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Dematipyriforma aquilaria gen. et sp. nov., a new hyphomycetous taxon from *Aquilaria crassna*

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Abstract – A hyphomycetous fungus producing dark muriform conidia, was isolated as endophyte from trunk of *Aquilaria crassna* in Laos. The fungus was compared to morphologically similar genera, such as *Annellophorella*, *Berkleasmium*, *Canalisporium*, and *Monodictys*. Morphological evidence, however, excluded the fungal taxon from any of these genera. Phylogenetic analysis of combined small subunit, 5.8S, and large subunit ribosomal DNA sequence data placed the fungus within the order Savoryellales (Sordariomycetes, Hypocreomycetidae), but on a distinct terminal clade along with other groups of the order. Based on morphological and molecular phylogenetic evidence, we therefore established the genus *Dematipyriforma* to accommodate the taxon, which was named *Dematipyriforma aquilaria*.

***Aquilaria crassna* / *Dematipyriforma aquilaria* / endophyte / molecular phylogeny / morphology / Savoryellales**

INTRODUCTION

Endophytic fungi are inhabiting plant organs that, at some time in their life, can colonize internal plant tissues without causing apparent harm to their host (Petrini 1991). Endophytic fungi have been found in various plant species and their different tissues, and are thought to be a rich source of fungal diversity (Arnold & Lutzoni 2007; Huang *et al.*, 2008; Hyde & Soytong 2008; Mitchell *et al.*, 2008; Sun & Guo 2010; Wang *et al.*, 2015).

Aquilaria spp. (Thymelaeaceae) are widely distributed in tropical rainforests from India eastward to New Guinea, and have long been a valuable resource in international trade due to the fragrant properties of agarwood, which is derived from the bark of *Aquilaria* spp. (Zich & Compton 2001). However, the mycoflora of endophytes associated with agarwood producing *Aquilaria* spp. is largely unexplored.

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During our research exploring the endophytic fungal assemblage associated with *Aquilaria crassna* Pierre ex Lecomt, a hyphomycetous fungus with muriform conidia and dark catenated chlamydospores was recovered. The morphology of the fungus appeared to be distinctive from genera with similar morphological features, e.g., *Annellophorella* Subram., *Berkleasmium* Zobel, *Canalisporium* Nawawi & Kuthub., and *Monodictys* S. Hughes. The current study aimed to describe the novel fungal taxon and to determine its phylogenetic placement within the Ascomycota.

MATERIALS AND METHODS

Fungus isolation and morphological study

Whitewood segments were collected from trunks of *A. crassna* in a forest 30 kilometers northwest to Vientiane Wattay International Airport (17º58'N, 102º36'E), Laos in late October, 2012. The plant materials from 38 matured *A. crassna* individuals were immediately placed in plastic bags, labeled, and transported to the laboratory. The samples were stored at 10°C and processed within 4 days of collection. The fungus isolation process followed the protocol described by Guo *et al.* (2003). The wooden segments of 5 × 5 × 4 mm were surface sterilized by consecutive immersions of 1 min in 75% ethanol, 3 min in 3.25% sodium hypochlorite, and 30 s in 75% ethanol. After surface sterilization, wooden segments were placed in 90 mm Petri dishes containing malt extract agar (MEA, 2%; Sigma, St Louis, MO). Streptomycin sulphate (50 mg/l, North China Medicine, Shijiazhuang, China) was added to the media to suppress bacterial growth. Petri dishes were sealed, incubated for 2 months at 25°C, and examined periodically. Unlike other endophytic fungi in our study, the newly described fungus emerged in a much slower way after nearly two months of incubation on only one of 304 incubated wooden segments. The fungal colony was then transferred to new Petri dishes with potato dextrose agar (PDA, 38 g/L; Solarbio Science and Technology Co. Ltd., Beijing, China) for morphological observation and preservation.

