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Dyfrolomycetaceae, a new family in the Dothideomycetes, Ascomycota

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Abstract – A new mangrove fungus collected in Tioman Island, Malaysia, is morphologically similar to marine species of *Saccardoella*. It also phylogenetically groups with *Saccardoella rhizophorae* in the *Dothideomycetes*, based on combined analysis of partial SSU, LSU rRNA and TEF1 gene sequences. The new fungus and *S. rhizophorae* form a well-supported clade with *Acrospermum* spp. in the Acrospermaceae. Both species therefore do not belong in *Saccardoella*, a genus with unitunicate asci. A new genus, *Dyfrolomyces*, is established to accommodate the new fungus (*Dyfrolomyces tiomanensis*) while the three marine *Saccardoella* species (*S. mangrovei*, *S. marinospora*, *S. rhizophorae*) are transferred to the new genus. *Dyfrolomyces* is characterized by forming a clypeus on substrates, with immersed perithecial ascomata, bitunicate/fissitunicate asci and multi-septate ascospores with/without a sheath. Since *D. rhizophorae* and *D. tiomanensis* do not cluster with any known families in the *Dothideomycetes*, a new family, *Dyfrolomycetaceae*, is introduced to accommodate the *Dyfrolomyces* species.

Ascomycota / marine fungi / *Saccardoella* / *Sordariomycetes* / *Xylariales*

INTRODUCTION

Saccardoella Speg. was introduced by Spegazzini (1879) and was typified by *Saccardoella montellica* Speg., which was described from *Quercus* sp. in Italy. *Saccardoella montellica* is characterized by having large ascomata with erumpent papillae, long, cylindrical, unitunicate asci with an apical ring and uniseriate

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ascospores having numerous septa and a sheath or polar appendage (Petrak 1962; Hyde 1992). There has been confusion over the nature of the asci as they are neither typically unitunicate nor bitunicate, thus the placement of the genus has been problematic (Mathiassen 1989; Hyde 1992). *Saccardoella* has been variously classified in the *Clypeosphaeriaceae*, *Xylariales* (Barr 1989), the *Pleurotremataceae*, a family with an unknown higher taxomomic position (Barr 1994) and 'Unitunicate *Ascomycota* genera *incertae sedis*' (Jones *et al.*, 2009).

Currently, there are 22 species epithets referred to *Saccardoella* (Index Fungorum 2013) with most described from aquatic environments. Hyde (1992) described the marine species including *S. mangrovei* K.D. Hyde, *S. marinospora* K.D. Hyde and *S. rhizophorae* K.D. Hyde from wood collected in mangroves of Australia and Thailand. Species from freshwater environments were subsequently described: *S. allequashensis* Fallah & Shearer, *S. aquatic* K.M. Tsui *et al.*, *S. horizontalis* Fallah & Shearer, *S. lacustris* Fallah & Shearer and *S. minuta* L. Cai & K.D. Hyde (Tsui *et al.*, 1998; Fallah and Shearer 2001; Cai *et al.*, 2002) and these have been recollected repeatedly (Cai *et al.*, 2003; Luo *et al.*, 2004). Suetrong *et al.* (2009) investigated the phylogeny of marine *Dothideomycetes* and included sequences of an isolate of *S. rhizophorae* from Oahu, Hawaii. Surprisingly, this fungus did not show any affinities to members of the *Sordariomycetes*, but grouped within the *Dothideomycetes* although it did not cluster with any known families and orders in the class.

Recently, a morphologically similar ascomycete to the three described marine *Saccardoella* species was collected in a mangrove area of Tioman Island, Malaysia. The LSU rRNA gene sequence of this fungus grouped with that of *S. rhizophorae* from GenBank and therefore confirmed the phylogeny of *S. rhizhophorae* as a dothideomycetous fungus. As a result, a new genus, *Dyfrolomyces*, is introduced to accommodate the three marine species of *Saccardoella* in the new family *Dyfrolomycetaceae*, *Pleosporomycetidae*, *Dothideomycetes*. The fungus from Tioman Island is described here as a new species in *Dyfrolomyces* as it is different in having spindle-shaped ascospores with 20-24 septa.

