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Morphological and molecular characterization of *Aquaticheirospora* and phylogenetics of Massarinaceae (Pleosporales)

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A morphologically interesting hyphomycete was collected from submerged wood in a stream in Doi Suthep-Pui National Park, Thailand. It is described as ***Aquaticheirospora lignicola*** **gen.** and **sp. nov.,** and is characterized by euseptate conidia with divergent arms, which are vertically inserted in different planes to a basal cell. The genus differs from other chirosporous genera in having synnematous conidioma and conidia that are produced on conidiogenous cells borne at the apices of synnemata. The morphological characterization of this new fungus is reported and compared with similar chirosporous genera. To investigate the teleomorphic and phylogenetic relationships of this new taxon, three different regions of the ribosomal gene [18S rDNA, 28S rDNA, and internal transcribed spacer (ITS) including 5.8S] were sequenced and analysed. The results of phylogenetic analyses based on 18S, 28S, and partial ITS including 5.8S rDNA, employing different tree-making methods, indicate that ***Aquaticheirospora lignicola*** is closely related to the ascomycetes family Massarinaceae (Order: Pleosporales). The Massarinaceae as currently circumscribed is monophyletic. *Massarina australiensis* and *M. bipolaris*, however, appear to belong to the Lophiostomataceae.

ADDITIONAL KEYWORDS: chirosporous fungi – fungi on wood – new genus – rDNA – saprobic fungi – systematics.

INTRODUCTION

Freshwater fungi represent a ubiquitous and diverse group of organisms, which colonize substrates found in aquatic or semi-aquatic environments (Luo *et al*., 2004; Fryar *et al*., 2005; Pascoal, Marvanová & Cássio, 2005; Sakayaroj, Phongpaichit & Jones, 2005; Vijaykrishna & Hyde, 2006). They appear to be taxonomically diverse, and flourish in various ecological niches (Shearer, 1993; Goh & Hyde, 1996; Gönzöl & Révay, 2003, 2004; Fryar *et al*., 2004a, b). So far, more than 1000 freshwater fungi have been documented and many more are being discovered as new habitats are being explored (Jeewon *et al*., 2003b; Tsui, Goh & Hyde, 2003; Tsui, Hodgkiss & Hyde, 2003; Ho, Hyde & Hodgkiss, 2004; Kodsueb *et al*., 2004; Tsui & Hodgkiss, 2004; Tsui & Hyde, 2004). Several novel and interesting fungi have been described from submerged wood in freshwater environments in both tropical and subtropical countries (Cai *et al*., 2003a, b; Fryar & Hyde, 2004; Luo *et al*., 2004; Tsui & Hyde, 2004). Recent fungal biodiversity studies in Thailand have also resulted in a number of new fungal taxa from aquatic habitats (Pinnoi *et al*., 2004; Pinruan *et al*., 2004a, b). During the examination of freshwater fungi occurring on submerged wood in Doi Suthep-Pui National Park, Thailand, we collected an unusual synnematous

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chirosporous hyphomycete. *Aquaticheirospora lignicola* gen. et sp. nov. is described and illustrated with interference light micrographs, and compared with similar genera. A synopsis of morphological characters of chirosporous genera is provided. In this study, we also determine the phylogenetic relationships of our new taxon based on three sequence data sets [18S rDNA, 28S rDNA, and internal transcribed spacer (ITS) including 5.8S] to establish its teleomorphic affinities with known ascomycetes.

MATERIAL AND METHODS

Collection and phenotypic characterization

Submerged wood (unidentified wood) was collected from a stream in Doi Suthep-Pui National Park, Chiang Mai, Thailand, during the rainy season of 2003. Samples were returned to the laboratory in individual plastic bags. High humidity was maintained by the addition of a paper towel moistened with sterile distilled water. Samples were incubated under ambient laboratory conditions (25–28 °C, fluctuating daylight, and fluorescent light conditions) and were examined microscopically for the presence of microfungi after 4–5 days and periodically for up to 1 month. Cultures of fungi were obtained where possible from single spore isolation (Choi, Hyde & Ho, 1999). Herbarium specimens and living cultures were deposited in The Hong Kong University Culture Collection (HKUCC).

DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA was extracted from mycelial cultures grown on potato dextrose agar (PDA) following a 2 × cetyltrimethylammonium bromide (CTAB) protocol (Jeewon, Liew & Hyde, 2004; Cai, Jeewon & Hyde, 2005). Partial sequences from three different regions of the rDNA molecule (characterized by different rates of evolution) were amplified. Primers pairs NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′), as defined by White *et al*. (1990), were used to amplify a region spanning approximately 1200 nucleotides from the small subunit (18S) of rDNA. LROR (5′-ACCC GCTGAACTTAAGC-3′) and LRO5 (5′-TCCTGAGG GAAACTTCG-3′) primer pairs, as defined by Vilgalys & Hester (1990), were used to amplify a segment of the large 28S subunit (about 950 nucleotides). In addition, primer pairs ITS4 (5′-TCCTCCGCTTAT TGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTC GTAACAAGG-3′), as defined by White *et al*. (1990), were used to generate about 600 nucleotides from the complete ITS (including 5.8S) regions. The amplification conditions were performed in a 50-µl reaction volume as follows: 1 × PCR buffer, 0.2 mM each dNTP, 0.3 µM of each primer, 1.5 mM $MgCl_2$, 0.8 units Taq Polymerase, and 10 ng DNA. PCR parameters for all the regions were as follows: initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 52 °C for 50 s, and 72 °C for 1 min, and final extension of 72 °C for 10 min. The characterization of PCR products was performed via agarose gel electrophoresis on a TAE 1% agarose gel containing ethidium bromide as the staining agent. DNA sequencing was performed using the primers as mentioned above in an Applied Biosystems 3730 DNA Analyser at the Genome Research Centre (University of Hong Kong). The novel rDNA sequences have been deposited in GenBank under accession numbers AY736377 (18S rDNA), AY736378 (28S rDNA), and AY864770 (ITS and 5.8S rDNA). The accession numbers of the other sequences used to construct the phylogenetic trees are shown in Table 1.

