.e. BUL–1–1: site 1, slide 1). See Table 1 for collecting sites details. All specimens collected by the authors (Leg.). All measurements, length × width in micrometres, for *Orphella* zygospores and diameter of the major outer spore coil × spore width (in micrometres) were made. Other measurements, as indicated in the text.

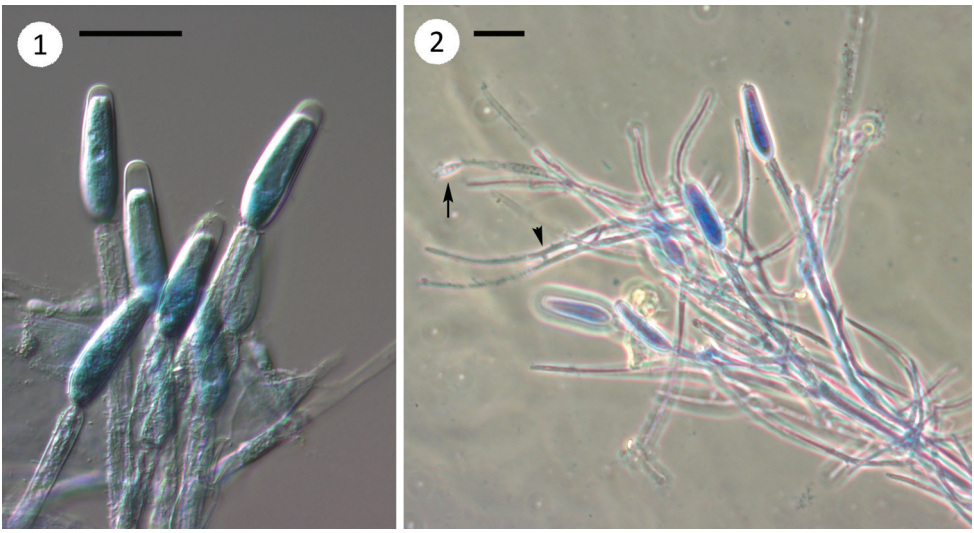
Results

Order Harpellales

***Genistellospora homothallica* Lichtw, 1972.**
Figs 1, 2

Specimens examined. Site 2: slides BUL–2–1, BUL–2–6, BUL-2-7 (zygo.), BUL–2–10; Site 3: slides BUL–3–1; site 4: slides BUL–4–10; Site 7: slides BUL–7–1, BUL–7–2, BUL–7–3; site 8: slides BUL–8–3; site 12: slide BUL–12–5.

Notes. *Genistellospora homothallica* is a cosmopolitan species and its Simuliidae hosts are widespread and common in varied environments (Lichtwardt et al. 2001), especially in fast flowing waters. This species has been previously documented from many countries in the Northern Hemisphere, including USA (Lichtwardt 1972), Canada (Moss and Lichtwardt 1976), United Kingdom (Lichtwardt 1986), Spain (Santamaria and Girbal 1998), France (Valle 2004), Italy (Valle et al. 2013) and Portugal (Valle 2013a). The species has also been recorded from Southern tropical regions, including Costa Rica (Lichtwardt 1997), Puerto Rico (White et al. 2000), Argentina (López-Lastra et al. 2005), Dominican Republic (Valle and Cafaro 2010), Chile (Lichtwardt and Arenas 1996) and Colombia (Barón and Valle 2018). Trichospores of *G. homothallica* are typically ovate-elongated, slightly asymmetrical, measuring 34–40 × 10.5–12 µm in our collections. Young zygospores were observed in one Bulgarian specimen (BUL–2–7) with the characteristic zygosporophore of the species, bearing a straight or reflexed thumb-like terminal cell measuring 43–58 µm length. Often, *G. homothallica* thalli were covered with thalli of the epithallic *Simuliomyces microsporus* Lichtw., as seen in Fig. 2 (arrows).



Figures 1, 2. *Genistellospora homothallica* and *Simuliomyces microsporus* from Simuliidae larvae. **1** Fertile branches of *G. homothallica* with terminal trichospores **2** fertile branches and trichospores of *G. homothallica* with an attached thallus of *S. microsporus* showing a trichospore (arrow) and conjugation tubes (arrowhead). Scale bars: 25 µm in all figures.

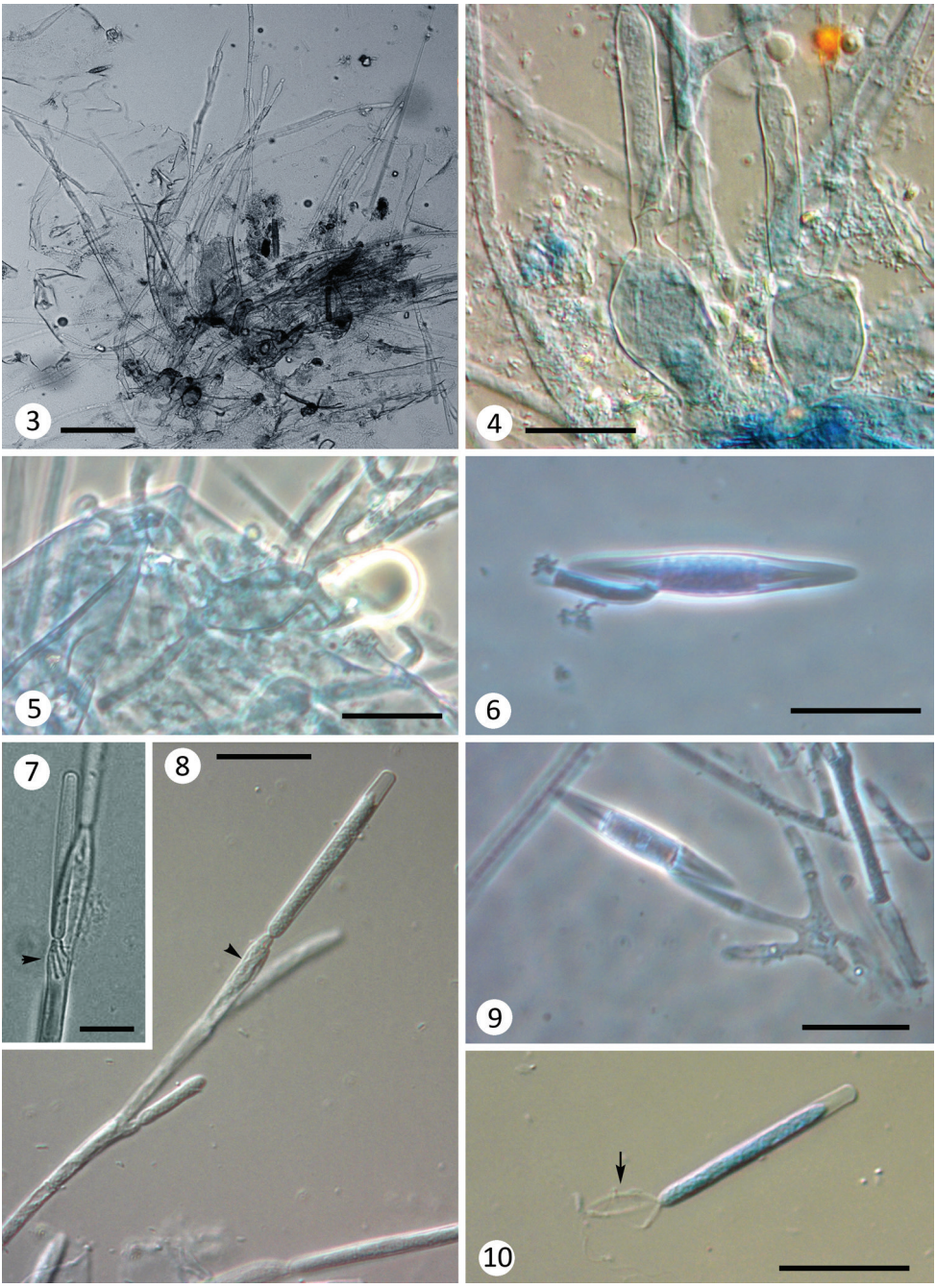
***Glotzia balkanensis* LG Valle & D Stoianova, sp. nov.**
MycoBank No: 834966
Figs 3–10

Holotype. Bulgaria, Sofia, Pasarel Village, Iskar River, 42.535885N, 23.508824E; 712 m a.s.l.; 19 Aug 2016; LG Valle and D Stoianova Leg; In the hindgut of *Baetis melanonyx* Pictet (Baetidae, Ephemeroptera); microscope slide BCB–BUL–2–2.

Paratypes. Same locality and date as the holotype; microscope slide BUL–2–3, BCB–BUL–2–4. Bulgaria, Sofia capital city, Darvenishka River, Park Vartopo, 42.645710N, 23.364568E; 585 m a.s.l.; 23 Aug 2016; LG Valle and D Stoianova Leg; In the hindgut of *Baetis melanonyx* (Baetidae, Ephemeroptera); microscope slide BCB-BUL–12–3.

Etymology. *Balkanensis*, from the Balkan Peninsula.

Description. Thalli measuring up to 600 µm long. Basal cell broadly inflated (18–30 µm diam.) and often branched (Fig. 3), bearing a small discoid secreted holdfast at the base or laterally to the basal cell axis (Fig. 4). Dichotomous branching above the basal cell; distal branches bearing spores (Figs 2, 8). Trichospores cylindrical, with a terminal refractive cap (not always visible), measuring 44–56 × 4.5–5.5 µm, with 3 appendages, one central long filiform appendage, coiled around two shorter (about 15–20 µm) and broader lateral appendages (Fig. 10). These appendages can be seen within the generative cell while still attached (Figs 7, 8 arrow-



Figures 3–10. *Glotzia balkanensis* sp. nov. from Baetidae nymphs. **3** thallus overview, note the inflated and branched basal cell structures **4, 5** detail of various swollen basal cells **6** loose zygospore with a collar **7, 8** trichospores on fertile branches, see the appendages inside the generative cell (arrowheads) **9** Zygospore arising from a conjugation tube **10** loose trichospore with a central long filiform appendage and two smaller lateral appendages. Scale bars: 50 μm (**3**), 25 μm (**4–10**).

head). Fertile branches bearing 3–4 generative cells, measuring 20–35 × 4–6 µm. Zygospores biconical, 48–60 × 7.5–9.5 µm, with a collar 5–10 (–16) × 4 µm, attached eccentrically and laterally (Type II) to a zygosporophore 20–30 µm long, arising from the conjugation tube in series of scalariform conjugations (Fig. 9). In the hindgut of Baetidae (Ephemeroptera) nymphs.

Notes. The genus *Glotzia* has nine species (including that described here), all of them sharing the characteristic cylindrical trichospores with a slightly globose cap and the peculiar 3-appendage arrangement observed also in *G. balkanensis*. This new species mostly resembles the type species *G. centroptili* described by Gauthier (1936) in French pools and streams of Dauphiné province (south-eastern France) from *Centroptilum luteolum* nymphs (Baetidae). This French species was recently rediscovered in Catalonia (Spain) also within *Centroptilum* sp. nymphs (Busquets et al. 2018). This second observation in Spain was important for providing new material to complete the original description, which was scant and had no photographs, only a drawing of a single specimen (Gauthier 1936). The specimens from Bulgaria can be differentiated from *G. centroptili* by spore characteristics. Trichospores of the Bulgarian species are longer than those observed in France or Spain (40 × 4 µm according to Gauthier (1936); 35–43 × 4–6 µm according to Busquets et al. (2018), up to 56 µm in the specimens reported here). All the fertile branches observed had a maximum of four generative cells in *G. balkanensis*, while up to seven have been reported in *G. centroptili*. Zygospores of *G. balkanensis* are quite similar to those of *G. centroptili* in length, but they have significantly larger diameter in the French species, 15 µm diameter (according to Gauthier 1936), while only 7.5–9.5 µm (8.4 µm average) in *G. balkanensis*. Unfortunately, the specimens of *Glotzia centroptili* collected from Spain, had no zygospores to compare with the new species, only trichospores were observed and, thus, we do not have a broad perspective of the zygosporic variation in this species, because apparently, the description of the type species was based on just a few specimens (Busquets et al. 2018). The presence of a collar on released zygospores was not described by Gauthier in *G. centroptili*. The species described here has a quite variable collar length, but in most zygospores, it is rather short (5–10 µm). Regarding thallial characteristics, both species are quite similar, but there are major differences in their fertile branches, generative cells and in the basal cell. The basal cell is much more swollen in the Bulgarian species, resembling (but not identical to) that of the Italian species *Glotzia distorta* LG Valle, Santam. & W Rossi which has different spore features (Valle et al. 2014). Most species of *Glotzia* are associated with Baetidae (Ephemeroptera), except for one species recorded in a New Zealand Plecoptera nymph, *Glotzia plecopterorum* Lichtw. (Williams and Lichtwardt 1990) and another species living within Dixidae (Diptera) larvae *Glotzia incilis* Strongman & MM White, (Strongman and White 2008). Actually, *Glotzia* is one of the Harpellid genera with a wider host range. *Glotzia centroptili* was recorded from *Centroptilum* (Baetidae) in France and Spain and *G. distorta* from the related *Procleon pennulatum* (= *Centroptilum pennulatum*) (Valle et al. 2014). However, *Glotzia balkanensis* has been recorded from a different host, *Baetis melanonyx*, but in the same family Baetidae. In fact, this is the first record of a Harpellid fungus within this host species.

Graminella bulbosa **Léger & Gauthier, 1937 ex Manier, 1962.**
Figs 11–13

Specimens examined. Site 2: slide BUL–2–5; Site 8: slides BUL–8–1, BUL–8–2.

Notes. This species is characterised by the unusual formation of vegetative propagules from the bulbous basal cells (Fig. 12), a feature only shared with the related genus *Gauthieromyces* (Lichtwardt 1983). *Graminella bulbosa* was described from France (Léger and Gauthier 1937; Manier 1962). The species is also known from Spain (Valle 2007), Portugal (Valle 2013a) and Italy (Valle et al. 2013). *Graminella bulbosa* has been reported associated with various species of *Baetis* and related genera, very frequently within the hindgut of *B. rhodani* (Pictet). This species of mayfly is common and widespread in Europe and it also hosted Bulgarian specimens of *G. bulbosa* in the surveyed rivers, together with *B. alpinus* (Pictet). In fact, the genus *Baetis* bears different Harpellid species, including the more common *Legeriomyces ramosus*, occasionally sharing the same gut lumen with *Graminella bulbosa.* Bulgarian specimens of *G. bulbosa* show the typical small and numerous trichospores (Fig. 13), measuring 8 –11 × 2 µm in our collections. These measurements are midway between *G. bulbosa* and *G. microsporus* (see discussion for further information). Unfortunately, only immature zygospores were observed (Fig. 11 arrowhead).

Harpella melusinae **Léger & Duboscq, 1929.**
Figs 14–15

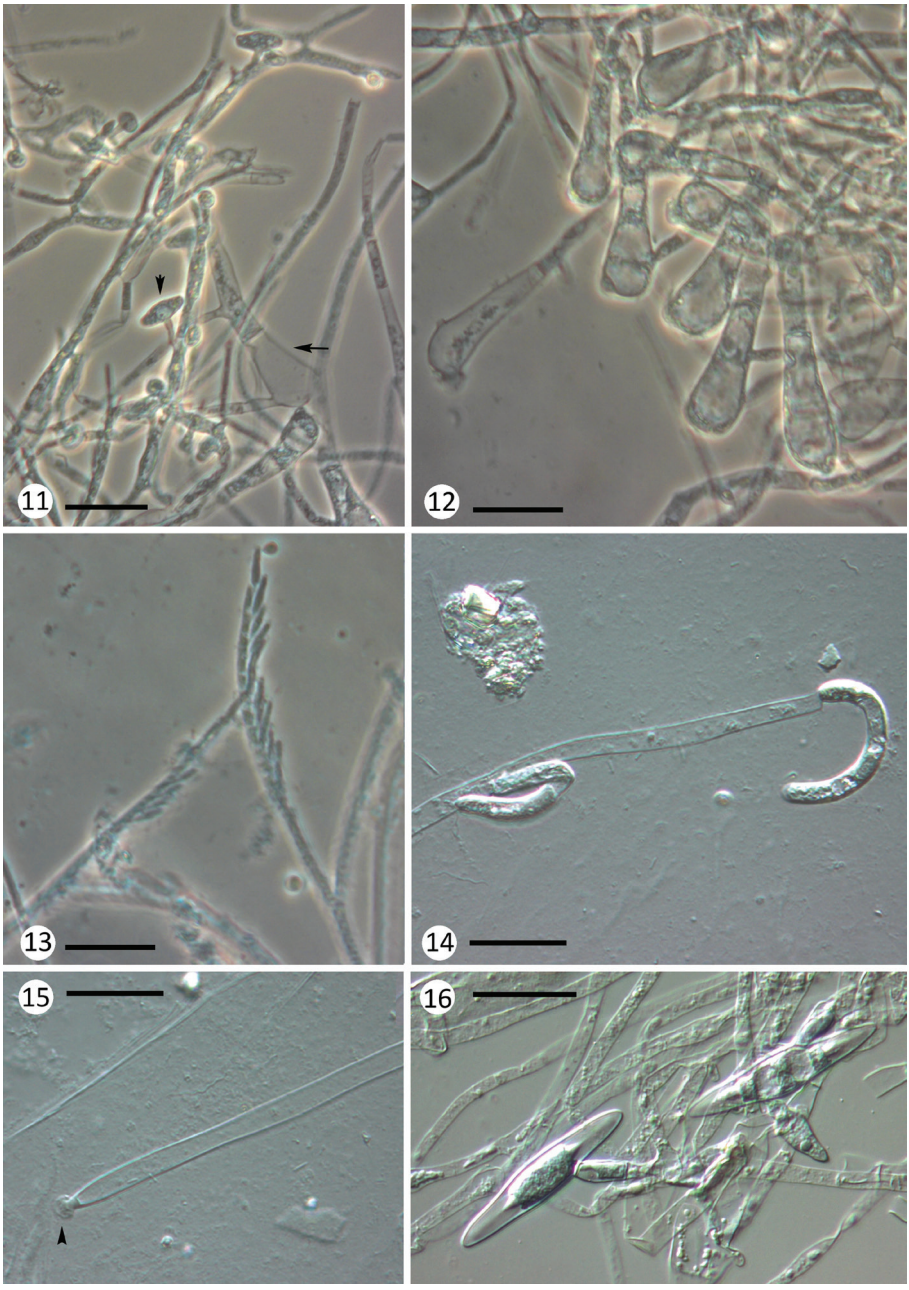
Specimens examined. Site 2: slides BUL–2–1, BUL–2–6, BUL–2–7, BUL–2–10; site 4: slides BUL–4–10; Site 7: slides BUL–7–1, BUL–7–2, BUL–7–3; Site 8: slides BUL–8–3; site 12 slide: BUL–12–5

Notes. A cosmopolitan or sub-cosmopolitan species, widespread in the Northern Hemisphere, it is common in European localities where their hosts were available. It has been found attached to the peritrophic matrix of Simuliidae larvae in different Bulgarian localities. Our specimens have the typical characteristics of the species, distinguishable on the basis of trichospore morphometry (Fig. 14) and holdfast structure (Fig. 15) (Léger and Duboscq 1929a). The trichospores in our specimens measured 50–60 × 6–7 µm and were variable in shape, from nearly straight to allantoid or slightly coiled.

Legeriomyces ramosus **Pouzar, 1972.**
Fig. 16

Specimens examined. Site 2: slide BUL–2–9; Site 11: slides BUL–11–1 (zygo.), BUL–11–2, BUL–11–3.

