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Three species of *Neofusicoccum* (Botryosphaeriaceae, Botryosphaeriales) associated with woody plants from southern China

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Abstract

Two new species, namely *N. sinense* and *N. illicii*, collected from Guizhou and Guangxi provinces in China, are described and illustrated. Phylogenetic analysis based on combined ITS, *tef1-α* and *TUB* loci supported their separation from other reported species of *Neofusicoccum*. Morphologically, the relatively large conidia of *N. illicii*, which become 1–3-septate and pale yellow when aged, can be distinguishable from all other reported species of *Neofusicoccum*. Phylogenetically, *N. sinense* is closely related to *N. brasiliense*, *N. grevilleae* and *N. kwambonambiense*. The smaller conidia of *N. sinense*, which have lower L/W ratio and become 1–2-septate when aged, differ from the other three species. *Neofusicoccum mangiferae* was isolated from the dieback symptoms of mango in Guangdong Province.

Key words – Asia – endophytes – Morphology– Taxonomy

Introduction

Neofusicoccum Crous, Slippers & A.J.L. Phillips was introduced by Crous et al. (2006) for species that are morphologically similar to, but phylogenetically distinct from *Botryosphaeria* species, which are commonly associated with numerous woody hosts world-wide (Arx 1987, Phillips et al. 2008). Some species of *Neofusicoccum* are reported to produce a *Dichomera* synanamorph, which may serve as a distinguishing characteristic from *Botryosphaeria* (Crous et al. 2006). There are 34 epithets included in *Neofusicoccum* according to Index Fungorum (2017), although most species previously described under *Fusicoccum* Corda are likely to reside in *Neofusicoccum* (Crous et al. 2006). Species of *Neofusicoccum* are differentiated on the basis of conidial dimensions, pigmentation of the culture media and DNA sequence data, although the taxonomic significance of some of the morphological characters has been questioned (Phillips et al. 2008), and patterns of septation and coloration in aged conidia discharged from pycnidia was regarded as a useful morphological feature to distinguish some species in *Neofusicoccum* and other genera of Botryosphaeriaceae (Slippers et al. 2004, Abdollahzadeh et al. 2013, Dissanayake et al. 2016).

In the course of an ongoing survey of biodiversity of Botryosphaeriaceae in China initiated in 2014, three Botryosphaeriaceous species that morphologically fit within *Neofusicoccum* were isolated from dieback symptoms as well as healthy tissues of some woody plants. The generic status of these isolates in *Neofusicoccum* was supported by their morphology and ITS, *tef1-α* and

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*TUB nu*DNA sequences phylogenetic analysis. Based on the combination of subtle morphological and molecular differences, two new species together with *N. mangiferae* are reported here.

Materials & Methods

Fungal isolation and morphology

Fresh material was collected from dieback symptoms and healthy tissues of some common tree species were collected from Guizhou, Guangdong and Guangxi provinces in Southern China from 2012 to 2016. Isolations were made from dead, diseased or healthy tissue of woody plants, and transferred to malt extract agar (MEA), and put in the ambient temperatures (about 28 °C) in the dark to establish colony characteristics, then transferred to synthetic nutrient-poor agar (SNA) with sterilized pine needles for three weeks to induce sporulation. Microscopic observations were made from material mounted in water. Photomicrographs were taken with a Nikon Coolpix 995 digital camera on a Leitz Orthoplan microscope. Measurements of conidia and conidiogenous cells were made from water mounts. Fungal isolates have been deposited at China General Microbiological Culture Collection Center (CGMCC) and herbarium specimens at the Mycological Herbarium of the Institute of Microbiology Chinese Academy of Sciences (HMAS).

DNA extraction, PCR amplification and sequencing

Colonies for DNA extraction were grown on MEA plates in darkness at 28 °C for 4–6 days until they completely covered the agar surface. DNA was extracted from the mycelium with CTAB plant genome DNA fast extraction kit (Aidlab Biotechnologies Co, Ltd, Beijing, China). The internal transcribed spacer of rDNA (ITS) was amplified and sequenced with primers ITS1 and ITS4 (White et al. 1990). Part of the translation elongation factor-1a (*tef1-α*) was amplified and sequenced with primers EF1-688F and EF1-1251R (Alves et al. 2008) and part of the *TUB* gene was amplified and sequenced with primers Bt2a and Bt2b (Glass & Donaldson 1995). DNA amplification and sequencing followed the protocol of Zhang et al. (2009).