Data analysis

Partial sequences from the 18S, 28S, and complete ITS regions, generated from the different primer sets, were assembled using Bioedit (Hall, 1999) and ClustalX (Thompson *et al*., 1997). Alignments were checked and then manually edited where necessary. Sequence homologies were analysed using BLAST to facilitate the selection of other fungal members to be used in the analyses. Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2002). For maximum parsimony (MP) analyses, characters were unordered and weighted equally and differentially. Trees were inferred using the heuristic search option with 10, 100, and 1000 random sequence additions. Gaps were treated as missing data and fifth character to increase the probability of finding all the most-parsimonious trees and to compare tree topologies (as outlined by Jeewon, Liew & Hyde, 2002, 2004; Jeewon *et al*., 2003a, b). Clade stability was assessed in a bootstrap analysis with 1000 replicates. For maximum likelihood (ML) analyses, a single tree generated under the MP criterion was used as a starting tree, and transition–transversion ratios, base frequencies, and shape parameter were estimated. Using these initial estimates of substitution rates and kinds, a heuristic search with tree bisection–reconnection (TBR) branch swapping was used to find the ML tree. The gamma model of site rate variation was used with no enforcement of a molecular clock. Initial branch lengths were obtained using Rogers–Swofford approximation methods. Neighbour-joining (NJ) analysis was carried out under different models of distance algorithms, including HKY85, JC, K2P, and F81, and the support

Table 1. Fungal taxa and GenBank accession numbers for rDNA sequences used in the phylogenetic analyses

Ingroups (18S)	18S rDNA	Ingroups (28S)	28S rDNA	Ingroups (ITS)	ITS, 5.8S
Aquaticheirospora lignicola	AY736377	*Aquaticheirospora lignicola*	AY736378	*Aquaticheirospora lignicola*	AY864770
Cochliobolus sativus	U42479	*Bimuria novae-zelandiae*	AY016356	*Alternaria helianthi*	AY154713
Cucurbidothis pityophila	U42480	*Cochliobolus heterostrophus*	AY544645	*Alternaria leucanthemi*	AY372684
Helicascus kanaloanus	AF053729	*Curvularia brachyspora*	AF279380	*Helminthosporium chlorophorae*	AF120259
Helminthosporium solani	AF120253	*Didymella cucurbitacearum*	AY293792	*Helminthosporium solani*	AF073919
Helminthosporium velutinum	AF120254	*Dothidea ribesia*	AY016360	*Helminthosporium solani*	AF145703
Keissleriella cladophila	AF164360	*Dothidea sambuci*	AY544681	*Helminthosporium velutinum*	AF145704
Kirschsteiniothelia elaterascus	AF053727	*Helicomyces roseus*	AY787932	*Leptosphaeria biglobosa*	AJ550892
Leptosphaeria maculans	U04238	*Karstenula rhodostoma*	AY787933	*Leptosphaeria maculans*	M96663
Leptosphaeria microscopica	U04235	*Letendraea eurotioides*	AY787935	*Massarina armatispora*	AF383955
Letendraea helminthicola	AY016345	*Letendraea helminthicola*	AY016362	*Massarina bipolaris*	AF383956
Lophiostoma caulium	AF164362	*Macroventuria anomochaeta*	AY787936	*Massarina corticola*	AF383957
Lophiostoma crenatum	U42485	*Massarina arundinacea*	DQ813509	*Massarina eburnea*	AF383959
Massarina arundinacea	DQ813513	*Massarina phragmaticola*	DQ813510	*Massarina fronisubmersa*	AF383960
Massarina australiensis	AF164364	*Phaeosphaeria avenaria*	AY544684	*Massarina ramunculicola*	AF383962
Massarina bipolaris	AF164365	*Phoma herbarum*	AF382386	*Massarina rubi*	AF383963
Massarina eburnea	AF164366	*Pleospora herbarum* var. *herbarum*	AY293791	*Neophaeosphaeria barrii*	AF466303
Massarina igniaria	DQ813511	*Pleospora ambigua*	AY787937	*Paraphaeosphaeria solitaria*	AF466301
Massarina phragmaticola	DQ813512	*Preussia terricola*	AY544686	*Phaeoseptoria musae*	AF439469
Massariosphaeria phaeospora	AF164368	*Pyrenophora tritici-repentis*	AY544672	*Phaeosphaeria avenaria*	U77357
Montagnula opulenta	AF164370	*Repetophragma ontariense*	DQ408575	*Phaeosphaeria caricicola*	AF439474
Ophiobolus herpotrichus	U43453	*Setomelanomma holmii*	AF525678	*Phaeosphaeria oryzae*	AF439495
Paraphaeosphaeria conglomerata	AF250824	*Setosphaeria monoceras*	AY016368	*Phaeosphaeria pleurospora*	AF439498
Paraphaeosphaeria glauco-punctata	AF250819	*Spirosphaera cupreorufescens*	AY616238	*Phaeosphaeria* sp.	AY345346
Paraphaeosphaeria michotii	AF250817	*Sporidesmiella fusiformis*	DQ408577	*Phoma tracheiphila*	AF272554
Paraphaeosphaeria pilleata	AF250821	*Stylodothis puccinioides*	AY004342	*Pleospora herbarum*	AY329169
Paraphaeosphaeria quadriseptata	AF250826	*Thaxteriella helicoma*	AY787939	*Saccharicola bicolor*	AF455415
Phaeosphaeria avenaria	AY544725	*Trematosphaeria heterospora*	AY016369	*Saccharicola taiwanensis*	AF439464