MATERIALS AND METHODS

Collection, identification and isolation: Driftwood/trapped wood was collected in a mangrove area of Tioman Island on 13 July 2010. Wood samples were placed in large Zip-lock plastic bags and incubated at room temperature in the laboratory. Ascomata of the new fungus (*D. tiomanensis*) on wood were cut open by a razor blade under an Olympus SZ61 stereomicroscope (Tokyo, Japan). Centrum material was transferred to a drop of sterile natural seawater on a glass slide. The morphology of asci and ascospores was observed under an Olympus BX51 microscope (Tokyo, Japan) and photographs taken with an Olympus DP20 Microscope Camera (Tokyo, Japan).

For isolation, a spore suspension of *D. tiomanensis* was made by transferring centrum material to a drop of sterile natural seawater on a sterilized glass slide. Spore mass was dispersed evenly in the drop of seawater with a sterilized forceps and its identification confirmed by observing under the compound microscope. More sterile natural seawater was added and dispensed onto the surface of a cornmeal seawater agar (CMAS) plate (Difco). The plate was incubated at 25°C for 1-3 days. Germinated single spores were picked up and

transferred to new CMAS plates. Cultures are deposited at Institute of Marine Biology, National Taiwan Ocean University and School of Science, Mae Fah Luang University.

Section of ascomata: Wood pieces with ascomata were cut out and fixed in FAA solution (5% formaldehyde and 5% glacial acetic acid in 50% ethanol) overnight at 4°C. The fixed samples were washed three times in 50% ethanol. Samples were then dehydrated in a graduated t-butanol/ethanol/water series (10/40/50, 20/50/30, 35/50/15, 55/45/0, 75/25/0, 100/0/0, 100/0/0, in percentage), and infiltrated gradually and embedded in paraffin. Paraffin sections (7 μm) were cut on a FRM-200P rotary microtome (Japan), floated on 42°C water-bath to relax compression and mounted on microscope slides. Dried sections were deparaffinised and rehydrated through a graded series of ethanol. The sections were then stained with 1% safranin O in 50% ethanol (10 sec) and 0.5% Orange G in 95% ethanol (30 sec). After washing and dehydration, each stained section was permanently mounted with a cover slip and Permount (Fisher, USA). Specimens were observed on the Olympus BX51 microscope with light micrographs taken.

Molecular analysis: The isolates were grown on potato dextrose seawater agar plates (Difco) for 2 weeks at 25°C. Mycelium was scrapped off from the agar surface and ground into powder in a mortar and pestle in liquid nitrogen. DNeasy Plant Mini Kit (Qiagen, California, USA) was used for genomic DNA extraction according to the manufacturer's instructions. Extracted DNA was used directly for PCR reactions with the following ingredients: 0.2 μM of each primer (NS1/NS4: White *et al.*, 1990, LROR/LR6: Bunyard *et al.*, 1994, TEF1-983F/ TEF1-2218R: Rehner and Buckley 2005), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 1 U of Taq Polymerase (Invitrogen). The amplification cycle consisted of an initial denaturation step of 94°C for 5 min followed by 35 cycles of (i) denaturation (94°C for 0.5 min), (ii) annealing (55°C for 0.5 min) and (iii) elongation (72°C for 0.5 min) and a final 11 min elongation step at 72°C. The PCR products were analysed by agarose gel electrophoresis and PCR product shipped to Genomics BioSci. & Tech., Taiwan, for purification and direct sequencing with the same primers.

Returned sequences were checked for ambiguity, assembled and deposited in GenBank. Sequences used in the phylogenetic analysis are listed in Table 1, which include sequences from the Dothideomycetes, other major classes and closest sequence matches after BLAST search in NCBI. Alignment was performed on the sequences in the program MUSCLE (Edgar 2004). The alignments of the partial nuclear SSU and LSU and TEF1 genes were entered into BEAUti v1.7.2 for prior settings and generation of XML files for Bayesian analysis in BEASTv.1.7.2 and analyzed simultaneously (Drummond and Rambaut 2007) with the following analytical settings: GTR, gamma+invariant sites, number of gamma categories set at 4, a strict clock, coalescent tree prior for populations of constant size as the speciation model, running 15 million generations with parameters and trees sampled every 1000 generations. The first 10% of the trees were treated as the burn-in and discarded based on the effective sample size (ESS) of the parameter statistics in Tracer v1.5 (Drummond and Rambaut 2007). A summary tree was produced using TreeAnnotator v1.7.2 (Drummond and Rambaut 2007) and viewed and edited in FigTree v1.3.1 (Rambaut 2009).

