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Amarenographium solium sp. nov. from Yanbu mangroves in the Kingdom of Saudi Arabia

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Abstract – During an ongoing study of marine fungi growing on *Avicennia marina* in the Red Sea coast of Saudi Arabia, a new coelomycete was collected in the genus *Amarenographium*. The new species is characterized by muriform, brown conidia with one polar gelatinous cap and sheath and holoblastic phialidic conidiogenesis. *Amarenographium solium* sp. nov. differs from the two known species of *Amarenographium* by the large size of its pycnidia, a thick (62-75 µm) two-layered peridial wall of the conidiomata that appears as textura epidermoidea in surface view and conidia with one apical appendage. Phylogenetic analysis of SSU and LSU rDNA sequences showed that the new species and thus genus *Amarenographium* grouped consistently with *Medicopsis romeroi* with high bootstrap support and form a basal clade to the families: Montagnulaceae and Trematosphaeriaceae, order Pleosporales, Dothideomycetes.

Ascomycota / marine fungi / phylogenetics / tropical fungi / taxonomy

INTRODUCTION

During a study of marine fungi growing on *Avicennia marina* (Forsk.) Vierh. on the Red Sea coast of Saudi Arabia, a *Amarenographium* species was found on 33.8% of the 245 wood samples examined from two mangrove stands near Yanbu City. The species could not be assigned to any of the known *Amarenographium* species, and is described herein as new. Other obligate marine fungi recorded on the same samples include *Julella avicenniae* (Borse) K.D. Hyde, *Lulworthia grandispora* Meyers, *Marinosphaera mangrovei* K.D. Hyde and *Swampomyces armeniacus* Kohlm. & Volkm.-Kohlm.

Previously referred to anamorphic fungi, these asexual ascomycetes or basidiomycetes can now be placed into a natural phylogenetic classification with the use of molecular data (Hyde *et al.,* 2011; Hawksworth *et al.*, 2011). This classification is possible when their various morphs are linked by cultural studies or via molecular phylogenetics (Hawksworth, 2012). The current number of marine fungi is 549 (Jones, 2011), while 144 asexual morphs have been recorded

from marine habitats, and all but one is thought to belong to Ascomycota (Abdel-Wahab & Bahkali, 2012). Asexual fungi in the phylum Ascomycota belong to five classes namely: Dothideomycetes, Eurotiomycetes, Leotiomycetes, Orbiliomycetes and Sordariomycetes (Jones, 2011; Abdel-Wahab & Bahkali, 2012).

The genus *Camarosporium* is a pycnidial asexual genus that is characterized by thick-walled, smooth, dark-brown, muriform conidia with or without a gelatinous sheath or polar pads. Conidiogenesis in *Camarosporium* is holoblastic annelidic (Nag Raj, 1993) *Camarosporium* species are saprobic or parasitic on higher plants. Around 100 terrestrial species have been described of which *Camarosporium palliatum* Kohlm. & E. Kohlm., *C. roumeguerii* Sacc. and *C. metableticum* Trail were described from plants growing along the seashore or in costal salt marshes (Kohlmeyer & Kohlmeyer, 1979). Eriksson (1982) transferred *C. metableticum* to a new genus *Amarenographium* based on the different morphology of the conidiophores which are much longer and branched, and phialidic conidiogenesis with solitary conidia that are provided with characteristic polar gelatinous appendages. Eriksson (1982) also noted the morphological similarity between *Amarenographium metableticum* (Trail) O.E. Erikss. *Amarenomyces ammophilae* (Lasch.) O. Erikss. and although the link has not been proven suggested these taxa might be the same biological species. Taylor *et al.* (2003) described a second species of *Amarenographium*, *A. sinense* Joanne E. Taylor, K.D. Hyde & E.B.G. Jones from a dead petiole of *Trachycarpus fortunei* (Hooker) Wendland, from Hubei Province in China.