All microscopic observations, measurements and photographs were taken with samples mounted in sterile water, using a compound microscope (Zeiss A×IO Imager A2, Carl Zeiss Microscopy, Göttingen, Germany) in which ZEN 2012 blue edition (Carl Zeiss Microscopy, Gottingen, Germany) was installed. Colony photographs were taken with a digital camera (Nikon Coolpix S80, Nikon Corporation Tokyo, Japan). Mophological diagnosis and precise notations of colors were in accordance with terminology provided in Ellis (1971) and Seifert *et al.*, (2011), and the color chart provided by Rayner (1970). Subcultures were grown on PDA plates for 2 wk and used for the molecular analysis. The culture referred to in this study was preserved in the China General Microbiological Culture Collection (CGMCC3.17268). Sporulating colonies were dried at 50°C for 24 h in an electric oven (DHG-9140A, Yiheng Instruments Co. Ltd, Shanghai, China), and deposited in Herbarium of Mycologicum Academia Sinica (HMAS) in Beijing, China.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from fresh cultures following the protocol of Guo *et al.*, (2000). Primers used for ribosomal DNA PCR amplification and sequencing were NS1 and NS4 (White *et al.*, 1990) for the partial small subunit (18S), LR0R and LR16 (Nugent and Saville 2004) for the partial large subunit (28S), and ITS4 and ITS5 (White *et al.*, 1990) for the 5.8S gene (5.8S).

PCRs were performed in 50 μl reactions, which contained PCR buffer (20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 20 mM Tris-HCl, pH8.4), 200 μM of each deoxyribonucleotide triphosphate, 15 pmols of each primer, c. 100 ng template DNA, and 2.5 units Taq DNA polymerase (Biocolor BioScience & Technology Company, Shanghai, China). The PCR cycling conditions for 18S, 5.8S, and 28S were as follow: 3 min initial denaturation at 95°C, followed by 35 cycles of 40 s denaturation at 94°C, 50 s primer annealing at 52°C, 1 min extension at 72°C, and a final extension of 10 min at 72°C. A negative control using purified water (Cascada Biowater; Pall, NY, US) instead of template DNA was included in the amplification process. PCR products were examined by agarose gel electrophoresis (75 V for 2 h) in a 0.8% (w/v) agarose gel in 1 × TAE buffer (0.4 M Tris, 50 mM NaOAc, 10 mM EDTA, pH 7.8). Products were visualized under UV light after staining with ethidium bromide (0.5 μg/ml). PCR products were purified using PCR Cleanup Filter Plates (MultiScreen® PCRμ96; Millipore, Billerica, USA) according to the manufacturer's protocol. Purified PCR products were directly sequenced with primer pairs as mentioned above in an ABI 3730-XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Each sequence was checked for ambiguous bases and assembled with BioEdit 7.0.0 (Hall 2004).

Phylogenetic analysis

Preliminary BLAST analyses with 18S, 5.8S and 28S rDNA sequences generated in this study indicated that the isolate belonged to Hypocreomycetidae (Sordariomycetes, Ascomycota). Therefore, in order to reveal the phylogenetic position of the new taxon, we selected the 18S, 5.8S and 28S sequences originated from taxa across four orders within Hypocreomycetidae as references in subsequent phylogenetic analysis. In addition, representatives of the Glomerellaceae and Reticulascaceae were chosen for outgroups. Sequences used in current study were listed in Table 1. The multilocus dataset was aligned with MAFFT v6.853b (Katoh & Toh 2008) and then manually adjusted using BioEdit 7.0.0 where necessary (Hall 2004). All sites were treated as unordered and unweighted, and gaps were treated as missing in the phylogenetic analyses. The alignment was deposited at TreeBase with http://purl.org/phylo/treebase/phylows/study/TB2:S19877.

A final dataset was prepared which included 18S, 5.8S, and 28S rDNA data from taxa for which at least two of the three genes were available, resulting in 32 taxa. Maximum parsimony and Bayesian analysis were performed following Lutzoni *et al.*, (2004). Maximum parsimony analysis was conducted with heuristic search algorithm using tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b10 (Swofford 2002). For each search, 1,000 replicates were performed by random stepwise sequence addition and all trees were saved per replicate. Statistical support for branches leading up to the clades was determined by bootstrap analyses using 1,000 replications with the same search settings. The tree length (TL), consistency index (CI), rescaled consistency index (RC), retention index (RI) and homoplasy index (HI) were calculated for each tree. The Kishino-Hasegawa (K-H) test was used to estimate the best tree topology.

