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Research Article

Morphology and Phylogeny of *Neoscytalidium orchidacearum* sp. nov. (Botryosphaeriaceae)

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Abstract A coelomycete with characters resembling the asexual morphs in the family Botryosphaeriaceae was isolated from a fallen leaf of an orchid collected in Thailand. Morphological and phylogenetic analyses placed the strain in *Neoscytalidium*. Phylogenetic relationships among *Neoscytalidium* species were inferred by analyzing internal transcribed spacers and large subunit of rRNA sequence data and indicate that our strain is a new species, which is introduced and illustrated herein as *Neoscytalidium orchidacearum* sp. nov.

Keywords Botryosphaeriales, Coelomycetes, New species, Phylogeny

In a partial taxonomic revision of the family Botryosphaeriaceae, Crous *et al.* [1] concluded that *Scytalidium* is polyphyletic and proposed the genus *Neoscytalidium* to accommodate *Scytalidium dimidiatum* (Penz.) B. Sutton & Dyko as *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers. Campbell and Mulder [2] introduced the new species *Scytalidium hyalinum* C. K. Campb. & J. L. Mulder as the cause of human dermatomycosis. Pavlic *et al.* [3] described *Neoscytalidium novaehollandiae* Pavlic, T. I. Burgess & M. J. Wingf with similar morphological characteristics to *N. hyalinum* and this was accepted in the genus supported by internal transcribed spacers (ITS) and EF1α sequence data [1]. It has been suggested that *Scytalidium dimidiatum* and *S. hyalinum* might be conspecific [4, 5]. Phillips *et al.* [5] agreed with the synonymy and made *Neoscytalidium dimidiatum* a synonym of *N. hyalinum*. However, the epithet *dimidiatum* (1882) is older than *hyalinum* (1977) and should take priority.

Neoscytalidium is characterized by oblong-obtuse to doliiform arthroconidia borne in dry powdery chains. A coelomycetous synasexual morph with stromatic and solitary conidiomata is also formed [5]. Conidia of the coelomycetous morph, that become 2-septate with a darker central cell, and large subunit of rRNA (LSU) sequence data distinguish *Neoscytalidium* from the polyphyletic genus *Scytalidium*.

Neoscytalidium has been reported from America, northwestern Australia, Niger, and Oman as a plant pathogen [3, 6-9] and as a human pathogen causing skin infections [2, 4]. One human pathogen, associated with rhinosinusitis in Iran, has been reported and based on a blast search in GenBank was regarded as *Neoscytalidium dimidiatum* [10].

In this work, a collection of *Neoscytalidium* from an orchid leaf collected in Thailand was studied in terms of morphology and phylogenetic analysis of ITS and LSU sequence data. This

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collection was confirmed to be divergent from other species of *Neoscytalidium* and a new species was introduced. Furthermore, the name of type species of *Neoscytalidium* is corrected.

MATERIALS AND METHODS

Collection and isolation. Fallen and decomposing leaves were collected from Sukhotai Province, Thailand, during August 2012, placed in plastic Zip lock bags and brought to the laboratory. The samples were studied with a stereomicroscope to locate the fruiting bodies. If the fruiting bodies were immature, the specimens were incubated in a sterile moist chamber (plastic containers with sterile tissue paper soaked with sterile distilled water) and examined at intervals. The specimens were divided into two parts. The first part was used for morphological study and single spore isolations prepared following the methods described in Chomnunti *et al.* [11] and Tangthirasunun *et al.* [12, 13]. The colonies were transferred to water agar and incubated at room temperature to promote sporulation. Colony characters and growth rates were determined on 2% potato dextrose agar (PDA). Growth was measured after 5 days at room temperature (25~27°C). Colonies were cut into 15-mm cubes and suspended in 2-mL screw cap microcentrifuge tube either with water for storage at 4°C or with 10% glycerol for storage at −20°C. Cultures are deposited at Mae Fah Luang University Culture Collection (MFLUCC) and Guizhou University Culture Center (GZUCC). Cultures suspended in 2-mL screw cap micro-centrifuge tube with liquid RG (Ricardo G. Maggi) medium were kept for storage at −80°C

