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Sequence data reveals phylogenetic affinities of *Acrocalymma aquatica* sp. nov., *Aquasubmersa mircensis* gen. et sp. nov. and *Clohesyomyces aquaticus* (freshwater coelomycetes)

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Abstract – Three coelomycetous ascomycetes, *Acrocalymma aquatic* sp. nov.*, Aquasubmersa mircensis* gen. & sp. nov., and *Clohesyomyces aquaticus* are reported from northern Thailand. All taxa are characterized, illustrated and subjected to awnalyses of LSU, SSU and/or ITS rDNA gene sequence data to establish their phylogenetic placement in the ascomycetes. *Acrocalymma* is a monotypic genus in a distinct lineage of Pleosporales which is basal to *Morosphaeriaceae* as confirmed by our combined SSU and LSU analysis. *Acrocalymma aquatica* sp. nov., differs in morphology and gene sequence data from other species in the genus. *Aquasubmersa mircensis* gen. et sp. nov. is phylogenetically unrelated to any other dothideomycetous genus and is introduced as a new genus. Sequence data from *Clohesyomyces aquaticus* facilitated its accommodation in *Lindgomycetaceae.*

Anamorph / appendage / DNA / nomenclature / sheath / submerged / teleomorph / Thailand

INTRODUCTION

A continually increasing wealth of knowledge is available on freshwater fungi in terms of distribution, ecology, and classification (Monchy *et al.*, 2011, Raja *et al.*, 2009, Rosique-Gil *et al.*, 2008, Simonis *et al.*, 2008, Sridhar *et al.*, 2010, Voronin, 2008). There has also been some phylogenetic research on the freshwater fungi, although this has mostly been confined to the ascomycetes and the asexual hyphomycetes (Letourneau *et al.*, 2010, Seena *et al.*, 2010, Zhang *et al.*, 2009). For example, many of the asexual helicosporous hyphomycetes belong in the Tubeufiaceae (Boonmee *et al.*, 2011), while the bitunicate ascomycetes (e.g.

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Amniculicola immersa Y. Zhang ter, J. Fourn., Crous & K.D. Hyde and *Amniculicola parva* Y. Zhang ter, J. Fourn., Crous & K.D. Hyde) are scattered amongst the Pleosporales (Liu *et al.*, 2011, Zhang *et al.*, 2011, Zhang *et al.*, 2012). There is however little data on the aquatic asexual coelomycetes.

Previous studies on freshwater coelomycetes have casually relegated them to "Coelomycetes spp." In the accounts of freshwater coelomycetes in the streams in Philippines (Cai *et al.*, 2003), China (Luo *et al.*, 2004) and Hong Kong (Ho *et al.*, 2002), this is particularly noticeable with 3, 9 and 4 taxa being listed as Coelomycetes respectively. Some coelomycete species are however, better known and are generally reported in freshwater fungi checklists. For instance, *Coeloanguillospora appalachiensis* Dyko & B. Sutton, *Monochaetiopsis lakefuxianensis* L. Cai, Jeewon & K.D. Hyde (= *Dyrithiopsis lakefuxianensis* L. Cai, Jeewon & K.D. Hyde), *Pestalotiopsis* spp. and *Tiarosporella paludosa* (Sacc. & Fiori ex P. Syd.) Höhn. are listed in the papers of Cai *et al.* (2003), Dyko & Sutton (1978), Ho *et al.* (2001), Hyde (1993, 2007), Jeewon *et al.*, (2003) and Luo *et al.* (2004).

Although the naming of freshwater coelomycetes is rather difficult due to a dearth of morphological characters available, we now have molecular sequence data at our disposal to characterise and identify the taxa. Therefore we should no longer list freshwater coelomycetes as "Coelomycete sp." but should name them. In this study, we isolated a new species of *Acrocalymma*, an unidentified species described herein as a new genus, and *Clohesyomyces aquaticus*. We characterised the taxa morphologically and also used DNA sequence-analysis to determine the phylogenetic relationship of the asexual morphs within the ascomycete taxonomic framework.

MATERIALS AND METHODS

Morphological study

Submerged wood was randomly collected from the lotic freshwater habitats in Chiang Mai Province, Thailand, in November 2010 and April 2011, following the procedures described in Kurniawati et al (2010) and Tsui et al. (2003). Samples were placed separately in Zip lock plastic bags with sterile moist tissue paper. The woody substrates were examined under a dissecting microscope for fruiting bodies after one week of incubation and until 2 months (Shearer *et al.*, 2004).

Observations and photomicrographs were made from material mounted in water or lactic acid (85%) using a Nikon ECLIPSE 80i microscope. Melzer's reagent (MEZ; 0.5 g iodine, 1.5 g IKI, 20 g chloral hydrate, 20 ml distilled water) and aqueous cotton blue were added to determine staining reactions of the ascus apical apparatus (Raja & Shearer, 2008). India ink was used to reveal gelatinous sheaths on or around ascospores. Measurements were made with the Tarosoft (R) Image Frame Work.

