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Deniquelata barringtoniae gen. et sp. nov., associated with leaf spots of *Barringtonia asiatica*

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Abstract

Deniquelata barringtoniae gen. et sp. nov. (*Montagnulaceae*) forms numerous ascomata on distinct zonate leaf spots of *Barringtonia asiatica* (*Lecythidaceae*). We isolated this taxon and sequenced the 18S and 28S nrDNA. The result of phylogenetic analysis based on 18S and 28S nrDNA sequence data indicate that the genus belongs in the family *Montagnulaceae*, *Dothideomycetes*, *Ascomycota*. The ascomata are immersed, dark brown to black, with bitunicate asci and brown, muriform ascospores. *Deniquelata* is distinguished from the other genera in *Montagnulaceae* based on its short, broad, furcate and pedicellate asci, verruculose ascospores with short narrow pseudoparaphyses with parasitic naturee and this is also supported by molecular data. A new genus and species is therefore introduced to accommodate this taxon. We used isolates of this species to show via pathogenicity testing that the taxon is able to cause leaf spots when leaves are pin pricked.

Key words: Dothideomycetes, foliar pathogen, Montagnulaceae, new genus, new species

Introduction

Among the classes of Ascomycota, Dothideomycetes is the largest and most diverse (Kirk et al. 2008, Zhang et al. 2012) and is characterized by bitunicate, usually fissitunicate asci (Schoch et al., 2009). The majority of members in this class are endophytes, epiphytes or saprobes with a small number occurring as lichens and hyperparasites (Kirk et al. 2008, Zhang et al. 2012). Many, especially asexual or mitosporic Dothideomycetes, are agents of plant diseases, causing serious problems to crop plants (Manamgoda et al. 2011, Wikee et al. 2011).

Besides marine and freshwater fungi (Suetrong et al. 2010), there have been a few taxonomic studies of Dothideomycetes of Thailand. Boonmee et al. (2011) illustrated new collections of *Tubeufiaceae*, including a new genus *Chlamydotubeufia* based on dictyochlamydosporous anamorphs and three new species; one each in *Acanthostigma*, *Tubeufia* and *Thaxteriella* based on phylogenetic analysis of rDNA sequence data (Boonmee et al. 2011). Based on combined dataset of 18S and 28S nrDNA sequences, Chomnunti et al. (2011) introduced *Phragmocapnias asiaticus*, *P. longicollus*, *P. penzigii*, *P. siamensis*, *Leptoxyphium cacuminum* and *Capnodium coartatum* in the family *Capnodiaceae* and epitypified the genus *Phragmocapnias* with a new collection of *P. betle*. Liu et al. (2011) reported two new genera, *Fussiroma* and *Neoastrosphaeriella*, in *Aigialaceae*, based on their distinguishing morphology and molecular phylogeny.

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Barringtonia asiatica is commonly distributed in tropics. All plant parts of this species are poisonous and seeds are commercially used as a fish poison. The leaf decoction of *B. asiatica* is used to treat stomach-aches and rheumatism, whereas seeds are known to be wormicidal (http://www.naturia.per.sg/buloh/plants/sea_poison.htm; Accession date: 21 June 2012).

We have been sampling Dothideomycete species from various hosts and substrates in northern Thailand (Boonmee et al. 2011, 2012, Chomnunti et al. 2011, 2012, Lui et al. 2011), since 2010. In this paper, we introduce a new genus *Deniquelata* found associated with necrotic lesions on leaves of *Barringtonia asiatica*, based on morphological and molecular studies. The new genus is compared with other genera in the family *Montagnulaceae*.

Material and Methods

Sample collection and specimen examination

Samples included fresh, living leaves of *Barringtonia asiatica* (*Lecythidaceae*), gathered from the garden of the senior author (K.D. Hyde) at 31M. 17, Baan Khuakhae, near Bandu, Muang District, Chiang Rai Province, Thailand, in 2011 and 2012. Material was brought to the laboratory in Zip lock plastic bags and examined under a Motic SMZ 168 dissecting microscope for fungal fruiting bodies. Hand sections of the fruiting structures were mounted in water for microscopic studies and photomicrography. The fungus was examined in a Nikon ECLIPSE 80i compound microscope and photographed by a Canon 450D digital camera fitted to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures were processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems Inc., The United States). Following a modified method of Chomnunti et al. (2011), isolations were made from single ascospores. Contents of the sectioned fruiting body were transferred to a drop of sterile water on a flame-sterilized slide. Drops of the spore suspension were pipetted and spread on a Petri-dish containing 2% water agar (WA) and incubated at 25°C overnight. Germinated ascospores were transferred singly to MEA media (Alves et al. 2006, Liu et al. 2011).