Sequence alignment and phylogenetic analysis

The combined ITS, *tef1-α* and *TUB nu*DNA sequence dataset was used to infer the phylogenetic relationships among the new species and other reported species of *Neofusicoccum*. Sequences generated were analyzed with other sequences obtained from GenBank (Table 1). A multiple alignment was done in MEGA v. 6 (Tamura et al. 2013). Prior to the phylogenetic analysis, ambiguous regions at the start and the end of the sequences were deleted and gaps manually adjusted to optimize alignment. For Bayesian analysis, the best-fit model of nucleotide evolution (GTR+I+G) was selected using the Akaike information criterion (AIC; Posada & Buckley 2004) in MrModeltest 2.3. The metropolis-coupled Markov Chain Monte Carlo (MCMCMC) approach was used to calculate posterior probabilities (Ronquist & Huelsenbeck 2003). A preliminary Bayesian inference (BI) analysis using MrBayes revealed that the MCMC (Huelsenbeck & Ronquist 2001) steady state was reached after less than 2,260,000 generations (the average standard deviation of split frequencies was constantly below 0.01). A conservative burn-in of 22,600 trees was chosen and a full analysis of 10,000,000 generations was carried out with sampling every 100 generations. Maximum Parsimony (MP) analysis was conducted in PAUP v. 4.0b10 (Swofford 2002). Trees were inferred using the heuristic search option with 1,000 random sequence additions and tree-bisection-reconnection (TBR) as the branch-swapping algorithm and gaps were treated as missing data. Maxtrees were set to 50,000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Trees were viewed in TREEVIEW (Page 1996). Nucleotide sequences generated in this paper were deposited in GenBank. Trees and alignments were deposited in TreeBase with study ID S20904.

Results

Phylogenetic analyses

The combined ITS, *tef1-α* and *TUB nu*DNA sequence dataset consisted of 73 strains and 1243 characters in the MP analysis. Of the included bases, 270 sites (21.7 %) were parsimony-informative. A heuristic search with random addition of taxa (1,000 replicates) treating gaps as missing characters generated 5000 equally parsimonious trees, each with similar topology (figures not shown). A single parsimonious tree (TL = 629, CI = 0.676, RI = 0.872, RC = 0.589, HI = 0.324) is shown in Fig. 1. Bayesian posterior probabilities (PP) support equal to or greater than 70 % from Bayesian analysis and maximum parsimony (MP) support values greater than 60% are shown with Bayesian PP followed by MP bootstrap (PP/MP) values at the nodes (Fig. 1). This tree resolved 37 clades corresponding to 35 known and two previously unknown species. Therefore, two new species are introduced here.

Taxonomy

Neofusicoccum illicii Y. Zhang ter., M. Zhang **sp. nov.** Fig. 2

MycoBank MB 819397; Facesoffungi number: FoF 02822.

Etymology – named after the host from which it was isolated, *Illicium verum*.

Ascomata not observed. *Conidiomata* stromatic, produced on pine needles on SNA within 14 d, solitary or in groups covered by mycelium, dark brown to black, 2/3–3/4 erumpent, ellipsoidal or spherical, 250–350 μm diam. *Paraphyses* not observed. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* holoblastic, hyaline, smooth-walled, cylindrical or claviform, sometimes forming a periclinal thickening, (9–) 10.5–13 (–16) × (2–) 2.6–3.1 (–3.5) μm. *Conidia* hyaline, thin walled, granular cytoplasm, broadly to narrowly fusiform, or nearly cylindrical, sometimes slightly curved, base truncate or sometimes node-like, initially non-septate, subsequently becoming 1–3-septate and pale yellow, (22–) 23.7–27.1 (–30) × (5–) 6.1–7.9 (–9) μm (av. of 30 conidia = 25.4 ± 1.2 × 7 ± 0.8 μm, L/W ratio = 3.6), usually constricted at the main septum. *Spermatia* not observed.

Cultural characteristics – Colonies on MEA grey-white (surface) and buff to light primrose (reverse), sometimes not reaching the edge of the plate, with a dense mat of aerial mycelium, covering a 30 mm Petri dish in 3 days, the margin crenulated irregularly.

Specimens examined – CHINA, Guangxi province, from healthy tissue of *Illicium verum*, 7 Sept. 2012, L. Wang (holotype: HMAS 266205; cultures ex-holotype: CGMCC 3.18310; CGMCC 3.18311; CGMCC 3.18312; CGMCC 3.18313).