Table 1. Taxa used in the phylogenetic analysis and their GenBank accession numbers

	GenBank accession numbers		
Taxa	*18S rDNA*	*28S rDNA*	*Tef1*
Acrospermum adeanum	EU940031	EU940104	–
Acrospermum compressum	EU940012	EU940084	–
Acrospermum gramineum	EU940013	EU940085	–
Aigialus grandis	GU479738	GU479774	GU479838
Anisomeridium phaeospermum	JN887374	JN887394	JN887418
Astrosphaeriella bakeriana	–	GU349015	GU349015
Botryosphaeria dothidea	DQ677998	DQ678051	DQ767637
Botryosphaeria stevenii	DQ678012	DQ678064	DQ677907
Capnodium coffeae	DQ247808	DQ247800	DQ471089
Davidiella tassiana	DQ678022	DQ678074	DQ677918
Delitschia winteri	DQ678026	DQ678077	DQ677922
Dendryphiella arenaria	DQ471022	DQ470971	DQ677890
Dothidea insculpta	DQ247810	DQ247802	DQ471081
Dyfrolomyces (Saccardoella) rhizophorae	GU479766	GU479799	GU479860
Dyfrolomyces (*Saccardoella*) *rhizophorae* BCC15481	KF160009	–	–
Dyfrolomyces tiomanensis	KC692155	KC692156	KC692157
Elsinoe veneta	DQ767651	DQ767658	DQ767641
Eupenicillium limosum	EF411061	EF411064	EF411070
Falciformispora lignatilis	GU371835	GU371827	GU371820
Geoglossum nigritum	AY544694	DQ471044	DQ471044
Gloniopsis praelonga	FJ161134	FJ161173	FJ161090
Guignardia citricarpa	GU296151	GU301815	GU349053
Helicascus nypae	GU479754	GU479788	GU479854
Herpotrichia juniperi	DQ678029	DQ678080	DQ677925
Hysterobrevium mori	–	GU301819	GU397338
Hysterographium fraxini	FJ161132	FJ161171	FJ161088
Keissleriella cladophila	GU296155	GU301822	GU349043
Lecanora hybocarpa	DQ782883	DQ782910	DQ782901
Leptosphaeria maculans	DQ470993	DQ470946	DQ471062
Lophium mytilinum	DQ678030	DQ678081	DQ677926
Mycopepon smithii	AF279399	AF279400	–
Mycosphaerella eurypotami	GU479761	GU301852	GU371722
Passalora ageratinae	JN938702	GU214453	–
Patellaria atrata	GU296181	GU301855	GU349038
Petriella setifera	DQ471020	DQ470969	DQ836911
Platystomum scabridisporum	GQ925832	GQ925845	GU479856
Rhizocarpon oederi	DQ983486	DQ986804	FJ772239
Roccellographa cretacea	DQ883705	DQ883696	DQ883733
Schismatomma decolorans	NG_013155	NG_027622	DQ883725
Spiromastix warcupii	DQ782882	DQ782909	DQ782900
Trichoglossum hirsutum	AY544697	AY544653	DQ471049
Trypetheliopsis kalbii	JN887391	JN887406	JN887435
Tubeufia paludosa	GU296203	GU301877	GU349024
Verruculina enalia	GU479770	GU479802	GU479863
Westerdykella cylindrica	AY016355	AY004343	DQ497610
Xylaria acuta	AY544719	AY544676	DQ471048

TAXONOMY

Dyfrolomycetaceae K.D. Hyde, K.L. Pang, Alias, Suetrong & E.B.G. Jones, **fam. nov.**