The type material is deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand and cultures are deposited at the Mae Fah Luang University Culture Collection (MFLUCC), BIOTEC Culture Collection (BCC) and the International Fungal Research & Development Centre, Kunming, China (IFRD), the latter under Material Transfer Agreement No. 4/2010 (MTA).

Pathogenicity test

An agar plug (1 cm diam) with mycelium was cut from the periphery of 20 day-old culture grown on Malt extract agar (MEA) medium (27°C). *In vitro* pathogenicity testing was carried out on 12 healthy leaves of *Barringtonia asiatica* obtained from the original collection site at Bandu, Chiang Rai; the upper and lower surfaces of leaves were sterilized with 70% ethanol. The leaves were separated into three groups, each with four leaves. The first group of leaves was wounded by pin-pricking and inoculated by tiny agar plugs (1 cm diam) with fungal mycelium. Agar plugs with fungal mycelium were placed on the surface of second group containing unwounded leaves. The third group of leaves was not pin-pricked and was inoculated with MEA agar plugs without fungal mycelium (control). The inoculated test leaves were maintained in sterile, moist plastic boxes for 14 days. Observations on the development of disease symptoms were made on a daily basis. The fungus was reisolated from the inoculated leaves producing symptoms.

DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on MEA for 28 days at 25°C in the dark. Genomic DNA was extracted from the growing mycelium using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China).

Polymerase chain reaction (PCR) was carried out using known primer pairs NS1 and NS4 were used to amplify a region spanning the small subunit rDNA (White et al. 1990). LROR and LR5 primer pairs were used to amplify a segment of the large subunit rDNA (Vilgalys & Hester 1990) and internal transcribed spacers (JX254654) was amplified by primer pairs ITS5 and ITS4 (White et al. 1990). The amplification procedure was performed in a 50 µl reaction volume containing 5–10 ng DNA, 0.8 units *Taq* polymerase, 1× PCR buffer, 0.2 mM dNTP, 0.3 µM of each primer with 1.5 mM $MgCl_2$ (Cai et al. 2009). Amplification conditions were setup for initial denaturation of 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 45 s at 48°C and 90 s at 72°C, and a final extension period of 10 min at 72°C (Phillips et al. 2008). The PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China).

Phylogenetic analysis

Blast searches were made to reveal the closest matches in GenBank. Sequences were aligned using Bioedit (Hall 1999) and ClustalX 1.83 (Thompson et al. 1997) and further analyzed manually. PAUP4.0b10 was used to conduct the parsimony analysis to obtain the phylogenetic trees. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were setup to 500 and branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony Tree Length [TL], Consistency Index [CI], Retention Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI] were calculated for trees generated under different optimality criteria. Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. Trees were figured in Treeview (Page 1996).

Results

Phylogeny of combined 18S and 28S nrDNA

The combined 18S and 28S nrDNA data set comprised 27 taxa including the new strains of *Deniquelata barringtoniae*, with *Dothidea sambuci* as the outgroup taxon and consisted of 1961 characters; of which 1673 characters were constant, 118 variable characters were parsimony-informative and 170 characters were parsimony-uninformative. Two MP trees were generated and the first of the most parsimonious tree was selected (FIG. 1), KHT showed length = 508 steps, CI = 0.671, RI = 0.759, RC = 0.510 and HI = 0.329.

The 27 taxa analyzed in the cladogram formed four familial clades, i.e. *Didymellaceae*, *Pleosporaceae*, *Massarinaceae* and *Montagnulaceae*. The two isolates of the new genus *Deniquelata* clustered in the family *Montagnulaceae* but were separated from other genera of the family in a clade with a relatively high bootstrap value (77%).

Taxonomy

The genus *Deniquelata*, typified by *D. barringtoniae*, is introduced in the family *Montagnulaceae*. The new taxon is well differentiated from other genera in the family based on molecular phylogeny and morphology. The morphological characters also differentiated *Deniquelata barringtoniae* from other genera in the family, as discussed below.