Table 1. GenBank accession numbers of isolates included in this study. Newly generated sequences are in bold

Taxa	*Culture collection number*	*GenBank Accession Numbers*		
		SSU	*ITS*	*LSU*
Order Hypocreales				
Bionectria ochroleuca	AFTOL-ID 187	DQ862044	–	DQ862027
Claviceps purpurea	t5	DQ119133	DQ119114	–
Cordyceps acridophila	MV2498	JQ895531	JQ958604	JQ895539
Elaphocordyceps ophioglossoides	NBRC 106332	JN941732	JN943322	JN941409
Hypocrea gelatinosa	NBRC 9060	JN941690	JN943357	JN941452
Nectria balansae	AR4446	JN939651	JN995620	JN939840
Niesslia exilis	CBS357.70	AY489686	–	AY489718
Sphaerodes retispora	CBS 994.72	GU199043	–	GU205261
Order Melanosporales				
Melanospora tiffanii	SMCD2222	FJ748918	FJ748921	FJ748915
Melanospora zamiae	ATCC 96173	AY057905	–	AY057906
Order Microascales				
Ceratocystis moniliformis	CBS155.62	EU984265	–	EU984305
Faurelina indica	CBS 126.78	DQ368657	GU291802	GU180653
Gondwanamyces capensis	AFTOL-ID 1907	FJ176834	–	FJ176888
Graphium fimbriisporum	CMW5606	AY148172	AY148180	–
Halosphaeria appendiculata	CBS 197.60	U46872	–	U46885
Microascus cirrosus	CBS217.31	EU984279	–	JQ434680
Order Savoryellales				
Ascotaiwania lignicola	NIL00006	HQ446285	HQ446342	HQ446365
Ascotaiwania sawadae	SS00051	HQ446283	HQ446340	HQ446363
Ascothailandia grenadoidia	NB-2010a	GQ390252	GQ390282	GQ390267
Canalisporium caribense	SS03839	GQ390253	GQ390283	GQ390268
Canalisporium elegans	SS00895	GQ390256	GQ390286	GQ390271
Canalisporium exiguum	SS00809	GQ390266	GQ390296	GQ390281
Canalisporium jinghongensis	SS03483	GQ390258	GQ390288	GQ390273
Canalisporium pallidum	SS00091	GQ390264	GQ390294	GQ390279
Canalisporium pulchrum	SS03982	GQ390262	GQ390292	GQ390277
Canalisporium sp.	SS03732	HQ446288	HQ446345	HQ446368
Dematipyriforma aquilaria	**CGMCC3.17268**	**KJ138622**	**KJ138621**	**KJ138623**
Savoryella lignicola	NF00204	HQ446300	HQ446357	HQ446378
Savoryella paucispora	SAT00866	HQ446303	HQ446360	HQ446381
Savoryella verrucosa	SS00042	HQ446295	HQ446352	HQ446373
Family Glomerellaceae				
Glomerella miyabeana	C1117.4	JN939845	JN943481	JN939918
Family Reticulascaceae				
Reticulascus clavatus	CBS 125296	GU180622	GU180627	GU180643

Bayesian analysis was conducted in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). The general time-reversible model (Lanave *et al.*, 1984; Rodriguez et. al. 1990) with mutation rates were approximated from an invariable gamma distribution with four categories (GTR + I + Γ), was determined as the best-fit evolutionary model by comparing different evolutionary models via MrModeltest 2.3 (Nylander 2004). The prior probability density was a flat Dirichlet (all values are 1.0) for both Revmatpr and Statefreqpr as default settings. Four simultaneous Markov Chain Monte Carlo chains were run for 10 million generations, sampled every 100 generations. Convergence and stationarity were confirmed using the standard deviation of split frequencies (approaching zero), the potential scale reduction factor (PSRF, approaching 1), and plots of generation versus log probability. At the end of the analysis, 5,000 trees were excluded as the "burn in" when calculating the posterior probabilities. Bayesian posterior probabilities (BPP) were obtained from the 50% majority rule consensus of the trees kept. If > 95% of the sampled trees contained a given clade, we considered it to be significantly supported by our data.

RESULTS

Taxonomy

Dematipyriforma L. Y. Sun, Hai-Yan Li, Xiang Sun & L.D. Guo, **gen. nov.**

MycoBank: MB 808026

Etymology: Referring to its dematiaceous, pyriform conidia.

Typus: *Dematipyriforma aquilaria*

Mycelium mostly immersed, composed of septate, smooth, thin-walled, pale brown to brown hyphae. *Conidiophores* micronematous, pale brown to brown, smooth, septate. *Conidiogenous cells* monoblastic, integrated, intercalary or terminal, pale brown to brown, determinate, cylindrical. *Conidia* solitary, pyriform, smooth, thin-walled, with transverse and often oblique or longitudinal, usually with a single small basal cell. *Chlamydospores* solitary when present, numerous, terminal or arise laterally from intercalary cells, with multiple transverse septa, variable in shape, dark colored, smooth, walls thickened.