Notes – Phylogenetically, *N. illicii* is sibling to all other species of *Neofusicoccum*, and basal to the clade comprising *N. algeriense*, *N. batangarum*, *N. brasillense*, *N. cordaticola*, *N. grevilleae*, *N. kwambonambiense*, *N. occulatum*, *N. parvum*, *N. ribis*, *N. sinense* and *N. umdonicola* (Fig. 1). Morphologically, the large conidia of *N. illicii* differentiate this species from *N. algeriense*, *N. batangarum*, *N. occulatum*, *N. parvum*, *N. umdonicola* and *N. sinense* (Crous et al. 2006, Pavlic et al. 2008, Begoude et al. 2010, Sakalidis et al. 2011, Berraf-Tebbal et al. 2014). The septate aged conidia with L/W radio of *N. illicii* are most comparable with *N. grevilleae*, while the small condiomata (< 200 μm) of *N. grevilleae* (Crous et al. 2011) differentiate it from *N. illicii*.

Neofusicoccum sinense Y. Zhang ter., M. Zhang **sp. nov.** Fig. 3

MycoBank MB 819396; Facesoffungi number: FoF 02821.

Etymology – The epithet *sinense* refers to China (from Latin *sinensia* = China), the country from which it is described.

Ascomata not observed. *Conidiomata* stromatic, produced on pine needles within 1–2 week, solitary or botryose covered with white mycelium, dark brown to black, initially immersed, 1/2 erumpent through the pine needles at maturity, spherical or elliptical or with central, black ostioles, 200 to 350 μm diam. *Paraphyses* not observed. *Conidiophore* not observed. *Conidiogenous cells* not observed. *Conidia* hyaline, thin walled, granular cytoplasm, narrowly to broadly fusiform, initially non-septate, subsequently becoming 1–2-septate, constricted at the septum, apex rounded,

with somewhat truncate base or sometimes curved node-like, (15.2–) 17.6–20.4 (–23) × (6.9–) 7.4–8 (–9) μm (av. of 20 conidia = 18.7 ± 1.5 × 7.7 ± 0.9 μm, L/W ratio = 2.4). *Spermatogenous cells* hyaline, slimy cylindrical, smooth-walled and radiating divergent to the surrounding, (10–) 12–13.5 (–15) × 3–5.2 μm, inflated near the base and somewhat tapering upward, apex usually attached to spermatia which is going to fall off. *Spermatia* hyaline, cylindrical, aseptate, sometimes with arc bending, 5 × 3 μm.

Cultural characteristics – Colonies on MEA iron-grey (surface) and olivaceous- grey (reverse) with extensive grey aerial mycelium, and smooth margins, attaining a radius of 20 mm after 3 days in darkness at 28°C, aerial mycelium growing upward like conical antenna and eventually form the bowl colony about 10 days, tapered tip and part with slowly atrophy then flattened with tufts, its color transition from white to grey or ash grey after 25 days.

Specimens examined – CHINA, Guizhou Province, Huangping County, Fengxiangzhai (altitude: 1,000 m), from branch of unknown dead woody plant, 20 Feb. 2016, J.J. Gan (holotype: HMAS 255209; culture ex-holotype: CGMCC 3.18315).

Notes – Phylogenetically, *N. sinense* is sibling to other species of *Neofusicoccum*, while closely related to *N. brasillense*, *N. grevilleae* and *N. kwambonambiense* (Fig. 1). Morphologically, the conidia of *N. sinense* are initially non-septate but subsequently become 1–2-septate. *Neofusicoccum brasiliense* failed to sporulate in culture, thus its description was based solely on molecular data (Marques et al. 2013). The small-sized conidiomata (< 200 μm), larger conidia (20–32 × 6–10 μm) and the phialidic conidiogenous cells of *N. grevilleae* are distinguishable from those of *N. sinense*. The presence of spermatia, smaller conidia and lower L/W ratio of *N. sinense* (18.7 × 7.7 μm, L/W = 2.4) are distinguishable from those *N. kwambonambiense* (22.3 × 6.3 μm, L/W = 3.6). Thus, a new species, *N. sinense*, is introduced here.

Neofusicoccum mangiferae (Syd. & P. Syd.) Crous, Slippers & A.J.L. Phillips, Stud. Mycol. 55: 248 (2006)

≡ *Dothiorella mangiferae* Syd. & P. Syd., Annls mycol. 14(3/4): 192 (1916)

Specimens examined – CHINA, Guangdong province, Yangchun, Kongtong mountain, *Mangifera indica*, 23 Jan. 2016, Z.P. Dou & Z.C. Liu (CGMCC 3.18314).