Deniquelata Ariyawansa & K.D. Hyde, *gen. nov.*
MycoBank MB 800703

Etymology: The generic epithet is from the combination of two Latin words *denique* and *lata*, meaning short and broad, in reference to the asci having a short, broad pedicel.

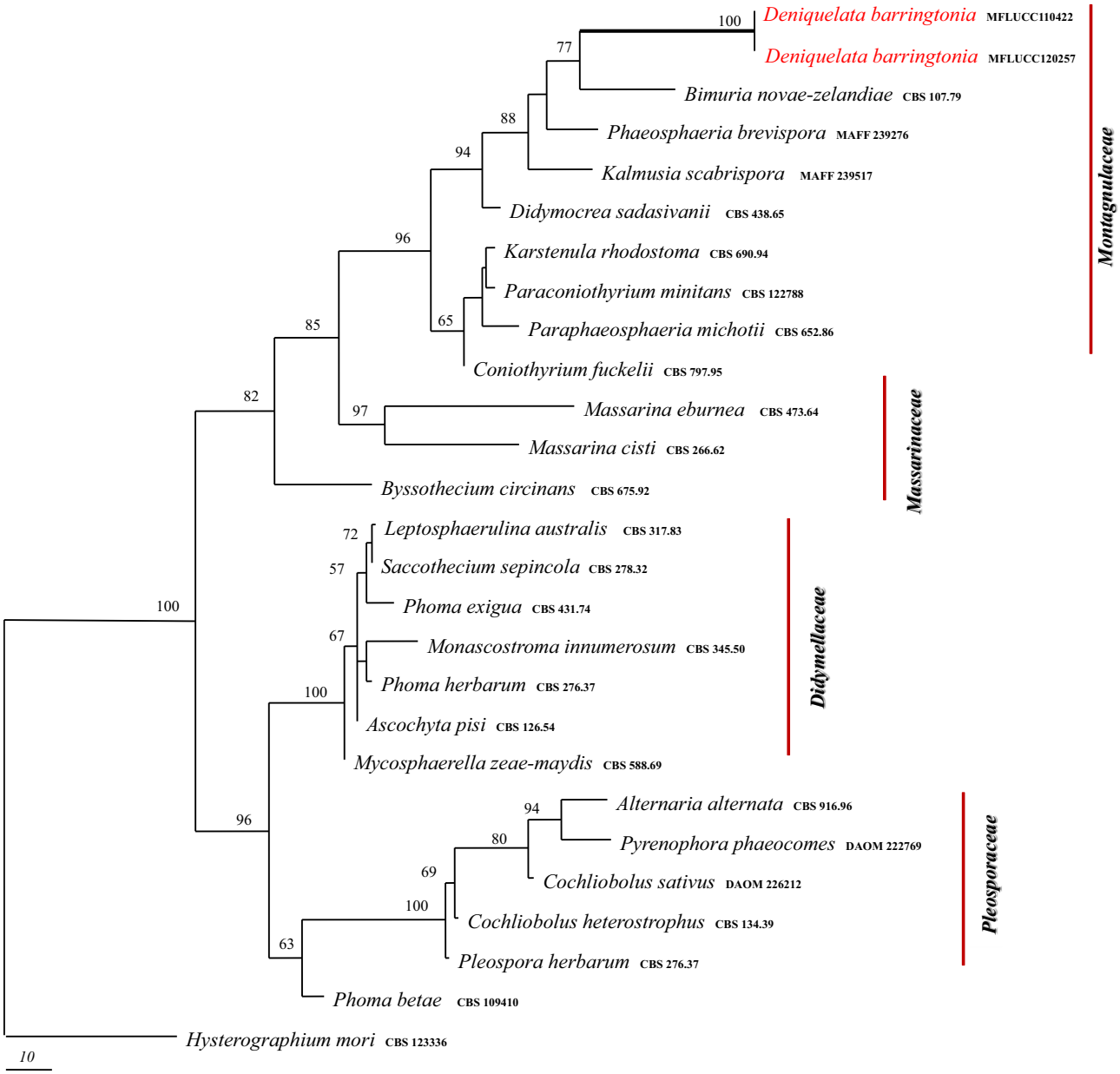

FIGURE 1. Gene tree constructed using MP method based on phylogenetic analysis of the nucleotide sequences of combined 18S and 28S nrDNA. Bootstrap support values >70% are shown below or above the branch. *Dothidea sambuci* is the outgroup taxon. The original isolate numbers are noted after the species names.