Isolations were made from single ascospores, following the methodology of Chomnunti *et al.* (2011), on 2% water agar (WA) (Biolab, S.A), with sterilized pine needles on the medium for the purpose of inducing sporulation. The herbarium specimens are deposited at Mae Fah Luang University (MFLU), Chiang Rai, Thailand. Fungi isolated in this study are deposited at Mae Fah Luang University Culture Collection (MFLUCC), Thailand and International Fungal Research & Development Centre Culture Collection (IFRDCC), China under MTA C10/2011 and MTA C19/2011.

DNA Extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA for 14 d at 25°C in the dark. Genomic DNA was extracted from the fresh mycelium using Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) according to the manufacturer's protocol (Hangzhou, P.R. China).

DNA amplification was performed by polymerase chain reaction (PCR). For nucleotide sequence comparisons fragments of two loci were analysed: LSU and SSU. Primer pairs LROR and LR5 (Vilgalys and Hester, 1990) for LSU, NS7R and NS24 (http://www.lutzonilab.net/primers/page244.shtml) for SSU were utilized to amplify. The amplifications were performed in a 50 µl reaction: 1 × PCR buffer, 0.2 mM dNTPs, 0.3 µM of each primer; 1.5 mM MgCl2, 0.8 units Taq Polymerase and 5-10 ng DNA (Jeewon *et al.*, 2004, Shenoy *et al.*, 2007b). The amplification conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, annealing at 55°C for 2 min and elongation at 72°C for 90 s, with a final extension period of 72°C for 10 min (Liu *et al.*, 1999). The PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide.

PCR products were then purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham Biosciences, Bucking hamshire, UK; product code: 27–9602–017). The sequences were carried out by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, P.R. China).

Sequence alignment and phylogenetic analysis

Sequences obtained from the forward and reverse primers (LROR and LR5, NS7R and NS24, ITS4 and ITS5) were manually aligned to obtain a consensus sequence using Bioedit 7.0.9 (Hall, 1999). Phylogenetic analyses were performed in PAUP v. 4.0b10 (Swofford, 2002) for Maximum-parsimony (MP) analyses. Prior to phylogenetic analysis, ambiguous sequence at the start and the end were deleted due to missing data in most sequences and gaps were adjusted manually wherever necessary.

MP analysis were performed using the heuristic searches option with random starting trees, random stepwise addition on 100 replicates, bootstrap (BT) analysis with 1000 replicates, gaps treated as missing data and a tree-bisection-reconnection (TBR) as the branch-swapping algorithm (Hillis & Bull, 1993). Parsimony tree scores for tree length (TL), the consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated in PAUP* (Figs 4). Trees were visualized with TreeView (Page, 1996).

RESULTS

Phylogenetic analysis

In Fig. 1, the LSU and SSU sequences for the isolates studied were combined and aligned with 59 strains of 18 families of Pleosporales and another four orders retrieved from GenBank. The combined dataset after alignment consisted of 1992 characters including gaps, of which 1957 sites are included in the