In this paper we introduce a new species of *Amarenographium* from mangroves in Saudi Arabia and compare it with other species in the genus and provide evidence for its placement in the Pleosporales.

MATERIALS AND METHODS

Details of collecting site: The site is named Yanbu royal commission zone and is composed of three areas of mangrove along the delta of Wadi Farrah, adjacent to the new industrial city of Madinat Yanbu Al-Sinaiyah, which is itself located 25 km south of Yanbu Al-Bahr. *Avicennia marina* is the dominant plant species that extends along 11 km of Red Sea coastline. Oil pollution poses the main threat to the area (Newton & Symens, 1994). The forest floor varies from sandy to muddy and the trees reach up to 4 m in height.

Sample collection: Intertidal decaying wood of *Avicennia marina* were collected from two sites inside Yanbu Royal Commission Zone on 17th November 2011 (N 24° 3´– E 38° 69´ and N23° 55´ – E38° 17´) and placed in clean plastic bags. Upon arrival in the laboratory, samples were washed using sterile sea water, examined for marine fungi, incubated in sterile humid plastic boxes and examined periodically for 3 months (Jones & Hyde, 1988).

Isolation in pure culture: To obtain single spore cultures, spore suspensions of the fruiting bodies were prepared in sterile sea water on a sterile slide under sterilized condition and small drops of this spore suspension placed on Corn Meal Sea water Agar (CMSA) (17 g CMA in 1 l seawater) in Petri-dishes and incubated at 25°C in the dark following a modified method of Chomnunti *et al.* (2011). Germinated spores were transferred to new CMSA plates and incubated at 25°C in the dark.

A spore squash was mounted in seawater for all measurements and photography. Pure cultures of the fungus are deposited at the Department of Botany and Microbiology, College of Science, King Saud University and Mae Fah Luang University international culture collection (MFLU).

DNA extraction, sequencing, and phylogenetic analysis: Single-spore isolates of the fungus were grown in YMG broth (4 g yeast extract, 10 g glucose, 10 g malt extract in 1 liter sea water) until sufficient mycelium was formed for DNA extraction. DNA extraction was done by using Microbial DNA extraction kit (MOBIO; Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The LSU ribosomal DNA was amplified using primers LROR and LR7 (Vilgalys & Hester, 1990). Small subunit rDNA was amplified using NS1 and NS4 (White *et al.* 1990) primers. Sequencing was made by Macrogen Inc., Korea using MGTM Taq-HF DNA Polymerase; cycling parameters: initial denaturation at 96°C for 3 min, 96°C for 15 s, 52°C for 45 s, 72°C for 1 min 30 s, and final elongation at 72°C for 7 min. Sequencing reactions are performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing is performed on each template using LROR and LR7 primers for LSU and NS1 and NS4 primers for SSU sequence. The fluorescent-labeled fragments are purified by the method that Applied Biosystems recommends as it removes the unincorporated terminators and dNTPs. The samples are injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

Sequences were aligned with others retrieved from GenBank using ClustalX (Thompson *et al.,* 1997) and optimized manually. Phylogenetic analyses were carried out using PAUP* (Swofford, 2002). Maximum-likelihood (ML) analysis (Felsenstein, 1981) was performed using heuristic searches with the random stepwise addition of 100 replicates and tree bisection-reconnection (TBR) rearrangements. The optimal model of nucleotide substitution for the ML analyses was determined using hierarchical likelihood ratio tests as implemented in Modeltest 3.7 (Posada & Crandall, 1998). The model selected as the best fit for SSU and LSU rDNA were TIM+I+G and TrNef+I+G respectively. Maximum-parsimony (MP) trees were obtained by 100 random addition heuristic search replicates using phylogenetic packages, and 1000 bootstrap replicates were performed employing 5 random addition heuristic searches. The posteriori probabilities were obtained by using the Bayesian phylogenetic inference on the program MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) with the GTR+I+G and SYM+I+G models for SSU and LSU rDNA respectively that was determined using MrModeltest 2.2 (Nylander, 2004). Five million generations were run in four chains with sampling every 100 generations, yielding 50 000 trees, of which the first 12 500 were discarded as "burn in".

