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
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'     | EU673330              | DQ377857              |
| Botryobambusa fusicoccum             | MFLUCC 11-0143' | JX646792              | JX646809              |
| Botryosphaeria agaves                | CBS 133992'     | JX646791              | JX646808              |
| Botryosphaeria agaves                | MFLUCC 10-0051  | JX646790              | JX646807              |
| Botryosphaeria dothidea              | CBS 115476'     | AY236949              | AY928047              |
| Botryosphaeria dothidea              | CBS110302       | AY259092              | EU673243              |
| Botryosphaeria fusispora             | MFLUCC 10-0098' | JX646789              | JX646806              |
| Botryosphaeria fusispora             | MFLUCC 11-0507  | JX646788              | JX646805              |
| Botryosphaeria ramosa                | CMW 26167'      | KF766168              | KF766333              |
| Cophinforma atrovirens               | MFLUCC 11-0425' | JX646800              | JX646817              |
| Cophinforma atrovirens               | CBS 117444      | KF531822              | DQ377855              |
| Diplodia mutila                      | CBS 112553      | AY259093              | AY928049              |
| Diplodia rosulata                    | CBS116470'      | EU430265              | DQ377896              |
| Lasiodiaphora pseudotheobromae       | CBS 116459'     | EF622077              | EU673256              |
| Lasiodiplodia theobromae             | CBS 164.96'     | AY640255              | EU673253              |
| Macrophomina phaseolina              | CBS 227.33      | KF531825              | DQ377906              |
| Macrophomina phaseolina              | CBS 460.70      | KF951639              | EU754169              |
| Neodeightonia phoenicum              | CBS 122528'     | EU673340              | EU673261              |
| Neodeightonia subglobosa             | CBS 448.91'     | 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        | AY19727               | DQ377925              |
| Neoscytalidium dimidiatum (Neoscytalidium hyalinum) | CBS 145.78' | KF531816              | DQ377922              |
| Neoscytalidium dimidiatum (Neoscytalidium hyalinum) | CBS 135.75 | KF51862               | –                     |
| Neoscytalidium novaehollandiae       | CBS122071', CMW 26170' | KF766207          | KF766374              |
| Neoscytalidium novaehollandiae       | CBS 122072      | EFS85535              | –                     |
| Neoscytalidium orchidacearum         | MFLUCC 12-0533' | KU179865              | KU179864              |
| Phaeobotryon mamane                  | CBS 122980'     | EU673332              | EU673248              |
| Tiarosporella tritici                | CBS 118719'     | KF531830              | DQ377941              |
| Tiarosporella urbis-rosarum          | CBS 130405'     | JQ239407              | JQ239420              |

Isolate from this study is indicated in bold.

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

*Ex-type strain.

*Ex-neotype strain.

*Ex-epitype strain.

*Ex-isotype strain.

*Ex-holotype strain.
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(Podospora anserina Genome Project, http://podospora.igmors.un-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. [19, 20]. PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide [21] 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.

RESUL TS

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.

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 dimidiate Penz., Michelia 2: 466 (1882).
≡ Scytalidium dimidiatum (Penz.) B. Sutton & Dyko, Mycol. Res. 93: 484 (1989).
≡ Fusicoccum dimidiatum (Penz.) D. F. Farr, Mycologia 97:
Huang et al. 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).](image)
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 *Fusarium 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

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Index Fungorum number: IF551726; Facesoffungi number: FoF 01362 (Fig. 2).

**Etymology:** The name *orchidacearum* refers to the host family (Orchidaceae).

**Holotype:** MFLU 13-0294.

Saprobitic 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, asetate 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 diameter of 48 mm on PDA in 5 days at room temperature (25–27°C).

**Material examined:** Thailand, Sukhotaï; 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 asexual coelomycetous morph

1. Conidiomata immersed, eventually erumpent      2
2. Conidiomata semi-immersed or superficial (Phillips et al. [5])                        Neoscytalidium novaehollandiae

2. Conidia hyaline                                Neoscytalidium dimidiatum

### 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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