Habit parasitic on living leaves. *Ascomata* immersed, solitary, scattered, globose to subglobose, dark brown to black, smooth-walled, with a distinct ostiole, apex somewhat papillate to depressed. *Peridium* several-layered, outer wall composed of small, dark brown to black, heavily pigmented, thick-walled, comprising cells of *textura angularis* and fusing with the host, inner wall consist with broad yellowish brown cells, inwardly lined by hamathecial tissues. *Hamathecium* of dense with pseudoparaphyses. *Pseudoparaphyses* broad, hyaline, embedded in a gelatinous matrix. *Asci* 8-spored, bitunicate, fissitunicate, clavate to broadly-clavate, with a short, broad, furcate pedicel, rounded at apex and with an ocular chamber. *Ascospores* biseriate, partially overlapping, oblong to narrowly oblong, reddish brown to dark yellowish brown, muriform, with three transverse septa and 1-2 vertical septa in the central cells when mature, constricted at the septa, verruculose, without a sheath. **Generic type:**

Deniquelata barringtoniae Ariyawansa & K.D. Hyde, *sp. nov.* FIGS. 1A–F, 3G–S
MycoBank MB 800704

Etymology: The specific epithet *barringtoniae* is based on the host genus from which the fungus was isolated. *Habit* pathogenic, causing large brown spots on living leaves of *Barringtonia asiatica* (FIG. 2A–E). *Ascomata* 150–180 µm high, 164–190 µm wide ($\bar{x}$ = 175 × 167 µm, n = 10), immersed, scattered, globose to subglobose, black to dark brown, smooth walled, with a papillate to depressed elongate ostiole (FIG. 3A,B) *Peridium* 9–17 µm diam ($\bar{x}$ = 12, n = 10), composed of 3–5 layers of brown to black, darkly pigmented, small, thick-walled, 2–5 µm wide cells of *textura angularis*, with outer wall fused with the host cells, inner wall consists of 2 layers of polygonal to rectangular, light brown-hyaline cells 1–4 µm diam. (FIG. 3C). *Hamathecium* composed of dense, 1–3 µm diam ($\bar{x}$ = 2, n = 20), broad, hyaline, septate pseudoparaphyses, surrounding the numerous asci and embedded in a gelatinous matrix (FIG. 3D). *Asci* (60–)68–80 × 1015 µm ($\bar{x}$ = 72 × 13 µm, n = 20), 8-spored, bitunicate, fissitunicate, clavate to broadly-clavate, with a 9–17 µm, short, broad, furcate, long pedicel, rounded at apex, ocular chamber up to 1–2 µm wide, 1–3 µm high (FIG. 3E–G). *Ascospores* 14–16 × 5–7 µm diam ($\bar{x}$ = 15 × 6 µm, n = 40), biseriate or distichously arranged, partially overlapping, oblong to narrowly oblong, straight or somewhat curved, reddish-brown, with three transverse septa and 1–2 vertical septa in the central cells, constricted at the primary and secondary septa at maturity, verruculose, straight or slightly inequilateral, without a gelatinous sheath (FIG. 3H–J).