RESULTS

Phylogenetic analysis

Small subunit dataset: The SSU dataset include 23 taxa of which 20 belong to the order Pleosporales, one belongs to the order Myriangiales and two belong to Dothideales and are used as outgroup taxa. The SSU dataset consisted of 1309

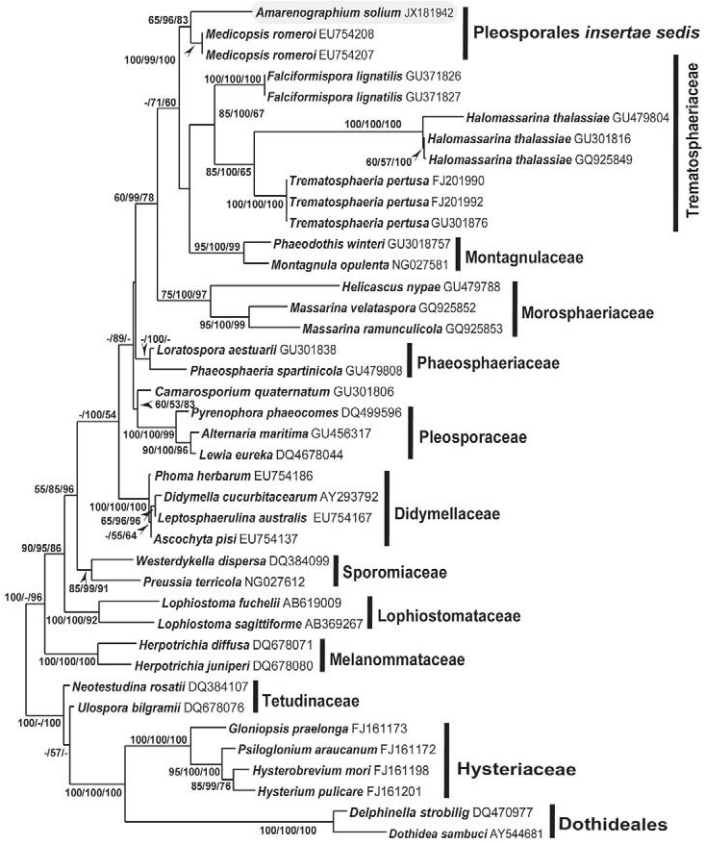

Fig. 1. Bayesian phylogenetic tree of *Amarenographium solium* and related taxa, based on the nucleotide sequences of the small subunit sequence (SSU). Numbers are bootstrap support for Bayesian pp, MP and ML respectively. The new species, *A. solium*, is highlighted.

total characters, of which 1157 are constant, 89 are variable and are parsimony-uninformative and 63 are parsimony informative characters. The nine most parsimonious trees were produced using a heuristic search, all produced trees with a tree length of 232 steps, a consistency index of 0.7198, a retention index of 0.7059 and a rescaled consistency index of 0.5081. Maximum likelihood analysis produced one tree (–ln likelihood = 3412.37068). ML, MP and Bayesian analyses produced trees with similar topologies. The Bayesian phylogenetic tree is shown in Figure 1.

Large subunit dataset: The LSU dataset include 35 taxa of which 33 belong to the order Pleosporales and two belong to Dothideales and used as outgroup taxa. The LSU dataset consisted of 707 total characters, of which 398 are constant, 111 are variable and are parsimony-uninformative and 198 are parsimony informative characters. The 4 most parsimonious trees were produced using a heuristic search, all produced trees with a tree length of 725 steps, a consistency index of 0.4786, a retention index of 0.7128 and a rescaled consistency index of 0.3411. Maximum likelihood analysis produced one tree (–ln likelihood = 5507.29986). ML, MP and Bayesian analyses produced trees with similar topologies. The Bayesian phylogenetic tree is shown in Figure 2.