Dematipyriforma aquilaria L. Y. Sun, Hai-Yan Li, Xiang Sun & L.D. Guo, **sp. nov.** **Fig. 1**

MycoBank: MB 808027

Etymology: Named after the host genus from which the taxon was collected, *Aquilaria*.

Holotypus: Vientiane, Laos, wood endophyte from *Aquilaria crassna*, October 10, 2012, Hai-Yan Li, Liang-Dong Guo (HMAS245108).

Colonies on PDA effuse, with aerial hypha, olivaceous to greyish, reverse greenish black, reaching 25 mm in diam. after 3 weeks of growth at 28°C; or grey olivaceous to olivaceous, reverse black, reaching 15 mm in diam. after 3 weeks of growth on MEA. *Mycelium* mostly immersed, hyphae subhyaline to pale brown, septate, branched, smooth, thin-walled, 1.3-2.0 μm wide. *Chlamydospores* intercalary or terminal, solitary, catenated, straight, or curved, brown to dark brown, sometimes

with pale intermediate cell, smooth, containing highly refractive granules, walls sometimes thickened, 1-2 µm thick, septa 1.5-2.2 µm thick with axial perforative canals when thickened wall present, with (0) 1-7 (11) transverse and occasionally 1 or more oblique or longitudinal septa, constricted at the septa conspicuously, (6) 15-63 × (6) 9-14 (16) µm, 40.6 × 10.3 µm on average (n = 30). *Conidiophores* micronematous, mononematous, subhyaline to pale brown, straight or flexuous, smooth, septate, 2-5 µm wide. *Conidiogenous cells* holoblastic, integrated, intercalary,