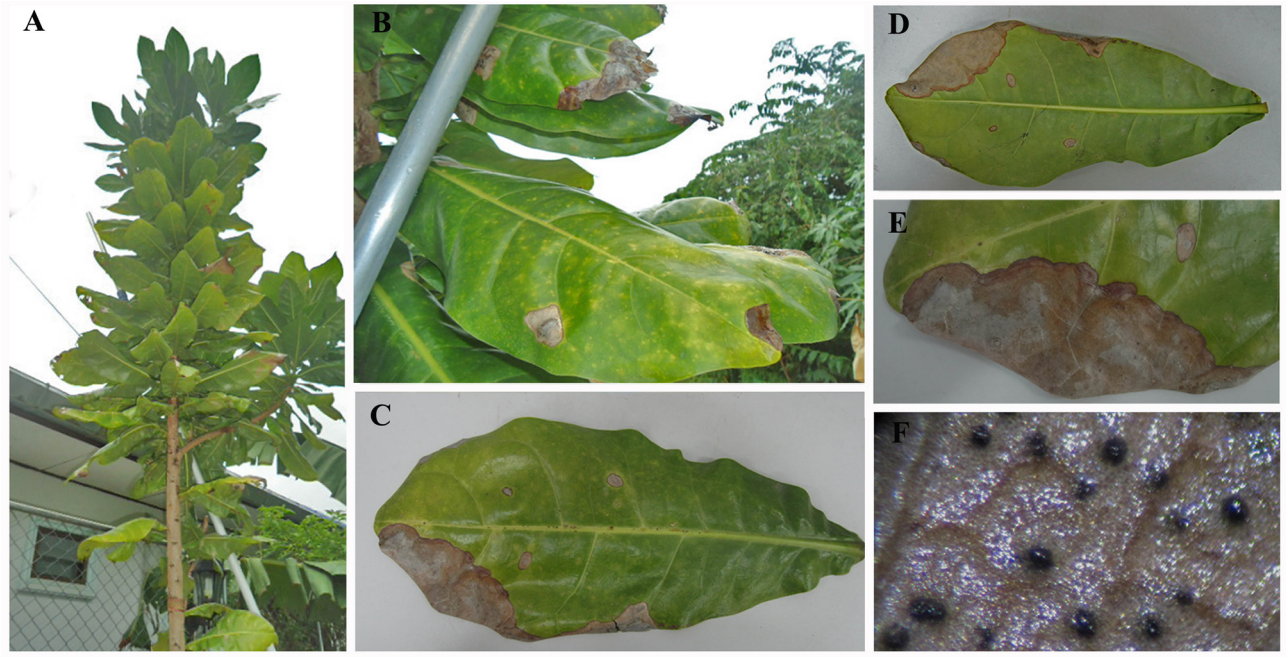

FIGURE 2. Habit of *Deniquelata barringtonia*. **A–E**. Leaves with characteristic leaf spots (Holotype). **F.** Immersed ascomata on leaf tissue.

Cultural characteristics: Ascospores germinated on WA within 18 h and germ tubes were produced from each cell (FIG. 3K). Colonies grew slowly on MEA, attaining 3 mm diam. After 14 days at 27°C, effuse, velvety, entire to slightly undulated at the edge and remaining white to pinkish white (FIG. 3L–S). After 6 months of incubation, the colonies on malt extract agar and water agar media co ntained only superficial, branched, septate, smooth, mycelia with no asexual-stage.

Material examined: THAILAND, Chiang Rai Prov., Muang District, Bandu, Baan Khuakhae, 31M.17 on leaf of *Barringtonia asiatica* (*Lecythidaceae*) 18 September 2011, K.D Hyde, RP0025 (MFLU 12-0303; **holotype**)—extype living culture (MFLUCC 110422**)**; Chiang Rai Prov., Muang District, Bandu, Baan Khuakhae, 31M.17, on leaves of *Barringtonia asiatica* (*Lecythidaceae*), 15 May 2012, H.A Ariyawansa, RP0025 (MFLU 12-0304, **paratype**)—extype living culture (MFLUCC 120257).