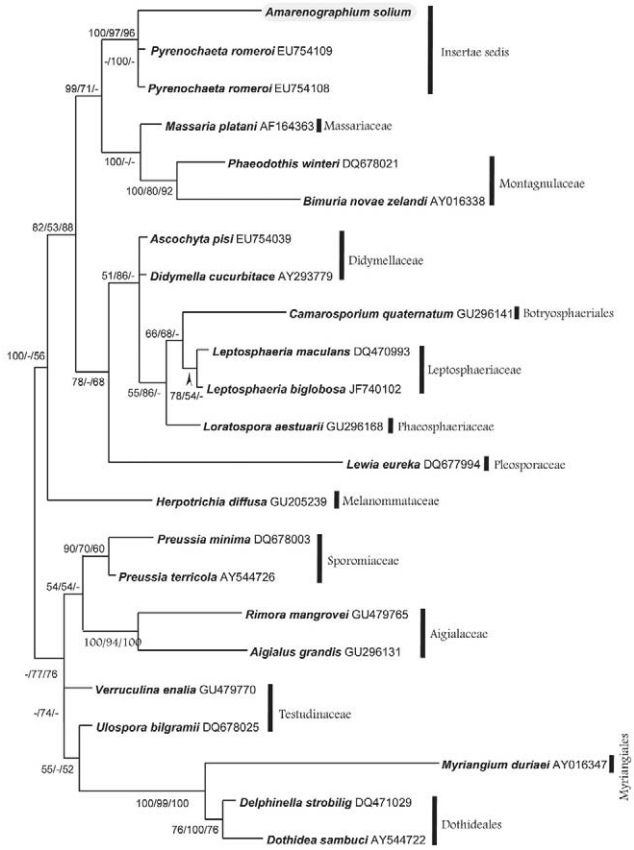

Fig. 2. Bayesian phylogenetic tree of *Amarenographium solium* and related taxa, based on the nucleotide sequences of the large subunit (LSU) sequence. Numbers are bootstrap support for Bayesian pp, MP and ML respectively. The new species, *Amarenographium solium*, is highlighted.

Amarenographium solium proved to be a member of the order Pleosporales *insertae sedis* and formed a sister taxon to *Medicopsis romeroi* (Borelli) Gruyter, Verkley & Crous forming a well supported clade in the SSU data set (100/97/96 for Bayesian/MP/ML respectively) and moderate supported clade in the LSU data set (65/96/83 for Bayesian/MP/ML respectively). Both SSU and LSU data sets produced similar phylogenetic trees topologies in all analyses performed. de Gruyter *et al.* (2012) included *Medicopsis romeroi* in the family Trematosphaeriaceae, however in this study *A. solium* and *M. romeroi* formed a basal clade to the families Trematosphaeriaceae and Montagnulaceae and might represent a new family but it is premature to introduce such a family at this stage.

Taxonomy

Amarenographium solium Abdel-Wahab, Hodhod, Bahkali & K.D. Hyde, **sp.nov.** **Figs 3-4**

MycoBank MB800681

Etymology: From the Latin adjective *solio*, in reference to the cap-like structure of the fungus.