Notes. This species was found on the hindgut lining of Baetidae hosts (*Baetis rhodani* Pictet, *Baetis melanonyx* (Pictet) and *B. alpinus* (Pictet). The species seems to



Figures 11–16. Various species of Harpellales. **11–13** *Graminella microspora* from Baetidae nymphs: **11** thallus overview, with inflated basal cell structures from which propagules arise or are extruded (note: one empty basal cell which has extruded its cellular content, arrow); young zygospores can also be observed (arrowhead) **12** a cluster of basal cells **13** fertile branch with a long series of minute trichospores; **14, 15** *Harpella melusinae* from Simuliidae larvae**: 14** generative cells and allantoids or curved trichospores **15** basal cell and small conic holdfast **16** *Legeriomyces ramosus* from Baetidae nymphs, zygospores and zygosporophores. Scale bars: 25 μm in all figures.

have a cosmopolitan distribution, at least in the Northern Hemisphere (Lichtwardt et al. 2001). In Europe, the species is common, with records from France, where it was originally described under the name of *Genistella ramosa* (Léger and Gauthier 1932) (Pouzar 1972), from United Kingdom (Moss 1979), Switzerland (Lichtwardt 1986), Spain (Valle and Santamaria 2002), Norway (White and Lichtwardt 2004), Sweden (Lichtwardt 1984), Portugal (Valle 2013a) and Italy (Valle et al. 2013). *Legeriomyces ramosus* has been reported also from India (Misra and Tiwari 2008), China (Strongman et al. 2010), from several localities in USA (Lichtwardt 1986) and Canada (Strongman 2010). Bulgarian specimens have, as is usual in this species, a broad range of trichospore variability, measuring 30–40 ×7–8.5 µm, with two appendages differing in length. Zygospores measure 50–61 × 9–12 µm in our collections (Fig. 16).

***Simuliomyces microsporus* Lichtw, 1972.**
Fig. 2

Specimens examined. site 2: slides BUL–2–1, BUL–2–6, BUL–2–10; site 3: slides BUL–3–1; Site 7: slide BUL–7–3(zygo.); Site 12: slide BUL–12–5.

Note. This species was obtained from the hindgut lining of Simuliidae larvae. Most of the observed specimens of *S. microsporus* were attached to thalli of both *Genistellospora homothallica* (Fig. 2) and *Paramoebidium chattoni*. All the individuals had trichospores and one of the specimens showed also the typical type-I zygospores' young (Fig. 2). All characteristics and measurements of Bulgarian *S. microsporus* match that of previous descriptions of the species in Europe (Moss 1970; Valle 2004, White and Lichtwardt 2004). This species, discovered in the USA (Lichtwardt 1972), has a sub-cosmopolitan distribution (see Lichtwardt et al. 2001), with a patchy distribution in the Northern Hemisphere and also Australia (Lichtwardt and Williams 1992).

***Smittium dipterorum* Lichtw., 1997.**
Fig. 17

Specimen examined. site 12: slides BUL–12-1; BUL–12-4.

Notes. This species was previously known from Costa Rica (Lichtwardt1997), Spain (Valle and Santamaria 2004), Dominican Republic (Valle and Cafaro, 2010) and Mexico (Valle et al. 2011) in the tract of Simuliidae and Chironomidae. Bulgarian specimens were associated with Chironomidae midges (*Chironomus* sp.). They had cylindrical-elongated trichospores measuring 16–19 × 3–4 µm, with a short collar about 2.5 µm, slightly flared outwards. The thallus is branched at the base, with verticillate apical branching (Fig. 17). Each fertile branch has 4–6 generative cells. Specimens from Bulgaria resemble most of those described from Spain.

***Spartiella barbata* Tuzet & Manier ex Manier, 1968.**
Fig. 18

Specimens examined. Site 7: slide BUL–7–4; Site 10: slide BUL–10–3.

Notes. This species was described from France (Tuzet and Manier 1950) in the hindgut of Baetidae nymphs. *Spartiella barbata* is distinguished by its lobulate basal cell, obpyriform trichospores, measuring 21–26.5 × 6.5–7.5 µm in our specimens (Fig. 18) and the presence of one appendage tightly coiled just after release and then eventually uncoiling into a long delicate filiform structure. *Spartiella barbata* seems to be more common in Europe, where it has been recorded from France (Tuzet and Manier 1950, Manier 1962b), United Kingdom (Lichtwardt 1986) and Spain (Valle 2007), with only one report of the species from North America, in Canada (Strongman 2010).

***Stachylina nana* Lichtw., 1984.**
Fig. 19

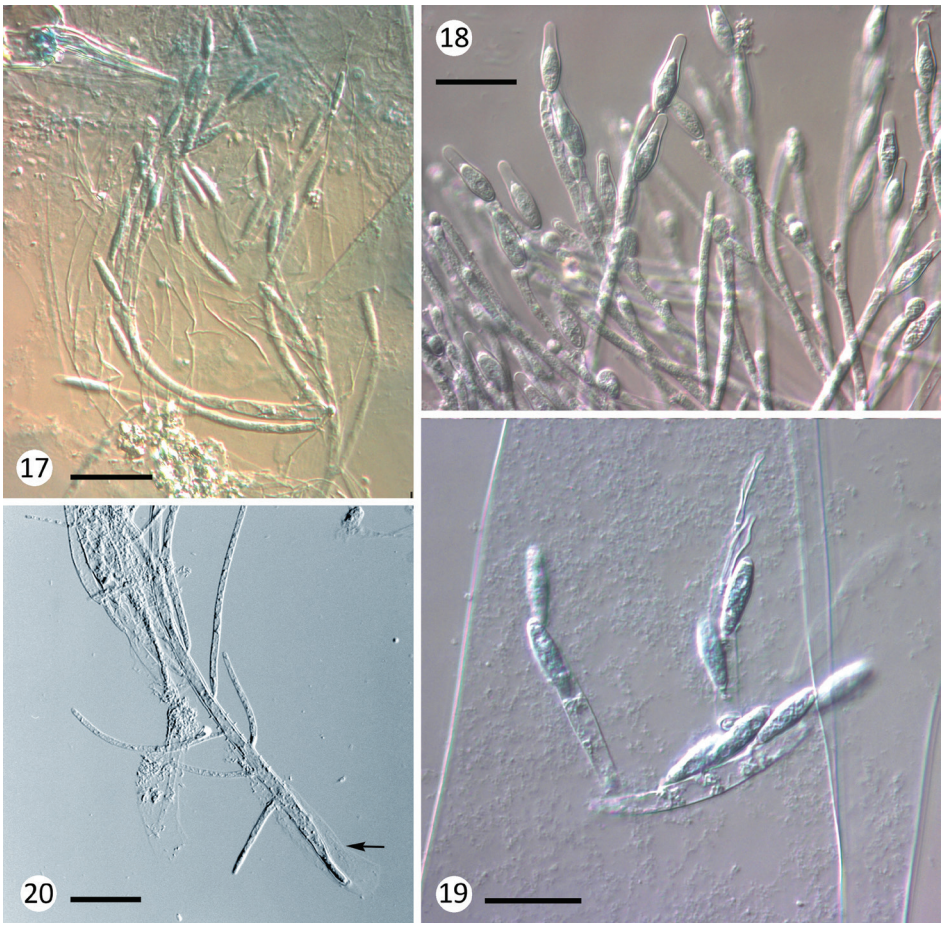
Specimens examined. Site 11: slide BUL–11–4.

Notes. *Stachylina nana* was found in the mid-gut of Chironomidae (*Chironomus* sp.) in the same host as *Smittium dipterorum*. Specimens of *S. nana* in Bulgaria had a small thallus, 60–70 × 7–8 µm, with 1–4(-5) generative cells. Trichospores measure 20–24 × 7–7.5 µm, without a collar. All measurements agree with the original description of the species (Lichtwardt 1984). This species was recorded before from Europe, including France (Lichtwardt 1984) and Spain (Santamaria and Girbal 1997); Asia, in Thailand (Hapsari et al. 2009), China (Strongman and Wang 2015) and also America, including Canada (Strongman 2007, 2010; Strongman and White 2008) and USA (Beach and White 2012). Probably, *S. nana* has a cosmopolitan distribution, although we only have patchy data from few surveyed countries.

***Stipella vigilans* Léger & Gauthier, 1932.**
Fig. 20

Specimens examined. Site 4: slides BUL–4–3, BUL–4–11.

Notes. *Stipella vigilans* was originally described from the French Alps in the hindgut of Simuliidae, together with the protozoan *Paramoebidium* sp. (Léger and Gauthier 1932). This species was also reported from Spain (Valle 2007), England (Moss 1970), Armenia (Nelder et al. 2005), Thailand (Hapsari et al. 2009). The species is easily distinguished by the particular basal cell, simple or forked, verrucose and narrowing in the most basal section, attached to the hindgut by means of a mucilaginous adhesive substance (holdfast) (Fig. 20). The trichospores of *S. vigilans* are also very characteristic, almost cylindrical, measuring 35–50 × 3–4.5 µm in our collections, although probably somewhat young, they fit the original description, according to Léger and



Figures 17–20. Various species of Harpellales. **17** *Smittium dipterorum* from Chironomidae larvae; thallus overview with fertile branches and trichospores **18** *Spartiell barbata* from Baetidae nymphs, fertile branches with trichospores **19** *Stachylina nana* from Chironomidae larvae, thallus overview with trichospores **20** *Stipella vigilans* from Simuliidae larvae, thallus with mucilaginous material at the basal cell (arrow). Scale bars: 25 μm in all figures.

Gauthier (1932). We did not see released trichospores, since none of them was mature enough in our collections. Trichospores in this species bear three petaloid appendages, which are visible inside the generative cells before detachment.

Order Orphellales

***Orphella catalaunica* Santam & Girbal, 1998.**
Fig. 21

Specimens examined. Site 4: slides BUL–4–1, BUL–4–5, BUL–4–7; site 7: slide BUL–7–6.

Notes. We found this species associated with *Leuctra hippopus* (Kempny 1899) in two Bulgarian rivers and streams. The specimens examined had the typical characteristics of the species, including the straight trichospores measuring 47–56 × 5–7 μm in our collections, with generative cells 21–26 μm long and a supporting cell 6–8 μm length (Fig. 21). All the characters of trichospores and accompanying cells fit the description of the species (Santamaria and Girbal 1998). Zygospores were not seen on this occasion. This species was described from Catalonia, Spain (Santamaria and Girbal 1998, Valle and Santamaria 2005) and has been reported also from Norway (White and Lichtwardt 2004), France (Valle 2013b) and Italy (Valle et al. 2014).

***Orphella coronata* Léger & Gauthier, 1931.**
Figs 22, 23

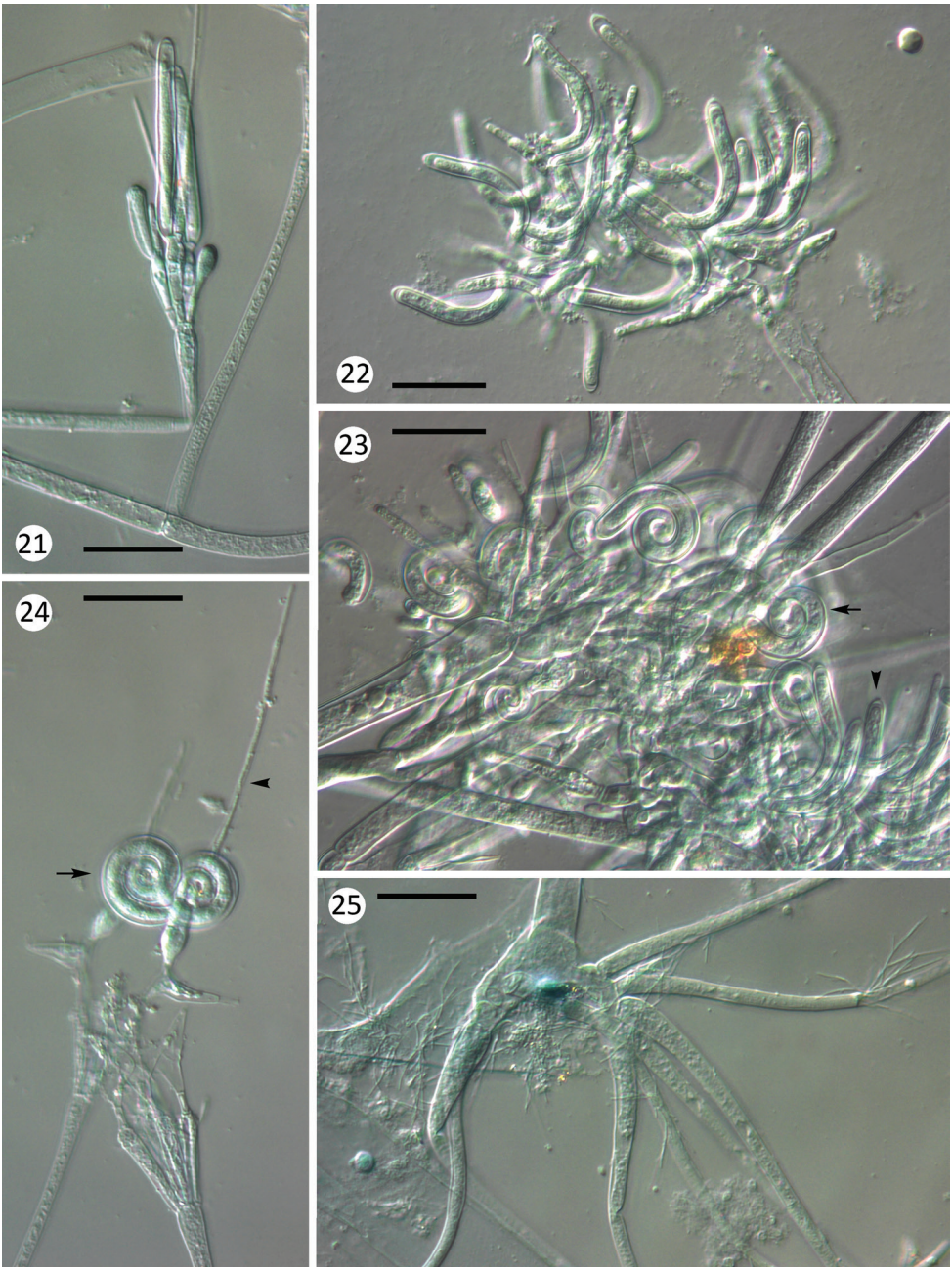
Specimens examined. Site 10: slide BUL–10–4 (zygo.)

Notes. Collections were made from the hindgut of *Protonemura montana* Kimmins nymphs, with a low infestation rate (2%). *Orphella coronata* has been reported from diverse localities in Europe, (e.g. France (Léger and Gauthier 1931, 1932), Norway (White and Lichtwardt 2004), Spain (Valle and Santamaria 2005), Portugal (Valle 2013a) and Italy (Valle et al. 2013)). *Orphella coronata* seems to be the rarest species of the genus in the Bulgarian streams surveyed, although many potential hosts were dissected from the Struma River. Fortunately, the only *Protonemura* infested specimen was carrying various mature thalli of *O. coronata*, so that we could observe trichospores and the typical heterothallically-formed helicoidal zygospores typical for the species, these being very important to discern and identify possible cryptic species (Valle and Santamaria 2005, Valle et al. 2014, White et al. 2018). Bulgarian specimens show the typical thallus with a bifurcate basal cell and allantoid trichospores (Fig. 22), measuring 36–41 × 5.5–6.5 μm in our collections, slightly smaller than previously reported (35–48 × 6–7.5 μm according to Valle & Santamaria 2004). Terminal cell measures 22–25 × 3–3.5 μm. Zygospores (Fig. 23) in our collections measure 26–32 × 6–7 μm, also somewhat smaller than those reported in the description of the zygospores (30–35 × 5–7 μm according to Valle & Santamaria 2004), but likely attributable to intraspecific variation; in fact, just a couple of thalli were found producing sexual spores in our collections and about 5 producing trichospores.

***Orphella helicospora* Santam & Girbal, 1998.**
Figs 24, 25

Specimens examined. site 4: slide BUL-4-1; Site 7: slide BUL-7-6; site 10: slides BUL-10-2, BUL-10-5.

Notes. Species were obtained from the hindgut lining of Leuctridae nymphs (mainly *Leuctra hippopus*). We found several thalli, most of them producing trichospores and one also bearing helicoidal zygospores, formed homothallically, measuring 25–27 × 5.5–6.5 μm, growing on a fusiform zygosporophore measuring 20–23 × 7–8.5 μm,



Figures 21–25. Various species of Orphellales. **21** *Orphella catalaunica* from Leuctridae nymphs, trichospores and accompanying cells **22, 23** *Orphella coronata* from Nemouridae nymphs **22** allantoid trichospores and accompanying cells **23** zygospores produced homothallically **24, 25** *Orphella helicospora* from Leuctridae nymphs: **24** homothallical zygospores and accompanying cells **25** basal cell and holdfast. Scale bars: 25 μm in all figures.

with a 3 μm long supporting cell and a sigmoid or reflexed intermediate cell about 20–24 μm long in the specimens seen (Fig. 24). All the characteristics of spores and accompanying cells fit those of the specimens reported from other localities in Europe, including Spain (Santamaria and Girbal 1998; Valle and Santamaria 2005), Norway (White and Lichtwardt 2004); Italy (Valle et al. 2013). Thallus has the characteristic basal cell (Fig. 25) and lateral subsidiary branches.

Order Amoebidiales (Mesomycetozoea)

***Paramoebidium angulatum* Valle, 2014a.**
Fig. 26

Specimens examined. Site 4: slide BUL-4-2

Notes. This species is characterised by having a thallus bent approximately at a right angle about one-quarter up the thallus from the holdfast; no other described species shares this feature. This species was originally described from the stonefly family Taeniopterygidae. Our specimens were observed in Nemouridae, a different family in the same Order of insects sharing all characteristics with *P. angulatum*, although most individuals were immature, measuring 350–380 × 29–33 μm, with the typical thallus with the right angle bend and discoid acellular holdfast, known previously only from France (Valle 2014a).