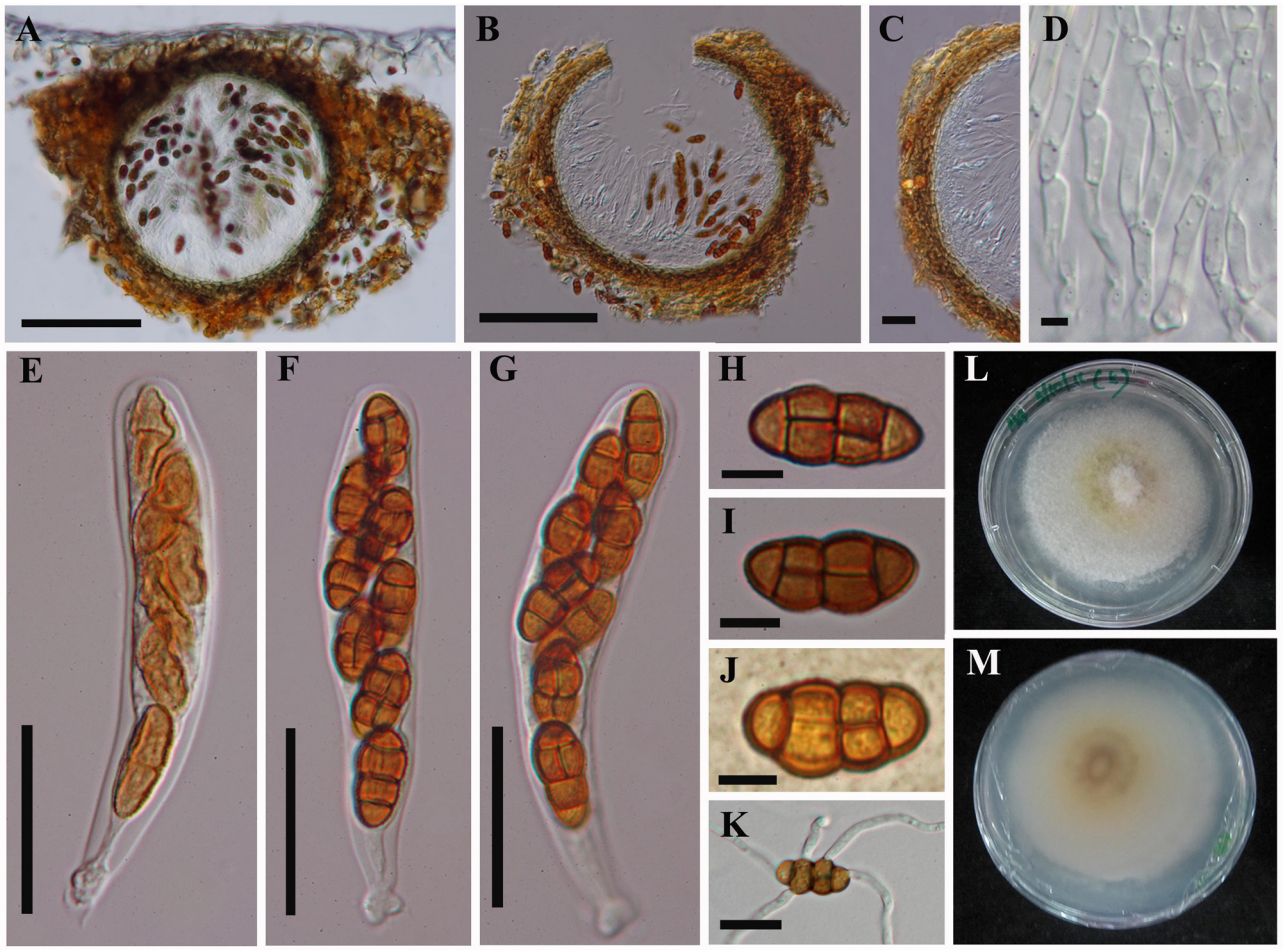

FIGURE 3. *Deniquelata barringtoniae* (holotype). **A–B.** Section of ascoma (TS). **C.** Section of peridium comprising a few layers of cells. **D.** Hyaline, pseudoparaphyses **E–G.** Eight-spored asci with short, broad pedicel at the base. **H–I.** Reddish-brown, muriform, ascospores. **J.** Ascospores stained in Indian Ink **K.** Germ tubes developing from ascospore cells **L–M.** Surface and lower view of colonies on MEA. **Scale bars:** C–D = 100 µm, E = 10 µm, F = 10 µm, G = 30 µm, H–I = 10 µm, J–L = 30 µm, M-P = 10 µm, Q= 20 µm.