Discussion

A few species of *Neofusicoccum* had been reported in China as plant pathogens. For instance, *N. parvum* causes gummosis of mango (Li et al. 2014), stem die-back of blueberries (Yu et al. 2013) and canker of *Juglans regia* seedlings (Yu et al. 2015). *Neofusicoccum vitifusiforme* causes blueberry blight (Kong et al. 2010), and *N. mangiferae* causes grapevine dieback in Henan and Anhui Provinces in China (Dissanayake et al. 2015). In this study, three species of *Neofusicoccum* were isolated from subtropical regions in China, and two of which, namely *N. illicii* and *N. sinense*, are new to science. *Neofusicoccum illicii* was isolated from *Illicium verum* as endophyte in Guangxi Province in China, while *N. sinense* was isolated from an unidentified dead woody plant as saprophyte in Guizhou province.

Botryosphaeriacous fungi are among the most severe pathogens that affect mango trees and fruits (Slippers et al. 2005). *Neofusicoccum mangiferae* has been widely reported as a pathogen of *Mangifera indica* worldwide wherever mangoes are grown, for instance in India (Sydow & Sydow 1916), Australia (Johnson et al. 1991, Johnson 1992, Slippers et al. 2005), United States (Mitra & Baldwin 1997), Puerto Rico (Serrato-Diaz et al. 2014) and China (this study). Besides *N. mangiferae*, other species of *Neofusicoccum*, such as *N. australe*, *N. brasilense*, *N. mediterraneum* and *N. parvum* have also been reported as prevalent pathogens of mango causing fruit stem-end rot, dieback, gummosis and blossom blight (Slippers et al. 2005, Adesemoye & Eskalen 2011, Abdollahzadeh et al. 2013, Barradas et al. 2013, Ismail et al. 2013, Li et al. 2013, 2014, Marques et al. 2013, Lopes et al. 2014, Krishnapillai et al. 2015).

Table 1 Details of *Neofusicoccum* strains and species considered in this study (newly generated sequences are indicated in bold).

Species	Strain	Host	Origin	GenBank Accession no.		
				ITS	*tef1-a*	*TUB*
Neofusicoccum algeriense	CBS 137504	*Vitis vinifera*	Algeria	KJ657702	KJ657715	KX505915
	CAA 322	*Eucalyptus globulus*	–	KX505906	KX505894	KX505916
N. andinum	CBS 117453	*Eucalyptus* sp.	Venezuela	AY693976	AY693977	KX464923
	CBS 117452	*Eucalyptus* sp.	Venezuela	DQ306263	DQ306264	KX464922
N. arbuti	CBS 116131	*Arbutus menziesii*	USA	AY819720	KF531792	KF531793
	CBS 117090	*Arbutus menziesii*	USA	DQ306263	KF531791	KF531794
N. australe	CMW 6837	*Acacia* sp.	Australia	AY339262	AY339270	AY339254
	CMW 6853	*Sequiadendron* sp.	Australia	AY339263	AY339271	AY339255
N. batangarum	CBS 124924	*Terminalia catappa*	Africa	FJ900607	FJ900653	FJ900634
	CBS 124923	*Terminalia catappa*	Africa	FJ900608	FJ900654	FJ900635
N. brasiliense	CMM 1338	*Mangifera indica*	Brazil	JX513630	JX513610	KC794031
	CMM 1269	*Mangifera indica*	Brazil	JX513629	JX513609	KC794032
N. buxi	CBS 116.75	*Buxus sempervirens*	Sweden	KX464165	KX464678	–
	CBS 113714	*Buxus sempervirens*	France	KX464164	KX464677	KX464954
N. cordaticola	CBS 123634	*Syzygium cordatum*	South Africa	EU821898	EU821868	EU821838
	CBS 123635	*Syzygium cordatum*	South Africa	EU821903	EU821873	EU821843
N. corticosae	CBS 120081	*Eucalyptus corticosa*	New South Wales	DQ923533	KX464682	KX464958
	CBS 118099	*Eucalyptus camaldulensis*	Australia	KX464168	KX464681	KX464957
N. cryptoaustrale	CMW 23785	*Eucalyptus* sp.	South Africa	FJ752742	FJ752713	FJ752756
	CMW 23786	*Eucalyptus* sp.	South Africa	FJ752744	FJ752714	FJ752753
N. eucalypticola	CBS 115766	*Eucalyptus rossii*	Australia	AY615143	AY615135	AY615127
	CBS 115679	*Eucalyptus rossii*	Australia	AY615141	AY615133	AY615125
N. eucalyptorum	CMW 10125	*Eucalyptus grandis*	South Africa	AF283686	AY236891	AY236920
	CMW 10126	*Eucalyptus grandis*	South Africa	AF283687	AY236892	AY236921
N. grevilleae	CBS 129518	*Grevillea aurea*	Australia	JF951137	–	–