Table 1. *Continued*

Ingroups (18S)	18S rDNA	Ingroups (28S)	28S rDNA	Ingroups (ITS)	ITS, 5.8S
Pleospora betae	U43466	*Tubeufia amazonensis*	AY787938	*Stemphylium herbarum*	AF071344
Pleospora herbarum	DQ247812	*Westerdykella cylindrica*	AY004343	**ITS outgroups**	
Pyrenophora trichostoma	U43459	**LSU outgroup**		*Apiosporina morbosa*	AY166451
Saccharicola bicolor	U04202	*Mycosphaerella suttoniae*	AF309587	*Botryosphaeria* sp.	AY513947
Septoria nodorum	U04236			*Venturia inopina*	AY177406
Setomelanomma holmii	AY161121				
SSU outgroups					
Dothidea ribesia	AY016343				
Dothidea sambuci	AY544722				

Figure 1. Micrographs of *Aquaticheirospora lignicola* (from holotype). A, Synnemata on host tissue. B, Synnemata formed in culture. C, Synnema from host tissue. D, E, Conidia of synnema from host tissue. F, G, Synnemata from culture. H–O, Conidia from synnema that formed in culture. Rhexolytic secession in D (arrowhead); conidiogenous cells in H–J (arrowhead); pore at the end of basal cell in K (arrowhead); formation of young conidium in L and M; brown vacuole in the apices of conidium in N (arrowhead). Bars: A, 200 μm; B, 150 μm; C, 100 μm; D, 30 μm; E, 15 μm; F, 45 μm; G, 50 μm; H–K, N–O, 25 μm; L, M, 15 μm.

for individual clades within the tree was assessed by 1000 replicates of bootstrapping. Bayesian analyses were performed as outlined in Shenoy *et al*. (2006) and not detailed here.

The Kishino–Hasegawa and Templeton tests were performed in order to determine whether the trees inferred under different optimality criteria were significantly different.

RESULTS

TAXONOMY

Aquaticheirospora *Kodsueb & W. H. Ho,* ***gen. nov.***

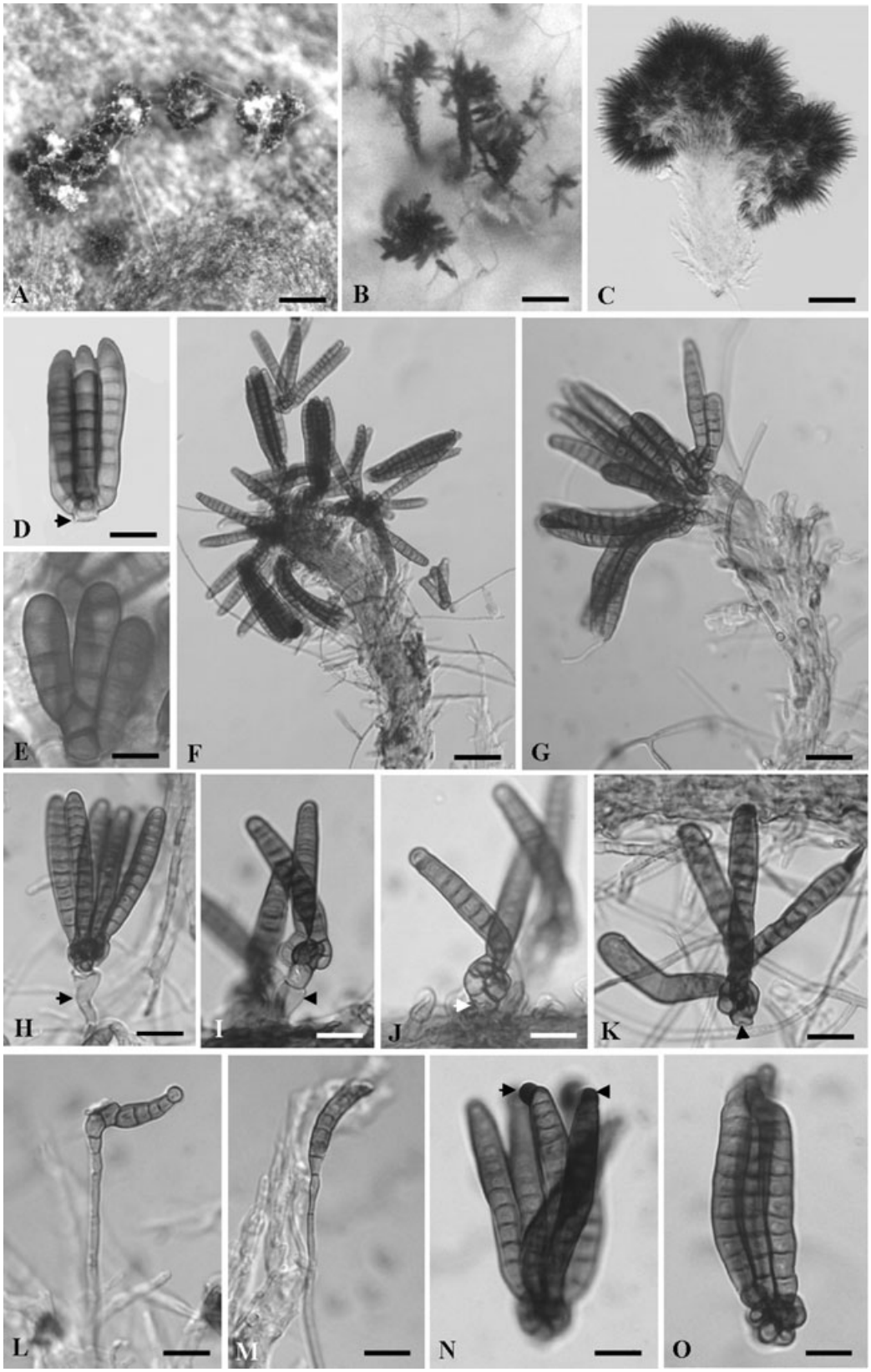
Coloniae in substrato naturali effusae, solitaria, atrae. Conidiomata synnemata, erectra, brunnea. Mycelium in substrato immersum, hyalina vel pallide brunnea. Setae nullae. Cellulae conidiogenae in conidiophoris incorporatae, monoblasticae, terminales, determinatae, hyalina vel pallide brunneae, oblongae. Conidia acrogenosae, holoblasticae, gregaria, hyalina vel pallide brunneae ubi immatura, brunneae ad maturita, chiriodea, euseptata, verticalia; cellulae basilares pallide brunneae, cuneiformes-truncatae, laeves, tenuitunicatae; rami discreti, ramosi, plerumque divergenti, cylindrici. Conidiorum secessio rhexolytica.

