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Neokalmusia didymospora sp. nov. (*Didymosphaeriaceae*) from bamboo

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1. Introduction

There has been considerable recent studies on the biodiversity and taxonomy of Dothideomycetes, the largest and most diverse class of Ascomycota (Schoch et al., 2006; Kirk et al., 2008; Hyde et al., 2013; Ariyawansa et al., 2014; Boonmee et al., 2014; Phookamsak et al., 2014; Thambugala et al., 2014; Wijayawardene et al., 2014a). The majority of Dothideomycetes species and genera are endophytes, saprobes or epiphytes on substrates such as woody plant debris, decaying leaves, herbivore dung (Hyde et al., 2013) or lichens (Lücking and Sérusiaux 2013; Lücking et al., 2013).

This manuscript is part of a study on the diversity and taxonomy of microfungi inhabiting bamboo (Poaceae, Bambusoideae) in northern Thailand and China. Several new taxa have already been described (Dai et al., 2012, 2014a, 2014b, 2014c; Liu et al., 2012). In this paper, we introduce a new ascomycetous genus which belongs to the family Didymosphaeriaceae (Pleosporales), based on morpho-molecular study.

2. Materials and Methods

2.1 Collection and isolation of fungi

Fallen and decomposing bamboo culms were collected from Mae Fah Luang University campus in Chiang Rai Province, Thailand. The samples were placed in plastic Ziplock bags and brought to the laboratory. The specimens were incubated in sterile moist chambers and examined at regular intervals until the resident fungi attained maturity and sporulated. The fungi were examined under stereoscope and compound microscopes and isolated by single spore isolation following the method of Chomnunti et al. (2011). The colonies were transferred to 1.5 ml microcentrifuge tubes with 2% potato-dextrose agar (PDA) to deposit at 4˚C and suspended in 2 ml screw cap microcentrifuge tube with 10% Glycerol to store at -20˚C. Microscopic observations and photomicrographs were made as described in Boonmee et al. (2011). Type materials are deposited at the herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU) and Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN). The living cultures are deposited at Mae Fah Luang University Culture Collection (MFLUCC), the Research Institute of Resource Insects, Chinese Academy of Forestry (IFRD) and Landcare Research, New Zealand (ICPM).

2.2 DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA for 30 d at 27˚C and genomic DNA was extracted from fresh mycelia, following the specification of Biospin Fungus Genomic DNA Extraction Kit (BioFlux®). ITS5 and ITS4, NS1 and NS4 (White et al., 1990) and LROR and LR5 (Vilgalys and Hester 1990) primers were used for the amplification of internal transcribed spacers (ITS), small subunit rDNA (SSU) and large subunit rDNA

(LSU) respectively. Polymerase chain reaction (PCR) amplification was carried out following the method of Phillips et al. (2008). Amplified PCR fragments were sequenced at Kunming Shuo Yang Technology Company, P.R. China. Generated new sequences of ITS, LSU and SSU regions are deposited in GenBank.

2.3 Sequence alignment and phylogenetic analyses

Blast searches at GenBank were carried out by both LSU and SSU rDNA sequences in order to reveal the closest taxa to our strain. Sequence data of Didymosphaeriaceae were selected from Ariyawansa et al. (2014) and Wijayawardene et al. (2014b) (Table 1). Further, we included other families in Pleosporales i.e. Massariaceae, Morosphaeriaceae, Lentitheciaceae and Trematosphaeriaceae (Hyde et al., 2013). The tree is rooted to Halojulella avicenniae (BCC 20173). Sequences were aligned using Bioedit (Hall, 2001) and ClustalX (Kohli and Bachhawat, 2003). Alignments were checked and manual adjustments were carried out when necessary. In the analysis, gaps were treated as missing data, and all characters were unordered and of equal weight (Begoude et al., 2010; Liu et al., 2011, 2012).