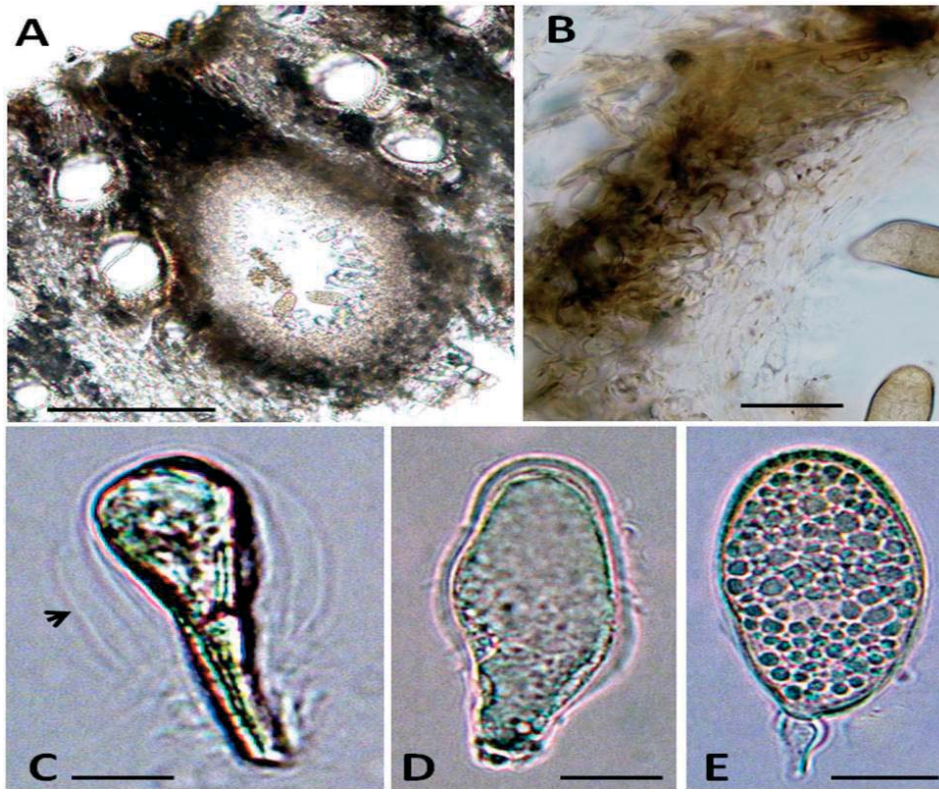

Fig. 3. A-E. *Amarenographium solium.* Bright field light micrographs (from holotype, mounted in water). **A.** Longitudinal section of pycnidium, **B.** Magnified part of the peridial wall that consists of two layers of flattened polygonal cells forming textura epidermoidea. **C-E.** Young conidia. Bars: **A** = 50 µm, **B-E** = 5 µm.

Pycnidia 320-400 µm high, 200-250 µm in diameter, subglobose, immersed, brown to black, ostiolate, with circular opening, mature pycnidia forming a black mass of the conidia on the surface of the wood (Fig. 3, A). **Peridium** 62-75 µm thick, comprising two layers, the outer layer 20-25 µm, with brown cells forming a *textura epidermoidea* and the inner layer 25-37.5 µm with hyaline, flattened, cells forming a *textura angularis* (Fig. 3, B). **Conidiophores** absent. **Conidiogenous cells** 5 × 7.5 µm, ampulliform, hyaline, lining the inner layer of the conidioma, holoblastic, phialidic, producing a single conidium. **Conidia** 35-50 × 12.5-20 µm (× = 42.5 × 15.7 µm, n = 100), muriform, with (3-) 5 (-7) transverse septa and (0-) 1 (-2) longitudinal septa clavate, with apex rounded and basal end acute or truncate, at first yellowish to olivaceous-brown, becoming brown at maturity (Fig. 3 C-E, Fig. 4 A-D), constricted at the central septum and slightly at the other septa, smooth with a gelatinous, striated, regular cap and sheath; the sheath is multi-layered in young conidia (Fig 3, C), swelling and forming fibrillar material in water (arrowed in Fig. 4, B-C) and eventually dissolving.