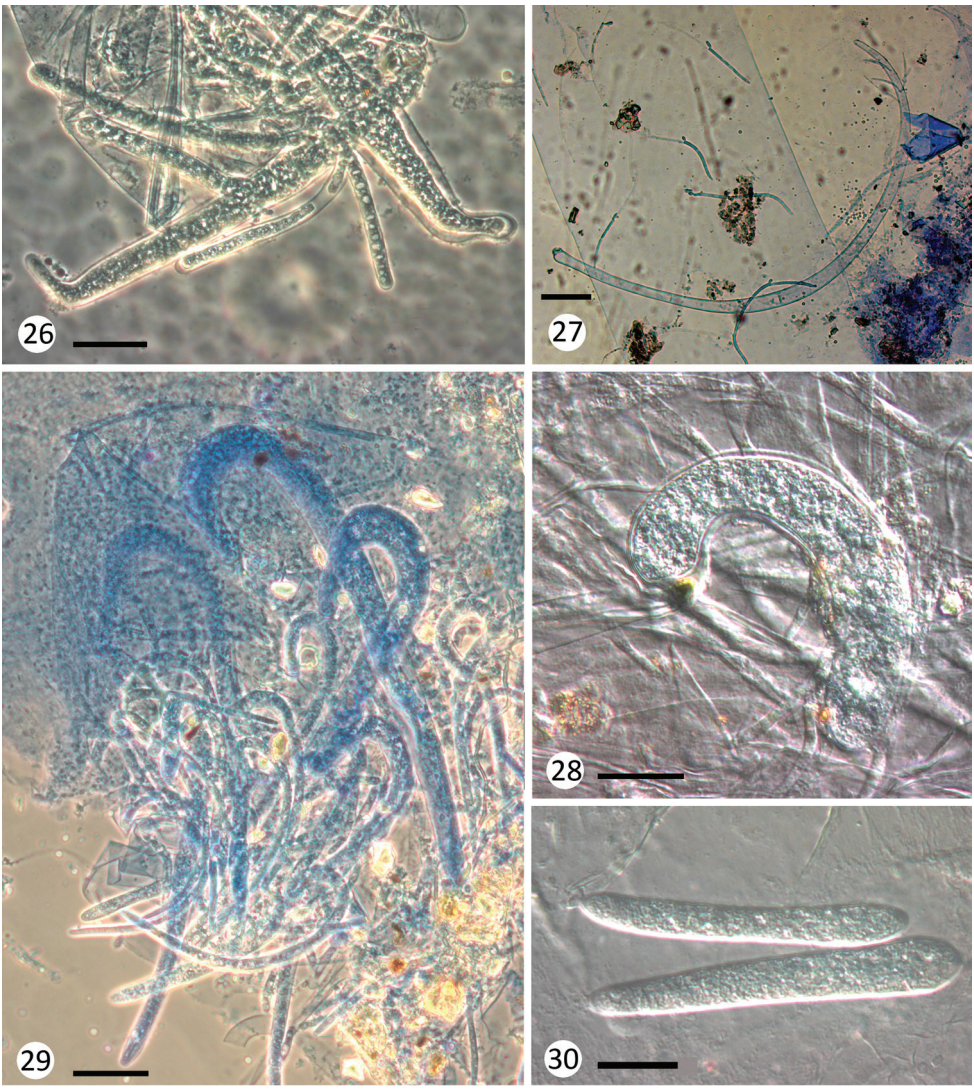
***Paramoebidium chattoni* Léger & Duboscq ex LG Valle, 2014b**
Fig. 27

Specimens examined. Site 2: slide BUL–2–6.

Notes. This species of *Paramoebidium* is common within Simuliidae hosts and is identifiable by having the widest diameter of thallus at the basal to middle sections, slightly tapering towards the distal end. It has a non-cellular holdfast located at the proximal end smaller in diameter (12–27 μm) than the thallus, cylindrical or slightly campanulate (Valle 2014b). Bulgarian species perfectly match with the original description (Léger and Duboscq 1948) and with that provided in the validation of the species by Valle (2014b). The species was recently reported from Colombia (Barón and Valle 2018). In our collections, *Simuliomyces microsporus* was often found attached to the thalli of this *Paramoebidium* (Fig. 27).

***Paramoebidium curvum* Lichtw., 1979.**
Fig. 28

Specimens examined. Site 2: slides BUL–2–1; BUL–2–6, BUL–2–7, BUL–2–10; Site 12: slide BUL–12–5.



Figures 26–30. Various species of Amoebidiales. **26** *Paramoebidium angulatum* from Nemouridae nymphs, thalli overview with typical angular shape at the upper section **27** *Paramoebidium chattoni* from Simuliidae larva, overview, with thin filaments of *Simuliomyces microsporus* **28** *Paramoebidium curvum* from Simuliidae larva, overview **29** *Paramoebidium hamatum* from Baetidae nymphs, overview of various thalli in different growth phases **30** *Paramoebidium inflexum* from Nemouridae nymphs, thallus overview. Scale bars: 50 µm (**26, 29, 30**), 100 µm (**27**), 25 µm (**28**).

Notes. This species was found attached to the posterior hindgut or anal gills of larval Simuliidae hosts, where we also found *P. chattoni*. In Bulgaria, the species was quite common in the black fly hosts dissected. All the individuals had the typical characteristics of the species (Dang and Lichtwardt 1979), with a holdfast placed on the

incurved section of the thallus. This species is known from USA (Dang and Lichtwardt 1979), Sweden (Lichtwardt 1984), Armenia (Nelder et al. 2005), Canada (Strongman and White 2008), Spain (Valle and Santamaria 2009, Valle 2014b), Italy (Valle et al. 2014). *P. curvum* is probably very common and cosmopolitan as their hosts, but their thalli can be easily overlooked when attached to or near to the anal gills.

***Paramoebidium hamatum* Bench & MM White, 2012**
Fig. 29

Specimens examined. Site 1: slides BUL–1–1, BUL–1–2.

Notes. *Paramoebidium hamatum* was described originally from USA in Chironomidae, Ameletidae and Baetidae (Ephemeroptera). In Bulgaria, it is associated with Baetidae nymphs (*Baetis* sp. *B. rhodani* and *B. melanonyx*). The species was recorded before in Europe (Spain, Busquets et al. 2018). Bulgarian specimens measured 180–300 × 12–25 μm, with the broader diameter at the proximal end, near the holdfast, thinner at the distal end (Fig. 29); cystospores observed, measuring about 10 × 4–4.5 μm. The species was identified by the curved portion at the basal one-eighth to one-third of the thallial length and by its holdfast characteristics (Bench and White 2012).

***Paramoebidium inflexum* Léger & Duboscq, 1929**
Fig. 30

Specimens examined. Site 4: slide BUL–4–6.

Notes. Species observed attached to the hindgut lining of *Protonemura montana,* measuring 280–340 × 40–60 μm in our collections. This species was described from France, associated with *Nemoura variegata* nymphs, in the stonefly family Nemouridae. The species has three different thallial morphologies (Léger and Duboscq 1929b, Duboscq et al. 1948). Subsequent to the revision of the species by Duboscq et al. (1948), this endobiont was not reported again, until now, probably because of the thallus variability and, thus, relatively difficult identification. We only saw one of the thallial morphologies described for the species, this being the stouter and shorter thallus type. The other two morphological types show longer and incurved thalli, one type being wider than the other (Duboscq et al. 1948). Neither was observed in our collections.

Discussion

The new species, *Glotzia balkanensis*, was the most remarkable taxon recorded in this study. As noted above, it is morphologically close to *G. centroptili* (Gauthier ex Manier and Lichtw.), recorded from the western Mediterranean (Gauthier 1936, Busquets et al. 2018) and to *G. distorta* from Italy (Valle et al. 2014), both species from *Centroptilum*

hosts. The new Bulgarian species is justified on the basis of spore and thallial characteristics and was found associated with a different host, *Baetis melanonyx*, in two distant sites in the Province of Sofia. Both sites were permanent non-tidal, smooth-flowing watercourses (EUNIS habitat type code C2.3, see Table 1). Moreover, both localities, where the new species was found, had in common a water pH of 7.2, the highest of all the watercourses we surveyed in Bulgaria, but different water temperatures (see Table 1). Another species of the genus *Glotzia* with similar characteristics is *Glotzia stenospora* MM White & Lichtw., from Norway. However this species has larger trichospores measuring 60–68 × 3–5 µm, with a higher length/width ratio (White and Lichtwardt 2004). Additionally, *G. stenospora* has larger zygospores than *G. balkanensis* (61–72 × 11–14 µm, according to White and Lichtwardt 2004). The other non-European species of *Glotzia* have smaller zygospores and most of them (except *G. coloradense* William & Lichtw.) also have smaller trichospores (Lichtwardt et al. 2001).

We gathered other Harpellid species from the guts of Baetidae nymphs, including *Graminella bulbosa*, *Legeriomyces ramosus* and *Spartiella barbata* and the Amoebidiales *P. hamatum*. The species *Graminella bulbosa* has been reported from western Europe, in France (Léger and Gauthier 1937, Manier 1962a), Spain (Valle 2003, 2007), Portugal (Valle 2013a) and Italy (Valle et al. 2014), with this being the first report in eastern Europe. The characteristics of the Bulgarian specimens are interesting indeed, since trichospore morphology is intermediate between two species: *G. bulbosa* and *G. microspora*. We have assigned the specimens to *G. bulbosa*, the type species, which probably is variable enough to actually include both species, as suggested by Valle (2004). Trichospores of *G. microspora* measure 6–8.5 x 2–2.5 µm, according to Lichtwardt and Moss (1984), while trichospores of Bulgarian *G. bulbosa* measure 8–11 × 2 µm. According to Léger and Gauthier (1937) and later validated by Manier (1962a, 1970), trichospores of French *G. bulbosa* measure 9–17 × 2–3 µm. Bulgarian specimens, like many other specimens collected by one of the authors (LGV) in diverse European localities, overlap the measurements of both species. This question will be addressed further in an upcoming paper. *Graminella* is very peculiar amongst Harpellales for its vegetative propagules, originated at, or near, the basal cell. Bulgarian specimens of *G. bulbosa* also had these swollen basal cells, which can extrude their cellular contents to act as vegetative propagules within the same gut. Our specimens were associated with *Baetis rhodani* (Pictet) and *B. alpinus*, both common hosts previously reported with this fungus.

Legeriomyces ramosus is a common endosymbiont of Baetidae nymphs and has a broad distribution, especially in the Northern Hemisphere (Lichtwardt et al. 2001). The Bulgarian specimens had the characteristic attributes of the species, including trichospore and zygospore morphology and thallial features. *Spartiella barbata* is often seen together with *L. ramosus*, both sharing the same host, as observed in some of our collections, although *S. barbata* is not as prevalent as *L. ramosus*. The trichospores of *S. barbata* are somewhat similar to those of *L. ramosus*, but more ovoidal and have a single appendage initially folded showing a knob at the proximal end (Manier 1968, Valle 2007). The basal cell of *S. barbata* is also characteristic, with bulbous swellings around the zone of attachment to the host cuticle, a feature not present in *L. ramosus* (Manier

1968, Valle and Santamaria 2002). *Spartiella barbata* has a noticeable preference for calcareous watercourses, but in this study, even though calcareous rocks were present on the river substrate, as in collection site 10 (see Table 1), the water where *S. barbata* specimens were collected, had a slightly acidic pH (6.8 in site 7 and 6.1 in site 10).

Amongst the Amoebidiales inhabiting Ephemeropteran nymphs, we collected and recognised *P. hamatum* from Baetidae hosts. This species described from America (Bench and White 2012) was recorded before from Europe in Italy (Valle et al. 2014) and then Spain (Busquets et al. 2018). Léger and Duboscq (1929b), in a paper dealing with *Paramoebidium,* named *P. arcuatum* from Baetidae nymphs, without providing additional information on the species (no description or illustrations). Subsequently, Duboscq et al. (1948) provided more information on this species, including the identity of Baetidae hosts where it was observed, a description and some line drawings (Duboscq et al. 1948). The characteristics of this species, described from France earlier, are like those of *P. hamatum* and, consequently, very similar to the specimens we have collected within Baetidae hosts in Bulgaria. Probably, *P. hamatum* is the same species named *P. arcuatum* (regarded as *nomen nudum* by Lichtwardt et al. 2001), but further investigation is needed to resolve this question. There are some other *Paramoebidium* species that were named by French authors at the first half of the 20th century, but were not validated or lacked a complete description, making a clear, conclusive identification very difficult.

Other species of *Paramoebidium* were observed from different Ephemeropteran families, including Leptophlebiidae (with some specimens resembling *P. hamatum*), Caenidae and Heptageniidae, but were not identified for the lack of enough material or mature specimens for study. These mayfly nymphs did not have associated Harpellales.

The examined Bulgarian Simuliidae (Diptera) held various species of Harpellales and Amoebidiales. Larval black flies inhabit a wide range of flowing waters, from the smallest streams to the largest rivers (Nelder et al. 2006) and have been collected in nearly all surveyed watercourses with high enough flow velocity (not in lentic and slow moving watercourses). Amongst the trichomycetes they had in our collections, *Genistellospora homothallica* was the most prevalent in the hindgut, occasionally accompanied by *Simuliomyces microsporus*, a smaller species growing epithallically on the robust structure of *G. homothallica*. On the other hand, *Harpella melusinae* was the most common in the mid-gut, attached to the chitinous peritrophic matrix. *Harpella melusinae* is unbranched, placed within the family Harpellaceae and easily identifiable by the coiled trichospores in a series of long generative cells (Lichtwardt et al. 2001). *Stipella vigilans* is not as common as *G. homothallica*, but both species share the hindgut of Simuliidae. The former species can be identified by the mucilaginous substance embedding the basal cell and type I zygospores, according to zygospore types designated by Moss (1975).

In the hindgut, near the anal gills or attached to them, *Paramoebidium curvum* appears also commonly in Simuliidae, this being identifiable by its curved and robust sac-like thallus with a prominent and eccentric holdfast (Dang and Lichtwardt 1979). Another *Paramoebidium* observed within Simuliidae hindguts in Bulgaria was *P. chattoni*, with a longer, arched thallus. Both species of *Paramoebidium* are quite common in

various Simuliidae species and have been reported from different countries (Lichtwardt et al. 2001, Valle 2014b). We also collected symbionts of Chironomidae larvae, but just two species: *Stachylina nana* from the mid-gut and *Smittium dipterorum* from the hindgut, both in *Chironomus* larvae. *Smittium dipterorum* was described from Costa Rica (Lichtwardt 1997) and then reported from the Dominican Republic and Mexico (Valle and Cafaro 2010, Valle et al. 2011). It was also recorded from Europe, in Spain (Valle 2007) and it is probably a widespread species, like *Stachylina nana*, but more rare (less prevalent), especially in Europe. There are insufficient data to determine whether this is or is not a cosmopolitan species.

The genus *Orphella* (Orphellales) is associated with Plecopteran nymphs, a host also present in our collections, especially in pristine, high altitude or mountain watercourses, since stoneflies nymphs are very susceptible to water pollution and also water temperature, preferring cold and clean streams and rivers with running water and aquatic vegetation and often pebble stones (Berthélemy 1963). We gathered three species in the Orphellales: *O. coronata* within the hindgut of *Protonemura montana* was the rarest of the *Orphella* species in Bulgaria; however, we were very lucky to obtain several thalli producing trichospores and zygospores from heterothallic conjugations. *Orphella coronata* is the only European *Orphella* with heterothallic sexual reproduction. Only the related North American counterparts with allantoid trichospores, *O. haysii* Lichtw. & Williams (USA) and *O. dalhousiensis* Strongman & MM White (Canada) show the same sexual behaviour (White et al. 2018). The Bulgarian *O. coronata* had slightly smaller trichospores and zygospores than reported before, but differences are not significant; all other characters of accompanying cells and thallial structure matched the description of the species, taking into consideration all recorded geographic variability (White et al. 2018). *Orphella catalaunica* was associated with *Leuctra hippopus* nymphs and most of the observed thalli were immature, except for a couple producing trichospores. No zygospores were observed, but the straight cylindrical trichospores, accompanying cells in fertile cap and thallial characteristics of Bulgarian specimens, fit with those of the original description and later records (Santamaria and Girbal 1998, Valle 2004, White et al. 2018). *Orphella helicospora* was also associated with Leuctridae hosts, in the same species as *O. catalaunica*, sometimes sharing the same gut, which is common for these species. In the case of *O. helicospora*, both trichospores and zygospores were observed and were in accordance with previous descriptions of the species, which has been reported before from various western and central European countries (Lichtwardt et al. 2001). Two species of *Paramoebidium* were also identified within Plecopteran hosts. *P. angulatum*, described from France in Taeniopterygidae has been documented here from a different plecopteran family, the second record of the species. On the other hand, *P. inflexum*, is a rare species described from France that has not been documented until now since Dubosq et al. (1948), in both occasions from Nemouridae nymphs. This is an interesting addition and probably further studies and surveys will allow the possibility of gathering the three different thallial structures described for *P. inflexum*, since only one was observed in our Bulgarian collections.

Conclusions

This short survey provided 18 new taxa for both Bulgaria and the Balkan Peninsula, including one new species, *Glotzia balkanensis*, with sporic features that allow a clear differentiation from other described species of the genus and which also shares characteristics with the other two European species. The morphological characteristics of *Graminella bulbosa*, collected in Bulgaria, have intermediate spore and thallial characteristics between those of *G. microspora* and *G. bulbosa*, as previously reported by Valle (2004), making mandatory a revision of the European species of the genus. Some rare or poorly known taxa were recovered, including *Paramoebidium inflexum* and *P. angulatum*. Amongst the Orphellales, three species have been reported from Bulgaria (*O. catalaunica*, *O. coronata*, *O helicospora*). They seem to have a broad distribution in Europe, from the western Mediterranean to the Balkans. Some other taxa, reported here, have a cosmopolitan distribution, such as those associated with Simuliidae larvae, including *Harpella melusinae*, *Genistellospora homothallica* and *Simuliomyces microsporus*. The species associated with the dipteran Chironomidae, *Stachylina nana* have a wide geographic distribution and that is possibly also true for *Smittium dipterorum*; unfortunately, it seems to be less prevalent and there are few reports for this species described in Costa Rica. Our findings support previous observations, revealing that dipteran hosts generally bear endosymbiont species more widely distributed than other insect groups. This may be related to the possibility of adult-mediated transport of fungal diaspores (Moss and Descals 1986, Labeyrie et al. 1996) and also the more restricted flight and dispersal capacity of stonefly and mayfly adults. Amongst Plecopteran hosts, there seems to be a more manifest species delimitation between the Old and New world, as in the case of *Orphella* (White et al. 2018) and also between the North and South Hemispheres. Similarly, the geographic distribution of Ephemeropteran endobionts may be important, but not so evident, as in Plecopteran-associated endobionts. However, these are general tendencies and much more effort has to be spent to improve our knowledge of this poorly-known group of cryptic organisms, regarding diversity, ecology, biology and biogeography. It is our aim to increase the knowledge of trichomycetes in this very interesting biogeographic region.

Acknowledgements

We are indebted to Yanka Vidinova for her help in the identification of Ephemeropteran hosts and to Violeta Tyufekchieva for identifying Plecoptera hosts. We are grateful also to Snejana Grozeva for the hospitality and the permission to use the equipment of the laboratory "Cytotaxonomy and Evolution" at the Institute of Biodiversity and Ecosystem Research, Sofia, Bulgaria. We also want to thank Nikolay Simov for his help with the choice of collection sites.

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MycoKeys 67: 81–94 (2020)
doi: 10.3897/mycokeys.67.50068
http://mycokeys.pensoft.net

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A taxonomic reassessment of the genus *Balsamia* from China

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Academic editor: T. Lumbsch | Received 12 January 2020 | Accepted 13 May 2020 | Published 4 June 2020

Citation: Xu Y-Y, Yan X-Y, Li T, Fan L (2020) A taxonomic reassessment of the genus *Balsamia* from China. MycoKeys 67: 81–94. https://doi.org/10.3897/mycokeys.67.50068

Abstract

Molecular analysis of the genus *Balsamia* was conducted with ITS and 28S sequences available, including newly gained sequences from Chinese specimens. Combined with the morphological examinations, a new hypogeous species, *Balsamia lishanensis* was described and illustrated from North China, which is morphologically characterized by reddish brown ascomata covered with fine warts, the whitish gleba with numerous small chambers, 3–5 layers peridium with reddish brown polygonal cells and the smooth and regular ellipsoid ascospores with one large oil drop. Two species previously described as *Barssia* were transferred to *Balsamia*. *Balsamia platyspora* was confirmed to be in existence in China based on newly collected specimen. A key to the Chinese *Balsamia* species was provided.

Keywords

Ascomycota, Helvellaceae, Hypogeous fungi, phylogeny, taxonomy

Introduction

The genus *Balsamia* Vittad. (*Helvellaceae*, *Pezizales*), with *B. vulgaris* Vittad. as the type species, was established in the early 19th century (Vittadini 1831), usually forming ectomycorrhizae with both broad leaf and conifer trees (Southworth et al. 2018; Hansen et al. 2019). Geographically, *Balsamia* species are widely distributed across Europe, North America, North Africa and Asia in the temperate regions of the northern hemisphere (Liu and Tao1990; Pegler et al.1993; Southworth et al. 2018; Hansen et al. 2019). Until now, nine *Balsamia* species have been reported from Europe (Vittadini

1831; Tulasne and Tulasne 1843; Berkely 1844; Tulasne and Tulasne 1851; Schulzer 1870; Petitberghien 1966; Ławrynowicz and Skirgiełło 1984; Kaounas et al. 2015; Hansen et al. 2019), twelve from North America (Southworth et al. 2018), and one from North Africa (Crous et al. 2014; Hansen et al. 2019) In China, this genus is poorly understood as only one species *Balsamia platyspora* Berk. is reported, based on morphological evidence (Liu and Tao 1990).