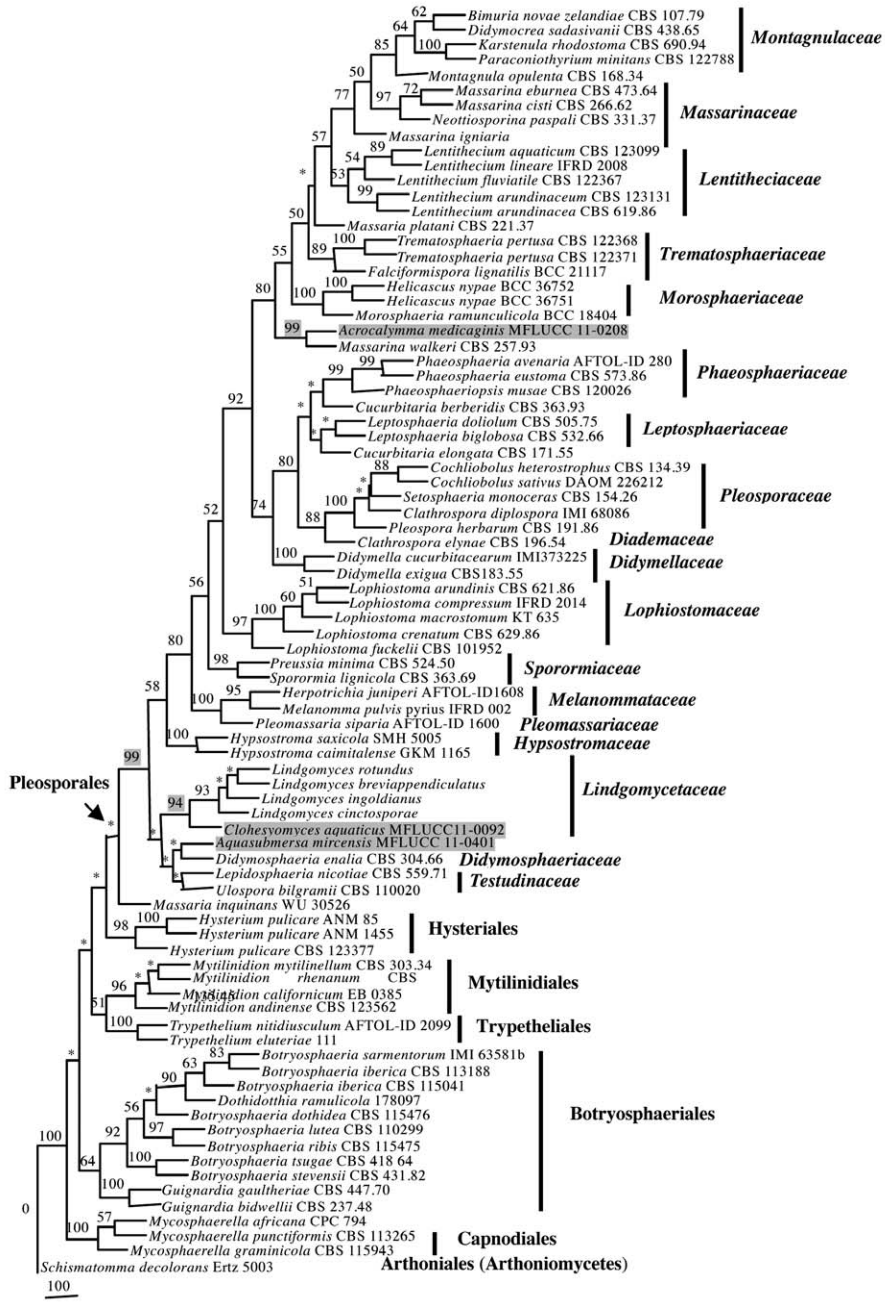

Fig. 1. Maximum parsimony tree generated from 18S and 28S rDNA and with gaps as missing data. Designated outgroup is *Schismatomma decolorans* (Ertz 5003). Bootstrap support values above 50% (based on 1,000 replicates) are shown on nodes, and those lower than 50% are marked as *. Newly generated sequences are highlighted. The strain numbers are noted after the species names.

Maximum parsimony (MP) analysis. Of the 1992 characters, 1343 were constant, while 195 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 419 parsimony informative characters resulted in a single tree with TL = 2111, CI = 0.407, RI = 0.731, RC = 0.298 and HI = 0.593. In Fig. 2, ITS sequences of *Acrocalymma aquatica* were aligned with 18 sequences of Pleosporales. Four hundred seventy three characters were consisted including gaps. Ninety one variable characters were parsimony-uninformative, while 211 parsimony informative characters resulted in a single tree with TL = 921, CI = 0.568, RI = 0.556, RC = 0.316 and HI = 0.432.

In Fig. 1, *Acrocalymma aquatica* clustered in a basal lineage to the *Morosphaeriaceae* with *Massarina walkeri* with 99% Maximum Parsimony bootstrap support values. A blast search of the unidentified species yielded no closely related sequenced taxa in GenBank and in the phylogenetic analysis it clustered in the *Didymosphaeriaceae* with poor support and therefore herein described as a new species in a new genus. *Clohesyomyces aquaticus* clustered in *Lindgomycetaceae* with strong (94%) bootstrap support.

Table 1. Newly generated species and sequences database accession numbers used in this study (type sequences are indicated in *)

Species	*Source of sequences*	*GenBank accession no.*		
		LSU	*SSU*	*ITS*
*Acrocalymma aquatica**	MFLUCC11-0208 = IFRDCC 2361	JX276952	JX276953	JX276951
*Aquasubmersa mircensis**	MFLUCC11-0401 = IFRDCC 2572	JX276955	JX276956	JX276954
Clohesyomyces aquaticus	MFLUCC11-0092 = IFRDCC 2360	JX276950	JX276949	JX276948

Taxonomy

Acrocalymma aquatica H. Zhang ter & K.D. Hyde, **sp. nov.** **Fig. 3**

MycoBank: 800877

Etymology: from the Latin "*aquaticus*", in reference to the freshwater habit.

Habit saprobic on submerged wood in a freshwater stream. *Pycnidia* 150-300 µm diam., globose, dark brown or black, semi-immersed to superficial, solitary, papillate, ostiole central, unilocular, with wall 12-18 µm thick, dark towards the outside with occluded cells, with wall cells becoming paler, elongated and hyaline towards the conidiogenous region. *Conidiophores* reduced. *Conidiogenous cells* 5-12 × 2.5-5 (-6) µm, determinate, discrete, commonly cylindrical, sometimes lageniform, with a narrow channel and apical periclinal thickening, formed from the inner cells of the pycnidial wall. *Conidia* 12-17 × 3-4 µm ($\bar{x}$ = 14.5 × 3.4 µm, n = 20), holoblastic, initially unicellular, becoming mostly one-euseptate, not constricted at the septa, cylindrical to fusoid, straight, hyaline, thin-walled with guttulate content, truncate at the base and becoming a little narrower at apex with a mucilaginous helmet-shaped appendage.

In culture, on PDA, slow growing, 20 mm diam. in 10 days at 25°C, grey from above, circular, raised, fairly dense, with a smooth edge, pale grey around the culture.