N. hellenicum	CERC 1947	*Pistachia vera*	Greece	KP217053	KP217061	KP217069
	CERC 1948	*Pistachia vera*	Greece	KP217054	KP217062	KP217070
N. illicii	CGMCC 3.18310	*Illicium verum*	Guangxi, China	**KY350149**	–	**KY350155**
N. illicii	CGMCC 3.18311	*Illicium verum*	Guangxi, China	**KY350150**	**KY817756**	**KY350156**
N. illicii	CGMCC 3.18312	*Illicium verum*	Guangxi, China	**KY350151**	**KY817757**	**KY350157**
N. illicii	CGMCC 3.18313	*Illicium verum*	Guangxi, China	**KY350152**	**KY817758**	**KY350158**
N. kwambonambiense	CBS 123639	*Syzygium cordatum*	South Africa	EU821900	EU821870	EU821840
	CBS 123641	*Syzygium cordatum*	South Africa	EU821919	EU821889	EU821859
N. lumnitzerae	CBS 139674	*Lumnitzera racemosa*	South Africa	KP860881	KP860724	KP860801
	CBS 139675	*Lumnitzera racemosa*	South Africa	KP860882	KP860725	KP860803
N. luteum	CBS 110299	*Vitis vinifera*	Portugal	AY259091	AY573217	DQ458848
	CBS 110497	*Vitis vinifera*	Portugal	EU673311	EU673277	EU673092
N. macroclavatum	WAC 12444	*Eucalyptus globulus*	Australia	DQ093196	DQ093217	DQ093206
	WAC 12446	*Eucalyptus globulus*	Australia	DQ093197	DQ093218	DQ093208
N. mangiferae	CBS 118531	*Mangifera indica*	Australia	AY615185	DQ093221	AY615172
	CBS 118532	*Mangifera indica*	Australia	AY615186	DQ093220	AY615173
	CGMCC 3.18314	*Mangifera indica*	Guangdong, China	KY350153	KY817759	KY350159
N. mangroviorum	CMW 41365	*Avicennia marina*	South Africa	KP860859	KP860702	KP860779
	CMW 42481	*Avicennia marina*	South Africa	KP860848	KP860692	KP860770
N. mediterraneum	CBS 121718	*Eucalyptus* sp.	Greece	GU251176	GU251308	GU251836
	CBS 121558	*Olea europaea*	Italy	GU799463	GU799462	GU799461
N. nonquaesitum	CBS 126655	*Umbellularia*	USA	GU251163	GU251295	GU251823
	PD 301	*Vaccinium corymbosum*	Chile	GU251164	GU251296	GU251824
N. occulatum	CBS 128008	*Eucalyptus grandis hybrid*	Australia	EU301030	EU339509	EU339472
	MUCC 286	*Eucalyptus pellita*	Australia	EU736947	EU339511	EU339474
N. parvum	CMW 9081	*Actinidia deliciosa*	New Zealand	AY236943	AY236888	AY236917
	CBS 110301	*Vitis vinifera*	Portugal	AY259098	AY573221	EU673095
N. pennatisporum	MUCC 510	*Allocasuarina fraseriana*	Australia	EF591925	EF591976	EF591959
N. pistaciae	CBS 595.76	*Pistacia vera*	Greece	KX464163	KX464676	KX464953
N. pistaciarum	CBS 113083	*Pistacia vera*	USA	KX464186	KX464712	KX464998
	CBS 113084	redwood	USA	KX464187	KX464713	KX464999

N. protearum	CBS114176	*Protea* sp.	South Africa	AF452539	KX464720	KX465006
N. ribis	CBS 115475	*Ribes* sp.	USA	AY236935	AY236877	AY236906
	CBS 121.26	*R. rubrum*	USA	AF241177	AY236879	AY236908
N. sinense	CGMCC 3.18315	unknown woody plant	Guizhou,China	**KY350148**	**KY817755**	**KY350154**
N. stellenboschiana	CBS 110864	*Vitis vinifera*	South Africa	–	–	KX465047
	CBS 282.70	*Arum italicum*	Spain	KX464225	KX464758	KX465051
N. terminaliae	CMW 26679	*Terminalia sericea*	South Africa	GQ471802	GQ471780	KX465052
	CMW 26683	*Terminalia sericea*	South Africa	GQ471804	GQ471782	KX465053
N. umdonicola	CBS 123645	*Syzygium cordatum*	South Africa	EU821904	EU821874	EU821844
	CBS 123646	*Syzygium cordatum*	South Africa	EU821905	EU821875	EU821845
N. ursorum	CMW 24480	*Eucalyptus arboretum*	South Africa	FJ752746	FJ752709	KX465056
	CMW 23790	*Eucalyptus arboretum*	South Africa	FJ752745	FJ752708	KX465057
N. viticlavatum	CBS 112878	*Vitis vinifera*	South Africa	AY343381	AY343342	KX465058
	CBS 112977	*Vitis vinifera*	South Africa	AY343380	AY343341	KX465059
N. vitifusiforme	CBS 110887	*Vitis vinifera*	South Africa	AY343383	AY343343	KX465061
	CBS 110880	*Vitis vinifera*	South Africa	AY343382	AY343344	–
Botryosphaeria corticis	CBS 119047	*Vaccinium corymbosum*	USA	DQ299245	EU017539	EU673107
Botryosphaeria dothidea	CBS 115476	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927