Recently, two new species of the genus *Barssia* have been described from China (Xu et al. 2018), their taxonomic position, however, needs to be reassessed because Hansen et al. (2019) synonymized *Barssia* under *Balsamia* based on their phylogenetic analysis from three loci (*28S*, *RPB2*, *EF-1a*) and morphological studies. More recently, an un-described *Balsamia* species is recognized when we check the specimens newly collected from north China. In this paper, both the molecular analyses and morphological examinations are conducted for the Chinese samples,, and our aims are:1) to illustrate the position of Chinese *Balsamia* species based on ITS and 28S sequences newly obtained from Chinese *Balsamia* collections with distinct features in this study as well as recently published and used sequences ; 2) to give a detailed characterization of a new species based on morphological features and phylogenetic evidences.

Materials and methods

Morphological studies

Collections were obtained and photographed in the field from Shanxi regions in China, and they were dried and deposited in BJTC (Herbarium, Biology Department, Capital Normal University). One specimen was studied from HMAS (Herbarium Mycologicum Academiae Sinicae, Institute of Microbiology, Chinese Academy of Sciences). Macroscopic characters were recorded from fresh specimens. Microscopic characters were observed in thin sections of dry specimens mounted in 3% KOH, Melzer's reagent (Dring 1971) or 0.1% (w/v) cotton blue in lactic acid. Thirty mature ascospores were measured, and the symbol Q is used to indicate length/width ratios of ascospores in side view.

DNA extraction, PCR amplification and DNA sequencing

Herbarium specimens were crushed by shaking for 30 s at 30 Hz 2–4 times (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube together with one 3 mm diam. tungsten carbide ball, and total genomic DNA was extracted using the modified CTAB method (Gardes and Bruns 1993). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) was amplified using primers ITS1f/ITS4 (White et al. 1990; Gardes and Bruns 1993). The 28S large subunit nrDNA (nrLSU) region was amplified using primers LR0R/LR5 (Vilgalys and Hester 1990). PCRs were

performed in a volume of 50 μl consisted of 4 μl of DNA template; 2 μl of (10 μM) per primer; 25 μl 2× Master Mix (Tiangen Biotech Co., Beijing). The procedure for PCR reaction was: an initial denaturation at 94 °C for 3 min; followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were sent to Beijing Zhongkexilin Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing and editing. Validated sequences are stored in the NCBI database (http://www.ncbi.nlm.nih.gov/) under the accession numbers provided (Table1). The other sequences used in the molecular phylogenetic analysis were downloaded from the NCBI database (Suppl. material 1).

Phylogenetic analyses

Two datasets, ITS and 28S, were compiled to identify *Balsamia* species and investigate relationships among species. The taxa *Tuber anniae* and *T. bellisporum* were selected as outgroups. The ITS and 28S sequences were aligned using the MAFFT v.7.110 online program under default parameters (Katoh and Standley 2013), and manually adjusted to allow maximum sequence similarity in Se-Al version.2.03a. (Rambaut 2000). Ambiguously aligned regions and gaps in alignment were excluded by Se-Al version.2.03a. (Rambaut 2000) before the phylogenetic analysis. Alignments were submitted to TreeBASE under accession number S25937. We conducted maximum likelihood (ML), most parsimonious (MP) and Bayesian inference (BI) analyses on the two datasets.

Maximum likelihood (ML) analysis of the dataset was carried out using RAxML 8.0.14 (Stamatakis 2014) and the GTRGAMMA substitution model with parameters unlinked. The ML bootstrap replicates (1000) were computed in RAxML using a rapid bootstrap analysis and search for the best-scoring ML tree. The ML trees were viewed with TreeView32 (Page 2001). Clades with bootstrap support (MLBS) ≥ 70% were considered as significant-supported (Hillis and Bull 1993).

A most parsimonious (MP) analysis was constructed with PAUP* 4.0b10. (Swofford 2002). The bootstrap values were generated using the following settings: 1000 replicate searches on all parsimoniously informative characters using 100 random sequence addition replications and TBR (tree-bisection reconnection) branch swapping

Table 1. Information on newly generated DNA sequences used in this study.

Fungal taxon	Specimen voucher	Locality	ITS	28S
Balsamia lishanensis	BJTC FAN587	Shanxi, China	MT232721	MT232903
	BJTC FAN591	Shanxi, China	MT232899	MT232911
	BJTC FAN676	Shanxi, China	MT232907	MT232902
	BJTC FAN689	Shanxi, China	MT232905	MT232914
	BJTC FAN697	Shanxi, China	MT232908	MT232912
	BJTC FAN714	Shanxi, China	MT232901	MT232913
	BJTC FAN1010	Shanxi, China	MT232900	MT232910
	HMAS 97115	Gansu, China	MT232904	MT232909
Balsamia platyspora	BJTC FAN557	Shanxi, China	MT232906	MT229143

algorithms in PAUP*. Tree statistics (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) were also calculated. Tree was viewed with TreeView32 (Page 2001). Clades with bootstrap support (MPBS) ≥ 70% were considered to be significant (Hillis and Bull 1993).

Bayesian inference (BI) analyses was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) based on the best substitution models determined by MrModeltest 2.3 (Nylander 2004), which were GTR+I+G for the ITS dataset and SYM+I+G for the 28S dataset. Two independent runs of four chains were conducted for 4 000 000 for ITS and 2 000 000 for 28S datasets Markov Chain Monte Carlo generations using the default settings and sampled every 100 generations. The temperature value was lowered to 0.20, burn-in was set to 0.25, and the run was automatically stopped as soon as the average standard deviation of split frequencies reached below 0.01. A 50% majority-rule consensus tree was constructed and visualized with TreeView32 (Page 2001). Clades with Bayesian posterior probability (BPP) ≥ 0.95 were considered as significantly supported (Alfaro et al. 2003).

Results

Phylogenetic analysis

For ITS dataset, we comprehensively collected the ITS sequences of *Balsamia* and the fungi previously described as *Barssia*, and sequences that are high similarity to *Balsamia*. For 28S dataset, we collected all sequences of *Balsamia* and the fungi previously described as *Barssia*, and representative sequence of other genera of *Helvellaceae*. Sequences of each locus were aligned and analyzed separately.

The 28S dataset contained 72 sequences (9 were newly gained in this study), and 4 from the outgroup *Tuber anniae* and *T. bellisporum*. The dataset had an aligned length of 886 characters, of which 578 were constant, 308 were variable, and 278 of these variable sites were informative. The maximum parsimony analysis resulted in one most parsimonious tree with a length (TL) of 842 steps, consistency index (CI) of 0.570, retention index (RI) of 0.896, homoplasy index (HI) of 0.430. MP, ML and BI analyses yielded similar tree topologies, and only the tree inferred from the MP analysis is shown (Fig. 1). The 28S sequences of *Balsamia* were grouped into a distinct clade with high supports (MPBS = 100%, MLBS = 100%, BPP = 1.00). The Chinese materials were well clustered in the *Balsamia* clade (Fig. 1), including the sequences of the fungi previously described as *Barrsia guozigouensis* L. Fan & Y.Y. Xu and *Barssia luyashanensis* L. Fan & Y.Y. Xu (Xu et al. 2018). Three distinct branches with strong supports can be recognized from Chinese collections, respectively representing *Balsamia guozigouensis*, *Balsamia luyashanensis*, and a new species *Balsamia lishanensis* proposed in this study. In addition, the Chinese sequence from BJTC FAN557 grouped together with a reliably identified sequence (MK100252) of *B. platyspora* (Hansen et al. 2019) with strong support value (MPBS = 99%, MLBS = 99%, BPP = 1.00), and they shared 99.83% 28S sequences similarity, indicating the Chinese specimen BJTC FAN557 was *B. platyspora*.

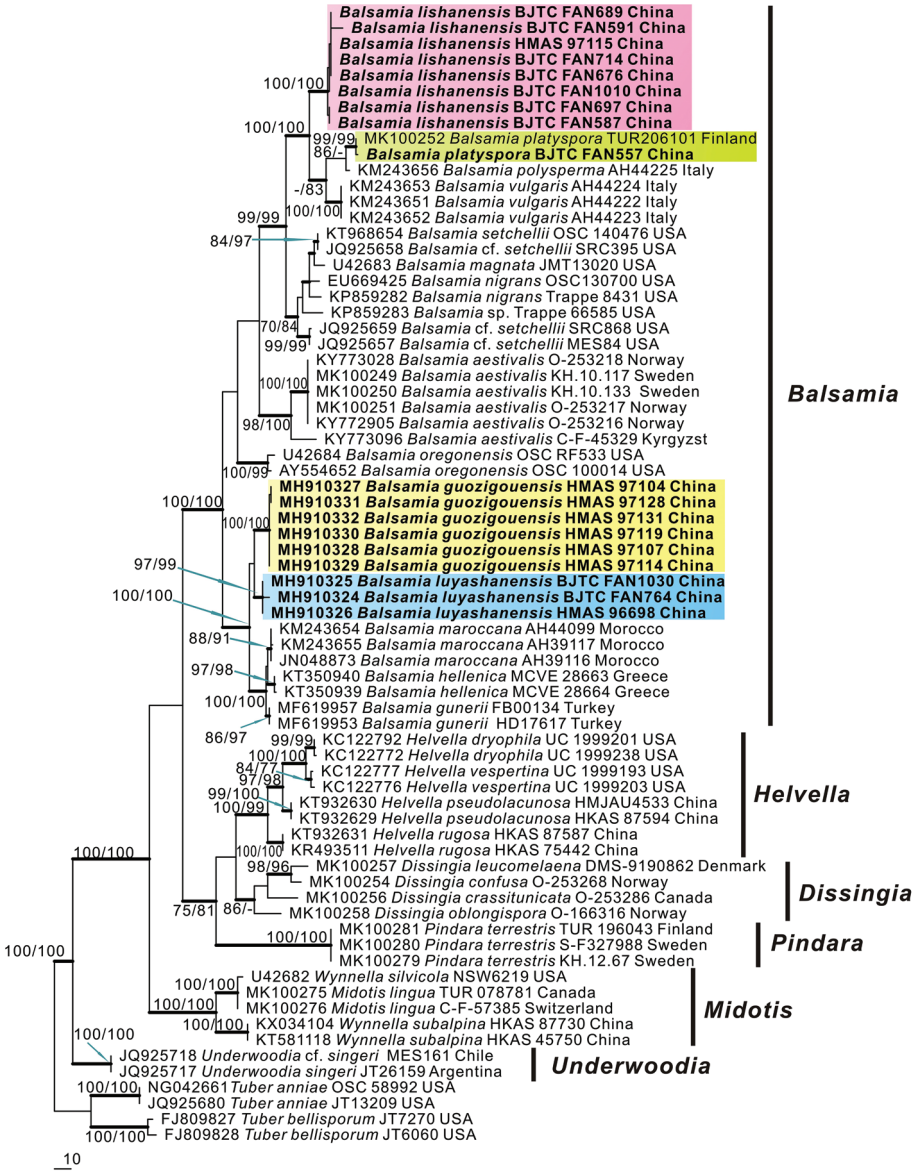

Figure 1. Phylogenetic tree generated from a maximum parsimonious analysis based on 28S sequences, showing the phylogenetic relationships of *Helvellaceae*. *Tuber anniae* and *T. bellisporum* are the outgroups. Maximum parsimonious bootstrap support values (≥ 70%) and maximum likelihood bootstrap support values (≥ 70%) are indicated above the nodes as MPBS/MLBS. Thick black branches received Bayesian posterior probabilities (BPP) ≥ 0.95. Novel sequences are printed in bold.

The ITS dataset contained 108 sequences (9 were newly gained in this study), and 4 from the outgroup *T. anniae* and *T. bellisporum*. The dataset had an aligned length of 1056 characters, of which 310 were constant, 745 were variable, and 622

of these variable sites were informative. The maximum parsimony analysis resulted in one most parsimonious tree with a length (TL) of 2220 steps, consistency index (CI) of 0.580, retention index (RI) of 0.900, homoplasy index (HI) of 0.420. MP, ML and BI analyses yielded similar tree topologies, and only the tree inferred from the Bayesian analysis is shown (Fig. 2). The ITS sequences of *Balsamia* were grouped into a distinct clade with high supports (MPBS = 100%, MLBS = 100%, BPP = 1.00), and the sequences from the Chinese collection unambiguously clustered in the *Balsamia*

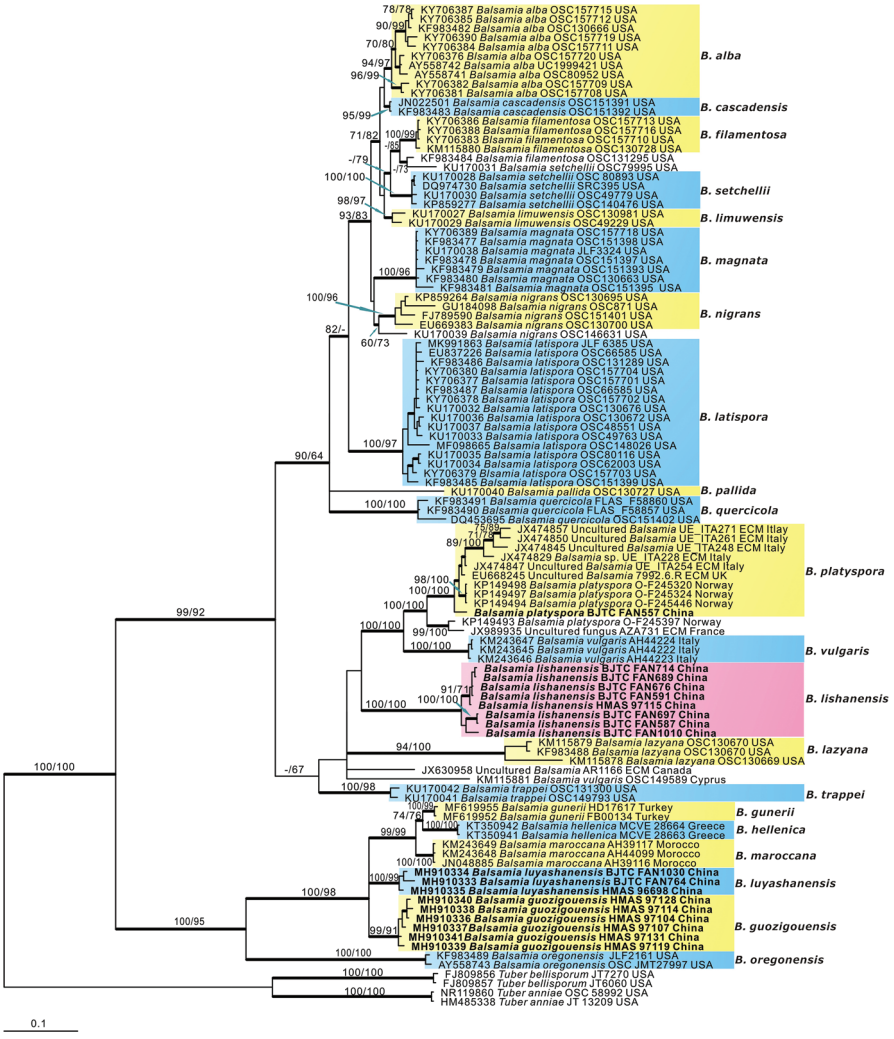

Figure 2. Phylogenetic tree generated from Bayesian analysis based on ITS sequences, showing the phylogenetic relationships of *Balsamia*. *Tuber anniae* and *T. bellisporum* are the outgroups. Maximum parsimonious bootstrap support values (≥ 70%) and maximum likelihood bootstrap support values (≥ 70%) are indicated above the nodes as MPBS/MLBS. Thick black branches received Bayesian posterior probabilities (BPP) ≥ 0.95. Novel sequences are printed in bold.

clade, including the sequences of the fungi previously described as *Barrsia guozigouensis* and *Barssia luyashanensis* (Xu et al. 2018) (Fig. 2). The sequences of all Chinese collections excepting specimen BJTC FAN557 were grouped into three independent clades with strong supports (Fig. 2), respectively representing *Balsamia guozigouensis*, *Balsamia luyashanensis* and a new species *Balsamia lishanensis* proposed in this study. The sequence of BJTC FAN557, which was identified as *B. platyspora* by morphology and 28S phylogeny (Fig. 1) in this study, formed a strong support clade together with nine European sequences isolated from ascomata of *Balsamia platyspora* or ectomycorrhizal root tips of *Balsamia*. These ten samples showed high sequences similarity so the clade was considered as representing *B. platyspora*.

Based on the above phylogenetic analyses (Figs 1, 2), we concluded that *Barrsia guozigouensis* and *Barssia luyashanensis* should be transferred to *Balsamia*. The clade of *B. lishanensis* was a distinct species and represented a new species. The specimen BJTC FAN557 should be recognized as the European *Balsamia platyspora*.

Taxonomy

***Balsamia lishanensis* L. Fan & Y.Y. Xu, sp. nov.**
MycoBank No: 834962
Figure 3

Etymology. *lishanensis*, Lishan Mountain, referring to the locality where the type specimen was collected.

Holotype. China. Shanxi Province, Yuanqu County, Lishan Mountain Shunwangping Scenic Area, alt. 2300 m, 17 October 2016, in soil under *Pinus armandii* Franch., M. Chen CM019 (BJTC FAN676).

Ascomata subglobose to irregularly subglobose, 3–14 × 2–12 mm in fresh, reddish brown when fresh, usually with some superficial furrows, surface covered with verrucose or fine warts, warts obtuse or pointed, 270–400 μm wide and 150–300 μm high. Odor light, mushroom flavor. Gleba solid, white to cream white, with numerous irregular canals and chambers of around 1 mm width. Peridium 150–350 μm thick, two-layered, outer layer pseudoparenchymatous, 90–190 μm thick, composed of 3–5 layers of reddish brown polygonal cells with 4-6 sides, cells 15–35 × 10–27 μm, walls 4.0–8.0 μm thick, the outermost cells reddish-brown, and gradually light-yellow to hyaline towards inner side; inner layer 60–150 μm thick, composed of interwoven hyphae, that is more or less parallel to the surface of peridium, hyphae hyaline, 2.5–6.0 μm wide. Paraphyses line the surface of chamber, arranged like a fence, 3–4 × 50 μm, but disorganized in the mature ascomata, usually not well-defined. Asci 8-spored, hyaline, citriform or fusiform, 55–80 × 27–38 μm (not including stalk), inamyloid, with a slender-stalk of 13.5–35 × 5–10 μm, spores irregularly arranged in ascus. Ascospores ellipsoid, smooth, hyaline, inamyloid, 20.6–25.6 × 12.9–15 μm (av. 23.5 × 14.0 μm), Q (L/I) = 1.55–1.80 (Qm = 1.68) (n = 30), usually containing one large oil drop and several small droplets.