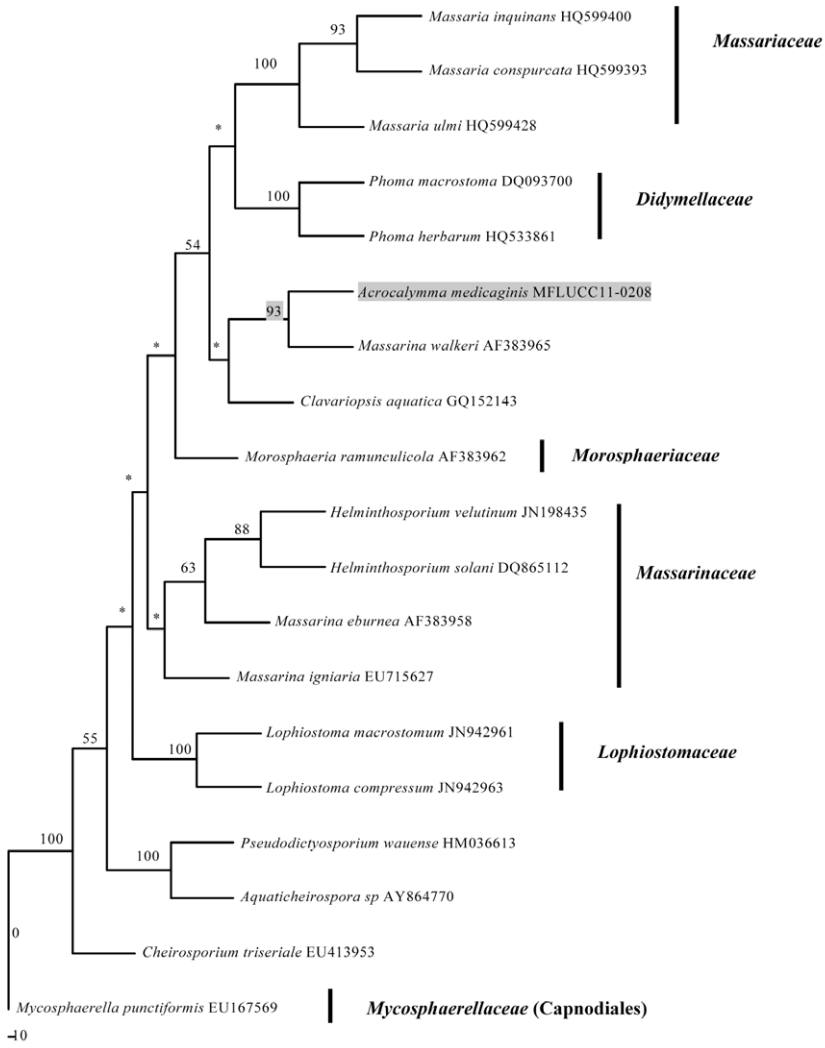

Fig. 2. Maximum parsimony tree generated from ITS rDNA and with gaps as missing data. Designated outgroup is *Mycosphaerella punctiformis* (EU167569). Bootstrap support values above 50% (based on 1,000 replicates) are shown on nodes, and those lower than 50% are marked as *. Newly generated sequences are highlighted. The GenBank accession numbers are noted after the species.

Mode of life: saprobic.
Habitat: on submerged wood in a freshwater stream.
Known distribution: Thailand.
Material examined: THAILAND, Chiang Mai, Doi Inthanon, on submerged wood, 16 November 2010, Huang Zhang d67 (MFLU 111113, **holotype**), ex-type living culture MFLUCC11-0208 = IFRDCC 2361; THAILAND, Chiang Mai, Doi Inthanon, on submerged wood, 16 November 2010, Huang Zhang i10 (MFLU111137, **paratype**), ex-paratype living culture MFLUCC11-0525 = IFRDCC 2461.