MycoBank MB 804662

Saprobic on wood in aquatic environments. **Ascomata** relatively large, solitary to gregarious, immersed, globose or subglobose, coriaceous, clypeate, ostiole rounded, papillate. **Peridium** broadest at the sides, comprising two layers, an outer layer of cells of *textura intricata* composed of host cells interspersed with fungal hyphae and an inner layer of thick-walled cells of *textura angularis*. **Hamathecium** comprising numerous, relatively narrow (up to 2 µm, wide), septate pseudoparaphyses embedded in a gelatinous matrix. **Asci** 8-spored, bitunicate, fissitunicate, cylindrical, with a relatively short pedicel and apically rounded or flattened with a distinct ocular chamber and ring-like subapical apparatus. **Ascospores** overlapping uniseriate, broadly fusiform, symmetrical, hyaline, multiseptate with wide septa (distoseptate?), smooth-walled, with/without a sheath.

Asexual state: Unknown.

Family type: *Dyfrolomyces* K.D. Hyde *et al.*

Dyfrolomyces K.D. Hyde, K.L. Pang, Alias, Suetrong & E.B.G. Jones, **gen. nov.**

MycoBank MB 804660

Saprobic on wood in aquatic or terrestrial environments. **Ascomata** relatively large, solitary to gregarious, immersed, globose or subglobose, coriaceous, clypeate, ostiole rounded, papillate. **Peridium** broadest at the sides, comprising two layers, an outer layer of cells of *textura intricata* composed of host cells inter-

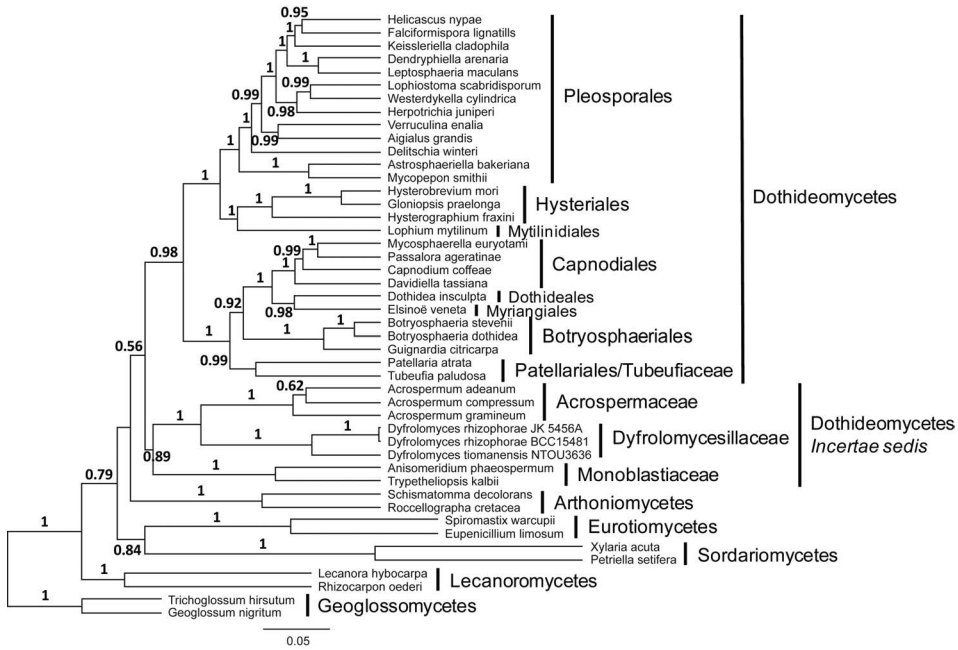


Fig. 1. A Bayesian likelihood tree based on a combined analysis of the partial 18S and 28S rRNA and TEF1 genes using BEASTv.1.7.2. Posterior probability (PP) is shown on the branches.

spersed with fungal hyphae and an inner layer of thick-walled cells of *textura angularis*. **Hamathecium** comprising numerous, relatively narrow (up to 2 μm, wide), septate pseudoparaphyses embedded in a gelatinous matrix. **Asci** 8-spored, bitunicate, fissitunicate, cylindrical, with a relatively short pedicel and apically rounded or flattened with a distinct ocular chamber and ring-like subapical apparatus. **Ascospores** overlapping uniseriate, broadly fusiform, symmetrical, hyaline, transseptate with wide septa (distoseptate?), smooth-walled, with/without a sheath.