Table 1. Strains and NCBI GenBank accession numbers of species used in this study

Species	Voucher/Culture	GenBank accession No.	
		ITS	LSU
Barriopsis fusca	CBS 174.26[a]	EU673330	DQ377857
Botryobambusa fusicoccum	MFLUCC 11-0143[a]	JX646792	JX646809
Botryosphaeria agaves	CBS 133992[b]	JX646791	JX646808
Botryosphaeria agaves	MFLUCC 10-0051	JX646790	JX646807
Botryosphaeria dothidea	CBS 115476[c]	AY236949	AY928047
Botryosphaeria dothidea	CBS110302	AY259092	EU673243
Botryosphaeria fusispora	MFLUCC 10-0098[a]	JX646789	JX646806
Botryosphaeria fusispora	MFLUCC 11-0507	JX646788	JX646805
Botryosphaeria ramosa	CMW 26167[a]	KF766168	KF766333
Cophinforma atrovirens	MFLUCC 11-0425[a]	JX646800	JX646817
Cophinforma atrovirens	CBS 117444	KF531822	DQ377855
Diplodia mutila	CBS 112553	AY259093	AY928049
Diplodia rosulata	CBS116470[a]	EU430265	DQ377896
Lasiodiplodia pseudotheobromae	CBS 116459[a]	EF622077	EU673256
Lasiodiplodia theobromae	CBS 164.96[b]	AY640255	EU673253
Macrophomina phaseolina	CBS 227.33	KF531825	DQ377906
Macrophomina phaseolina	CBS 460.70	KF951639	EU754169
Neodeightonia phoenicum	CBS 122528[a]	EU673340	EU673261
Neodeightonia subglobosa	CBS 448.91[a]	EU673337	DQ377866
Neoscytalidium dimidiatum	CBS 251.49	KF531819	DQ377923
Neoscytalidium dimidiatum	CBS 499.66	KF531820	DQ377925
Neoscytalidium dimidiatum	UTHSCSA DI 14-340	KM357894	KM357895
Neoscytalidium dimidiatum	IP127881	AY819727	DQ377925
Neoscytalidium dimidiatum (*Neoscytalidium hyalinum*)	CBS 145.78[d]	KF531816	DQ377922
'*Neoscytalidium dimidiatum*'	CBS 135275	KF571862	–
Neoscytalidium novaehollandiae	CBS122071[a], CMW 26170[a]	KF766207	KF766374
Neoscytalidium novaehollandiae	CBS 122072	EF585535	–
Neoscytalidium orchidacearum	**MFLUCC 12-0533[e]**	**KU179865**	**KU179864**
Phaeobotryon mamane	CBS 122980[a]	EU673332	EU673248
Tiarosporella tritici	CBS 118719[a]	KF531830	DQ377941
Tiarosporella urbis-rosarum	CBS 130405[a]	JQ239407	JQ239420

Isolate from this study is indicated in bold.

ITS, internal transcribed spacer; LSU, large subunit of rRNA; –, absent.

[a]Ex-type strain.

[b]Ex-neotype strain.

[c]Ex-epitype strain.

[d]Ex-isotype strain.

[e]Ex-holotype strain.

(*Podospora anserina* Genome Project, http://podospora.igmors.u-psud.fr/) at the Institute of Genetics and Microbiology (IGM, NTCL code), University Paris-Sud 11, France. The pure cultures were used for molecular analysis. The second part of the sample was used as herbarium material and is deposited at MFLU herbarium (Mae Fah Luang University, Chiang Rai, Thailand) with duplicates at GZUH herbarium (Guizhou University, Guiyang, China). Facesoffungi numbers and Index Fungorum numbers are as outlined in Jayasiri *et al.* [14] and Index Fungorum [15].