Combined ITS, LSU and SSU gene sequence data were used in the analyses. All sequences obtained from GenBank and used by Schoch et al. (2009), Suetrong et al. (2009), Zhang et al. (2012), Hyde et al. (2013), Ariyawansa et al. (2014), Boonmee et al. (2014), Verkley et al. (2014) and Wijayawardene et al. (2014b) are listed in supplementary Table 1. Multiple sequence alignments were generated with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html). All introns and exons were aligned separately. Regions containing many leading or trailing gaps were removed from the ITS, SSU and LSU alignments prior to tree building. The alignments were checked visually and improved by eye wherever necessary.

Maximum likelihood analyses including 1000 bootstrap replicates were run using RAxML v. 7.2.6 (Stamatakis and Alachiotis 2006, 2008). The online tool Findmodel (http:// www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to determine the best nucleotide substitution model for each partition. The best scoring tree was selected with a final likelihood value of -19492.551787. The resulting replicates were plotted on to the best scoring tree obtained previously. Maximum Likelihood bootstrap values (ML) equal or greater than 50 % are given below or above each node in red (Fig. 1).

The model of evolution was performed by using MrModeltest 2.2 (Nylander, 2004). Posterior probabilities (PP) (Rannala and Yang, 1996; Zhaxybayeva and Gogarten, 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003). Six simultaneous Markov chains were run for 3000000 generations and trees were sampled every 100th generation and 30000 trees were obtained. The first 6000 trees, representing the burn-in phase of the analyses, were discarded while the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01) (Cai et al., 2006; Ariyawansa et al., 2014). Bayesian Posterior Probabilities (BYPP) equal or greater than 0.90 is

given below or above each node (Fig. 1).

Trees were visualized with TreeView (Page, 1996).

3. Results

3.1 Phylogeny of the combined ITS, LSU and SSU gene dataset

Partial nucleotide combined sequences of ITS, LSU and SSU were used to determine the taxonomic placement of our strain. The ITS, LSU and SSU datasets were used in the phylogenetic analyses for generic placement (Fig. 1).

The dataset comprises 66 sequences of one newly sequenced taxon and 55 taxa including one outgroup taxon (Table 1). Results of the partition-homogeneity test (P = 0.107) indicate that the ITS, LSU and SSU genes trees reflect the same underlying phylogeny. Therefore, these datasets were combined and analyzed using several tree-building programs, the resulting trees compared and the best tree is presented in Fig. 1. The phylogenetic trees generated by Maximum likelihood (ML) and Bayesian analyses of combined ITS, LSU and SSU gene regions have shown that new taxon clusters within the family Didymosphaeriaceae and same clade within genus Neokalmusia (Fig. 1). Bootstrap support (BS) values of ML and the Bayesian posterior probabilities (PP) from MCMC analyses are shown in Fig 1. New sequences are deposited in GenBank (Table 1).

3.2 Taxonomy

Neokalmusia Kaz. Tanaka, Ariyawansa & K.D. Hyde, Fungal Diversity 68(1): 92 (2014)
Index Fungorum number: IF550700
Facesoffungi number: FoF 00050

Notes: Neokalmusia, introduced by Ariyawansa et al. (2014), is typified by N. brevispora (Nagas. a Y. Otani) Kaz. Tanaka, Ariyawansa & K. D. Hyde. This genus is established to accommodate two bambusicolous taxa, N. brevispora and N. scabrispora (Ariyawansa et al., 2014). Neokalmusia is characterised by immersed, subglobose to oblong ascomata with several perithecia in a row, a clypeus-like structure composed of thin-walled cells and verrucose ascospores (Tanaka et al., 2005; Ariyawansa et al., 2014).

Key to species of Neokalmusia

1. Clypeus black, raised, ascus pedicel shorter than 45 µm, ascospores 3–7-septate **2**
1. Clypeus flattened, orange to brown, asci pedicel longer than 45 µm, ascospores 1-septate ..... **Neokalmusia didymospora**
2. Ascospores with 3 septa ..... **N. brevispora**
2. Ascospores with 3–7 septa ..... **N. scabrispora**

Table 2 Comparison of species in the genus Neokalmusia.