Etymology: From the Welsh word 'dyfol' meaning 'aquatic' and the Greek word 'myces' meaning 'fungus'.

Asexual state: Unknown.

Generic type: *Dyfrolomyces tiomanensis* K.L. Pang *et al.*

Dyfrolomyces tiomanensis K.L. Pang, S.A. Alias, K.D. Hyde, Suetrong & E.B.G. Jones, **sp. nov.** **Figs 2-8**

MycoBank: MB 804661

Ascomata 565–(615)–667 × 283–(374)–446 μm, pyriform, some with a broad flattened base, immersed, clypeate, ostiolate, papillate, coriaceous, dark-coloured, solitary or gregarious. **Clypeus** 326–(414)–500 × 152–(163)–179 μm, extending outwards around the papilla. **Papilla** 251 × 55 μm, black, conical, without periphyses. **Peridium** 16–(27)–36 μm, two-layered, an outer layer of cells of *textura intricata* composed of host cells interspersed with fungal hyphae and an inner layer of thick-walled cells of *textura angularis*, cells 2–(6)–9 × 1–(3)–7 μm. **Hamathecium** comprising numerous, hypha-like pseudoparaphyses, attached at the base and top of the ascoma, branching and anastomosing above the asci, and in a gelatinous matrix. **Asci** 316–(323)–333 × 12–(16)–17 μm, 8-spored, cylindrical, thin-walled, fissitunicate, short-pedicellate, apically rounded, with a faint ring-like subapical apparatus, asci forming at the base of the ascoma. **Ascospores** 69–(74)–82 × 9–(10)–11 μm, overlapping uniseriate, hyaline at maturity, spindle-shaped, 20–24-septate, slightly constricted at the septa.

Etymology: In reference to the place of discovery of the holotype, Tioman Island, Malaysia.

Holotype: MALAYSIA: Tioman Island, on a piece of unidentified mangrove wood, 13 July 2010, K.L. Pang, MFLU13-00063, sections of the ascomata; MFLUCC13-0440, an ex-type culture.

Known geographical distribution: Tioman Island, Malaysia.

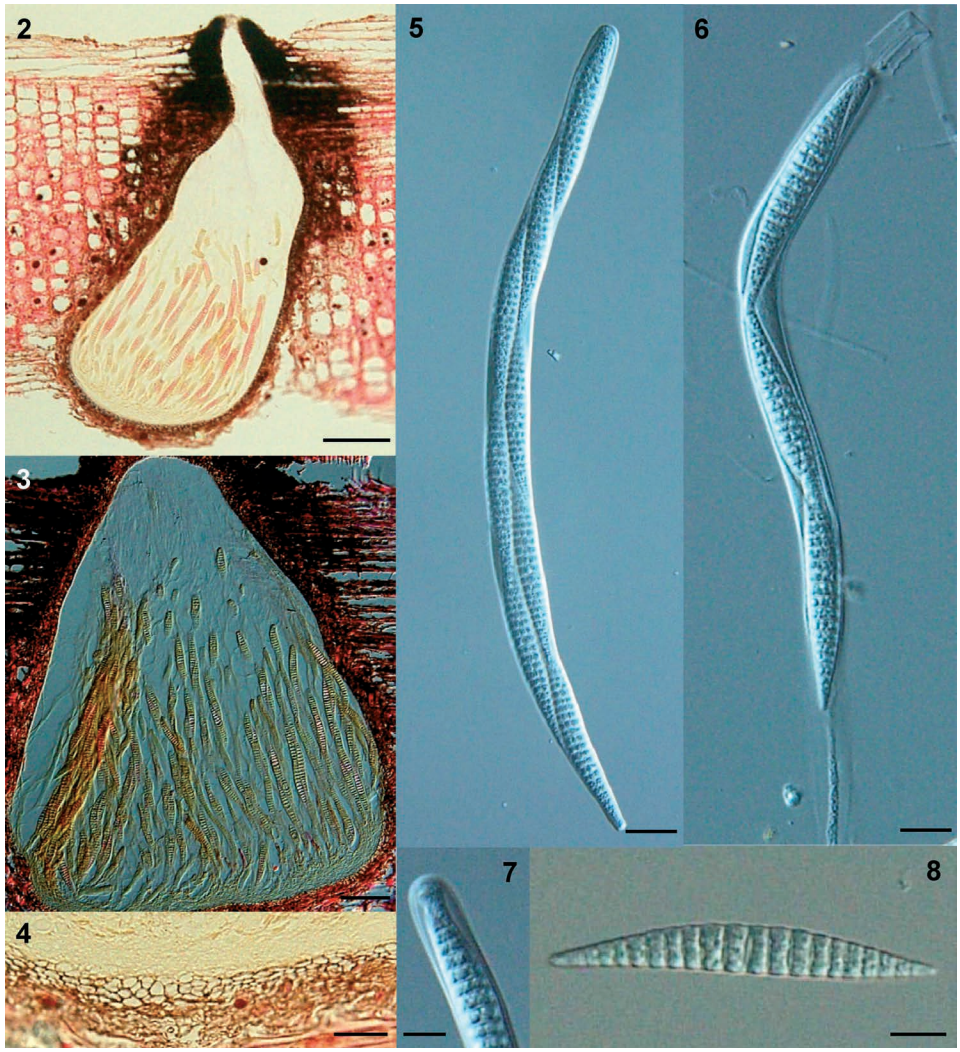
Substrata: Decaying mangrove wood.