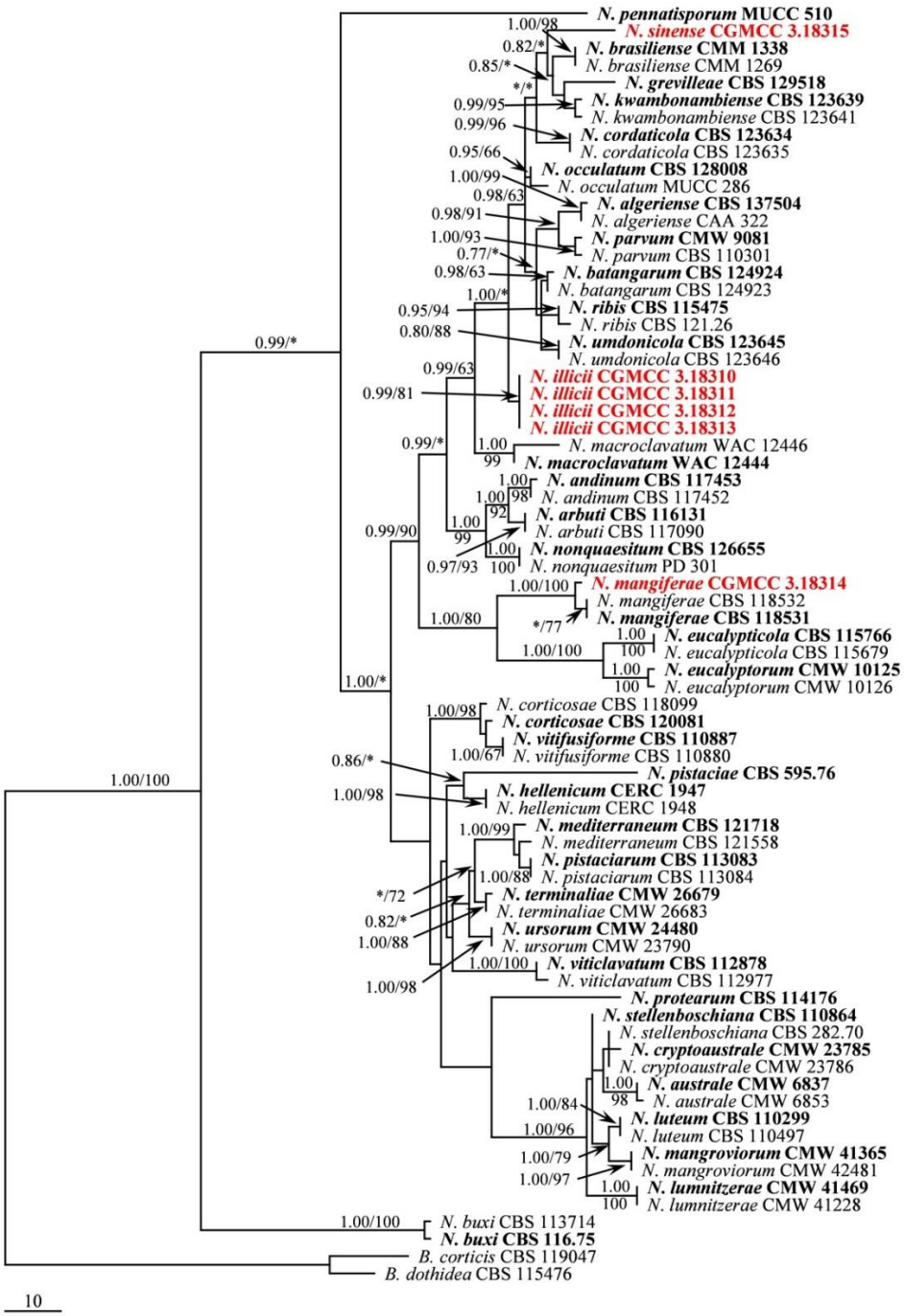

Figure 1 – Maximum parsimony tree obtained from combined ITS, *tef1-α* and *TUB* sequence data of *Neofusicoccum* species. The tree is rooted to *Botryosphaeria corticis* (CBS 119047) and *B. dothidea* (CBS 115476). Bayesian posterior probabilities (PP) support above 70 % and maximum parsimony (MP) support values above 60 are shown with Bayesian PP followed by MP bootstrap (PP/MP) values at the nodes. Ex-type strains are printed in bold face and new isolates in red bold face.