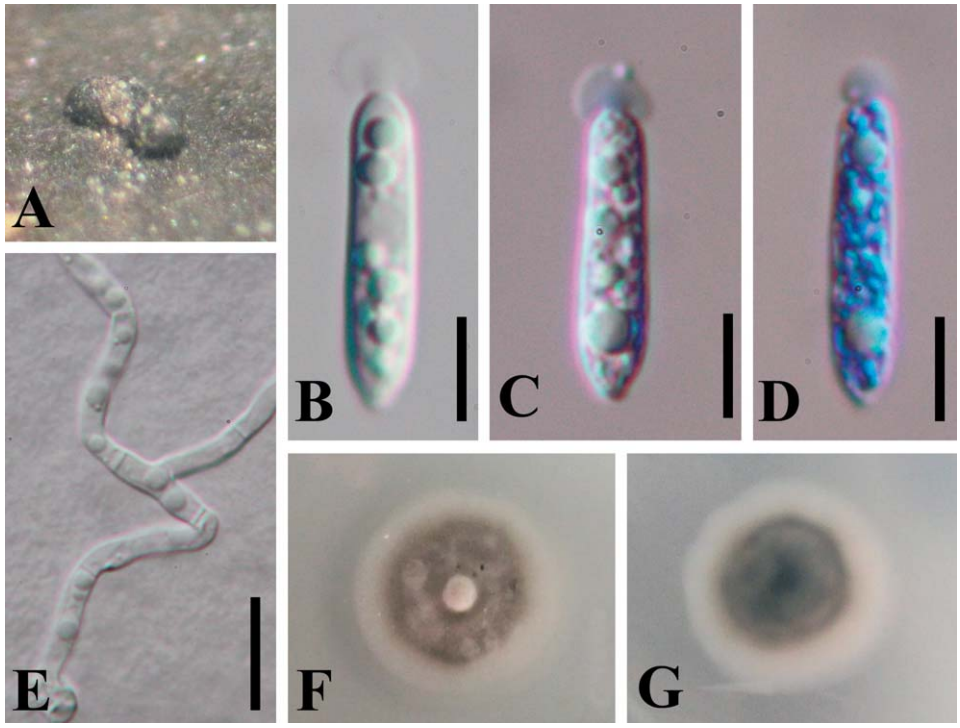

Fig. 3. **A-G.** ***Acrocalymma aquatica*** **(holotype) A.** Pycnidia on wood surface. **B-D.** Conidia with single apical appendage. C, D. Conidia in cotton blue. **E.** Germinating conidium. **F-G.** Cultures in PDA. F, front view; G, reverse. Scale bars: B-D = 5 µm, E = 10 µm.

Notes: *Acrocalymma medicaginis* Alcorn & J.A.G. Irwin, described as *Stagonospora meliloti* (Lasch) Petr., causes root and crown rot disease of Lucerne (*Medicago sativa* L.) (Alcorn & Irwin, 1987). Alcorn & Irwin (1987) reexamined the type material and introduced the new genus *Acrocalymma* to accommodate it. *A. medicaginis* is characterized by solitary, papillate, globose pycnidia, absence of conidiophores, determinate, discrete conidiogenous cells and holoblastic, aseptate, cylindrical and hyaline conidia with a helmet-shaped mucilaginous appendage at each end (Alcorn & Irwin, 1987). The genus is monotypic and the sexual morph of *A. medicaginis* is reported to be *Massarina walkeri* Shoemaker, C.E. Babc. & J.A.G. Irwin, formed in pure culture of *A. medicaginis* (Shoemaker *et al.*, 1991).

Our new collection fits well with *Acrocalymma* but can be distinguished from *A. medicaginis* by conidia having a single appendage at the apex (Fig 3). *A. aquatica* has smaller conidia (12-17 × 3-4 µm vs. 11-21 × 3.5-5 µm). Furthermore, their habitats are different. *A. medicaginis* is a plant pathogen which causes root and crown rot disease of Lucerne (*Medicago sativa* L.) (Alcorn & Irwin, 1987), while *A. aquatica* is saprobic in submerged wood. The phylogram from analysis of ITS sequence data (Fig. 2) shows that *A. aquatica* is related to *Massarina walkeri* with 93% bootstrap support. *Acrocalymma medicaginis* was also reported as saprobic from peat swamp forest (Pinnoi *et al.*, 2006) and this needs reexamination.

Recently, a relatively narrow familial concept was established for *Massarinaceae* and *Massarina walkeri* was excluded (Zhang *et al.*, 2012). In the

phylogram generated from LSU and SSU sequence data (Fig. 1), *Massarina walkeri* (= *Acrocalymma medicaginis*) and*haeriaceae* (Suetrong *et al.*, 2009). Only one name can be used for *Massarina walkeri*/*Acrocalymma medicaginis* which is a distinct genus (Hawksworth *et al.*, 2011, 2012) and *Massarina* is already a good genus occupied by *Massarina eburnea* (Tul. & C. Tul.) Sacc., the generic and family type (Zhang *et al.*, 2012). In this case, it is therefore felt appropriate to adopt the name *Acrocalymma* for this genus and place *Massarina walkeri* as synonymy. *Acrocalymma aquatica* is a second species in this genus.

Acrocalymma medicaginis Alcorn & J.A.G. Irwin, *Trans. Br. mycol. Soc.* 88(2): 163 (1987)

=*Massarina walkeri* Shoemaker, C.E. Babc. & J.A.G. Irwin, *Can. J. Bot.* 69(3): 569 (1991)

MycoBank: 130085

Aquasubmersa K.D. Hyde & H. Zhang ter, **gen. nov.**

MycoBank: 800875

Etymology: *aqua* = water; *submersa* = submerged; refers to submerged habit of this genus.

Habit on submerged plant substrate. *Pycnidia* subglobose to ellipsoidal, dark brown to black, semi-immersed to superficial, solitary, unilocular, with outer layer of wall brown and cells occluded, with the inner layers of the wall hyaline and elongated cells. *Conidiophores* reduced. *Conidiogenous cells* determinate, discrete, lageniform, hyaline, smooth, formed from the inner cells of the pycnidial wall. *Conidia* holoblastic, unicellular, ellipsoidal, hyaline, thin-walled, guttulate.

Type species: *Aquasubmersa mircensis* H. Zhang ter & K.D. Hyde, **sp. nov.**

Aquasubmersa mircensis H. Zhang ter & K.D. Hyde, **sp. nov.** **Fig. 4**

MycoBank: 800876

Etymology: mir = from, MRC (Mushroom Research Centre) "*ensis*", the location of the fungus.