New combinations:

Dyfrolomyces rhizophorae (K.D. Hyde) K.D. Hyde, K.L. Pang, Alias, Suetrong & E.B.G. Jones, **comb. nov.**
≡ *Saccardoella rhizophorae* K.D. Hyde, Mycologia 84(5): 806 (1992)
MycoBank: MB 804663

Dyfrolomyces mangrovei (K.D. Hyde) K.D. Hyde, K.L. Pang, Alias, Suetrong & E.B.G. Jones, **comb. nov.**
≡ *Saccardoella mangrovei* K.D. Hyde, Mycologia 84(5): 803 (1992)
MycoBank: MB 804665

Dyfrolomyces marinospora (K.D. Hyde) K.D. Hyde, K.L. Pang, Alias, Suetrong & E.B.G. Jones, **comb. nov.**
≡ *Saccardoella marinospora* K.D. Hyde, Mycologia 84(5): 806 (1992)
MycoBank: MB 804664



Figs 2-8. *Dyfrolomyces tiomanensis* (holotype). **2.** Immersed, pyriform ascoma with blackened clypeus. **3.** Asci forming at the base of ascoma with pseudoparaphyses attached at the base and top of ascoma. **4.** Peridium comprising two layers, an outer layer of cells of *textura intricata* composed of host cells interspersed with fungal hyphae and an inner layer of thick-walled cells of *textura angularis*. **5.** Cylindrical ascus. **6.** Dehiscence of the ascus. **7.** Faint ring-like subapical apparatus. **8.** Spindle-shaped ascospore with 20 septa. Scale bars: **2** = 100 µm, **3** = 50 µm, **4-6** = 20 µm, **7-8** = 10 µm.

Key to *Dyfrolomyces* species:

1a. Ascospores spindle-shaped, lacking a sheath..... *D. tiomanensis*
1b. Ascospores ellipsoidal to fusiform, with a sheath..... 2
2a. Ascospores with 7 to 9 septa..... *D. mangrovei*
2b. Ascospores with fewer than 7 septa..... 3
3a. Ascospores with 4 to 6 septa..... *D. rhizophorae*
3b. Ascospores consistently with 3 septa..... *D. marinospora*

RESULTS AND DISCUSSION

A combined analysis of the 18S and 28S regions of the rRNA and the partial TEF1 genes was run and the phylogenetic tree resulting from the Bayesian analysis is shown in Figure 1 with posterior probabilities. The mean likelihood is -43733.7774. The initial BLAST search for sequences of *Dyfrolomyces tiomanensis* suggested that it was a member of the *Dothideomycetes* (results not shown) and the closest sequence matches were included in the phylogenetic analysis. Two isolates of *Dyfrolomyces* (*Saccardoella*) *rhizophorae* and the new fungus *D. tiomanensis* form a monophyletic group but they do not group with the core taxa of the *Dothideomycetes*. *Dyfrolomyces* rather clusters with taxa of the *Acrospermaceae* (*Dothideomycetes incertae sedis*) with a strong posterior probability, while taxa of the *Monoblastiaceae* form a sister clade to the *Acrospermaceae* and *Dyfrolomyces* spp.