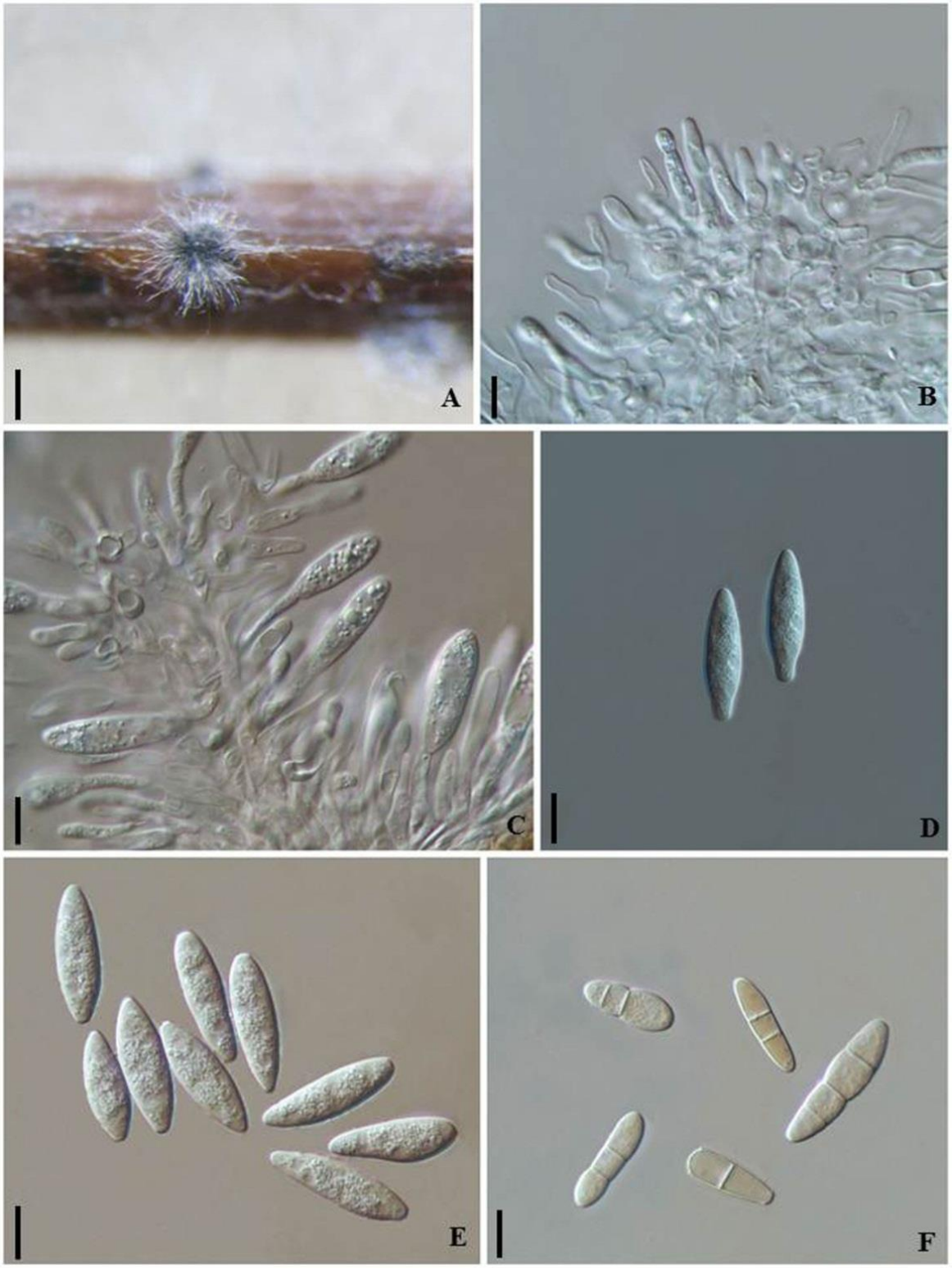

Figure 2 – *Neofusicoccum illicii* (from holotype: HMAS 266205). A. Conidiomata developing on pine needles in culture. B, C. Conidiogenous cells with developing conidia. D. Hyaline, aseptate conidia with a node-like base. E. Hyaline, aseptate conidia. F. Yellowish, 1–3-septate, senescent conidia. Scale bars: A = 250 μm, B–F = 10 μm.