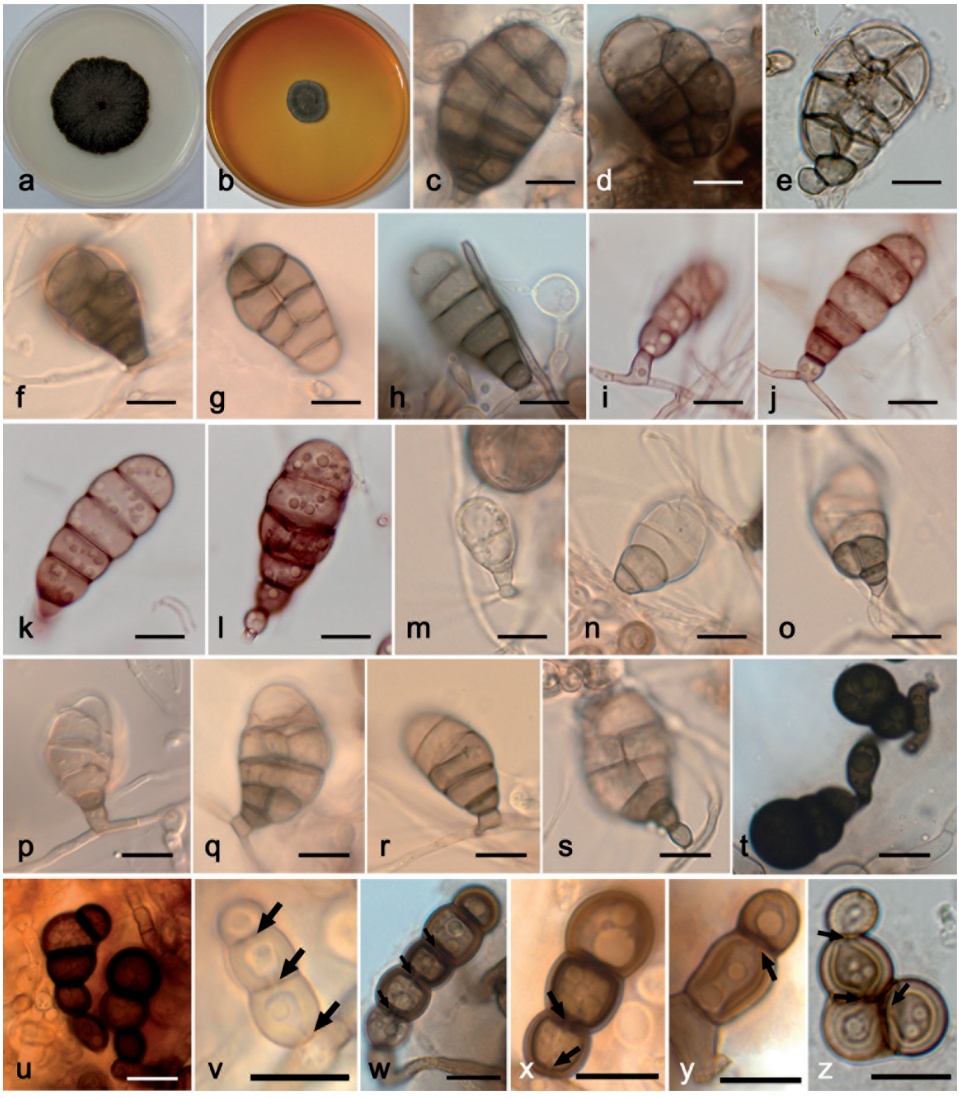

Fig. 1. Morphology of *Dematipyriforma aquilaria*. **a.** Colony on PDA. **b.** Colony on MEA. **c-l.** Conidia. **m-s.** Immature conidia with acropetal pigmentation. **t.** Chlamydospores with merely 1 or 2 transverse septa. **u.** Chlamydospores with pale intermediate cell. **v-z.** Chlamydospores with thickened wall and axial perforative canals which were indicated by arrows. Scale bars indicate 10 µm.

pale brown to brown, determinate, cylindrical, smooth, thin-walled; *Conidial secession* rhexolytic. *Conidia* solitary, intercalary, smooth, thin-walled, pyriform or enlongated pyriform, rounded at the apex, pale grey olivaceous to pale brown, acropetal pigmentation in conidium formation, muriform, 5-9 (13) cells, 4-5 transverse septa and 0-2 longitudinal septa, slightly constricted at the septa, 25-37.5 × 15-22.5 µm, 32.9 × 18.8 µm on average (n = 30); apex with 1-2 cells, 2.5-12.5 × 10-17 µm; basal cell single, subglobose, thin walled, subhyaline to very pale brown, 2.5-7.5 × 3-6 µm.

Phylogenetic analyses

The 18S, 5.8S and 28S sequences of *D. aquilaria* and 29 reference taxa across four orders (Hypocreales, Melanosporales, Microascales, Savoryellales) of Hypocreomycetidae were included in the multilocus phylogeny analyses to determine the taxonomic placement of *D. aquilaria* in Hypocreomycetidae, with *Glomerella miyabeana* (Glomerellaceae) and *Reticulascus clavatus* (Reticulascaceae) as outgroups. As a result of excluding ambiguously aligned regions, the final multilocus matrix consisted of 1691 characters.

Of the 1691 characters, 1065 (63.0%) were constant, 493 (29.2%) were parsimony informative, and 133 (7.9%) were parsimony uninformative. In the parsimony analysis, a strict consensus tree was obtained from two equally parsimonious trees with TL = 1594, CI = 0.5307, HI = 0.4693, RI = 0.7884, and RC = 0.4184. A similar topology was recovered from Bayesian analysis. The Bayesian tree is displayed in Fig. 2, with Bayesian posterior probabilities and parsimony bootstrap values at each nodes. The phylogenetic analyses revealed that the new taxon, *D. aquilaria*, clusters together with 13 taxa of the Savoryellales within the Hypocreomycetidae (Sordariomycetes) with strong support (Fig. 2). Furthermore, *D. aquilaria* forms a unique terminal clade within Savoryellales. We therefore proposed that the new genus represented by *D. aquilaria* was a member of the Savoryellales.

DISCUSSION

The newly described genus *Dematipyriforma* closely resembles *Annellophorella*, *Berkleasmium*, *Canalisporium*, and *Monodictys* in morphology as it produces dematiaceous, solitary dictyoconidia (Hughes 1958; Moore 1959; Ellis 1971; Nawawi & Kuthubutheen 1989; Sri-Indrasutdhi *et al.*, 2010; Seifert *et al.*, 2011). The genus *Annellophorella* accommodates hyphomycetes taxa with semi-macronematous conidiophores and pigmented dictyoconidia, and shares similar features with *Dematipyriforma* in dark conidia with transverse and often oblique or longitudinal septa (Ellis 1971). However, *Annellophorella* produces percurrent conidiogenous cells, where the conidiogenous cell is determinate in *Dematipyriforma* (Ellis 1971; Seifert *et al.*, 2011). *Monodictys* was proposed to accommodate ten wood and dead bark inhabiting taxa, previously placed *Acrospeira*, *Coniosporium*, *Dicoccum*, *Epochnium*, *Hyphelia*, *Melanconium*, *Papulaspora*, *Sporidesmium* and *Stemphylium* (Hughes 1958). Members in the genus possess micronematous or semi-macronematous conidiophores, and solitary, dark, muriform conidia, as with