Name and reference	Ascomata	Ascomata wall	Asci	Ascospores
Neokalmusia brevispora (Ariyawansa et al., 2014)	190–440 µm diam., 200–400 µm high, clypeus black, rise.	15–20 µm thick.	80–118 × 10.5–15 µm, cylindrical to clavate, pedicel 10–30 µm	18–24(-26.5) × 4–7 µm, fusiform, 3-septate, verrucose.
N. scabrispora (Ariyawansa et al., 2014)	130–500 µm diam., 200–300 µm high, clypeus black, rise.	7.5–20 µm thick.	123.5–160 × (15.5-)17–22 µm ,clavate, a short stipe.	29–40.5 × 7–10 µm, fusiform to ellipsoid, 5–7 -septate, verrucose.
N. didymospora (This study)	350–600 µm diam., 100–300 µm high, clypeus orange to brown, flatten, slightly raised cracks at the centre.	30–45 µm thick.	125–160 × 9.5–14 µm, cylindrical, pedicel 45–80 µm.	13.5–16 × 5.0–6.5 µm, broad fusiform, 1-septate, verrucose.

Neokalmusia didymospora D.Q. Dai & K.D. Hyde, **sp. nov.** (Fig. 2)

Index Fungorum: IF550836

Facesoffungi number: FoF 0061

Etymology: With reference to its didymospores.

Holotype: MFLU 14–0197

Saprobic on decaying bamboo culms. **Sexual morph:** Clypeus orange to brown on host surface with ascomata breaking through slightly raised cracks at the centre. Ascomata 350–600 µm diam., 100–300 µm high, solitary, scattered or in groups of 2–5, immersed under the host epidermis, subglobose, light brown, coriaceous, ostiolate at the centre. Peridium comprising host and fungal tissues, laterally 25–30 µm thick, composed of brown and thick-walled to hyaline and thin-walled cells of textura angularis. Hamathecium composed of dense, long, 1.5–2 µm broad, septate, branched pseudoparaphyses between and above the asci. Asci 125–160 × 9.5–14 µm ($\bar{x}$ = 142.2 × 12.3 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical, with a shallow apical chamber and a long furcate pedicel 45–80 × 3–7.5 µm ($\bar{x}$ = 61.3 × 5.3 µm, n = 20). Ascospores 13.5–16 × 5.0–6.5 µm ($\bar{x}$ = 14.7 × 5.9 µm, n = 20), 1–2-seriate, broad fusiform, with larger upper cell and elongated lower cell, with narrowly rounded or pointed ends, golden brown, 1-septate, deeply constricted at the septum, distinctly verrucose on the surface. **Asexual morph:** unknown.

Colonies in culture: Ascospores germinating on PDA within 36 h and germ tubes

produced from both ends. Colonies growing slowly on PDA, reaching 10 mm in 2 weeks at 28°C, effuse, velvety to hairy, circular, white to brown from above, dark brown in media from below. Mycelium immersed in the media, septate, branched, smooth, hyaline.

Material examined: THAILAND, Chiang Rai, Mae Fah Luang University, on dead culm of bamboo, 1 August 2011, Dong-Qin Dai, DDQ00098 (MFLU 14–0197, **holotype**; **isotype** in KUN under the code of HKAS 83862), ex-type living culture = MFLUCC 11–0613 = IFRDCC 2553 = ICMP.

Notes: Neokalmusia didymospora is characterized by immersed, orange to brown, thin-walled ascomata, bitunicate, fissitunicate, cylindrical asci with long pedicel and brown, 1-septate ascospores. This fungus shares similar characters with N. brevispora and N. scabrispora in having immersed ascomata, a thin-walled peridium containing host and fungal tissue and verrucose ascospores (Tanaka and Harada, 2004; Ariyawansa et al., 2014). However, N. didymospora differs from N. brevispora and N. scabrispora in ascomata forming an orange to brown, flat, clypeus on the host surface, long-pedicellate asci and 1-septate ascospores.