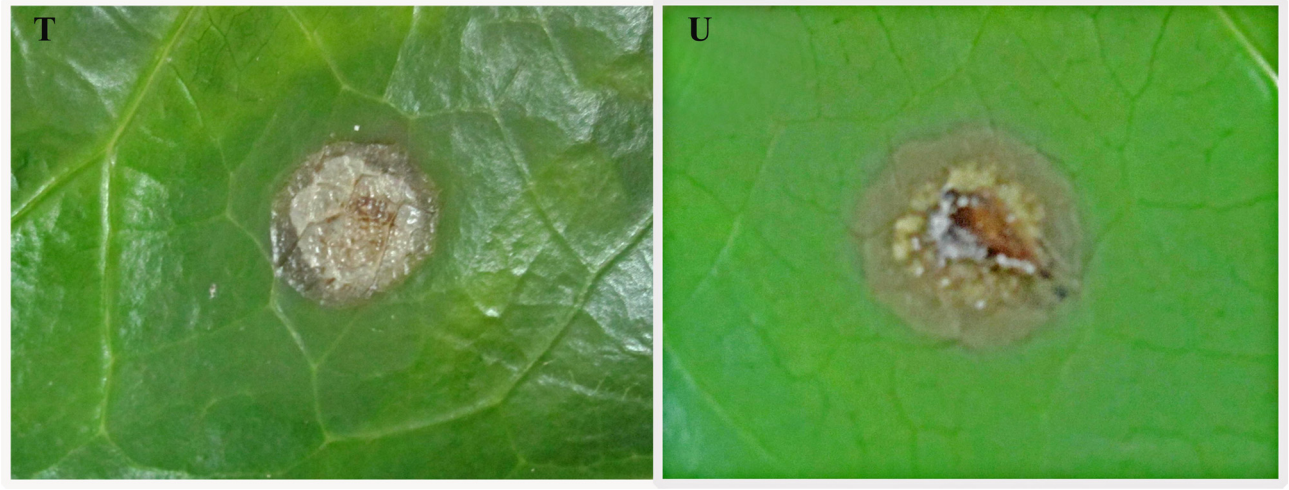

FIGURE 4. Symptoms appearing on pin pricked leaves of *D. barringtonia* inoculated with agar plugs.

Distribution: On living leaves of *Barringtonia asiatica* Thailand.

Pathogenicity test (FIG. 4): Leaves wounded by pinpricking inoculation technique, initially developed small, circular, ash-coloured spots which subsequently transformed into brown spots. After 10 days of incubation, the spots expanded to 2 mm diam. The spots further enlarged and became sunken causing soft decay of the leaf tissues, surrounded by white mycelium. The pathogen, re-isolated from the leaf spots was

found to be identical with the original strain and thus confirmed its pathogenicity. No symptoms were observed on leaves inoculated by agar plugs with fungal mycelium but without wounding and also in the case of controls. The experiment was carried out using four replicates and repeated three times. In all cases, the results were similar.

Discussion

Barr (2001) introduced a new family, *Montagnulaceae* in the order Pleosporales (the largest of the Dothideomycetes, comprising almost 25% of the species) for genera and species with ascomata immersed under a clypeus and having pseudoparenchymatous peridium with small cells, cylindrical or oblong, fissitunicate, pedicellate asci and brown, muriform ascospores. Three genera, viz. phragmosporous *Kalmusia*, dictyosporous *Montagnula* and didymosporous *Didymosphaerella* were recognized (Barr 2001). Combined analysis of LSU, SSU, RBP2 and TEF1 genes, however, has shown *Montagnulaceae* to comprise the genera *Bimuria, Didymocrea, Kalmusia*, *Karstenula, Montagnula, Didymosphaerella* and *Paraphaeosphaeria*. Some species of *Phaeosphaeria, Paraconiothyrium* and *Letendraea* also cluster in *Montagnulaceae* and this family now accommodates species which are saprobic in terrestrial or aquatic environments (Schoch et al. 2009, Zhang et al. 2009, 2012, Lumbsch & Huhndorf 2010).

The phylogenetic analysis of the nucleotide sequences of combined 18S and 28S nrDNA provides good evidence that *Deniquelata* belongs in *Montagnulaceae*, where it forms a distinct clade representing the new genus separated parallel from other genera of the family with a high bootstrap value (77%) (FIG. 1). *Deniquelata* differs considerably from the type of *Montagnula* (*M. infernalis*) both in the phylogenetic tree and also morphologically. *Deniquelata* is a pathogenic genus with fruiting bodies scattered in the necrotic host tissues. Ascomata are immersed, with a relatively thin peridium (2–5 µm) comprising 2–3 layers of small, thick-walled cells of *textura angularis*, fusing at the outside with the host tissues. Pseudoparaphyses are relatively wide (1–3 µm) and dense, asci have broad, short, bifurcate pedicels, and ascospores are conspicuously reddish brown at maturity. In *Montagnula infernalis* ascomata are clustered in circular groups on leaves, with a much thicker (40–55 µm), brown to dark-brown peridium comprising two distinct layers, asci have long stretching pedicels and ascospores are brown (Zhang et al. 2012). *Bimuria*, a monotypic genus represented by *B. novae-zelandiae* (Hawksworth et al. 1979, Zhang et al. 2009), occurs in terrestrial environment as a saprobe and differs from *Deniquelata* in having asci with short and small knob-like pedicel and ascospores without vertical septum and verrucose wall (Zhang et al. 2012). *Deniquelata* differs from *Kalmusia* (typified by *K. ebuli*), by long, narrowed, asci with a furcate pedicel and ascospores having a verruculose wall and 3 distosepta (Zhang et al. 2012). A synopsis of characters of genera of *Montagnulaceae* is provided in Table 1. Pathogenicity test results showed that *Deniquelata* caused lesion on pin-pricked leaves of *Barringtonia asiatica*, indicating that *Deniquelata* is probably an opportunistic pathogen.