Morphological study. Specimens were sectioned free-hand with a razor-blade, the sections mounted in water and examined with a light microscope. Photomicrographs of thin or useful sections of the fruiting bodies and contents were taken with a Nikon ECLIPSE 80i compound microscope equipped with a Nikon 600D digital camera (Nikon, Tokyo, Japan). Structures were measured using Image Frame Work program (ver. 0.9.7). Besides water, 70% lactic acid, 3% KOH, or lactophenol cotton blue were used as mountants or stains. Photoplates were prepared with Photoshop CS5.

DNA extraction, PCR amplification and sequencing. Isolates were grown on PDA for 30 to 45 days at room temperature. Genomic DNA was extracted from fresh mycelia following the protocol described by Lecellier and Silar [16]. Primers ITS1 and ITS4 [17] and LROR and LR7 [18] were used to amplify the ITS and part of the LSU rRNA genes. PCR reaction mixtures and amplification conditions were as described by Tangthirasunun *et al.* [12, 13]. PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide [19] and sequenced by Beckman Coulter Genomics (Danvers, MA, USA; Grenoble, France).

Phylogenetic analysis. BLAST searches of the National Center for Biotechnology Information (NCBI) were used to check for sequence homologies for the assembled consensus sequences and for preliminary identification of the isolates used in the analysis. Sequences of the available allied taxa were obtained from GenBank (Table 1). Sequences were aligned with Bioedit 7.0.9.0 [20] and improved in MAFFT v6 [21], with the online sequence alignment editor under the default settings of MAFFT ver. 7 (http://mafft.cbrc.jp/alignment/server/index.html). A maximum likelihood (ML) analysis was performed with RAxML GUI ver. 1.3 [22] with 1,000 rapid bootstrap replicates using the GTR + gamma model of nucleotide substitution. The tree was rooted to *Tiarosporella tritici* and *Tiarosporella urbis-rosarum*. Phylogenetic trees were viewed with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and the final tree prepared in Adobe Illustrator CS5.

RESULTS

Phylogenetic analysis. The phylogenetic tree of most genera of Botryosphaeriaceae (Fig. 1) inferred from the ITS, LSU dataset using ML analysis supports the monophyly of *Neoscytalidium*. Our strain from dead orchid leaves clustered with CBS 135275, which was isolated from a human with rhinosinusitis, but is distinct from ex-type isolates of *N. dimidiatum* and *N. novaehollandiae*.