4. Discussion

The family Didymosphaeriaceae, introduced by Munk (1953) and typified by Didymosphaeria Fuckel, can be placed in the order Pleosporales. Hyde et al. (2013) included Appendispora, Didymosphaeria and Phaeodothis in Didymosphaeriaceae. Wijayawardene et al. (2014b) introduced two new coelomycetous genera. Ariyawansa et al. (2014) synonymized Montagnulaceae under Didymosphaeriaceae. Based on these recent studies (Ariyawansa et al., 2013, 2014; Wijayawardene et al., 2014b), 16 genera are accepted in Didymosphaeriaceae, viz. Alloconiothyrium, Bimuria, Deniquelata, Didymocrea, Didymosphaeria, Kalmusia, Karstenula, Letendraea, Montagnula, Neokalmusia, Paracamarosporium, Paraconiothyrium, Paraphaeosphaeria, Phaeodothis, Pseudocamarosporium and Tremateia. In our study, we have included all of these genera of Didymosphaeriaceae in the phylogenetic tree (Fig. 1) to determine the placement of our taxon.

The dataset of combined LSU, SSU and ITS genes were used in the analysis. The new taxon Neokalmusia didymospora is embedded within the Didymosphaeriaceae (71%/0.92 MLBS/BYPP support (Fig. 1) and can be distinguished phylogenetically from other species of Neokalmusia (54%/0.95 MLBS/BYPP support) (Fig. 1). Morphological studies show N. didymospora differing phylogenetically from the closest species, N. brevispora and N. scabrispora (Ariyawansa et al., 2014). These species (N. brevispora and N. scabrispora), have raised, black clypeus-like structures forming a dehiscent, black hemisphere on host surface and 3–7-septate ascospores (Tanaka and Harada, 2004; Ariyawansa et al., 2014). Neokalmusia didymospora, however, differs with ostiole of ascomata rising through the host epidermis tissue and forming an orange, flat area, and 1-septate ascospores.