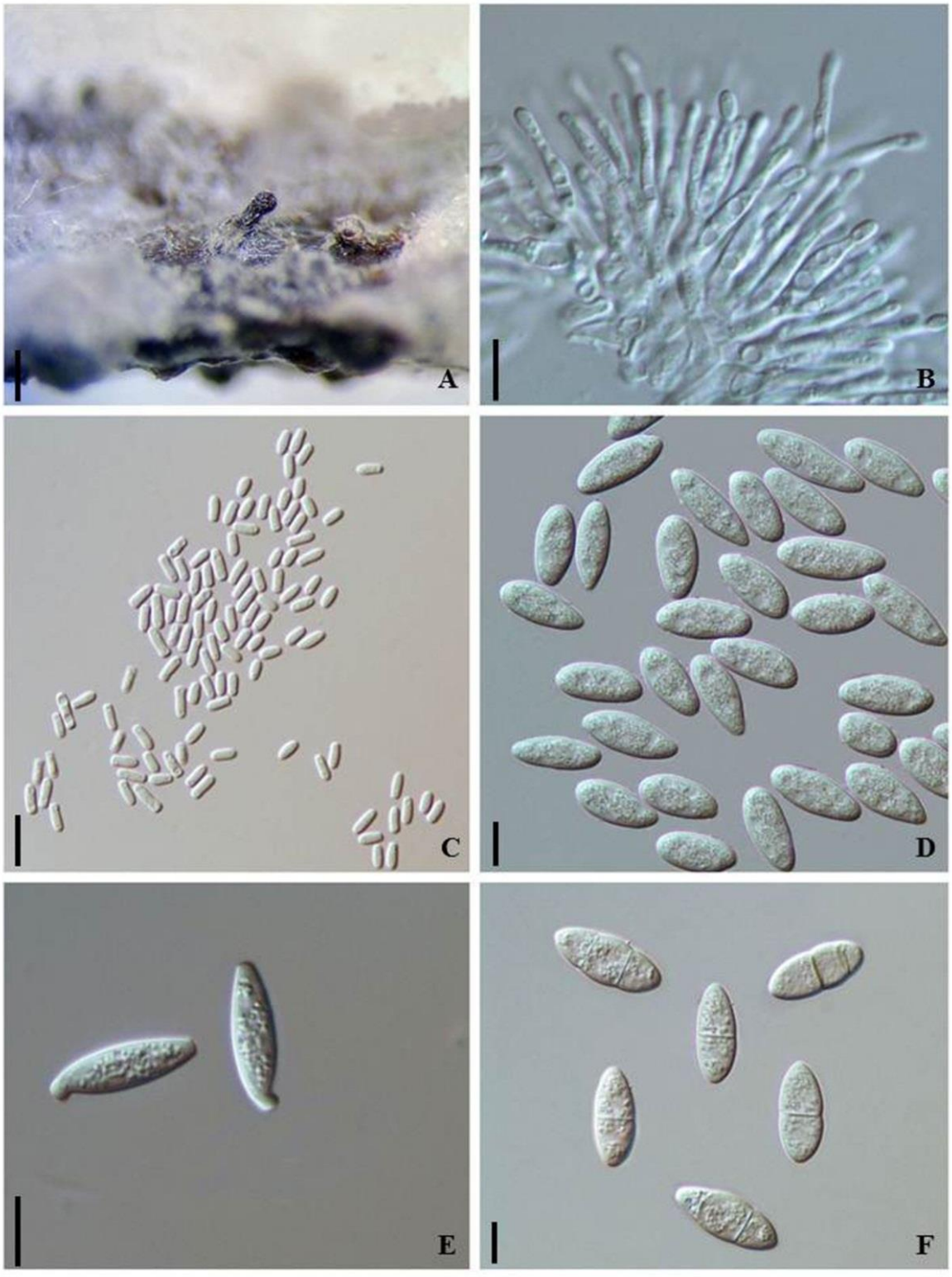

Figure 3 – *Neofusicoccum sinense* (from holotype: HMAS 255209). A. Conidiomata formed on pine needles in culture. B. Spermatogenous cells with developing spermatia. C. Spermatia. D. Hyaline, aseptate conidia. E. Hyaline, aseptate conidia with a curved node-like base. F. 1- or 2-septate, hyaline, senescent conidia. Scale bars: A = 300 μm, B–F = 10 μm.

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