Etymology: In reference to the freshwater habitat and shape of the conidia of the fungus.

Mycelium immersed in the substratum, hyaline to pale brown. Conidiomata synnema, erect, brown. Conidiogenous cells monoblastic, determinate, hyaline or pale brown, oblong. Conidia acrogenous, holoblastic, hyaline to pale brown when immature and brown when mature, chiroid, euseptate, arms vertically inserted in different planes, on a basal cell; basal cells pale brown, cuneiform truncate, smooth, thin-walled; arms discrete, mostly divergent, cylindrical. Conidial secession rhexolytic.

Aquaticheirospora lignicola *Kodsueb & W. H. Ho,* ***sp. nov.*** *(Fig. 1)*

Coloniae in substrato naturali effusae, solitaria, atrae. Conidiomata synnemata, erect, 510 μm. Mycelium in substrato immersum, hyalina vel pallide brunnea. Cellulae conidiogenae in conidiophoris incorporatae, monoblastica, terminales, determinata, hyalina vel pallide brunnea, oblonga, 14.5×9.5 μm. Conidia acrogenosa, holoblastica, gregaria, hyalina vel pallide brunneae ubi immatura, brunneae ad maturita, chiriodea, 65–85(–100) × (16–)22–60(–75) μm ($\bar{\chi} = 77 \times 41$ μm, $N = 30$), euseptata, cum (3–)6–8(–10) rami praedita, verticalia; cellulae basilaris pallide brunnea, cuneiforma-truncata, 8×7 μm ($N = 10$), laevia, tenuitunicata; rami discreti, non



ramosi, divergenti, cylindrici (25–)55–75(–100) × 7–10(–15) μm ($\bar{\chi}$ = 68 × 8.25 μm, *N* = 30) (4–)10–13(–17) euseptati. Conidiorum secessio rhexolytica.

Etymology: The name *lignicola* is derived from the words *lignum*, meaning wood, and *cola*, meaning habitat, referring to the habitat in which this fungus has been found.

Colonies on PDA, black at the centre, with a yellowish-white, thick periphery, edge smooth and slightly raised, underside yellow, reaching 1 cm in diameter after 1 week. Mycelium immersed in the substratum, hyaline to pale brown. Synnemata, erect, forming after 6 weeks, white to pale brown and becoming dark brown when mature, up to 510 μm long. Conidiogenous cells monoblastic, determinate, hyaline to pale brown, oblong, 14.5 × 9.5 μm. Conidia acrogenous, holoblastic, gregarious, hyaline to pale brown when immature, brown when mature, chiroid, 65–85(–100) × (16–)22–60(–75) μm ($\bar{\chi}$ = 77 × 41 μm, *N* = 30), euseptate, with (3–)6–8(–10) arms vertically inserted in different planes, on a basal cell; basal cells pale brown, cuneiform truncate, 8 × 7 μm (*N* = 10), smooth, thin-walled; arms discrete, unbranched, mostly divergent, cylindrical (4–)10–13(–17) euseptate (25–)55–75(–100) × 7–10(–15) μm ($\bar{\chi}$ = 68 × 8.25 μm, *N* = 30). Conidial secession rhexolytic.

Teleomorph: Unknown.

Substratum: Wood submerged in streams.

Known distribution: Thailand.

Holotype: THAILAND; Chiang Mai Province, Doi Suthep-Pui National Park, on submerged wood, 21.viii.2003, *R. Kodsueb* [HKU(M) *17493*, isotype CMU026511. Living cultures ex holotype HKUCC 10304].

Note: The above descriptions are from material grown in culture on PDA. Slight differences in conidial characters were observed when the fungus was examined on the substrate. On the substrate, conidial arms were tightly packed, usually with 3(–5) often wider arms, whereas, in culture, conidia had (3–)5(–10) longer and narrower divergent arms, and the apical cell of some arms have a brown vacuole. The vacuole varied in shape from globose to irregular shape, some having rough ornamentation. The vacuole became larger when the conidia germinated.

PHYLOGENETIC ANALYSES

Partial 18S rDNA, 28S rDNA, and complete ITS regions amplified from *Aquaticheirospora lignicola* were 1044, 881, and 548 nucleotides in length, respectively. The comparative sequence analysis based on BLAST search revealed sequence similarities of 92–97% to other bitunicates fungi, especially *Leptosphaeria*, *Massarina*, *Paraphaeosphaeria*, *Phaeosphaeria*, and *Pleospora* (order Pleosporales).

The 18S rDNA data set consisted of 36 taxa and 1059 characters. Unweighted MP analysis and treating gaps as missing data yielded 86 trees [total length (TL), 273; consistency index (CI), 0.656; retention index (RI), 0.833; rescaled consistency index (RC), 0.546; homoplasy index (HI), 0.344]. Weighted MP (with a transition–transversion ratio of 1.5 : 1) generated four trees, which were similar in topology and not significantly different from each other (TL, 331.5; CI, 0.671; RI, 0.840; RC, 0.564; HI, 0.329). The most-parsimonious tree with bootstrap and Bayesian support on the branches is shown in Figure 2. Treating gaps as fifth character and ML analyses resulted in trees of identical topologies (results not shown). NJ analyses, however, yielded trees in which the major clades uniting different families were not fully resolved (results not shown).