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Figure 1

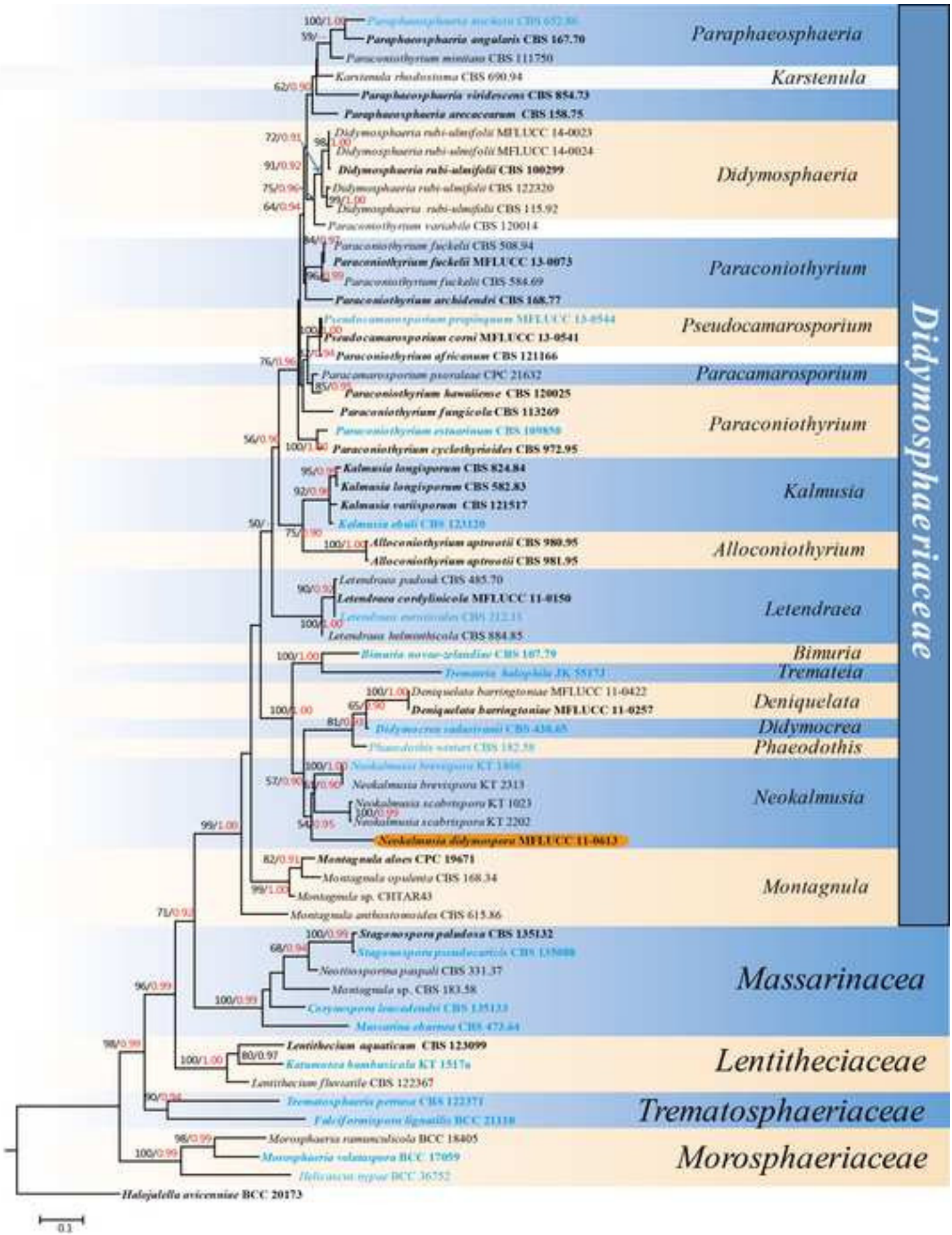

Figure 2

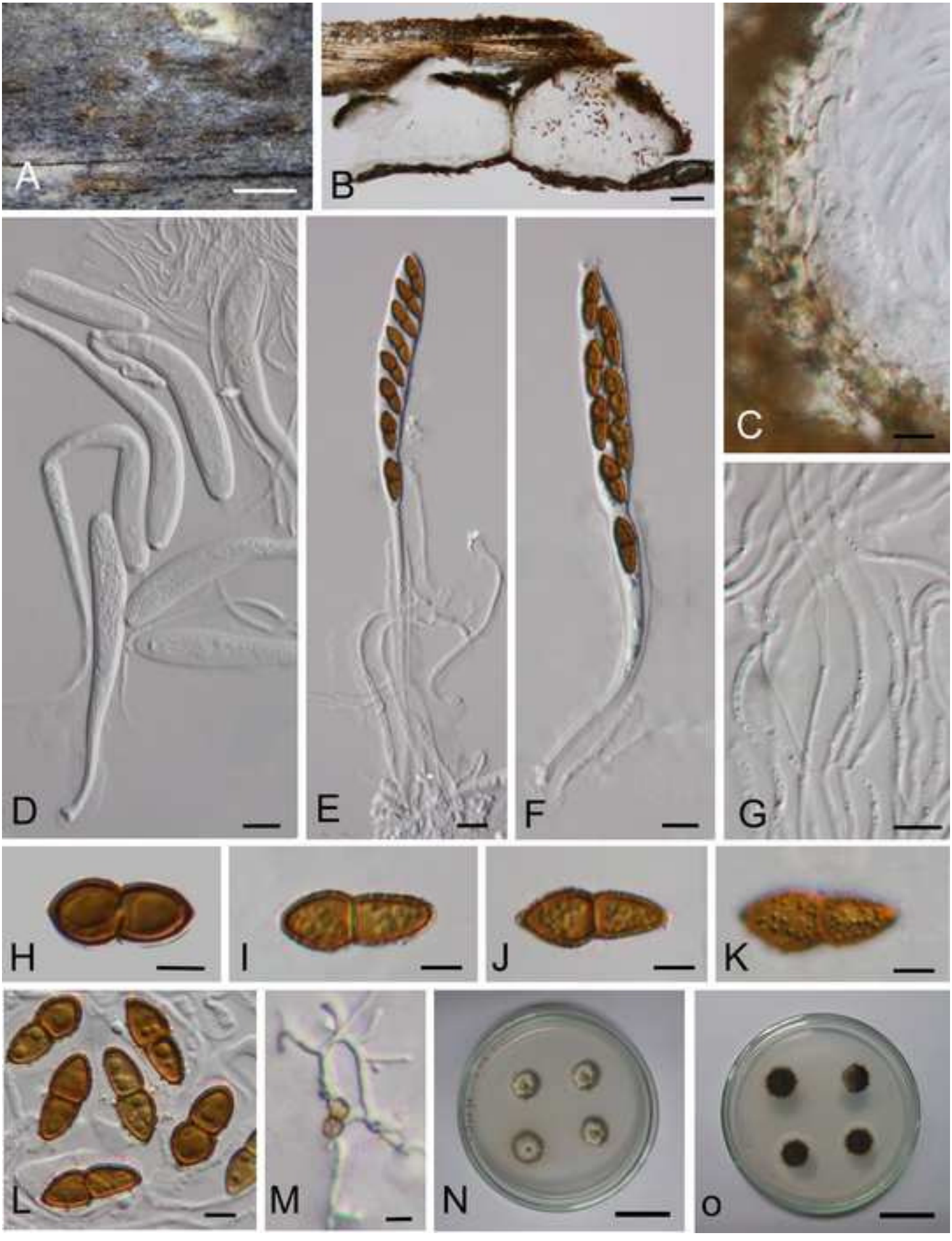

Figure 1 Phylogenetic tree generated from maximum likelihood (ML) and Bayesian analysis based on combined ITS, LSU and SSU sequences. Data were analyzed with random addition sequence and treating gaps as missing data. Bootstrap support (BS) values above 50% are shown at nodes. Hyphen ("--") indicates a value lower than 50 % (BS). The original isolate numbers codes are noted after the species names. The tree is rooted with Halojulella avicenniae (BCC 20173). Ex-type or ex-epitype strains are in bold. The type species of each genus is indicated in blue. Newly generated taxon is in orange background.

Figure 2 Neokalmusia didymospora (**holotype,** MFLU 14–0197). A. Ascomata immersed in bamboo host. B. Section of ascoma. C. Peridium of ascoma. D, E. Asci. F. Immature asci. G. Pseudoparaphyses. H–L. Ascospores. K. Ascospore with verrucose surface. M. Germinating ascospore. N, O. Cultures on PDA. Scale bars: A = 1mm, B = 100 µm, C = 10µm, D–M = 10 µm, N-O = 25 mm.

Keywords: Dothideomycetes, ITS, LSU, SSU, taxonomy

Abstract: A new ascomycetous species, Neokalmusia didymospora, inhabiting decaying bamboo, is introduced based on morpho-molecular studies. Neokalmusia didymospora is characterized by orange to brown clypeus, immersed, subglobose ascomata, bitunicate, cylindrical asci and 1-septate, brown ascospores. Maximum-likelihood and Bayesian analyses of combined ITS, LSU and SSU gene sequence data show that N. didymospora belongs in Didymosphaeriaceae, Pleosporales. The new species is compared with other morphologically and phylogenetically similar species.