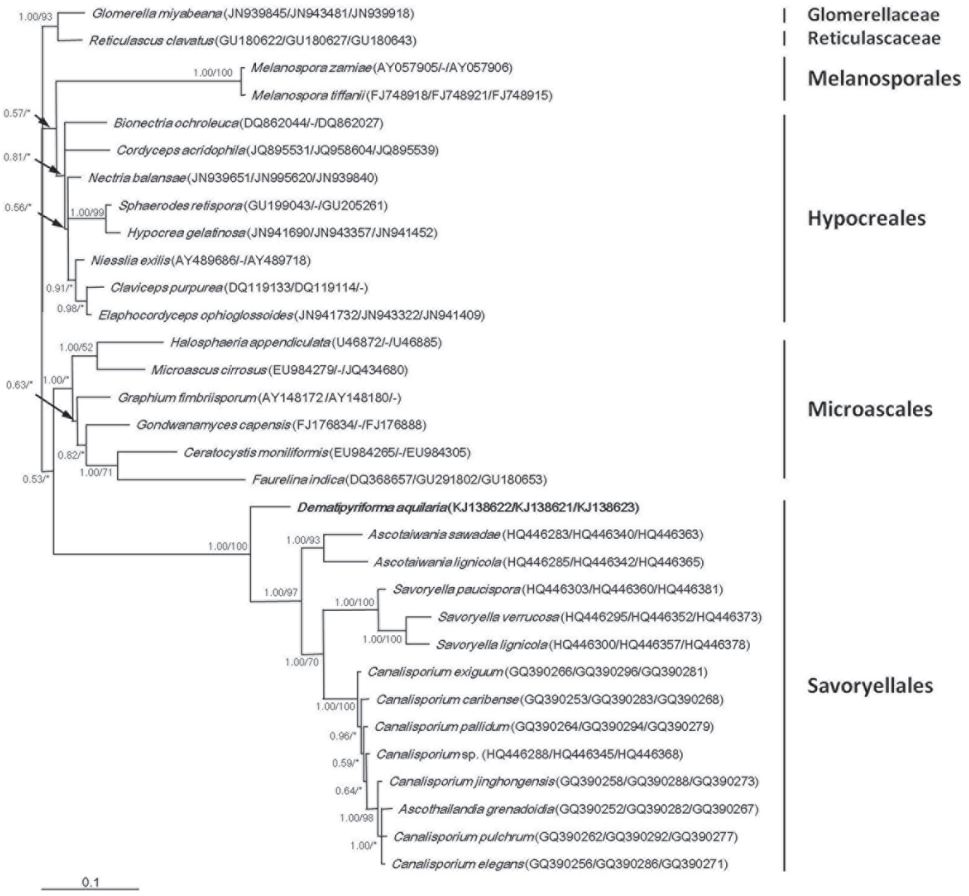

Fig. 2. Bayesian tree based on 18S + 5.8S + 28S rDNA combined sequences. The tree was rooted with *Glomerella miyabeana* and *Reticulascus clavatus*. Bayesian posterior probabilities are given above nodes, and Bootstrap values higher than 50% from maximum parsimony analysis are indicated as below nodes. * indicates lack of support or support less than 50% for a particular clade. The access numbers in GenBank of 18S, 5.8S, 28S are shown from left to right in parentheses. Scale bar indicates 0.1 expected changes per site.

Dematipyriforma. However, the basal cell of mature conidia in *Monodictys* are commonly paler, which is in contrast to the relatively even pigmentation of the conidia in *Dematipyriforma*. Moreover, *Dematipyriforma aquilaria* forms continuous transverse septa, and with cells arranged in rows when oblique or longitudinal septa are present, while cells of conidia in *Monodictys* appeared to be arranged in a relatively irregular way.