Single spore isolates of *Amarenographium solium* growing on CMSA are grey to black with tufts of grey aerial mycelium and deeply immersed black mycelium, one month old colony are 4-10 mm in diameter. Pycnidia are produced

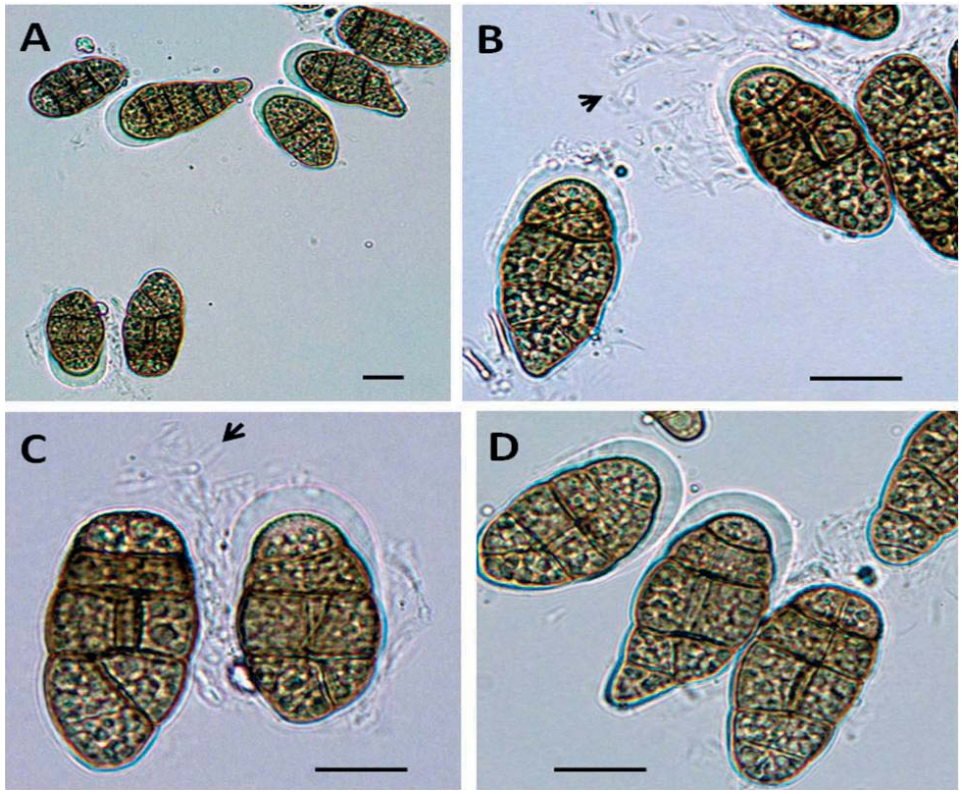

Fig. 4. *Amarenographium solium*. Bright field light micrographs (from holotype, mounted in water). **A-D:** Different shaped conidia with apical polar cap and gelatinous sheath. Note: Fibril material that formed from gelatinous sheath when mounted in water (see arrows in B & C). Bars: **A-D** = 5 µm.

abundantly in pure cultures after 2 months incubation that are immersed with black mass of the conidia produced on the surface of the agar. Pycnidial and conidial dimensions in cultures are similar to those recorded from natural wood.

Type: Saudi Arabia, Yanbu, on decayed wood of *Avicennia marina* at mangrove stand on the Red Sea coast, 17 November 2011, M. A. Abdel-Wahab (Holotype, MFLU12-0059), ex-type culture: MFLUCC12-0087; Saudi Arabia, Yanbu Industrial City mangroves, on decayed wood of *A. marina*, 17 November 2011, M.S. Hodhod, (Paratype, MF100; living culture MFCC100, Culture collection at Department of Botany and Microbiology, King Saud University, Saudi Arabia).