The 28S data set of 31 taxa formed an aligned data matrix of 896 characters in length and consisted of 189 (21%) parsimony informative sites. Weighted MP (with a transition–transversion ratio of 1.75 : 1) produced only one tree (TL, 795.25; CI, 0.592; RI, 0.773; RC, 0.457; HI, 0.408; Fig. 3). Similar results were obtained with a transition–transversion ratio of 2 : 1 and unweighted parsimony. ML analyses yielded identical tree topologies with a –ln likelihood of 4446.27919, shape parameter of 0.5626, and estimated base frequencies as follows: A, 0.2394; C, 0.2201; G, 0.3116; T, 0.2289 (results not shown). Bayesian values generated from Bayesian analyses are shown on the lower nodes.

Only 270 nucleotides were used in the ITS and 5.8S data set (entire 5.8S and the last 75 nucleotides of ITS1 and first 25 nucleotides from ITS2), as other regions were too variable and difficult to align. Both unweighted and weighted MP analyses resulted in one tree each, which were similar in topology and not statistically different. Figure 4 shows the tree generated from the unweighted parsimony (TL, 602.5; CI, 0.485; RI, 0.695; RC, 0.337; HI, 0.515). The bootstrap support based on 1000 replicates is shown on the upper nodes.

DISCUSSION

TAXONOMY

Morphologically, *Aquaticheirospora* is quite distinct from other anamorphic chirosporous genera. In addition to *Aquaticheirospora*, there are 27 other genera

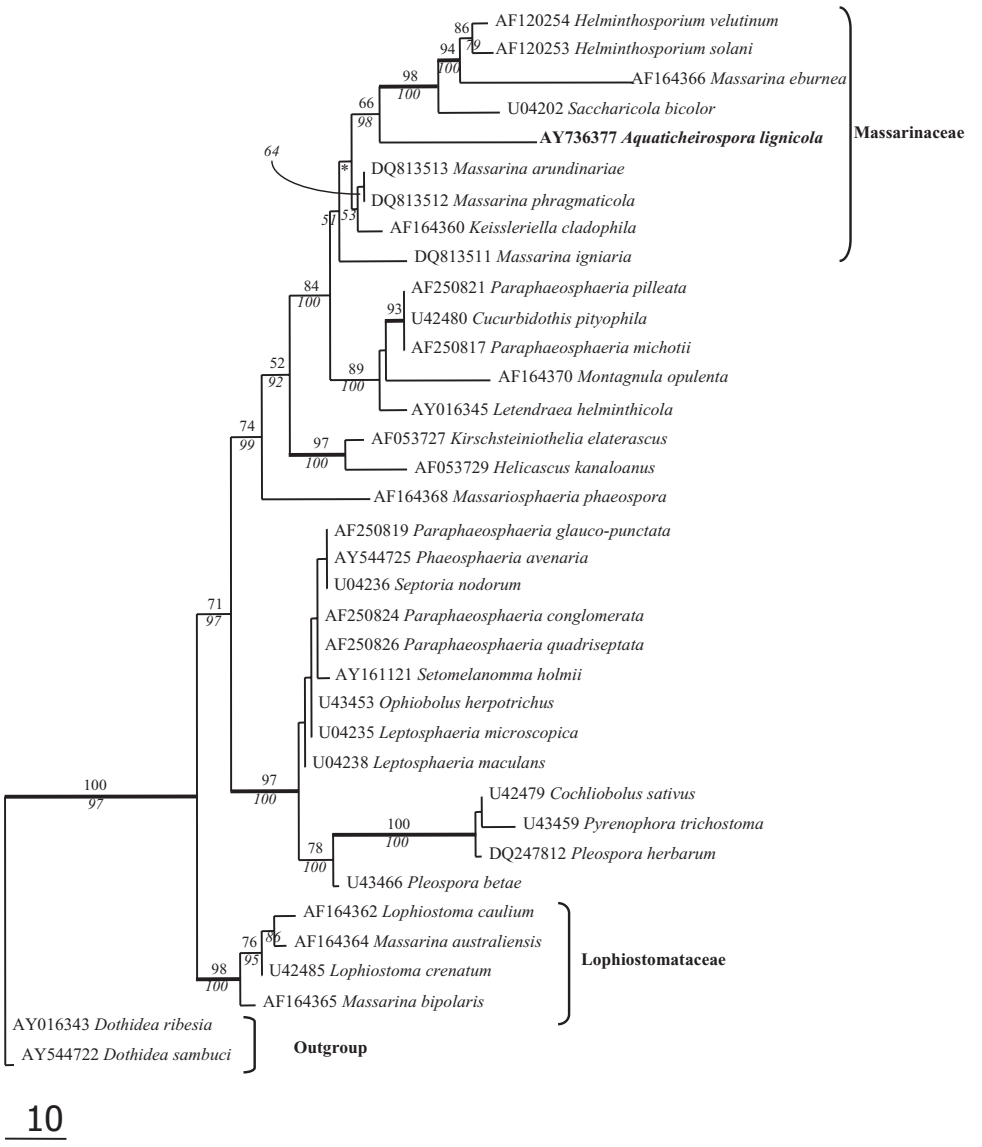


Figure 2. Maximum parsimony tree generated with the 18S rDNA data set [length, 273; consistency index (CI), 0.656; retention index (RI), 0.833; rescaled consistency index (RC), 0.546; homoplasy index (HI), 0.344]. Designated outgroups are *Dothidea ribesia* and *Dothidea sambuci*. Bootstrap support based on 1000 replicates for each clade is shown on the upper node and Bayesian support is shown on the lower node. The thickened lines represent bootstrap support above 90%.

that produce chiroid conidia (Ho, Hodgkiss & Hyde, 2000). Two of these genera (*Cheiromyces* and *Cheiromycina*) have distoseptate conidia, whereas all the others, including *Aquaticheirospora*, have euseptate conidia. *Aquaticheirospora* is unique in that the conidiomata are synnematous.