The genus *Berkleasmium* produced sporodochial colonies and pigmented muriform conidia (Moore 1958, 1959). *Canalisporium* was afterward established to accommodate *B. caribense* Hol.-Jech. & Mercado and *B. pulchrum* Hol.-Jech. & Mercado of which the cell lumina in conidia were connected by canals, as well as a new taxon *Canalisporium elegans* Nawawi & Kuthub by Nawawi and Kuthubutheen (1989). Nine species were subsequently placed in *Canalisporium* (Goh *et al.*, 1998;

Goh & Hyde 2000; Cai *et al.*, 2003; Ferrer & Shearer 2005; Sri-Indrasutdhi *et al.*, 2010; Zhao *et al.*, 2012). Sri-Indrasutdhi *et al.* (2010) showed that the newly described *Ascothailandia grenadoidea* Sri-indrasutdhi, Boonyuen, Sivichai & E.B.G. Jones (as "*grenadoidia*") was the ascomycete teleomorph of *Canalisporium grenadoidia* using a single ascospore gemination technique. Boonyuen *et al.*, (2011) placed the genus *Ascothailandia* Sri-indrasutdhi, Boonyuen, Sivichai & E.B.G. Jones in Savoryellales, a newly proposed order in Sordariomycetes, containing the genera *Savoryella* E.B.G. Jones & R.A. Eaton and *Ascotaiwania* Sivan. & H.S. Chang. Critical morphological dissimilarities distinguish *Dematipyriforma* from *Berkleasmium* and *Canalisporium* in spite of the solitary, pigmented, muriform conidia occurring in all the three genera. *Berkleasmium* produces macronematous conidiophores, while the conidiophores are unambiguously micronematous in *Dematipyriforma*. In addition, no hilum was observed in *Dematipyriforma* while *Berkleasmium* conidia were often found with a protruding hilum. Septa of conidia showed primary morphological differences between *Dematipyriforma* and *Canalisporium*. *Canalisporium* possesses thick walls with narrow canals connecting adjacent cells through the septa, while *Dematipyriforma* produces thin-walled conidia without canals. Moreover, the conidial cells in *Canalisporium* are arranged in flattened dorsoventrality, and in vertical columns with continuous longitudinal septa. Nevertheless, the conidium cells in *Dematipyriforma* are not complanate and ordered in horizontal rows with continuous transverse septa. In addition, no chlamydospores in *Canalisporium* have been reported, while chlamydospores in *Dematipyriforma* are commonly found in culture. Furthermore, we did not observe sporodochia of *Dematipyriforma* in pure culture, which were present in *Berkleasmium* and *Canalisporium*.

Dematipyriforma produced no meiotic state in the current study, and hence we could not determine its taxonomic placement via morphology. Phylogenetic analyses of the 18S+5.8S+28S dataset indicated that *D. aquilaria* formed a strongly supported clade together with, yet conspicuously distinct from, all other members of Savoryellales, *viz*., *Ascothailandia*, *Ascotaiwania*, and *Savoryella*. The genus *Dematipyriforma* is not comparable with *Savoryella* and *Ascotaiwania*, as no anamorph of the latter have been reported. Nevertheless, molecular data proved that the taxon represents an independent genus instead of being a member in any o fthe described genera in Savoryellales. Therefore, we propose *Dematipyriforma* as a new genus in Savoryellales, supported by morphologial and molecular phylogenetic evidence.

While the majority of members of the Sordariomycetes are terrestrial, and life in aquatic habitats is considered a derived character for the class (Samuels & Blackwell 2001; Zhang *et al.*, 2006), Savoryellales is an order in which most taxa are aquatic. All members, except for 3 species of *Ascotaiwania* for which sequence data is currently unavailable, were described and reported from freshwater and marine habitats (Boonyuen *et al.*, 2011). The exceptions are *A. licualae* from a dead petiole of *Licuala ramsayi* (F. Muell.) Domin (Fröhlich & Hyde 2000), *A. palmicola* from dead leaves of *Iriartea* sp. (Hyde 1995), and *A. mauritiana* from prop root of *Pandanus palustris* Thouars in rivulet (Dulymamode *et al.*, 2001). Considering *Ascotaiwania* was the closest neighbor with *Dematipyriforma* in Savoryellales, our discovery implies that *D. aquilaria* and the three *Ascotaiwania* species were most likely keystone taxa representing the evolutionary transition of Savoryellales from terrestrial to aquatic habitats. Unfortunately, sequence data from *A. licualae*, *A. palmicola* and *A. mauritiana* were not available, which prevented additional phylogenetic analyses to further evaluate this question.

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