DISCUSSION

Amarenographium solium is characterized by the large size of the conidia with one apical appendage and by a thick, two layered peridial wall that forms a *textura epidermoidea* in surface view. *Amarenographium solium* differs from

Table 1. Synopsis of pycnidia and conidia characters of *Amarenographium* and marine *Camarosporium* species

	A. solium	*A. metableticum*	*A. sinense*	*C. roumegurii*	*C. palliatum*
Pycnidia					
Size (µm)	400 × 200	150-215 × 210-330	150-216 × 114-152	90-210 × 85-260	120-190 × 80-160
Color	Brown to dark brown	Black	Dark brown	Yellow brown to blackish	Light or dark brown to blackish
Shape	Subglobose	Subglobose	Subglobose	Globose	Subglobose
Conidia					
Size (µm)	35-50 × 12.5-20	(20-) 27.5-39 × 11-15.5	22-34 × 8-13	10-20 (-20) × 7-13	20-34 × 9-20
Sheath	Present	Absent	Present	Absent	Present
Cap-like	Only at the apex	Found at both ends	Only at the apex	Absent	Absent
Basal appendage	Absent	Present	Present	Absent	Absent
Microconidia	Absent	Present	Absent	Absent	Absent
Transverse septa	(3-) 5 (-7)	(3-7)	(12-17)	(1-3)	(3-)5(-6)

A. sinense and *A. metableticum* in having larger conidial dimensions. *Amarenographium sinense* is characterized by stromatic conidiomata and by having conidia that have 7-12 transverse septa and a pimpled wall (Taylor *et al*., 2003). *A. metableticum* differs from *A. solium* in having two polar gelatinous caps and smaller pycnidial and conidial dimensions (Table 1). Conidiogenesis in *A. solium* is similar to that found in *A. metableticum* which is holoblastic, and phialidic with solitary conidia (Kohlmeyer & Kohlmeyer, 1979).

Amarenographium solium is similar to *Camarosporium* species in producing muriform conidia in immersed pycnidia (Table 1). However, conidiogenesis in *Camarosporium* species is holobalstic annelidic or phialidic, conidia are thick-walled and lack conidial polar appendages and most of their species produce micro- and macroconidia and are terrestrial species (Sutton, 1980). Two *Camarosporium* species have been described from plants growing along the sea shore or in coastal salt marshes namely: *C. palliatum* Kohlm. & Kohlm. and *C. roumeguerii* Sacc. (Kohlmeyer & Kohlmeyer, 1979). *A. solium* differs from the two *Camarosporium* species in having larger pycnidia, in conidial dimensions and in having polar apical gelatinous caps.

Phylogenetic analyses of SSU and LSU rDNA sequences of *A. solium* placed it as sister taxon to *Medicopsis romeroi* (Borelli) Gruyter, Verkley & Crous (de Gruyter *et al*., 2012). *Medicopsis romeroi* is a human pathogen with setose superficial pycnidia, septate branched conidiophores and hyaline unicellular conidia (Badali *et al.* 2010, de Gruyter *et al*., 2010, 2012). The type species of *Camarosporium*, *C. quaternatum* is placed in a distant clade from the family Trematosphaeriaceae within Pleosporales (Figs 1-2).

When Eriksson (1982) introduced *Amarenographium* he noted the likelihood that *Amarenographium* and *Amarenomyces* were the same biological species, however the connection was based on similar morphology with no proven links. If these genera are proven to be congeneric it will be necessary to adopt one name for these genera and since *Amarenomyces* (Eriksson, 1981) is the older name and takes precedence over *Amarenographium* (Eriksson, 1982), it would be

necessary to transfer all *Amarenographium* species to *Amarenomyces*; this would not involve may changes.

Dothideomycetes species with muriform ascospores were previously clumped in the *Pleosporales*, however they were separated into several distinct genera in different families, based on other morphological characters (e.g. *Cucurbitaria*: *Cucurbitariaceae,* Zhang *et al*., 2009). Recent molecular data have shown muriform brown ascospores have evolved on numerous occasions and are phylogenetically scattered across the Dothideomycetes (Schoch *et al*., 2009; Suetrong *et al*., 2009; Morin *et al*., 2010; Voglmayr & Jaklitsch, 2011; Zhang *et al*., 2012).

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