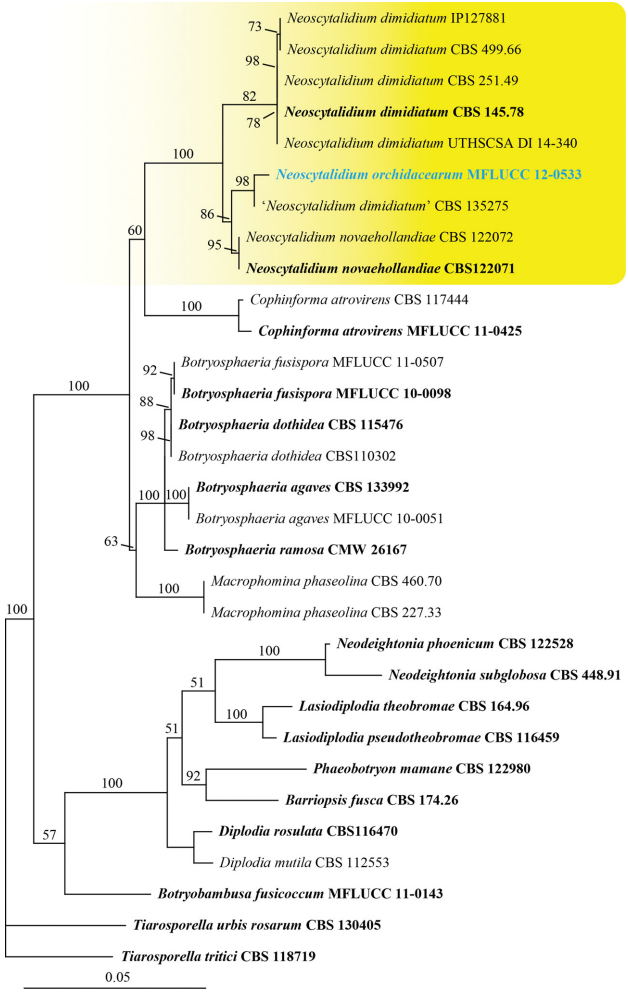

Fig. 1. Maximum likelihood phylogenetic tree (lnL = –4,448.525379) estimated from analysis of combined internal transribed spacer and large subunit of rRNA sequence data for 31 strains of Botryosphaeriaceae. Bootstrap support values for maximum likelihood greater than 50% are indicated above the nodes. Ex-type strains are indicated in bold. Isolate from this study is indicated in blue.

Taxonomy.
Phillips *et al.* [5] erroneously placed *N. hyalinum* as the type species of *Neoscytalidium*. This error is corrected here:

Neoscytalidium dimidiatum (Penz.) Crous & Slippers, Stud. Mycol. 55: 244 (2006).
Basionym: *Torula dimidiata* Penz., Michelia 2: 466 (1882).
≡ *Scytalidium dimidiatum* (Penz.) B. Sutton & Dyko, Mycol. Res. 93: 484 (1989).
≡ *Fusicoccum dimidiatum* (Penz.) D. F. Farr, Mycologia 97:

740 (2005).
= *Hendersonula toruloidea* Nattrass, Trans. Br. Mycol. Soc. 18: 197 (1933).
= *Neoscytalidium hyalinum* (C. K. Campb. & J. L. Mulder) A. J. L. Phillips, Groenewald & Crous, SIM 76: 148 (2013).
= *Scytalidium hyalinum* C. K. Campb. & J. L. Mulder, Sabouraudia, 15: 163 (1977).

Notes: *Hendersonula toruloidea* was introduced by Nattrass [23] based on multilocular and black stromata. Later Campbell and Mulder [2] reported *Scytalidium hyalinum* that can cause the same skin diseases as *Hendersonula toruloidea*. Sutton and Dyko [24] re-examined all the specimens belonging to the genus *Hendersonula*. They transferred *H. toruloidea* to *Nattrassia* and designated it