Acknowledgements

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TABLE 1. Synopsis of morphological features of genera of *Montagnulaceae*.

Species	Ascomata (µm)	Peridium (µm)	Paraphyses (µm)	Asci size and shape (µm)	Ascospores size, shape, color and septa(µm)
Deniquelata barringtonia	150–180 × 164–189 immersed, globose to subglobose, scattered, smooth without an ostiole, black to dark brown	7–9	1–3	67–78 × 10–15, clavate to broadly-clavate, short, broad furcate pedicel	13–16 × 5–7, oblong to narrowly oblong, reddish brown to dark yellowish brown, with three transverse septa and 1-2 vertical septa, verruculose
Kalmusia (*K. ebuli*)	290–360 × 300–520 Solitary, scattered, or in small groups, immersed to erumpent, globose or subglobose, black	15–40	1–1.5	75–125 × 10–15, clavate, long, narrowed, furcate pedicel	15–18 × 5.5–6.5, narrowly ovoid to clavate, pale brown, 3-distoseptate, verrucose
Bimuria (*B. novae-zelandiae*)	200–310, solitary, scattered, semi-immersed or superficial, globose, dark brown to black	5–8	2.5–4	80–95 × 20–32.5, broadly clavate, short and small knob-like pedicel	55–68 × 25–28, broadly ellipsoid, muriform ,dark brown, (5-)7 transverse septa, verrucose
Montagnula (*M. infernalis*)	220–280 × 250–310, immersed to erumpent, gregarious or clustered, globose to subglobose, black	40–55	2–4.5	153–170 × 17.5–21.5, cylindro-clavate to clavate, long, narrowed pedicel	24–29 × 9–11, width oblong to narrowly oblong, reddish brown to dark yellowish brown, five transverse septa and one vertical septum in each middle cells, verrucose

From Zhang *et al.* 2012

TABLE 2. Taxa used in the phylogenetic analysis and their corresponding GenBank numbers

Species	Culture/voucher	LSU	SSU
Bimuria novae-zelandi	CBS 107.79	AY016356	AY016338
Byssothecium circinans	CBS 675.92	AY016357	AY016339
Cochliobolus heterostrophus	CBS 134.39	AY544645	AY544727
Deniquelata barringtoniae	MFLUCC 110422	JX254655	JX254656
Didymocrea sadasivani	CBS 438.65	DQ384103	DQ384066
Kalmusia scabrispora	MAFF 239517	AB524593	AB524452
Karstenula rhodostoma	CBS 690.94	GU301821	GU296154
Leptosphaerulina australis	CBS 317.83	FJ795500	GU296160
Massarina eburnea	CBS 473.64	GU301840	GU296170
Paraconiothyrium fuckelii	CBS 797.95	GU237960	GU238204
Paraconiothyrium minitans	CBS 122788	EU754173	EU754074
Letendraea padouk	CBS 485.70	AY849951	GU296162
Montagnula opulenta	CBS 168.34	DQ678086	AF164370
Neottiosporina paspali	CBS 331.37	EU754172	EU754073
Dothidea sambuci	DAOM 231303	NG027611	NG012432
Paraphaeosphaeria michotii	CBS 652.86	GQ387581	GQ387520
Phaeosphaeria brevispora	MAFF 239276	AB524600	AB524459
Phoma betae	CBS 109410	EU754178	EU754079
Phoma exigua	CBS 431.74	EU754183	EU754084
Pleospora herbarum	CBS 191.86	DQ247804	DQ247812
Pyrenophora phaeocome	DAOM 222769	DQ499596	DQ499595

From Zhang *et al.* 2012

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