Habit saprobic on submerged wood in a freshwater stream. *Pycnidia* 130-170 μm high, 150-250 μm diam., subglobose to ellipsoidal, dark brown to black, semi-immersed to superficial, solitary, unilocular, ostiolate, with wall up to 20 μm thick, with outer layer of the wall brown and cells occluded, the inner layers of the wall hyaline and elongated cells; ostiole dark-brown, circular, centric, short papillate. *Conidiophores* reduced. *Conidiogenous cells* 10-14 μm long, 4-7 μm wide, determinate, discrete, lageniform, hyaline, smooth, formed from the inner layer of cells of the pycnidial wall. *Conidia* 16-21 × 8-11 μm ($\bar{x}$ = 18.3 × 9.4 μm, n = 10), holoblastic, initially unicellular, ellipsoidal, some with papillate bases, hyaline, thin-walled with guttulate content.

In on PDA, colony circular, slow growing, 20 mm in 20 days, at 25°C, grey from above, black from reverse, raised, felty-woolly, fairly dense, aerial with an sinuate edge, slightly staining agar to brown around the culture.

Mode of life: saprobic.

Habitat: on submerged wood in a freshwater stream.

Known distribution: Thailand.

Material examined: THAILAND, Chiang Mai, Mushroom Research Centre, on submerged wood, 21 April 2011, Huang Zhang m3 (MFLU 111001, **holotype**), ex-holotype living culture MFLUCC11-0401 = IFRDCC 2572.

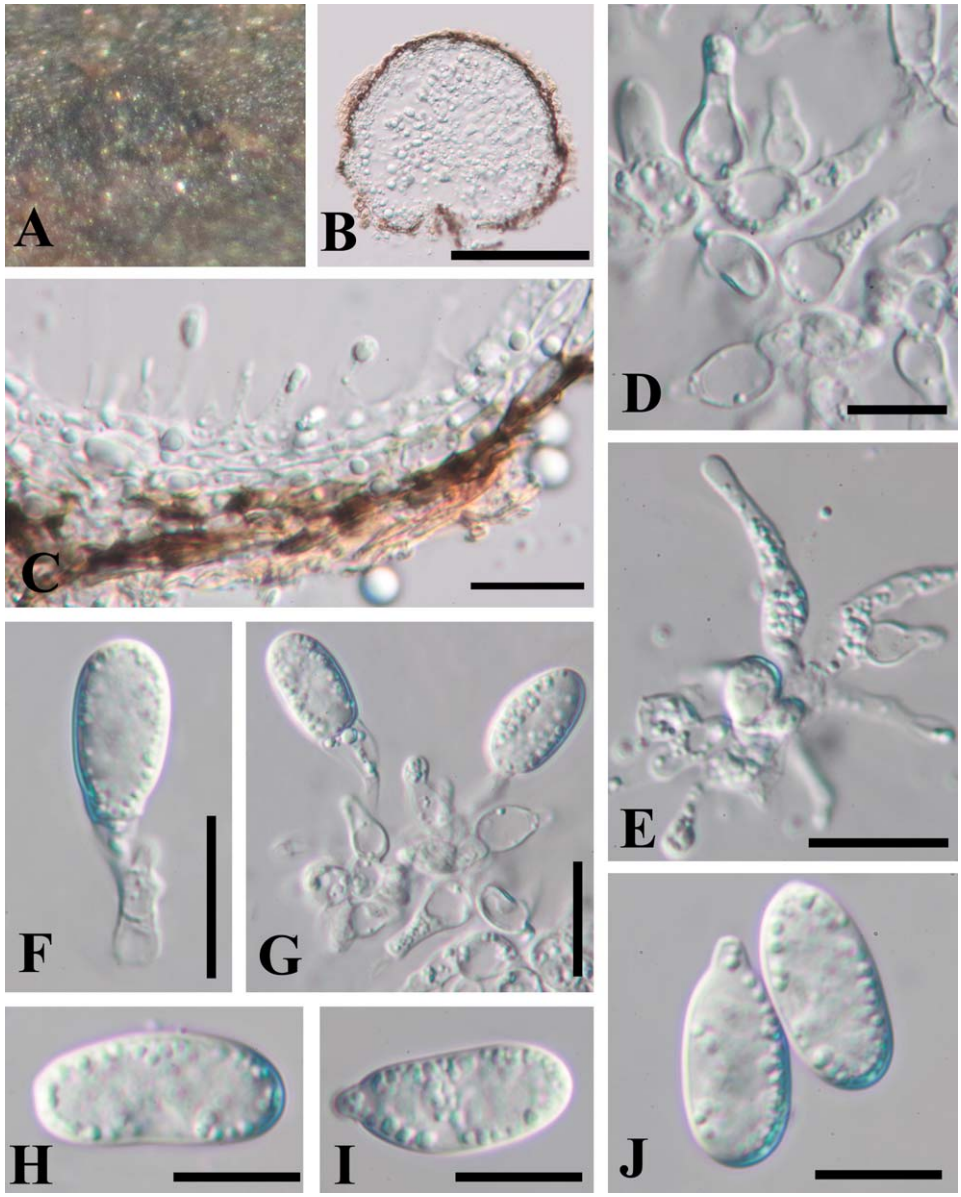

Fig. 4. **A-J.** *Aquasubmersa mircensis* (holotype). **A.** Pycnidium on wood surface. **B.** Section of pycnidium. **C.** Peridium with conidiogenous cells. **D-E.** Conidiogenous cells. **F-G.** Conidiogenous cells with conidia. **H-J.** Conidia. Scale bars: B = 100 µm, C, E-G = 15 µm, D, H-J = 10 µm.