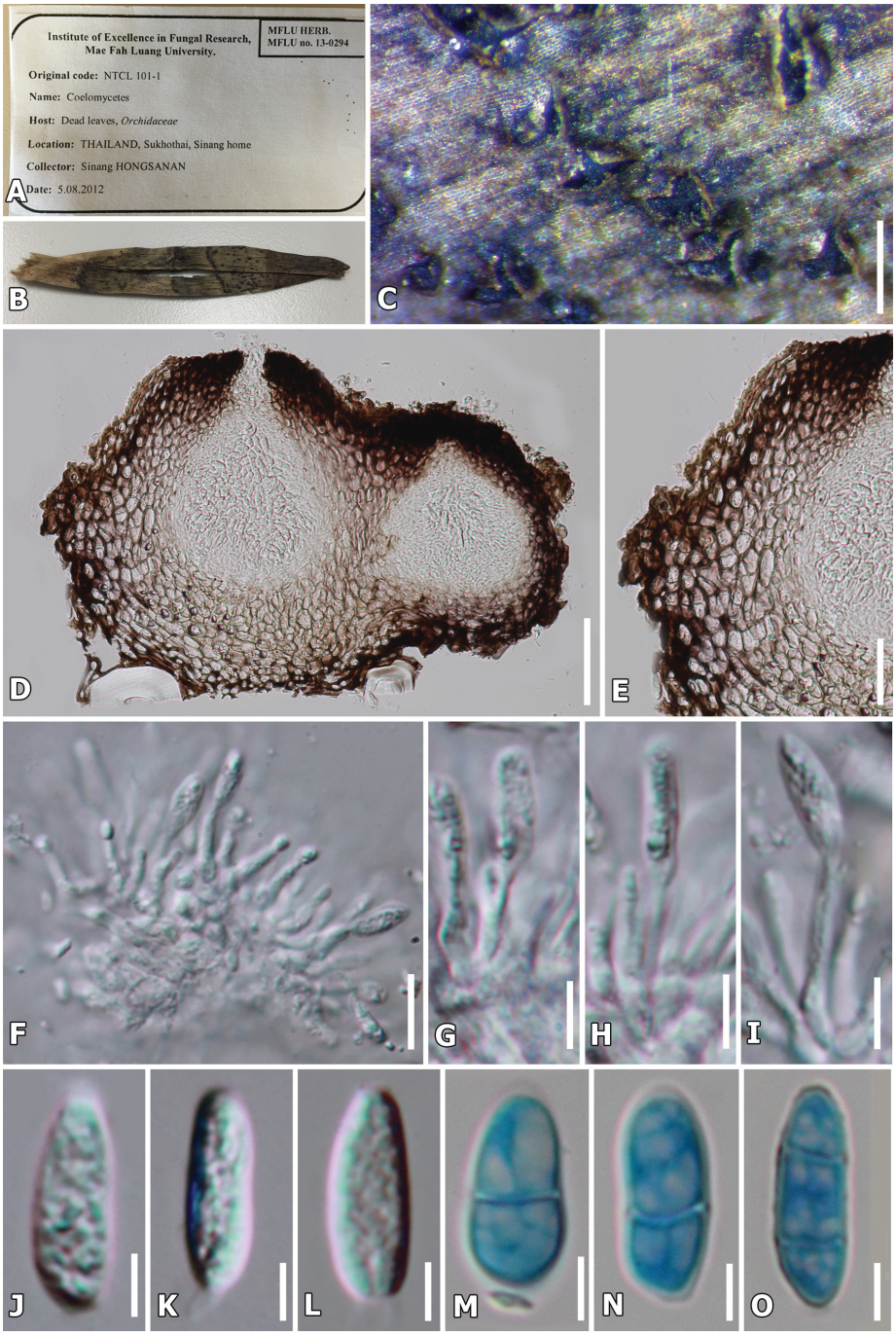

Fig. 2. *Neoscytalidium orchidacearum* (MFLU 13-0294, holotype). A, Herbarium label; B, Herbarium specimen; C, Conidiomata on host; D, Conidiomata in vertical section; E, Wall of conidioma; F~I, Conidiogenous cells with developing conidia; J~O, Conidia. Notes: M~O, Stained in lactophenol cotton blue (scale bars: C = 500 μm, D = 50 μm, E = 30 μm, F = 10 μm, G~I = 5 μm, J~O = 3 μm).

as the type species with the name *N. mangiferae*. Sutton and Dyko [24] accepted *Scytalidium dimidiatum* as the synanamorph of *Nattrassia mangiferae* that was described under the basionym of *Torula dimidiata*. However, based on a phylogenetic analysis, Farr *et al.* [25] regarded *Fusicoccum dimidiatum* as the correct name for *Scytalidium dimidiatum* and *Nattrassia mangiferae*. Crous *et al.* [1] concluded that *Scytalidium* is polyphyletic and introduced *Neoscytalidium* to accommodate *S. dimidiatum* as *N. dimidiatum*. Madriada *et al.* [4] suggested that *Scytalidium dimidiatum* and *S. hyalinum* could be synonyms and introduced the new variety *Neoscytalidium dimidiatum* var. *hyalinum* (C. K. Camp. & J. L. Mulder) Madrid *et al.* Phillips *et al.* [5] agreed that *N. dimidiatum* and *N. hyalinum* are conspecific species and combined them under *N. hyalinum*. However, *N. dimidiatum* is the oldest name and should take priority. This error is corrected here.