Saccardoella has been variously assigned to the *Clypeosphaeriaceae* (Barr 1990), and unitunicate *Ascomycetes incertae sedis* (Jones *et al.*, 2009). Suetrong *et al.* (2009) sequenced a strain of *Saccardoella rhizophorae* isolated by Dr. J. Kohlmeyer from collections made in Hawaii. This sequence did not group with any known taxon in the *Dothideomycetes*, but formed a unique clade that could not be referred to any family or order. In this study, the monophyly of *S. rhizophorae* and the new marine fungus, *S. tiomanensis*, confirms the placement of the former species in the *Dothideomycetes* (Suetrong *et al.*, 2009) and suggests that marine *Saccardoella* constitutes a disparate group from *S. montellica*, the type species of the genus in the *Sordariomycetes*. *Saccardoella montellica* is a sordariomycetous taxon with unitunicate asci, while *D. rhizophorae* and *D. tiomanensis* have bitunicate asci. Ascomata of *S. montellica* are partly immersed in wood; asci are 4-spored, long cylindrical and "iodine fluorescent"; and ascospores are 100-130 µm long and 20-30 septate, with apical spines (Spegazzini's drawing: http://www.cybertruffle.org.uk/spegazzini/eng/002103a_.htm). The iodine positive reaction of the asci was not mentioned in the protologue (Spegazzini 1879) or subsequent papers (Petrak 1962; Barr 1990; Hyde 1992; Tsui *et al.*, 2006) and this has caused considerable confusion. The iodine positive ring may indicate that *Saccardoella montellica* belongs to the *Xylariales*. We cannot confirm this, as LPS will no longer loan Spegazzini's type material, but deduce from the drawing provided by Spegazzini (1879). The asci and ascospores of the *Dyfrolomyces* species are significantly different to those of *S. montellica*. Asci of *S. montellica* are 4-spored while those of *Dyfrolomyces* species are 8-spored. One needle-like appendage is present at polar position in ascospores of *S. montellica* while this is lacking in those of *Dyfrolomyces* species but a sheath may be present. Therefore, any dothideomycetous elements in *Saccardoella* should be transferred to one or more new genera.

Consequently, a new genus, *Dyfrolomyces*, is introduced to accommodate *D. tiomanensis* while the three marine *Saccardoella* species are transferred to the new genus. Ascomatal structure of *D. tiomanensis* is similar to other three described marine species of *Dyfrolomyces*, while the species can be differentiated based on ascospore characteristics, in particular ascospore septation. Ascospores of *D. tiomanensis* are spindle-shaped with 20-24 septa, those of *D. mangrovei*, *D. marinospora* and *D. rhizophorae* have 7-9, 3 and 4-6 septa, respectively.

A new family *Dyfrolomycetaceae* is established for *Dyfrolomyces* in the *Dothideomycetes*. Phylogenetically, *Dyfrolomyces* forms a well-supported monophyletic clade with *Acrospermum* spp. but ecologically and morphologically, they are very different. *Acrospermum* is a terrestrial genus growing on grass, while

Dyfrolomyces spp. are marine taxa growing on decaying mangrove wood. Ascomata of *Acrospermum* are stalked and ascospores are filiform with some species having length over 1000 µm (Minter *et al.*, 2007; Hyde *et al.*, 2013). Ascomata of *Dyfrolomyces* are immersed forming a clypeus on the wood surface and ascospore shape is broadly ellipsoidal. However, functional dehiscence of the bitunicate asci in *Acrospermum* species has not been observed (Minter *et al.*, 2007), an observation previously found in the marine *Saccardoella* species. In this study, a clear dehiscence of an ascus was observed for *D. tiomanensis* (Fig. 6), confirming the bituncate nature of the asci. The freshwater species of *Saccardoella* will need to be recollected and sequenced to establish their taxonomy affinities.

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