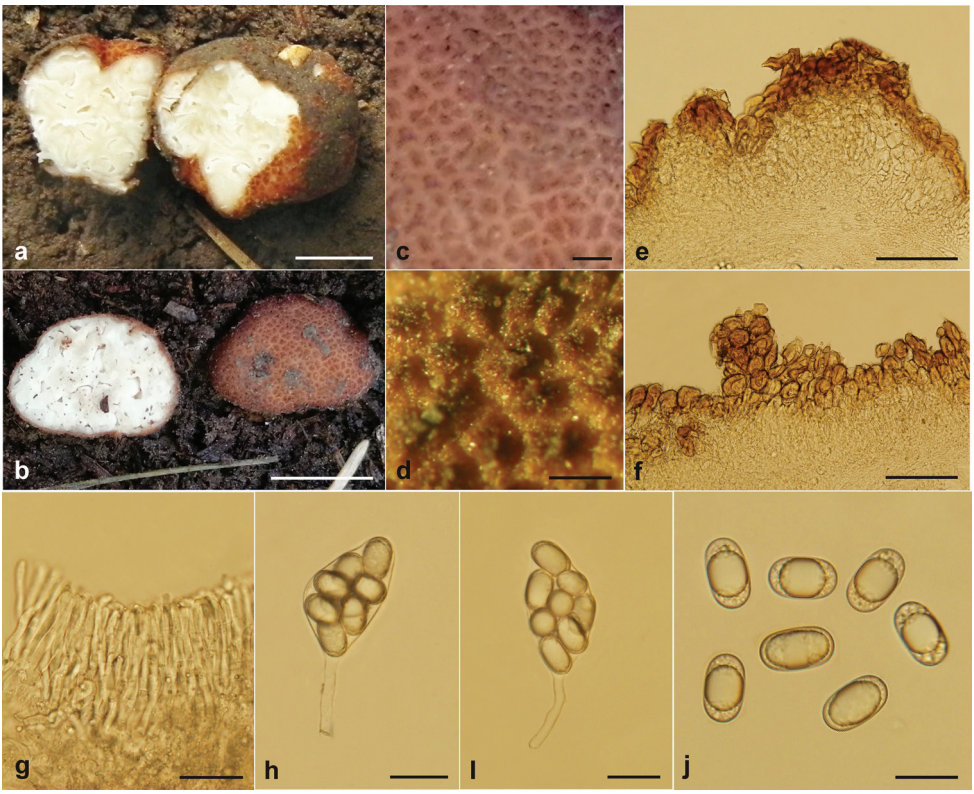

Figure 3. *Balsamia lishanensis* (BJTC FAN676, holotype) **a, b** ascomata **c** warts (when fresh) **d** warts (when dry) **e, f** peridium **g** paraphyses **h, i** mature ascus **j** ascospores. Scale bars: 5 mm (**a, b**); 500 μm (**c**); 300 μm (**d**); 100 μm (**e, f**); 25 μm (**g, h, i**); 20 μm (**j**).

Other materials examined. China. Shanxi Province, Yuanqu County, Lishan Mountain Shunwangping Scenic Area, alt. 2300m, 16 August 2016, in soil under *Pinus armandii* Franch., K.B. Huang HKB003 (BJTC FAN587); *ibid.*, 16 August 2016, in soil under *Pinus armandii* Franch., B.D. He HBD014 (BJTC FAN 591); *ibid.*, 17 October 2016, in soil under *Pinus armandii* Franch., K.B. Huang HKB039 (BJTC FAN689); *ibid.*, 17 October 2016, in soil under *Pinus armandii* Franch., X.Y. Sang SXY015 (BJTC FAN697); *ibid.*, 17 October 2016, in soil under *Pinus armandii* Franch., K.B. Huang HKB031 (BJTC FAN714); China. Shanxi Province, Ningwu County, Xiaoshidong Village, Guancen Mountain, alt. 2000m, 12 October 2017, in soil under *Picea asperata* Mast., L.J. Guo GLJ001 (BJTC FAN1010); China. Gansu Province, Bailongjiang Forestry Bureau, Seventh Forest Farm, alt. 2500m, 14 July 2002, in soil under *Pinus* sp., D.J. Ren & M.S. Song 02-034 (HMAS 97115).

Notes. *Balsamia lishanensis* was diagnosed by the combination of reddish brown ascomata covered with fine warts, the whitish gleba with numerous small chambers open to 1 mm, 3–5 layers peridium reddish brown polygonal cells and the smooth and regular ellipsoid ascospores with one large oil drop. There are four *Balsamia* spe-

cies similar to *B. lishanensis* in morphology. Of them, *B. vulgaris* differed by its large ascospores of (23–) 26–32 (–36) × 11.5–14 (–16) µm, *Balsamia lazyana* and *B. trappei* by their narrow ascospores, which are 19.5–27 × 8–11.5 µm in *B. lazyana* and 24–26 × 11.5–13.5 µm in *B. trappei*, *B. platyspora* by its short-ellipsoid ascospores of 19–22–28 × 12–13–16 µm (ca. 20 × 13 µm). Phylogenetic analysis revealed that the sequences of *B. lishanensis* were grouped into an independent clade with strong support value (Figs 1, 2). DNA analysis showed that *B. lishanensis* shared less than 87.19% identity in ITS sequence with other *Balsamia* species. These supported the erection of the new species.

Balsamia platyspora **Berk. Ann. Mag. Nat. Hist.13: 358(1884)**
Figure 4

Materials examined. China. Shanxi Province, Yuanqu County, Lishan Mountain Shunwangping Scenic Area, alt. 2200m, 16 August 2016, in soil under *Pinus armandii* Franch., Y.W. Wang WYW012 (BJTC FAN557).

Notes. *Balsamia platyspora* is distributed in Europe, North America and Asia (Berkely 1844; Gilkey 1939). In China, it is reported as early as 1990 from Shanxi Province based on morphological evidences (Liu and Tao 1990), but unfortunately, we have been unable to find the voucher specimen. In this study, our molecular analysis based on 28S sequences (Fig. 1) and ITS sequences (Fig. 2), and morphological studies confirmed the occurrence of this species in China based on the new collections from the Shanxi Province where this species was harvested originally by Liu and Tao (1990). *Balsamia platyspora* is mainly characterized by its minor subglobose ascomata,

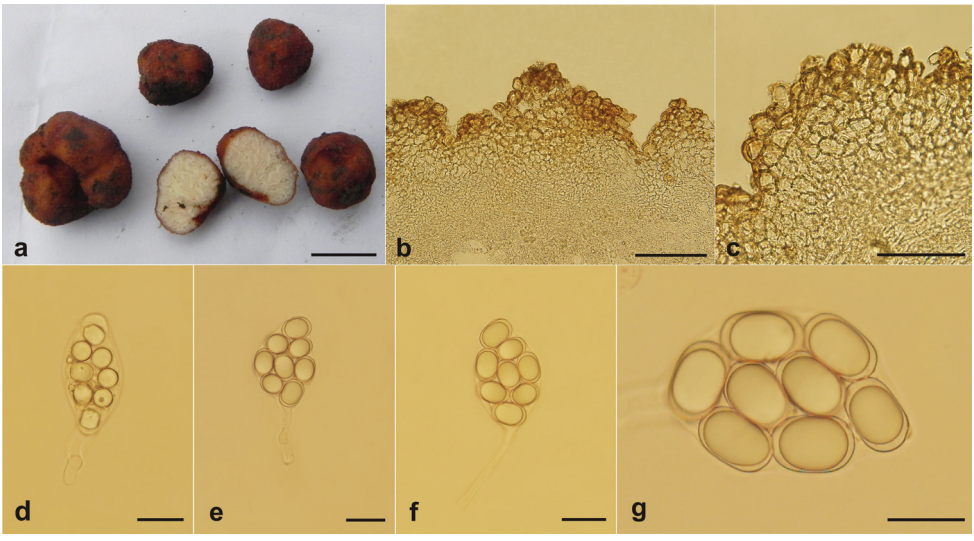

Figure 4. *Balsamia platyspora* (BJTC FAN557) **a** ascomata **b** peridium **c** warts **d** immature ascus **e, f** mature ascus **g** ascospores. Scale bars: 1 cm (**a**); 100 µm (**b, c**); 25 µm (**d, e, f**); 20 µm (**g**).

reddish brown to dark brown warts, white to yellowish white gleba with around 1 mm chambers, citriform or broadly elliptic asci, shortly elliptic ascospores 19–22–28 × 12–13–16 µm (ca. 20 × 13 µm) (Berkeley 1844; Hawker 1954; Pegler et al.1993), our specimen well matched the characteristics.

Balsamia guozigouensis **(L. Fan & Y.Y. Xu) L. Fan & Y.Y. Xu., comb. nov.**
MycoBank No: 834963

Basionym. *Barssia guozigouensis* L. Fan & Y.Y. Xu, Phytotaxa 374(2): 135 (2018).

Holotype. China. Xinjiang Autonomous Region, Huocheng County, Guozigou Forest Park, alt. 1800m, in soil under *Picea schrenkiana* Fisch. & C.A. Mey., 11 August 2003, W.P. Wu & M. S. Song 060 (HMAS 97107).

Illustrations – Xu et al. (2018: Fig. 4)

Notes. This species is recently described from Xinjiang Autonomous Region, China, under *Picea schrenkiana* Fisch. & C.A. Mey. So far it is known only from the type locality. *Balsamia guozigouensis* can be recognized by its distinctly warty ascomata, solid gleba with small and irregular chamber and irregularly clavate asci. Phylogenetically, it was closely related to *B. luyashanensis* (Fig. 2), but the latter differs in its ascomata with fine warts and gleba without chambers (Xu et al. 2018).

Balsamia luyashanensis **(L. Fan & Y.Y. Xu) L. Fan & Y.Y. Xu., comb. nov.**
MycoBank No: 834964

Basionym. *Barssia luyashanensis* L. Fan & Y.Y. Xu, Phytotaxa 374(2): 134 (2018).

Holotype. China. Shanxi Province, Ningwu County, Qiuqiangou Village, Luyashan Mountain, alt. 2100m, 25 August 2017, in soil under *Picea* sp., M. Chen CM023 (BJTC FAN764).

Illustrations – Xu et al. (2018: fig. 3)

Notes. *Balsamia luyashanensis* is also recently described from the Luyashan Mountain of Shanxi Province, China, under *Picea* sp. So far it is known only from the type locality. The species can be recognized by its red brown ascomata with fine warts, gleba without chambers and irregularly clavate asci (Xu et al. 2018). The species was similar in appearance of ascomata to *B. gunerii* and *B. hellenica* but *B. gunerii* can be separated by its subglobose to ovoid ascospores and gleba with irregularly sinuous, labyrinth-like veins (Doğan et al. 2018; Hansen et al. 2019), while *B. hellenica* by its ovoid ascospores (Kaounas et al. 2015; Hansen et al. 2019).

Key to Chinese species of *Balsamia*

1 Ascomata with an obvious apical depression **2**
– Ascomata without an obvious apical depression **3**

2 Surface with distinct warts, solid gleba scattered with some small and irregular chambers ..... ***B. guozigouensis***
– Surface with fine warts, solid gleba without chambers..... ***B. luyashanensis***
3 Ascospores long-ellipsoid, (20.6–25.6 × 12.9–15 μm, Q = 1.55–1.80) ..... ***B. lishanensis***
– Ascospores short-ellipsoid, 19–22–28 × 12–13–16 μm (ca. 20 × 13 μm) (Hawker 1954) ..... ***B. platyspora***

Acknowledgements

Dr. J.Z. Cao was appreciated for collecting specimens and providing valuable suggestions. The study was supported by the National Natural Science Foundation of China (No. 31750001) and the Beijing Natural Science Foundation (No. 5172003).

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Supplementary material 1

Table S1. Taxa used in this study and their GenBank accession numbers for ITS and 28S sequence data

Authors: Yu-Yan Xu, Xiang-Yuan Yan, Ting Li, Li Fan

Data type: Microsoft Word Document (.docx)

Link: https://doi.org/10.3897/mycokeys.67.50068.suppl1

MycoKeys 67: 95–124 (2020)
doi: 10.3897/mycokeys.67.52716
http://mycokeys.pensoft.net

RESEARCH ARTICLE

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Diversity and toxigenicity of fungi and description of *Fusarium madaense* sp. nov. from cereals, legumes and soils in north-central Nigeria

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Academic editor: C. Gueidan | Received 31 March 2020 | Accepted 15 April 2020 | Published 8 June 2020

Citation: Ezekiel CN, Kraak B, Sandoval-Denis M, Sulyok M, Oyedele OA, Ayeni KI, Makinde OM, Akinyemi OM, Krska R, Crous PW, Houbraken J (2020) Diversity and toxigenicity of fungi and description of *Fusarium madaense* sp. nov. from cereals, legumes and soils in north-central Nigeria. MycoKeys 67: 95–124. https://doi.org/10.3897/mycokeys.67.52716

Abstract

Mycological investigation of various foods (mainly cowpea, groundnut, maize, rice, sorghum) and agricultural soils from two states in north-central Nigeria (Nasarawa and Niger), was conducted in order to understand the role of filamentous fungi in food contamination and public health. A total of 839 fungal isolates were recovered from 84% of the 250 food and all 30 soil samples. Preliminary identifications were made, based on macro- and micromorphological characters. Representative strains (n = 121) were studied in detail using morphology and DNA sequencing, involving genera/species-specific markers, while extrolite profiles using LC-MS/MS were obtained for a selection of strains. The representative strains grouped in seven genera (*Aspergillus*, *Fusarium*, *Macrophomina*, *Meyerozyma*, *Neocosmospora*, *Neotestudina*

and *Phoma*). Amongst the 21 species that were isolated during this study was one novel species belonging to the *Fusarium fujikuroi* species complex, *F. madaense* **sp. nov.**, obtained from groundnut and sorghum in Nasarawa state. The examined strains produced diverse extrolites, including several uncommon compounds: averantinmethylether in *A. aflatoxiformans*; aspergillimide in *A. flavus*; heptelidic acid in *A. austwickii*; desoxypaxillin, kotanin A and paspalitrems (A and B) in *A. aflatoxiformans*, *A. austwickii* and *A. cerealis*; aurasperon C, dimethylsulochrin, fellutanine A, methylorsellinic acid, nigragillin and pyrophen in *A. brunneoviolaceus*; cyclosporins (A, B, C and H) in *A. niger*; methylorsellinic acid, pyrophen and secalonic acid in *A. piperis*; aspulvinone E, fonsecin, kojic acid, kotanin A, malformin C, pyranonigrin and pyrophen in *A. vadensis*; and all compounds in *F. madaense* sp. nov., *Meyerozyma*, *Neocosmospora* and *Neotestudina*. This study provides snapshot data for prediction of food contamination and fungal biodiversity exploitation.

Keywords
Aflatoxins, chemotaxonomy, food safety, *Fusarium*, mycology, secondary metabolites

Introduction

Fungi are ubiquitous and diverse, inhabiting various environments including agricultural soils and the crops grown on them (Stajich et al. 2009). Fungi in soil can contaminate, invade and colonise crops on the field during pre-harvest stages and can remain present during the post-harvest processing stages. Depending on the processing steps, these fungi may later spoil foods during storage or in households or markets when storage conditions are sub-optimal and climatic conditions are favourable for their growth (Prange et al. 2005, Taniwaki et al. 2018). Thus, fungal contamination and colonisation of crops could directly lead to pre- and post-harvest food losses, mycotoxin contamination and indirectly to public health risks from consumption of mycotoxin-contaminated foods (Avery et al. 2019). Additionally, soil could serve as a reservoir for pathogenic fungi, constituting public health hazards to farmers who spend much of their time on farms and have direct contact with agricultural soils. On the positive side, beneficial fungi, including biological control strains and species of industrial relevance, are also present in agricultural soils, waiting to be explored (Donner et al. 2009, Bautista-Rosales et al. 2013, Bandyopadhyay et al. 2016).

Proper characterisation of fungi is fundamental to effectively determine their ecology and roles in the environment. In Nigeria, several studies have focused on fungal contamination of food crops (Adebajo et al. 1994, Bankole et al. 2003, Marley et al. 2004, Afolabi et al. 2006, Adejumo et al. 2007, Atehnkeng et al. 2008, Makun et al. 2009, 2011, Fapohunda et al. 2012, Abdus-Salaam et al. 2016, Oyedele et al. 2017, Ezekiel et al. 2013a, 2013b, 2014, 2016, 2019, Frisvad et al. 2019, Akinfala et al. 2020) and soil (Donner et al. 2009, Bandyopadhyay et al. 2019, Ezekiel et al. 2019). Many of these reports focused mainly on characterising aflatoxigenic *Aspergillus* species, because of their high incidence and their ability to produce aflatoxins and less on other mycotoxins produced by other fungal genera and species. Thus, studies on characterisation of other fungi including *Fusarium*, a genus also comprising im-

portant mycotoxin producers, have rarely been conducted in Nigeria (Marley et al. 2004, Afolabi et al. 2006, Adejumo et al. 2007, Makun et al. 2011, Fapohunda et al. 2012). Regardless of the fungal genera studied, the application of robust taxonomic tools comprising a combination of phenotypic characterisation, DNA sequence-based methods and extrolite profiling for fungal identification is scarce (Frisvad et al. 2019, Ezekiel et al. 2020, Akinfala et al. 2020). This comprehensive approach is valuable due to the high precision, based on the use of species-specific DNA markers (Houbraken et al. 2011, 2012, Samson et al. 2014, 2019).

Therefore, in view of the need to understand the roles of fungi in food contamination and other processes, we conducted a mycological investigation into agricultural crops (foods) commonly consumed and available in agrarian households and soils on which the crops were grown in two north-central states (Nasarawa and Niger) in Nigeria. The two states were selected for this study, based on previous reports (Adetunji et al. 2014, Abdus-Salaam et al. 2015, Oyedele et al. 2017) that implicated these states and/or the agro-ecological zone to which they belong (Southern Guinea Savanna) as regions of moderate-to-high aflatoxin and fumonisin contamination in foods. Consequently, it was necessary to study the fungal diversity in these states.

Materials and methods

Food and soil sampling

Various food (n = 250) and soil (n = 30) samples were collected in two states (Nasarawa and Niger) in north-central Nigeria. Samples were collected in September 2018 (harvest season) and January 2019 (storage season). The distribution of samples by sampling season were: harvest (food, n = 143) and storage (food, n = 107; soil, n = 30). Samples were collected from households within one week of harvest and after three months of storage (storage samples). In each state, food samples (1 kg per sample) were collected from households within three randomly selected communities that are at least 5–20 km apart: Mada station, Tundun Adabu and Yelwa Doma in Nasarawa state and Diko, Nubwa Koro and Sabon Wuse in Niger state. The food samples collected included: cowpea (n = 7); groundnut (n = 53), maize (n = 142), millet (n = 1), rice (n = 23) and sorghum (n = 24). Soil samples were collected from the farmlands belonging to five randomly selected households in each community. Sampled fields were at least 1 km apart. In each field, one composite sample (90–100 g) was collected by traversing the field and taking five subsamples from random points. The depth of soil sampling was 3–4 cm.

Food samples were placed in polyethylene bags whilst soil samples were placed in paper bags. All food samples were fragmented in an electric blender (MX-AC400, Panasonic, India) and stored at 4°C prior to analysis within 48 h. Soil samples were transferred to plastic bags and clods were crushed using a mortar and pestle. Soil samples were then homogenised by hand-mixing prior to immediate fungal analysis.

Mycological studies of food and soil

Fungal isolation

Filamentous fungi, present in the food and soil samples, were isolated and enumerated using the dilution plating technique described by Samson et al. (1995). The fragmented samples (10 g each) were diluted in sterile distilled water (90 ml). Each mixture was homogenised on a vortex mixer for 2 min prior to surface-plating of 100 µl on malt extract agar (MEA; Oxoid, UK). The inoculated plates were incubated at 25 °C for 3 to 5 d. The number of fungal colonies on the plates was counted and the colony forming units per gram (CFU/g) of the analysed samples calculated. Distinct colonies, appearing on the isolation plates, were carefully transferred to freshly prepared MEA plates and incubated at 25 °C for 7 d. All pure cultures were stored at 25 °C on MEA slants in 4 ml vials covered with sterile distilled water.