Aquaticheirospora produces chirosporous conidia similar in appearance to those of species of *Cheiromyces*, *Chelisporium*, *Dictyosporium*, *Digitodesmium*, *Prostemium*, *Psammina*, *Sirothecium*, and *Tetranacrium*. However, these genera differ in the type of conidiomata: in *Aquaticheirospora*, conidia are produced on synnemata; in *Sirothecium*, *Prostemium*, and *Tetranacrium*, conidiomata are eustromatic; in *Cheiromyces*, *Dictyosporium*, and *Digitodesmium*, conidiomata are sporodochial; and, in *Psammina*, conidiomata are acervular. The conidia of *Aquaticheirospora* are released by rhexolytic secession that leaves a basal frill on the conidia (Fig. 1D), and is similar to conidial secession in *Cheiromyces* and *Dictyosporium* (Goh, 1999; Ho *et al*., 2000). Most *Dictyosporium* conidia are complanate with tightly packed arms arranged in the same plane (Tsui *et al*., 2006), whereas, in *Aquaticheirospora*, arms are arranged in

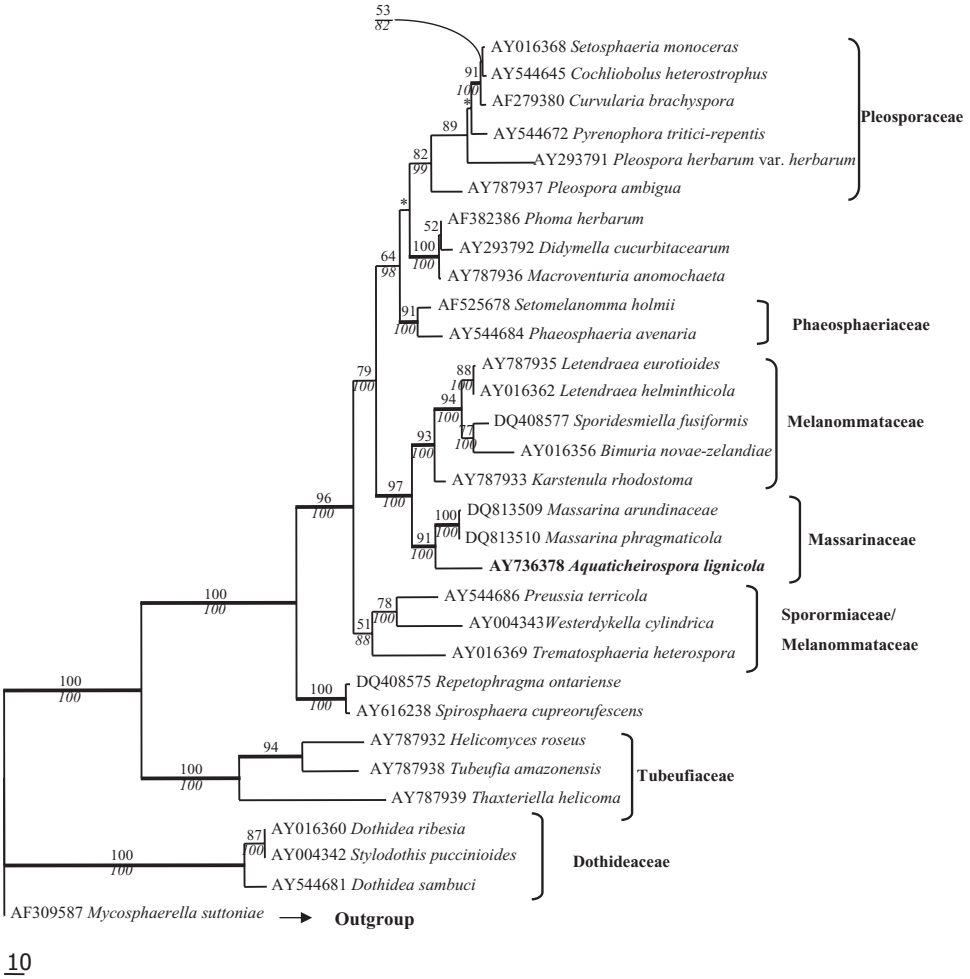


Figure 3. Maximum parsimony tree generated with the 28S rDNA data set [length, 795.25; consistency index (CI), 0.592; retention index (RI), 0.773; rescaled consistency index (RC), 0.457; homoplasy index (HI), 0.408]. Designated outgroup is *Mycosphaerella suttoniae*. Bootstrap support based on 1000 replicates for each clade is shown on the upper node and Bayesian support is shown on the lower node. The thickened lines represent bootstrap support above 90%.

different planes, as in *Cheiromyces*, *Digitodesmium*, and *Sirothecium* species. A synopsis of distinguishing characters of *Aquaticheirospora* and similar genera is given in Table 2.

Phylogeny of *Aquaticheirospora lignicola* based on rDNA sequence analyses

Molecular data provide conclusive evidence to support the association of this new hyphomycete taxon with other teleomorphic genera in the Pleosporales. There were no conflicting results between the different data sets with respect to the phylogeny of *A. lignicola*. Phylogenies based on sequences generated from 18S rDNA and ITS regions suggest that *A. lignicola* is phylogenetically related to *Leptosphaeria bicolor* D.Hawksw., W.J.Kaiser & Ndimande (= *Saccharicola bicolor*), *Massarina eburnea* (Tul. & C. Tul.) Sacc., and *Helminthosporium* species, which are all currently accommodated in the family Massarinaceae (Eriksson & Hawksworth, 2003). The family Massarinaceae (Pleosporales) was established by Munk (1956) to accommodate *Massarina*, which is characterized by aggregated, immersed to erumpent, pseudothecioid ascomata, cellular pseudoparaphyses, bitunicate cylindrical asci, and hyaline, fusiform to long ellipsoid ascospores with a mucilaginous sheath or appendages (Hyde, 1995; Aptroot, 1998). The family was synonymized to Lophiostomataceae (Barr, 1992; Kirk *et al*., 2001). Recently, Massarinaceae was resurrected following the redescription of *Leptospharia bicolor* as a new genus (*Saccharicola*) and its phylogenetic affinities with *Keissleriella cladophila* (Niessl) Corbaz, *M. eburnea*, and the anamorphs *Helminthosporium*