Notes: *Aquasubmersa mircensis* cannot be included in any previously described genus and therefore is ascribed to a new genus, *Aquasubmersa.* It is similar to *Diplodia* in its hyaline and unicellular conidia, but the conidia in *Diplodia* become two-celled and brown at maturity (Lazzizera *et al.*, 2008). In *Aquasubmersa,* the pycnidia wall is thinner; the conidia are hyaline at maturity

and some have papillate bases. *Aquasubmersa* differs from *Neottiosporina* which has 2-3 euseptate, cylindrical conidia with an apical gelatinous appendage which is formed from the gelatinous sheath (Sutton & Marasas, 1976).

The phylogram resulting from analysis of the LSU and SSU genes of *Aquasubmersa mircensis* and other taxa retrieved from GenBank representing a selection of families and orders in the Dothideomycetes is presented in Fig. 1. The information from Maximum Parsimony tree shows that *A. mircensis* clustered in Pleosporales with 99% support (Fig. 1), and sits with species from several families of Pleosporales with poor support.

Clohesyomyces aquaticus K.D. Hyde, *Aust. Syst. Bot.* 6(2): 170 (1993) **Fig. 5**

Habit saprobic on submerged wood in a freshwater stream. *Pycnidia* 200-300 µm long, 150-240 µm diam., subglobose to ellipsoidal, dark brown to black, semi-immersed, solitary, unilocular, ostiolate, with wall 12-20 µm thick, composed of thick-walled cells *textura angularis*, brown towards the outside and hyaline towards the inner region; ostiole dark-brown, circular, eccentric, short papillate. *Conidiophores* reduced. *Conidiogenous* cells up to 14 µm long, determinate, discrete, cylindrical to subcylindrical, hyaline, smooth, occaswionally with a wide channel, with marginal periclinal thickening and short collarette, formed from the inner cells of the pycnidial wall. *Conidia* 15-20 × 7-10 µm ($\bar{x}$ = 17.8 × 8 µm, n = 10), holoblastic, initially unicellular, becoming mostly one-euseptate, not constricted at the septum, ellipsoidal, rounded at apex, some with truncate bases, hyaline, thin-walled, guttulate, surrounded by an irregular mucilaginous sheath.

In PDA, colony circular, slow growing, 30 mm in 25 days, at 25°C, grey from above, yellow to orange from below, raised, felty-woolly, fairly dense, aerial with an smooth edge, slightly staining agar to yellow around, with numerous partly immersed conidiomata.

Mode of life: saprobic.

Habitat: on submerged wood in a freshwater stream.

Known distribution: Australia, China (Yunnan), Thailand

Material examined: THAILAND, Chiang Mai, Doi Inthanon, on submerged wood, 16 November 2010, Huang Zhang d66 (MFLU 111112); THAILAND, Chiang Mai, Doi Inthanon, on submerged wood, 16 November 2010, Huang Zhang d66 (MFLU 111114), living culture MFLUCC11-0092 = IFRDCC 2360.

Notes: *Clohesyomyces* was described from a freshwater habitat in Australia by Hyde (1993). Subsequently it was found in Yunnan, China (Cai *et al.*, 2006) and also Thailand (Vijaykrishna *et al.*, 2006). It is characterized by solitary, immersed, subglobose to ellipsoidal pycnidia, absence of conidiophores, determinate, discrete, cylindrical conidiogenous cells and holoblastic, unicellular, hyaline conidia becoming one-euseptate and surrounded by an irregular mucilaginous sheath (Hyde, 1993). The genus is monotypic represented by *C. aquaticus* K.D. Hyde. Information on sexual morph of *Clohesyomyces* is lacking.

This species was first considered to be *Diplodia* because of the hyaline and unicellular conidia. The irregular mucilaginous sheath in Indian Ink and hyaline conidia at maturity led us to identify it as *Clohesyomyces aquaticus* (Hyde, 1993). Our collections differ from the holotype in having smaller conidia (our collection vs. holotype: 15-20 × 7-10 µm vs. 21-31 × 9.5-12 µm) and may represent a new species. Other characters are similar.