Characterisation of fungal isolates

Fungal isolates from the food and soil samples were characterised, based on morphological characteristics, DNA sequence data and/or secondary metabolites. The strains were first cultivated on MEA and assessed for macro- and microscopic characters, which were then compared with descriptions in appropriate keys (Frisvad and Samson 2004, Leslie and Summerell 2006, Pitt and Hocking 2009, Samson et al. 2011, 2019). Phenotypically similar isolates were grouped and selected isolates representing each group were identified using a sequence-based approach. For *Fusarium* and *Neocosmospora* spp., colony features and growth rates were assessed using MEA, oatmeal agar (OA), potato dextrose agar (PDA; recipes in Crous et al. 2019) and synthetic nutrient-poor agar (SNA; Nirenberg 1976); and micromorphology was studied using carnation leaf agar (CLA; Fisher et al. 1982) and SNA following protocols described elsewhere (Leslie and Summerell 2006, Sandoval-Denis et al. 2018). For the molecular analysis, DNA was extracted from each selected isolate previously cultivated on MEA at 25 °C for 5 d. Parts of the β-tubulin (*BenA*) and calmodulin (*CaM*) genes of the *Aspergillus* isolates were amplified and sequenced as previously described (Houbraken et al. 2011, 2012, Samson et al. 2019). The ITS regions, a part of the translation elongation factor 1 alpha (*TEF-1α*) and/or the RNA polymerase II second largest subunit (*RPB2*) gene of all the other fungal species were amplified and sequenced in accordance with Groenewald et al. (2005), Groenewald et al. (2013), Chen et al. (2015), Chen et al. (2017) and O'Donnell et al. (1998, 2010). Additionally, partial fragments of the *BenA*, *CaM*, *TEF-1a* and RNA polymerase II largest subunit (*RPB1*) were generated for a subset of *Fusarium* strains, according to O'Donnell et al. (1998, 2009, 2010) and Woudenberg et al. (2009). All generated sequences were compared with the sequences present in the NCBI database and internal curated databases of the Westerdijk Fungal Biodiversity Institute (WI) for confirmation of species identities. The identified isolates are maintained in the working culture collection of WI ("DTO culture collection") and in the

culture collection of WI ("CBS culture collection"). All newly-generated sequences are deposited in GenBank (Suppl. material 1: Table S1).

To further explore the species diversity and determine the presence of putative novel taxa amongst the fusaria, phylogenetic analyses were carried out, based on *BenA, CaM, RPB1, RPB2* and *TEF-1a* sequences. A first analysis, based on partial *RPB2* sequences, was intended to determine the generic distribution of the Nigerian isolates. A second multi-locus analysis, based on the five gene regions above-mentioned, was used to determine the genetic exclusivity of an undescribed phylogenetic clade belonging to the *Fusarium fujikuroi* species complex (FFSC, O'Donnell et al. 2015, Choi et al. 2018). Additional sequences of type and reference strains were retrieved from GenBank and included in the analyses (Suppl. material 1: Table S2). Sequences of the individual loci were aligned using MAFFT v. 7.110 (Katoh et al. 2017). The individual gene datasets were assessed for incongruency prior to concatenation using a 70% reciprocal bootstrap criterion (Mason-Gamer and Kellogg 1996). Phylogenetic analyses were based on Maximum-Likelihood (ML) and Maximum-Parsimony (MP). For ML, randomised accelerated (sic) ML (RAxML) for high performance computing (Stamatakis 2014) was used on the CIPRES Science Gateway portal (Miller et al. 2012) and clade stability was tested with a bootstrap analysis (BS) using default parameters. For MP, PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford 2003) was used and phylogenetic relationships were estimated by heuristic searches with 1000 random addition sequences with tree-bisection-reconnection and branch swapping option set to 'best trees' only. All characters were weighted equally and alignment gaps treated as missing data. Tree length (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) were calculated. Clade stability was assessed by bootstrap analyses, based on 1000 replications.

For extrolite profiling, each representative *Aspergillus* isolate was grown on Czapek yeast autolysate (CYA) agar, MEA and yeast extract sucrose (YES) agar and the selected *Fusarium* and *Neocosmospora* strains were grown on OA and PDA prior to extraction (Yilmaz et al. 2014, Samson et al. 2019). The culture media were incubated for 7 and 14 d at 25 °C. The agar plug extraction method of Filtenborg et al. (1983) with modifications as described by Smedsgaard (1997) was applied to extract cultural compounds. Extraction solvent for the agar plugs included ethylacetate/dichloromethane/methanol (3:2:1, v/v/v) containing 1% formic acid. All extracts were air-dried prior to LC–MS/MS screening as described below.

LC-MS/MS extrolite analysis of agar plug extracts

Extrolites of fungal cultures were determined by a dilute and shoot LC–MS/MS method (Sulyok et al. 2020). The air-dried extracts were dissolved in 1 ml (ratio 1:1, v/v) of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) and then diluted with acetonitrile/water/acetic acid 20:79:1, v/v/v in a 1:1 (v/v) ratio prior to injection into the LC–MS/MS instrument. The QTrap 5500 LC–MS/MS System (Applied Biosys-

tems, Foster City, CA, USA), equipped with TurboIonSpray electrospray ionisation (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany) was applied to screen the compounds. Chromatographic separation was performed at 25 °C on a Gemini C18–column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 × 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). The chromatographic method, chromatographic and mass spectrometric parameters are as described by Sulyok et al. (2020). ESI-MS/MS was conducted in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ± 20 s and ± 26 s in the positive and the negative modes, respectively. The identified positive analytes were confirmed by the acquisition of two MRMs per analyte. This yielded 4.0 identification points, according to European Commission decision 2002/657 (EC 2002). Additionally, the LC retention time and the intensity ratio of the two MRM transitions were in agreement with the related values of an authentic standard within 0.03 min and 30%, respectively, following the criteria for mycotoxin identification as laid down in SANTE 12089/2016.

Data analysis

The IBM SPSS v21.0 (SPSS Inc., IL, USA) was applied for data analysis. Data on fungal load were first normalised by a logarithm to base 10 transformation of the original data prior to the calculation of mean values. Means were tested for significance by One-way ANOVA ($\alpha = 0.05$). Means of the concentrations (µg/kg) of the extrolites, produced by the fungal strains in culture media, were also calculated.

Results and discussion

Distribution of fungi in food and soil

Fungal propagules were recovered from 84% (n = 209) of the 250 food samples and from all of the soil samples (n = 30). The fungal load in the food samples was significantly ($p < 0.05$) higher at harvest (range: 2.00–6.22; mean: 4.07 ± 0.95 Log_{10}CFU/g) than in storage (range: 2.00–4.60; mean: 3.44 ± 0.69 Log_{10}CFU/g). The load of fungal propagules in the soil samples ranged 2.70–4.20 (mean: 3.45 ± 0.34 Log_{10}CFU/g). Variations observed in fungal load during the two seasons (harvest and storage) may be attributed to the sampling environment and nature of samples. For example, harvest samples were recently collected from the field where crops are in contact with soil and a large diversity of fungal propagules were present (Bankole et al. 2006), whereas storage conditions are often controlled (crops stored individually in local granaries), thereby leading to lower fungal densities (Williams et al. 2014). In addition, harvest

samples were not yet "cleaned" (threshed or deshelled) and so harboured more viable fungal propagules compared to samples collected from storage bins which were already threshed, deshelled and bagged. A similar fungal load found in soil samples in the present study was previously reported for 55 soil samples collected from maize fields (range: 55–3736 CFU/g = 1.74–3.57 Log_{10}CFU/g) across three agro-ecological zones of Nigeria (Donner et al. 2009). A total of 839 fungal isolates were recovered from the food and soil samples and grouped, based on similarities in phenotypic characters. Representative isolates (n = 121) selected from the groups clustered into seven genera (Fig. 1) and 21 species (Fig. 2C), based on a polyphasic taxonomic scheme. The overall incidences of the recovered fungal genera in decreasing order of magnitude were: *Aspergillus* (60%), *Fusarium* (17%), *Neotestudina* (12%), *Neocosmospora* (8%), *Phoma* (2%), *Macrophomina* (1%) and *Meyerozyma* (1%). The overall highest incidence of *Aspergillus* in the samples, as recorded in the present study, agrees with previous studies from different locations and substrates (Atehnkeng et al. 2008, Donner et al. 2009, Diedhiou et al. 2011, Makun et al. 2011, Ezekiel et al. 2013a, 2013b, 2016, 2019, Probst et al. 2014, Oyedele et al. 2017). To the best of our knowledge, we present the first report of *Neotestudina* from Nigerian soil.

Based on the fungal isolates recovered from food and soil samples and identified in this study, sample type-specific fungal incidences were estimated as 40.5%, 28%, 14.9%, 9.9%, 4.9% and 1.7% in soil, maize, sorghum, groundnut, cowpea and rice, respectively. Aspergilli were widely distributed in soil and food, although a higher proportion of isolates (35.6%) was recovered from soil compared to the individual foods. Nine *Aspergillus* species, belonging to two sections, were recovered in this study (Fig. 2). The species include: *A. aflatoxiformans*, *A. austwickii*, *A. cerealis*, *A. flavus* and *A. tamarii* in section *Flavi* (Frisvad et al. 2019) and *A. brunneoviolaceus*, *A. niger*, *A. piperis*

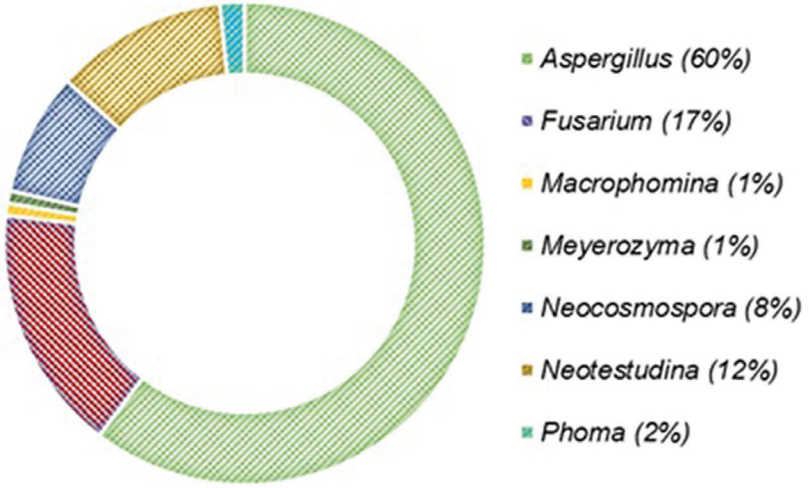

Figure 1. Overall incidence of fungal genera recovered from food and soil in two states in north-central Nigeria.

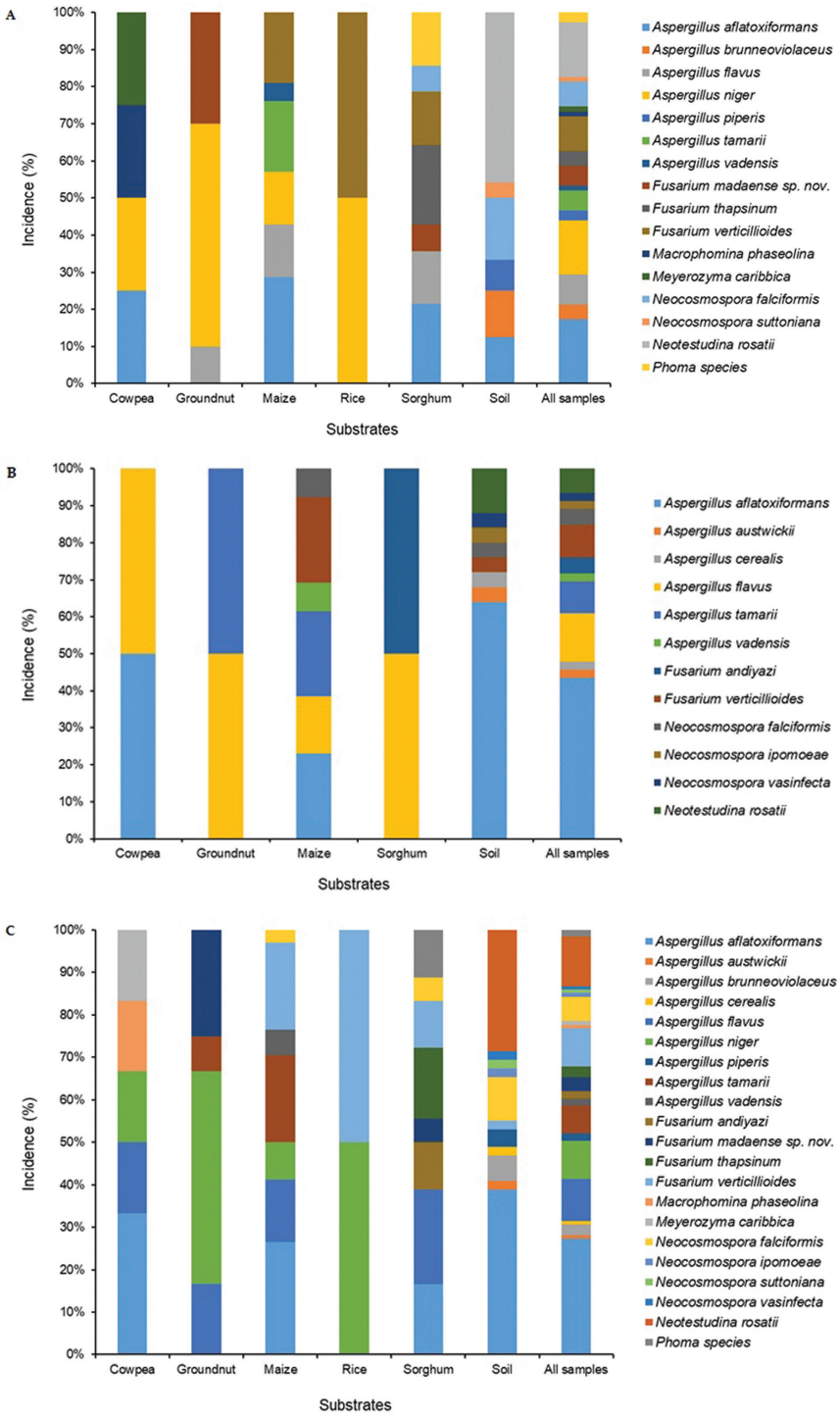

Figure 2. Distribution of fungal species in food and soil in two states **A** Nasarawa state **B** Niger state **C** combined/both states) in north-central Nigeria.

and *A. vadensis* in section *Nigri* (Samson et al. 2007, Perrone et al. 2011, Varga et al. 2011). *Aspergillus aflatoxiformans* (27.3%) was the predominant species found in this study, based on the identified representative isolates (Fig. 2C). However, the predominance of *A. aflatoxiformans* here contradicts several previous reports that presented *A. flavus* as the predominant *Aspergillus* species in food and crops in Nigeria and elsewhere (Atehnkeng et al. 2008, Donner et al. 2009, Ezekiel et al. 2013a, 2013b, 2016, 2019, Probst et al. 2014, Oyedele et al. 2017). The disparity between our finding and previous reports is explainable and owes to bias during sub-culturing and selection of fungal isolates for molecular identification as less emphasis was given to *A. flavus* isolates. With respect to location, *A. austwickii* and *A. cerealis* were recovered only from soil in Niger state, while *A. brunneoviolaceus*, *A. niger* and *A. piperis* were recovered only in food and soil from Nasarawa state. Here, we present for the first time, *A. vadensis* as isolates from Nigerian food.

Four *Fusarium* species (*F. andiyazi*, *F. madaense* sp. nov., *F. thapsinum* and *F. verticillioides*), belonging to FFSC, were identified. All the *Fusarium* spp. were recovered from food samples, except *F. verticillioides* that was found in both food (maize, rice and sorghum) and soil (Fig. 2C). *Fusarium andiyazi* was isolated from sorghum in the Nasarawa state (Fig. 2A), *F. andiyazi* was specific to sorghum from Niger state (Fig. 2B) and *F. madaense* sp. nov. was recovered from groundnut and sorghum from Nasarawa state (Fig. 2A). The diversity of *Fusarium* observed in this study is remarkable, as we present unique occurrences of *F. madaense* sp. nov. and *F. thapsinum* from Nigerian grains, in addition to the previously reported *F. andiyazi* and *F. verticillioides* (Marley et al. 2004, Afolabi et al. 2006, Makun et al. 2009, 2011). *Fusarium verticillioides* and other members of the FFSC, including those found in the present study (*F. andiyazi*, *F. proliferatum*, *F. pseudonygamai*, *F. subglutinans* and *F. thapsinum*), have been documented in cereals and natural environments in different countries (Fandohan et al. 2005, Ncube et al. 2011, Leyva-Madrigal et al. 2014, O'Donnell et al. 2015, Moussa et al. 2017, Choi et al. 2018, Chala et al. 2019). The specificity of *F. andiyazi* and *F. thapsinum* to sorghum, which we observed here, agrees with literature (Marley et al. 2004, Pena et al. 2018, Chala et al. 2019). The additional discovery of *F. madaense* sp. nov. in groundnut and sorghum in this study emphasises the need to adopt adequate and robust characterisation approaches in fungal studies, as well as to conduct large-scale fungal biodiversity studies of food and soil in the country.

Macrophomina phaseolina, *Meyerozyma caribbica* and *Phoma* species were isolated only from food in Nasarawa state (Fig. 2A, C). *Macrophomina phaseolina* and *M. caribbica* were specific to cowpea, while *Phoma* species were recovered from sorghum. *Macrophomina phaseolina* is a common pathogen of legumes, including cowpea and causes charcoal rot and root rot (Amusa et al. 2007, Oladimeji et al. 2012, Sarr et al. 2014, Khan et al. 2017). *Meyerozyma caribbica* (anamorph *Candida fermentati*) is a halophilic and rhizospheric yeast with biological control potential against phytopathogenic fungi (Bautista-Rosales et al. 2013), while *Phoma* is a genus of mostly phytopathogens (Chen et al. 2015, 2017). Future studies may explore the role of *M. caribbica* in biological control of mycotoxigenic fungi found in this study. *Neotestudina rosatii* was present

only in soil (Fig. 2C). This fungus was actually first described in Africa in 1961 as the agent for maduromycosis (Segretain and Destombes 1961) and has been associated with the same human disease in two African countries, Senegal and Somalia (Baylet et al. 1968, Destombes et al. 1977). Four species of *Neocosmospora* (*N. falciformis*, *N. ipomoeae*, *N. suttoniana* and *N. vasinfecta*) were recovered from food and soil (Fig. 2C). *Neocosmospora ipomoeae* and *N. vasinfecta* and *N. suttoniana* were specific only to soils in Niger and Nasarawa states, respectively, while *N. falciformis* was found in food and soil in both states (Fig. 2A, B). *Neocosmospora* (formerly '*Fusarium*' *solani* species complex, FSSC) comprises common pathogens of plants, humans and animals (Sandoval-Denis and Crous 2018). For example, *N. falciformis* (syn. *F. falciforme*) is known to be associated with diverse cutaneous and subcutaneous fungal infections (Dignani and Anaissie 2004, Garcia et al. 2015). This species is frequently found in equine ocular infections and in canines and reptiles (O'Donnell et al. 2016). Recently, Sandoval-Denis and Crous (2018) described *N. suttoniana*; this species is implicated in uncommon human eye infections in Africa and the USA (O'Donnell et al. 2008).