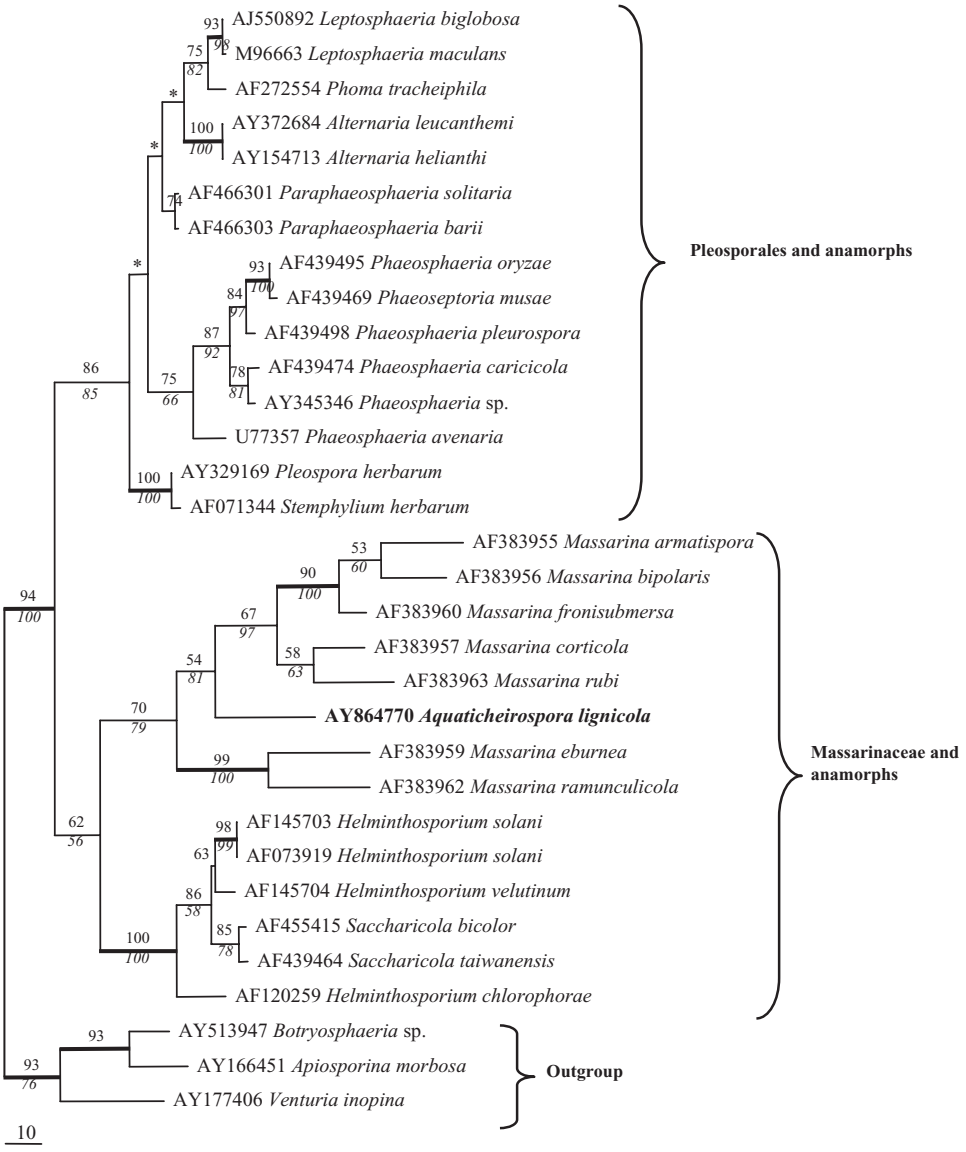


Figure 4. Tree generated from unweighted parsimony with the internal transcribed spacer (ITS) rDNA data set [length, 602.5; consistency index (CI), 0.485; retention index (RI), 0.695; rescaled consistency index (RC), 0.337; homoplasy index (HI), 0.515]. Designated outgroups are *Apiosporina morbosa*, *Botryosphaeria* sp., and *Venturia inopina*. Bootstrap support based on 1000 replicates for each clade is shown on the upper node and Bayesian support is shown on the lower node. The thickened lines represent bootstrap support above 90%.

solani Durieu & Mont. and *H. velutinum* G.K.Link based on 18S rDNA sequence analysis (Eriksson & Hawksworth, 2003). In this paper, we placed our new taxon in the family Massarinaceae as circumscribed by Eriksson & Hawksworth (2003). The phylogenetic relationships of this family in Pleosporales, based on morphology and molecular characters, have already been described by Oliver *et al*. (2000), Morales *et al*. (1995), and Eriksson & Hawksworth (2003), and are not detailed here. Basically, the molecular results shown here are congruent with previously published phylogenies. *Aquaticheirospora lignicola* clusters with *S. bicolor* and with other members of the Massarinaceae with a bootstrap confidence of 66% (18S rDNA-based phylogeny; Fig. 2) and 54% (ITS-based phylogeny; Fig. 4). 28S rDNA phylogeny also supports the inclusion of *A. lignicola* within the Pleosporales, and its affinity with members of the family Massarinaceae is resolved with high bootstrap support (91%; Fig. 3). The three gene regions could not be analysed