Neoscytalidium orchidacearum S. K. Huang, N Tangthirasunun, J. C. Kang & K. D. Hyde, **sp. nov.**
Index Fungorum number: IF551726; Facesoffungi number: FoF 01362 (Fig. 2).
Etymology: The name *orchidacearum* refers to the host family (Orchidaceae).
Holotype: MFLU 13-0294.
Saprobic on dead leaves. Sexual morph: Undetermined. Asexual morph: Hyphomycetous asexual morph not seen. Coelomycetous asexual morph: Conidiomata 200~500 µm diam, stromatic, immersed, eventually erumpent, unilocular to multilocular (2~4-loculate), glabrous, brown to black, globose to subglobose, papillate. Ostiole central, short, lined with periphyses (Fig. 2D). Wall of conidiomata 30~80 µm thick, membranaceous, composed of dark brown, or brown to hyaline cells of *textura angularis* (Fig. 2E). Conidiophores reduced to conidiogenous cells. Conidiogenous cells 6~15.5 × 1.5~3 µm (n = 30) enteroblastic, phialidic, cylindrical to subcylindrical, hyaline, smooth-walled, arising from the inner layers of conidioma (Fig. 2F~I). Conidia (10~) 12~13 (~15) × 3~5 (~6) µm (n = 50), ellipsoidal to oval, hyaline, smooth, guttulate, aseptate becoming 2~3-septate (Fig. 2J~O).
Culture characteristics: *Colonies* cream or white from above and reverse, with filamentous form or margin, flat, and attaining a diam of 48 mm on PDA in 5 days at room temperature (25~27°C).
Material examined: Thailand, Sukhotai; on dead leaves of orchid (Orchidaceae), 5 Aug 2012; S Hongsanan (MFLU 13-0294, holotype), *ibid.* (GZUH 15113001, isotype); ex-type living culture MFLUCC 12-0533 and GZUCC 15113001.

Key to genera of *Neoscytalidium* based on the syn-asexual coelomycetous morph

1. Conidiomata immersed, eventually erumpent ····· 2
1. Conidiomata semi-immersed or superficial (Phillips *et al.* [5]) ····· *Neoscytalidium novaehollandiae*
2. Conidia central cell dark brown, end cells hyaline to pale brown (Phillips *et al.* [5]) ····· *Neoscytalidium dimidiatum*
2. Conidia hyaline····· *Neoscytalidium orchidacearum*

DISCUSSION

In this study, a new species, *Neoscytalidium orchidacearum,* is introduced based on ML phylogenetic analysis (Fig. 1) and characters of the coelomycetous asexual morph, such as black and erumpent conidiomata, and ellipsoidal to oval and hyaline conidia. We were not able to find the hyphomycetous asexual morph or the sexual morph. We also corrected the taxonomic status of the type species of *Neoscytalidium*.

Although *Neoscytalidium orchidacearum* is similar to the other two species in the genus, namely *N. dimidiatum* and *N. novaehollandiae*, on the grounds of the phylogeny (Fig. 1) we introduce it as a new species. *Neoscytalidium* is usually found on woody plants, such as *Arbutus*, *Grevillea* and *Mangifera* in Africa, America, and Australia and in humans it produces mostly chronic superficial infections of skin, nail and nose [2-4, 6-10]. This is the first report of the genus on leaves of an orchid.

Bakhshizadeh *et al.* [10] isolated a fungus associated with rhinosinusitis in a human patient, which they referred to as *Neoscytalidium dimidiatum.* This isolate was characterized by 1~2 septate, brown conidia in the coelomycete morph and holothallic fragmentation of undifferentiated hyphae on Sabouraud dextrose agar [10]. In our study, ITS sequence data placed it close to *N. orchidacearum*. This isolate was not available to us and we could not make any detailed morphological study and DNA-based studies. Although this isolate may represent another species in *Neoscytalidium* we refrain from naming it until this isolate can be studied in detail.

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