Extrolites produced in fungal cultures

The elucidation of extrolite patterns from fungal strains grown on mycological media, using the highly sensitive LC-MS/MS technique, remains the gold standard chemotaxonomic approach to fungal characterisation (Frisvad et al. 2007, 2018, Samson et al. 2019). In this study, extrolite production in solid media was examined by LC-MS/MS in strains belonging to 20 of the 21 identified fungal species. Cultures of *A. tamarii* and *M. phaseolina* were not included in extrolite analysis. Whereas all compounds were quantitatively determined, aflatrem, asparason A, aspulvinone E, aurasperons, desoxypaxillin, fonsecin, nigragillin, paspalin, paspalinin, paspalitrems and tensidol B were only semi-quantified in the cultures due to lack of a quantitative standard. All the examined fungal strains/species produced at least three (Tables 1, 2) and as many as 33 compounds in *A. aflatoxiformans* (Table 1). Brevianamid F and cyclo(L-Pro-L-Tyr) were detected in examined cultures and cyclo(L-Pro-L-Val) was present in all except three *Neocosmospora* species (*N. falciformis*, *N. ipomoeae* and *N. suttoniana*). These three compounds, found in almost all fungal species in this study, were the only metabolites detected in cultures of *M. caribbica* and *Phoma* sp. in addition to tryptophol in *M. caribbica* (data not shown). Brevianamid F, cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Val) were previously reported in cultures of *A. niger*, *A. tamarii*, *Paecilomyces* and *Talaromyces* from cocoa beans processing in Nigeria (Akinfala et al. 2020).

Aspergillus metabolites

The extrolite patterns of the *Aspergillus* species, isolated and identified in this study, except *A. tamarii* which was not evaluated, are shown in Table 1. Members of the section *Flavi* produced metabolites (aflatoxins and their biosynthetic pathway precursors,

asparason A, cyclopiazonic acid, desoxypaxillin, kojic acid, kotanin A, paspalin and paspalitrems) consistent with previous reports (Frisvad et al. 2019, Uka et al. 2019, Ezekiel et al. 2020). However, some new findings are reported herein. For example, desoxypaxillin, heptelidic acid, kotanin A and paspalitrems were previously reported in *A. flavus* (Uka et al. 2019, Kovac et al. 2020, Ezekiel et al. 2020), but not in *A. aflatoxiformans*, *A. austwickii* and *A. cerealis*. Hence, we present the first report of desoxypaxillin, kotanin A (mean: 534 µg/kg) and paspalitrems in the three S-type sclerotium (minisclerotium) producing species and heptelidic acid (10.2 mg/kg) only in *A. austwickii*. In addition, averantinmethylether (9.4 µg/kg) and aspergillimide (21.3 µg/kg) are two uncommon compounds found only in cultures of one strain of *A. aflatoxiformans* and *A. flavus*, respectively. Similar to a recent report (Ezekiel et al. 2020), two of the four *A. flavus* strains, examined in this study, produced sporogen AO1 (mean: 974 µg/kg), confirming the production of this compound in *A. flavus*. One of the four *A. flavus* strains, DTO 421-G6, did not produce aflatoxins, any of its pathway metabolites, cyclopiazonic acid or kojic acid, but produced cyclosporins A, B, C and H. Cyclosporin production has been reported in *Aspergillus terreus* (Sallam et al. 2003), *Neocosmospora solani* (Sawai et al. 1981) and *Tolypocladium* (El-Enshasy et al. 2008). It is, therefore, suggested that strain DTO 421-G6, whose origin is sorghum grain from Sabon Wuse in Niger state, may be a prospective candidate for biological control of aflatoxins in view of its inability to biosynthesise aflatoxins, its pathway metabolites and cyclopiazonic acid. The biological control product, aflasafe, commercially available for aflatoxin control in Nigeria, contains strains of *A. flavus* original to Niger state and which possesses a similar inability to secrete the aforementioned metabolites (Bandyopadhyay et al. 2016, 2019).

The members of *Aspergillus* section *Nigri* (*A. brunneoviolaceus*, *A. niger*, *A. piperis* and *A. vadensis*) secreted a total of 31 extrolites (Table 1). However, only three extrolites, aurasperons, nigragillin and pyrophen, were common to all four species within this section. Varga et al. (2011) placed *A. brunneoviolaceus* in the *A. aculeatus* clade, whilst *A. niger*, *A. piperis* and *A. vadensis* were grouped into the *A. niger* clade. In the present study, members of the *A. niger* clade shared only four (aspulvinone E, fonsecin, malformins and pyranonigrin) of the compounds. Obviously, high variability in the types of metabolites produced was recorded amongst these closely-related species (Frisvad et al. 2007, Samson et al. 2007). Strains of *A. brunneoviolaceus* (syn. *A. fijiensis*) liberated several known extrolites: aspergillimide (mean: 30.1 mg/kg), emodin (mean: 541 µg/kg), endocrocin (mean: 23 mg/kg), iso-rhodoptilometrin (mean: 340 µg/kg), meleagrin (mean: 7.6 mg/kg), oxaline (mean: 17.3 mg/kg), paraherquamide E (mean: 6 mg/kg) and secalonic acid D (mean: 64.7 mg/kg) (Varga et al. 2011, Vesth et al. 2018, Ezekiel et al. 2020). However, citreorosein and tryprostatin B, two compounds recently reported to be produced by *A. brunneoviolaceus* from *garri* (farinated cassava) in Nigeria (Ezekiel et al. 2020), were not detected in cultures of the present strains. Nonetheless, six uncommon compounds (aurasperon C, dimethylsulochrin (mean: 1.9 mg/kg), fellutanine A (152 µg/kg), methylorsellinic acid (mean: 1.2 mg/kg), nigragillin and pyrophen (mean: 190 µg/kg)) were produced by strains examined in the present study. Of

Table 1. Extrolite production in *Aspergillus* cultures.

Extrolites	*Aspergillus aflatoxiformans*	*Aspergillus austwickii*	*Aspergillus brunneoviolaceus*	*Aspergillus cerealis*	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus piperis*	*Aspergillus vadensis*
3-Nitropropionic acid	+	+	-	+	+	-	-	-
Aflatoxicol	+	-	-	-	+	-	-	-
Aflatoxin B_1	+	+	-	+	+	-	-	-
Aflatoxin B_2	+	+	-	+	+	-	-	-
Aflatoxin G_1	+	+	-	+	-	-	-	-
Aflatoxin G_2	+	+	-	+	-	-	-	-
Aflatoxin M_1	+	+	-	+	+	-	-	-
Aflatrem	+	+	-	+	+	-	-	-
Asparason A	+	+	-	+	+	-	-	-
Asperfuran	+	+	-	+	+	-	-	-
Aspergillimide	-	-	+	-	+	-	-	-
Aspulvinone E	-	-	-	-	-	+	+	+
Aurasperon B	-	-	-	-	-	-	+	+
Aurasperon C	-	-	+	-	-	-	+	+
Aurasperon G	-	-	-	-	-	-	+	+
Averantin	+	+	-	+	+	-	-	-
Averantinmethylether	+	-	-	-	-	-	-	-
Averufin	+	+	-	+	+	-	-	-
Brevianamid F	+	+	+	+	+	+	+	+
Citreorosein	-	-	+	-	-	-	-	-
cyclo(L-Pro-L-Tyr)	+	+	+	+	+	+	+	+
cyclo(L-Pro-L-Val)	+	+	+	+	+	+	+	+
Cyclopiazonsäure	+	+	-	+	+	-	-	-
Cyclosporin A	-	-	-	-	+/-	+	-	-
Cyclosporin B	-	-	-	-	+/-	+	-	-
Cyclosporin C	-	-	-	-	+/-	+	-	-
Cyclosporin H	-	-	-	-	+/-	+	-	-
Demethylsulochrin	-	-	+	-	-	-	-	-
Desoxypaxillin	+	+	-	+	+	-	-	-
Emodin	-	-	+	-	-	+	-	-

Extrolites	*Aspergillus aflatoxiformans*	*Aspergillus austwickii*	*Aspergillus brunneoviolaceus*	*Aspergillus cerealis*	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus piperis*	*Aspergillus vadensis*
Endocrocin	-	-	+	-	-	+	-	-
Fellutanine A	-	-	+	-	-	-	-	-
Fonsecin	-	-	-	-	-	+	+	+
Heptelidic acid	-	+	-	-	+	-	-	-
Iso-Rhodoptilometrin	-	-	+	-	-	+	-	-
Kojic acid	+	+	-	+	+	+	-	+
Kotanin A	+	+	-	+	+	+	-	+
Malformin A	-	-	-	-	-	+	-	-
Malformin C	-	-	-	-	-	+	+	+
Meleagrin	-	-	+	-	-	-	-	-
Methylorsellinic acid	-	-	+	-	-	-	+	-
Nidurufin	+	+	-	+	+	-	-	-
Nigragillin	-	-	+	-	-	+	+	+
Norsolorinic acid	+	+	-	+	-	-	-	-
O-Methylsterigmatocystin	+	+	-	+	+	-	-	-
Oxaline	-	-	+	-	-	-	-	-
Paraherquamide E	-	-	+	-	-	-	-	-
Paspalin	+	+	-	+	+	-	-	-
Paspalinin	+	+	-	+	+	-	-	-
Paspalitrem A	+	+	-	+	+	-	-	-
Paspalitrem B	+	+	-	+	+	-	-	-
Pyranonigrin	-	-	-	-	-	+	+	+
Pyrophen	-	-	+	-	-	+	+	+
Secalonic acid D	-	-	+	-	-	-	+	-
Sporogen AO1	-	-	-	-	+	-	-	-
Sterigmatocystin	+	+	-	+	+	-	-	-
Tensidol B	-	-	-	-	-	+	-	-
Versicolorin A	+	+	-	+	+	-	-	-
Versicolorin C	+	+	-	+	+	-	-	-
Versiconal acetate	+	+	-	+	-	-	-	-

Extrolite produced (+); Extrolite not produced (-).
Produced only by the non-aflatoxigenic strain (+/-).

all the extrolites found in cultures of the four species within the section *Nigri*, aspergillimide, dimethylsulochrin, fellutanine A, meleagrin, oxaline and paraherquamide E were specific to only *A. brunneoviolaceus*, whilst cyclosporins (A, B, C and H) and tensidol B were unique to *A. niger*. This is the first report of cyclosporin production in *A. niger*; only 2/9 of the strains, DTO 422-H5 and DTO 422-H6, were implicated here. In addition, we observed emodin (48 µg/kg) and endocrocin (262 µg/kg) production in only one strain (DTO 421-I7) of *A. niger*. All other compounds found in cultures of *A. niger* in the present study are known extrolites (de Vries et al. 2005, Samson et al. 2007, Nielsen et al. 2009, Perrone et al. 2011, Akinfala et al. 2020).

Two strains of *A. piperis,* screened in this study, secreted compounds agreeable to those previously documented in literature (Samson et al. 2007, Ezekiel et al. 2020). Amongst the extrolites found in the present study are those being reported here for the first time in *A. piperis*: methylorsellinic acid (mean: 1.7 mg/kg), pyrophen (mean: 50 mg/kg) and secalonic acid (24.3 µg/kg) (Table 1). de Vries et al. (2005) examined extrolite production in one strain of *A. vadensis* and found aurasperon B, asperazine, nigragillin and a more polar kotanin-like compound. Here, we report aspulvinone E, aurasperons (B, C and G), fonsecin, kojic acid (mean: 281 µg/kg), kotanin A (91 µg/kg), malformin C (mean: 857 µg/kg), nigragillin, pyranonigrin (mean: 72 mg/kg) and pyrophen (mean: 52.4 mg/kg). The complexity in small molecule chemical profiles observed in the species belonging to the section *Nigri* suggests a high degree of close-relatedness amongst these species (Samson et al. 2007, Nielsen et al. 2009, Perrone et al. 2011).

Extrolites from *Fusarium* and its related fungal species

A total of 15, 12 and 11 extrolites were found in cultures of *Fusarium*, *Neocosmospora* and *Neotestudina* (Table 2). With the exception of *N. vasinfecta*, gibepyron D production was shared by all examined strains of these three genera; higher quantities were found in cultures of *F. madaense* sp. nov. (mean: 4.9 mg/kg) and *F. thapsinum* (mean: 4.2 mg/kg). Gibepyron D is an oxidised derivative of gibepyrone A that has been reported in *F. fujikuroi*, *F. oxysporum* and *F. proliferatum* (Wang et al. 2011, Liu et al. 2013, Janevska et al. 2016). Thus, its production by three fungal genera suggests ancestral relatedness of a gene cluster encoding production of this compound (Janevska et al. 2016). Fumonisins (FA_1 (mean: 616 µg/kg), FB_1 (mean: 3.4 mg/kg), FB_2 (mean: 2 mg/kg) and FB_3 (mean: 1.4 mg/kg)), fusarin C (mean: 27 mg/kg) and fusarinolic acid (mean: 84,856 mg/kg) were exclusively produced by the *Fusarium* species examined in this study. Fumonisins were produced as expected only by *F. verticillioides,* although the cultures of two strains, DTO 421-G2 and DTO 424-H5, did not contain any of the fumonisins. Fumonisin production is a signature in this species as well as in other selected members of the FFSC not found in the present study (Makun et al. 2011, Ncube et al. 2011, de Oliveira Rocha et al. 2011, Fanelli et al. 2012, Rocha et al. 2016, Choi et al. 2018).

All the species of *Fusarium*, except *F. andiyazi*, produced fusarin C in this study. Beauvericin, bikaverin, deoxyfusapyron and fusapyron were found in cultures of certain

Table 2. Extrolite production in cultures of *Fusarium* and related genera.

Extrolites	***Fusarium andiyazi***	***Fusarium thapsinum***	***Fusarium verticillioides***	***Fusarium madaense* sp. nov.**	***Neocosmospora falciformis***	***Neocosmospora ipomoeae***	***Neocosmospora suttoniana***	***Neocosmospora vasinfecta***	***Neotestudina rosatii***
Beauvericin	-	-	-	+	-	-	-	-	+
Bikaverin	+	+	+	+	-	-	-	-	+
Brevianamid F	+	+	+	+	+	+	+	+	+
cyclo(L-Pro-L-Tyr)	+	+	+	+	+	+	+	+	+
cyclo(L-Pro-L-Val)	+	+	+	+	-	-	-	+	+
Cyclosporin A	-	-	-	-	+	-	-	+	-
Cyclosporin B	-	-	-	-	+	-	-	+	-
Cyclosporin C	-	-	-	-	+	-	-	+	-
Cyclosporin D	-	-	-	-	+	-	-	+	-
Cyclosporin H	-	-	-	-	+	-	-	+	-
Deoxyfusapyron	+	-	-	+	-	-	-	-	+
Fumonisin A_1	-	-	+	-	-	-	-	-	-
Fumonisin B_1	-	-	+	-	-	-	-	-	-
Fumonisin B_2	-	-	+	-	-	-	-	-	-
Fumonisin B_3	-	-	+	-	-	-	-	-	-
Fusapyron	+	-	-	+	-	-	-	-	+
Fusaric acid	+	+	+	+	-	-	-	+	+
Fusarin C	-	+	+	+	-	-	-	-	-
Fusarinolic acid	+	+	+	+	-	-	-	-	-
Gibepyron D	+	+	+	+	+	+	+	+	+
Radicicol	-	-	-	-	-	-	-	+	-
Sulochrin	-	-	-	-	-	-	-	-	+
Tryptophol	-	-	-	-	+	-	+	-	+

Extrolite produced (+); Extrolite not produced (-).

species of *Fusarium*, *Neocosmospora* and *Neotestudina*. Specifically, *F. madaense* sp. nov. and *N. rosatii* produced all four aforementioned compounds together with the other three species of *Fusarium* for bikaverin and *F. andiyazi* for deoxyfusapyron and fusapyron. The immunosuppressant cyclosporins [A (mean: 42.2 mg/kg), B (mean: 27.3 mg/kg), C (mean: 29.9 mg/kg), D (mean: 4.6 mg/kg) and H (mean: 32.9 mg/kg)] were specific to *Neocosmospora* and were found only in cultures of *N. falciformis* and *N. vasinfecta*. Radicicol (323 mg/kg) and sulochrin (1.8 mg/kg) were found in one strain of *N. vasinfecta* and *N. rosatii*, respectively. Based on the recorded chemical profiles in this study, the three studied genera are closely related chemotaxonomically. However, *F. madaense* sp. nov. and *N. rosatii* seem to be more closely related than the other species. This is the first chemotaxonomic profiling of *F. madaense* sp. nov., *Meyerozyma*, *Neocosmospora* and *Neotestudina*.

Phylogenetic analyses of *Fusarium* and *Neocosmospora* and description of a novel *Fusarium* species

A first phylogenetic analysis, based on partial *RPB2* sequences, was conducted to identify Nigerian isolates morphologically compatible with *Fusarium* and *Neocosmospora* spp. (Fig. 3). The analysis included 659 positions of 78 isolates, including the two outgroup taxa (*Fusicolla aquaeductuum* NRRL 20686 and *Fusicolla* sp. NRRL 22136), of which 284 bp were constant sites, 375 bp were variable and 336 bp were parsimony-informative. The ingroup taxa included representative isolates of 40 species from 17 species complexes of *Fusarium* and seven species of *Neocosmospora*. Four isolates (CBS 146648, 146651, 146656 and 146669) clustered in a partially supported, putative novel clade, closely related to *F. andiyazi*; the latter taxon, however, clustered in an unresolved phylogenetic position.

To further determine the relationship between the putative novel clade and *F. andiyazi*, a second analysis was conducted which encompassed 4456 positions of five loci (*BenA* 525 bp, *CaM* 545 bp, *RPB1* 978 bp, *RPB2* 1 735 bp and *TEF-1a* 673 bp), of which 3417 were constant (*BenA* 406 bp, *CaM* 421 bp, *RPB1* 777 bp, *RPB2* 1 379 bp and *TEF-1a* 434 bp), 1018 were variable (*BenA* 118 bp, *CaM* 120 bp, *RPB1* 201 bp, *RPB2* 356 bp and *TEF-1a* 223 bp) and 614 were parsimony informative (*BenA* 64 bp, *CaM* 63 bp, *RPB1* 132 bp, *RPB2* 236 bp and *TEF-1a* 119 bp). The final alignment included 44 isolates, representing 35 *Fusarium* spp. from the three biogeographical phylogenetic clades of FFSC (African, American and Asian clades, O'Donnell et al. 1998) plus two outgroups (*F. oxysporum* NRRL 20433 and NRRL 22902) (Fig. 4). The multi-locus phylogeny confirmed the previous results. The putative novel clade (CBS 146648, 146651, 146656 and 146669) was resolved as a fully supported phylogenetic lineage (MP BS = 100, ML BS = 100), sister to a moderately-supported clade (MP BS 97, ML BS 100), encompassing the ex-type strain of *F. andiyazi* (CBS 119857), plus four additional representative isolates of the latter species, two of them (CBS 146647 and 146657) being obtained in this study. The novel phylogenetic lineage is here recognised as *Fusarium madaense* sp. nov.