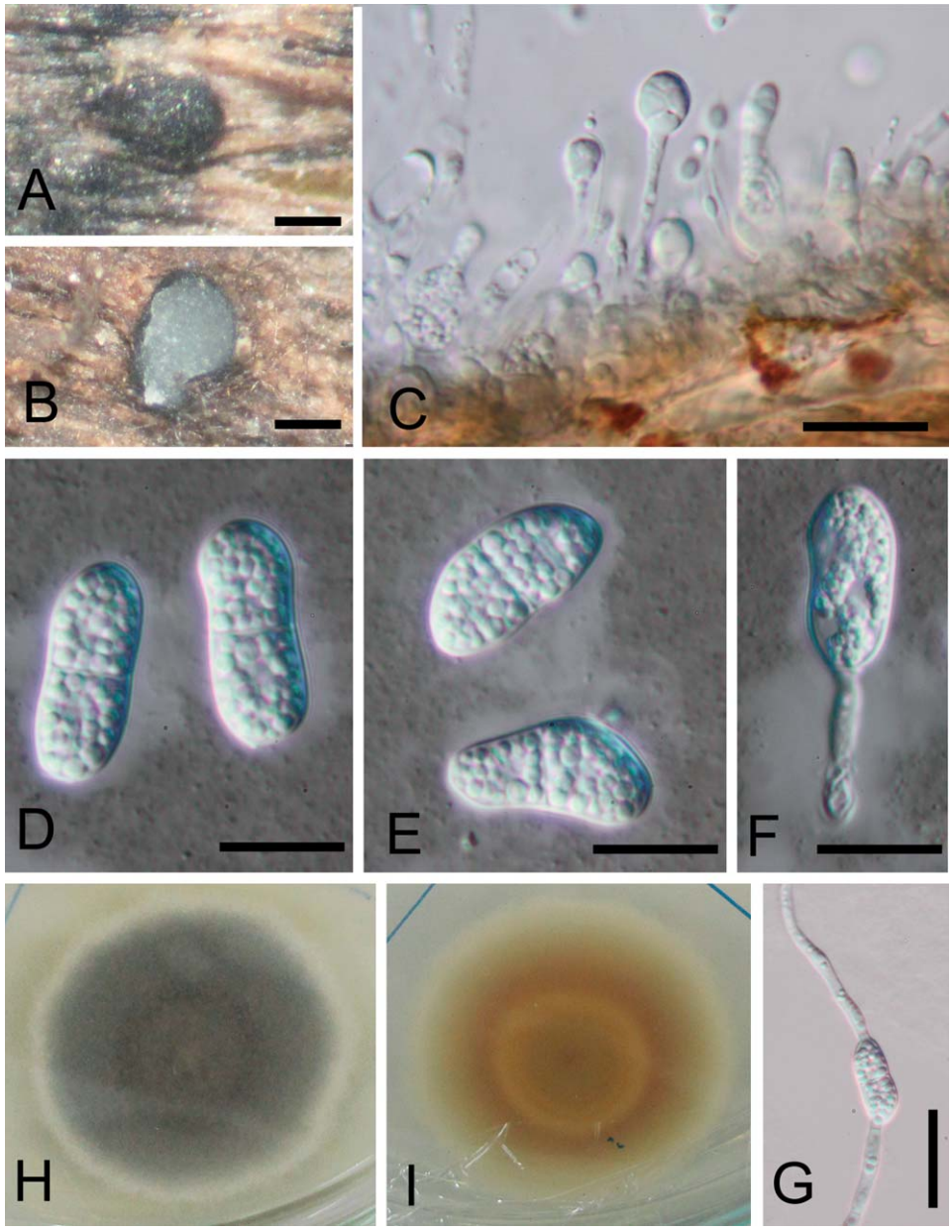

Fig. 5. **A-G. *Clohesyomyces aquaticus*. A.** Pycnidium on wood surface. **B.** Section of pycnidium with mass of conidia. **C.** Peridium with conidiogenous cells. **D-E.** Mature conidia with septa. **F.** Conidium with conidiogenous cell. **G.** Germinating conidium. **H-I.** Culture in PDA, H, front; I, reverse. Scale bars: A-B = 100 µm, C-F = 10 µm, G = 20 µm.

There is presently no report on the sexual morph of *Clohesyomyces.* Our molecular analysis, based on combined LSU and SSU gene data (Fig. 1), indicates that *C. aquaticus* clusters with *Lindgomycetaceae* in a well-supported clade (94%).

This suggests that *C. aquaticus* belongs to *Lindgomycetaceae* (Pleosporales) and this is the second report for an asexual morph in the family (Abdel-Aziz & Abdel-Wahab, 2010). *Clohesyomyces aquaticus* is also newly recorded from freshwater habitats in Thailand (Zhang *et al.*, 2011).

DISCUSSION

Different names were previously given to the asexual and sexual states of the same biological species having different morphologies because of the prevailing dual classification system used in the fungal systematics (Shenoy *et al.*, 2007, Shenoy *et al.*, 2010). With the use of molecular data, it has become common to link the various sexual morphs. The dual naming system has ended and mycologists are now working towards giving pleomorphic fungi single names (Hawksworth *et al.*, 2011, 2012). This is a major challenge for mycologists in the coming years (Hyde *et al.*, 2011, Wijayawardene *et al.*, 2012).

This study makes a small step towards achieving this goal. In this study, *Aquasubmersa mircensis* is introduced as a new genus linked to Pleosporales, *Clohesyomyces aquaticus* belongs in *Lindgomycetaceae*, while *Acrocalymma* is placed in Pleosporales.

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