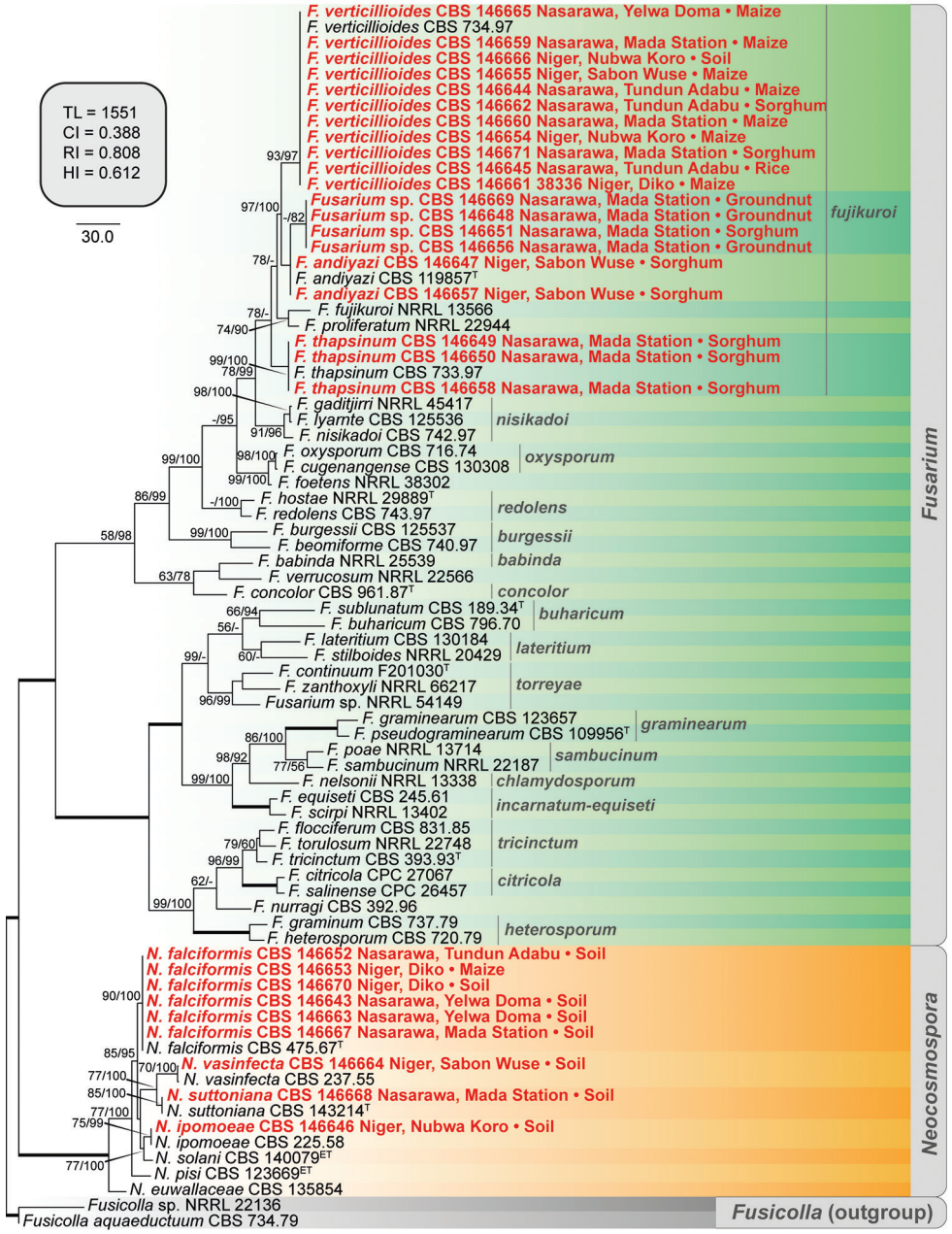

Figure 3. The first of 1000 equally parsimonious trees obtained from Maximum-Parsimony (MP) analysis of *RPB2* sequences of 76 isolates of *Fusarium* and *Neocosmospora* spp. Numbers on the nodes are MP bootstrap values (BS) and Maximum-Likelihood BS values above 70%. Branch lengths are proportional to distance. Ex-type and ex-epitype strains are indicated with ^{T} and ^{ET}, respectively. The names of 17 species complexes of *Fusarium* are shown in grey. Nigerian isolates obtained in this study are shown in red together with their geographical origin and source of isolation. The internal square shows MP statistics as follows: TL = tree length, CI = consistency index, RI = retention index and HI = homoplasy index.

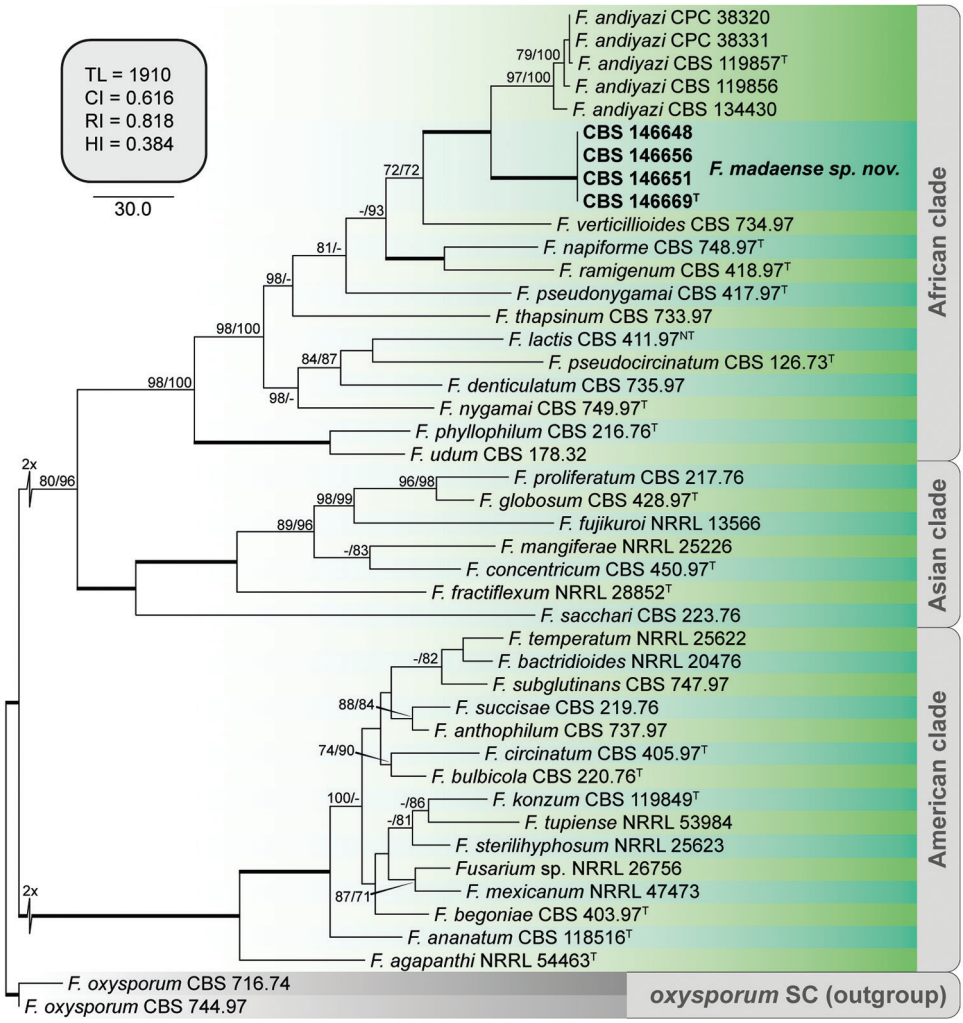

Figure 4. The first of 24 equally parsimonious trees obtained from Maximum-Parsimony (MP) analysis of *BenA, CaM, RPB1, RPB2* and *TEF-1a* sequences of 42 isolates of *Fusarium* spp. Numbers on the nodes are MP bootstrap values (BS) and Maximum-Likelihood BS values above 70%. Branch lengths are proportional to distance. Ex-type strains are indicated with T. Strains corresponding to new species described here are shown in **bold**. The internal square shows MP statistics as follows: TL = tree length, CI = consistency index, RI = retention index and HI = homoplasy index.

Taxonomy

Fusarium madaense **Ezekiel, Sand.-Den., Houbraken & Crous, sp. nov.**
MycoBank No: MB835266
Figure 5

Diagnosis. Different from *F. thapsinum* by the absence of napiform microconidia. Different from *F. andiyazi*, *F. thapsinum* and *F. verticillioides* by its lighter colony pig-

mentation, growth rates, microconidial septation, presence of true chlamydospores and secondary metabolite patterns.

Type. Nigeria, Nasarawa, Mada Station, from groundnut (*Arachis hypogaea*), Sep. 2018, C.N. Ezekiel, holotype CBS H-24346, ex-holotype strain CBS 146669 = CPC 38344 = 12B(3)2.

Description. Colonies grown in the dark at 24°C. On MEA and PDA with an average radial growth rate of 5.9–6.5 mm/d and filling an entire 90 mm Petri dish in 7 d. Surface white to pale rosy buff, flat, velvety to felty with abundant patches of white aerial mycelium; margin regular, filiform. Reverse pale saffron to peach, a pale bay diffusible pigment can be scarcely produced. On OA, occupying an entire 90 mm Petri dish in 7 d. Surface white to pale rosy buff, flat, velvety to felty with abundant patches of white aerial mycelium; margin regular. Reverse pale luteous to saffron. On SNA, reaching 24–25 mm diam. in 7 d. Surface white, velvety, with scarce aerial mycelium, margins filiform. Reverse white.

Conidiophores on aerial mycelium straight, erect, septate, smooth- and thin-walled, commonly simple or reduced to conidiogenous cells, borne laterally on hyphae or laterally branched at various levels, bearing terminal single monophialides; *phialides* subulate to subcylindrical, smooth- and thin-walled, (17–)25.5–39.5 µm long, (2–)2.5–3.5 µm at widest point, periclinal thickening and collarettes inconspicuous or absent; *microconidia* hyaline, clavate, smooth- and thin-walled, 0–3-septate, (7–)9–15(–21) × (2–)2.5–4(–5) µm, arranged in long chains at the tip of monophialides. *Sporodochia* pale to bright orange, formed abundantly on the surface of carnation leaves and on agar surface. *Conidiophores* in sporodochia, 21–60 µm tall, simple or irregularly and verticillately branched, bearing terminal, single monophialides or groups up 2–3 monophialides; *sporodochial phialides* doliiform to subcylindrical, (10.5–)13–18(–20.5) × (2.5–)3–4(–4.5) µm, smooth- and thin-walled, with conspicuous periclinal thickening and an often short apical collarette. *Sporodochial conidia* lunate to falcate, tapering towards apical and basal ends, moderately curved dorsiventrally or with an almost straight ventral part; apical cell more or less equally sized than the adjacent cell, apically slightly elongated to papillate; basal cell distinctly notched, (0–)1–5(–6)-septate, hyaline, thin- and smooth-walled. Aseptate conidia: (38–)38.5–42(–44) × 3.5–4.5 µm; one-septate conidia: (37.5–)40–48(–53) × 3.5–4(–4.5) µm; two-septate conidia: 43 × 3.7 µm; three-septate conidia: (29–)38–48.5(–61.5) × (3–)4–4.5(–5) µm; four-septate conidia: (45–)46.5–54(–59) × (3.5–)4–4.5(–5) µm; five-septate conidia: 47.5–55.5(–60) × 4–4.5 µm; six-septate conidia: 55.5 × 4.5 µm; overall (29–)38.5–50(–61.5) × (3–)4–4.5(–5) µm. *Chlamydospores* present on MEA, PDA and SNA, globose to subglobose, hyaline, smooth and thick-walled, (6–)6.5–8.5(–10) µm diam., terminal or intercalary in the aerial hyphae, solitary or in chains

Distribution. Nigeria.

Etymology. Name refers to Mada Station, a locality in Nasarawa State, Nigeria, where the species was found.

Additional isolates examined. Nigeria, Mada Station, from groundnut (*Arachis hypogaea*), Sept 2018, C.N. Ezekiel, CBS 146648 = CPC 38321 = 12B(3), CBS 146656 = CPC 38330 = 12B(5); from sorghum, Jan 2019, C.N. Ezekiel, CBS 146651 = CPC 38324 = 7S(6).

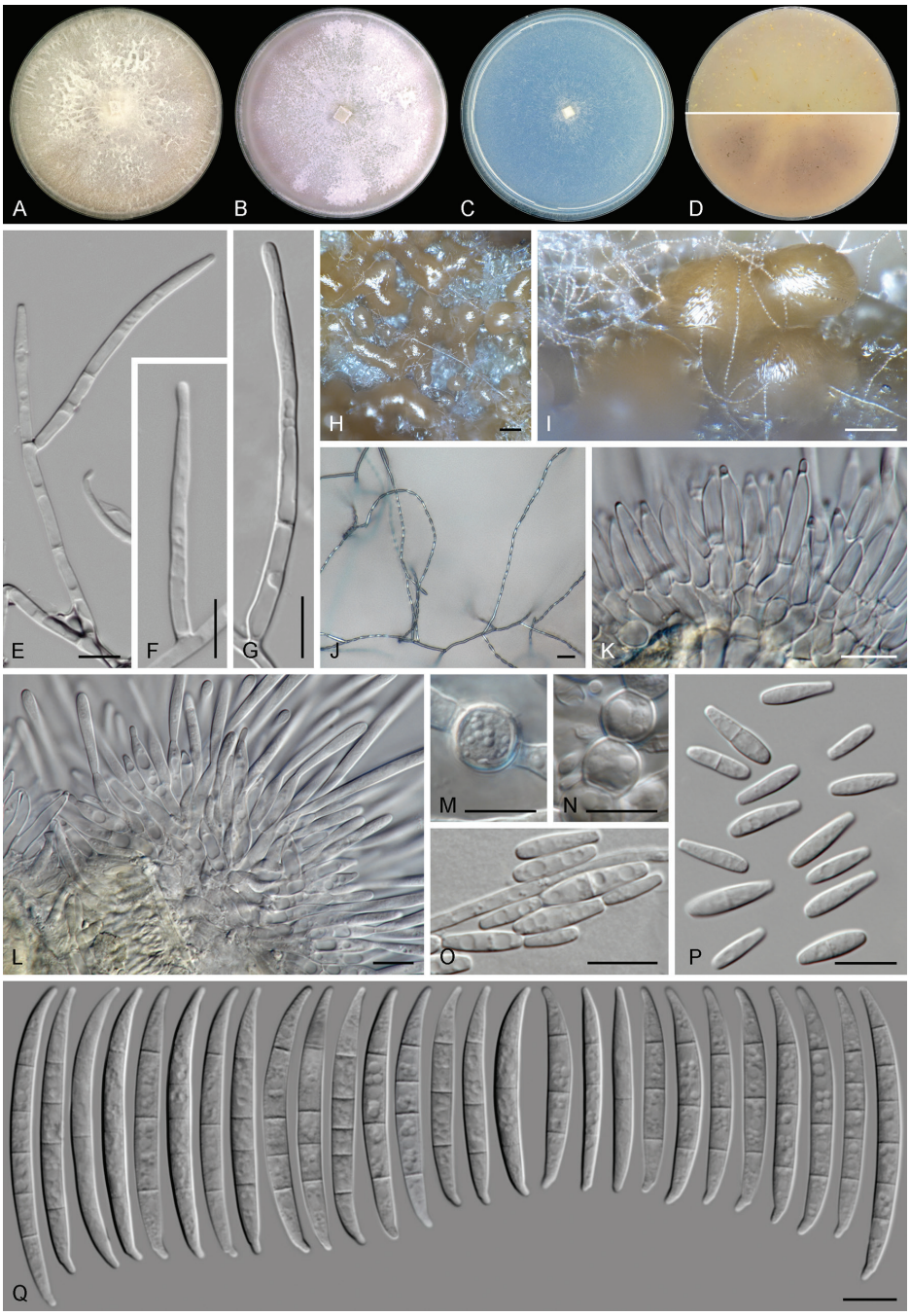

Figure 5. *Fusarium madaense* sp. nov. (ex-type culture CBS 146669). **A–C** aspect of colonies on PDA, OA and SNA, respectively, after 14 d at 24°C in the dark **D** colony reverse on OA (up) and PDA (down) after 14 d at 24 °C in the dark **E–G, J** aerial conidiophores and phialides **H, I** sporodochia formed on the surface of carnation leaves **K, L** sporodochial conidiophores **M, N** chlamydospores **O, P** microconidia **Q** sporodochial conidia. Scale bars: 100 µm (**H, I**); 20 µm (**J**); 10 µm (all others).

Notes. Although clearly recognisable based on genetic markers, *Fusarium madaense* is hardly distinguishable from its closer relatives, based on morphological features only. The novel species is characterised by abundant long, slender and slightly curved macroconidia, a morphology typical of the FFSC. The overall morphology of *F. madaense* is similar to that of *F. andiyazi*, *F. thapsinum* and *F. verticillioides*; all species are characterised by clavate microconidia formed in long chains from relatively long monophialides. Moreover, the four mentioned species are known to be pathogenic on sorghum (Leslie and Summerell 2006) and have been isolated here from the same geographical regions. Nevertheless, some morphological features of *F. madaense* can provide an indication of its identity. These include a pale saffron colony pigmentation on OA and PDA, not developing the intense purple colour typical of *F. andiyazi* and *F. verticillioides*, nor the yellow pigmentation of *F. thapsinum*; the presence of up to 3-septate clavate microconidia vs. the up to 2-septate and aseptate microconidia of *F. andiyazi* and *F. verticillioides*, respectively; and the aseptate, but also rarely napiform microconidia of *F. thapsinum* (Nirenberg 1976, Klittich et al. 1997, Marasas et al. 2001). In addition, *F. madaense* can be differentiated from *F. andiyazi*, its closest morphological and phylogenetic relative, by its slightly faster growth rates on PDA, somewhat wider macroconidia and the presence of true chlamydospores.

The proposal of the novel species *F. madaense* and its differentiation from *F. andiyazi*, *F. thapsinum* and *F. verticillioides* is also supported by secondary metabolite profiling of all the above-mentioned species, as found in this study. *Fusarium madaense* was the only beauvericin-producing species in our dataset. Nevertheless, it has been reported that *F. verticillioides* strains can produce trace levels of this toxin (Leslie et al. 2004, Leslie and Summerell 2006). The alpha-pyrones deoxyfusapyron and fusapyron were produced only by *F. madaense* and its closest relative *F. andiyazi*; by contrast, fusarin C was produced by *F. madaense*, *F. thapsinum* and *F. verticillioides*, but not by *F. andiyazi*.

Conclusions

We have shown the importance of applying robust taxonomic approaches to fungal characterisation in this study. Here, diverse fungal species, including those not previously reported from Nigerian food and soil, as well as a novel *Fusarium* species, *F. madaense* sp. nov., were identified and described. Several of these species possess mycotoxigenic, as well as plant, human and animal pathogenic, potential. We further elucidated the secondary metabolite profiles of strains within the identified fungal species. A handful of small molecule compounds were found in the cultures of the strains, including several compounds not previously reported from some strains; a few could serve as species-specific chemotaxonomic markers. Overall, we provide snapshot data on the fungal biodiversity in two north-central Nigerian states. The findings of this study are valuable to guide researchers to predict mycotoxin contamination of crops/food and possible sources of fungal infections in humans and animals, as well as to find, where unavailable and implement where available, strategies towards the control of problematic fungi and the adverse effects they may pose.

Acknowledgements

The authors are thankful to Oluwaseun T. Ojuri, Oluwabunmi Ogunniran and Dr. Isaac M. Ogara for being helpful during sampling and isolation of fungi. CN Ezekiel deeply appreciates the International Foundation of Sciences (Sweden) for partly supporting this study through an Individual Grant (Number: I-3-E-6046-1).

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Supplementary material 1

Tables S1, S2

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Data type: COL

Explanation note: **Table S1.** Strain details and GenBank accession numbers of molecularly identified isolates included in this study. **Table S2.** Strain data and accession numbers of additional strains included in phylogenetic analyses of *Fusarium* and related taxa.

Link: https://doi.org/10.3897/mycokeys.67.52716.suppl1