Table 2. Characteristic comparison between *Aquaticheirospora*, *Cheiromyces**, *Dictyosporium*†, *Digitodesmium*‡, *Prostemium*§, *Psammina*§, *Sirothecium*¶, and *Tetranacrium*§

	Aquaticheirospora	*Cheiromyces*	*Dictyosporium*	*Digitodesmium*	*Sirothecium* / *Chelisporium*	*Prostemium*	*Psammina*	*Tetranacrium*
Conidiomata	Synnemata, erect	Sporodochial, superficial	Sporodochial, superficial	Sporodochial, superficial	Eustromatic, unilocular, immersed to semi-immersed	Acervular to eustromatic	Acervular, separate	Eustromatic, subcuticular, becoming superficial, separate or gregarious
Conidiophores	Unbranched, septate, filiform, confined to the basal cell	Absent	Micronematous, mononematous or absent	Semi-macronematous, mononematous	Hyaline, repeatedly and irregularly branched, septate, vertically orientated, formed from the inner cells of the conidiomatal wall	Hyaline, unbranched, septate, filiform, confined to the basal cell	Irregularly branched, septate, ramifying over the acervular tissue	Absent
Conidiogenous cells	Monoblastic, determinate	Monoblastic, determinate	Monoblastic, determinate	Monoblastic, determinate	Monoblastic, integrated, apical, lateral or comprising the conidiophore	Holoblastic, integrated, determinate	Holoblastic, determinate, integrated	Holoblastic, discrete, determinate
Conidia	Solitary, holoblastic, euseptate, chiroid, arms inserted on the basal cells in different planes	Solitary, holoblastic, distoseptate, chiroid, arms inserted on basal cells in different planes	Solitary, holoblastic, euseptate, chiroid, branched from the base, in most species flattened in one plane	Solitary, holoblastic, euseptate, chiroid, with arms inserted on basal cells in different plane	Solitary, holoblastic, euseptate, chiroid, closely branched, with 2–4 vertical divergent arms inserted in different planes	Smooth, consisting of 10–14, radiating, 2–3 transversely euseptate arms connected to a central cell	Smooth, up to 6 septate arms radiating from a central complex of short branched cells	Tetraradiate, consisting of 1 vertical arm and 3 ± equidistant horizontal arms
Conidial basal cells	Truncate	Truncate	Truncate	Truncate	Truncate	None	None	Truncate
Conidial arms	Discrete, unbranched, mostly divergent, cylindrical, euseptate	Discrete, distoseptate, each arm with an inflated apical cell	Tightly packed arms	Discrete, euseptate, mostly with a narrowed, recurved apical cell	Euseptate, slightly constricted at the septa, occasionally branched, apical cell obtuse	Data not available	Data not available	Arising from a central globose cell, euseptate, tapered towards the apex
Conidial secession	Rhexolytic	Rhexolytic	Rhexolytic	Schizolytic	Data not available	Data not available	Data not available	Data not available

* Sutton (1985); Ho *et al*. (2000).
† Goh (1999).
‡ Kirk (1981); Ho, Hyde & Hodgkiss (1999).
§ Sutton (1980).
¶ Morgan-Jones, Nag Raj & Kendrick (1972); Sutton (1980, 1985).

in combination because of inadequate sequences available in GenBank for this group of fungi.

Phylogenies based on DNA sequence analyses have been shown to be important in the classification of many anamorphs and their integration with teleomorphs (Rehner & Samuels, 1995; Taylor, 1995; Jacobs & Rehner, 1998; Jeewon *et al*., 2002; Shenoy *et al*., 2006). In this study, the hypothesis that *A. lignicola* is the anamorph of a member of the Massarinaceae is assessed. Pairwise sequence alignment of the partial 18S rDNA gene (1059 nucleotides) reveals that *A. lignicola* shares 98% and 96% similarities to *Keissleriella cladophila* and *S. bicolor*, respectively. Massarinaceae is known to possess both coelomycetous and hyphomycetous anamorphs (Sivanesan, 1984; Shoemaker & Babcock, 1990). For instance, *M. tetraploa* Scheuer and *M. lacustris* (Fuckel) Leuchtm. have been linked to *Tetraploa aristata* Berk. & Broome (hyphomycete) and *Stagonospora* sp. (coelomycete), respectively. The anamorph of *M. eburnea* is still obscure, although it has been referred to *Ceratophoma*-like anamorphs, whereas *Keissleriella* is recognized to produce *Dendrophoma*-like anamorphs (Bose, 1961). *Saccharicola bicolor* has been linked to *Stagonospora*-like anamorphs, but a *Phoma*-like anamorph has also been reported (O'Neill & Farr, 1996; Venkatasubbaiah *et al*., 1998; Eriksson & Hawksworth, 2003). *Helminthosporium* has been accepted as an anamorphic genus within the Pleosporales (Oliver *et al*., 2000). In this study, *H. solani* and *H. velutinum*, both hyphomycetous taxa, belong to the Massarinaceae, as does *A. lignicola*. However, *Helminthosporium* spores superficially resemble *Bipolaris*, *Drechslera*, and *Exserohilum* spores and are distinct from those of *Aquaticheirospora*. Recently, a new coelomycetous genus, *Paraconiothyrium* (anamorph of *Paraphaeosphaeria*), characterized by pycnidial, unilocular conidiomata with circular and central ostiole, brown conidia, and conidiophores which are absent, was described and accommodated in the Pleosporales (Verkley *et al*., 2004). Another recent phylogenetic study also indicated that other hyphomycetous fungi (including *Cheiromoniliophora*, *Dictyosporium*, *Digitodesmium*, *Kamatia*, and *Pseudodictyosporium*), sharing similar morphologies to *Aquaticheirospora*, also have affinities to the family Massarinaceae (Tsui *et al*., 2006).

The phylogenies generated in this study also shed meaningful taxonomic insights into the systematics of *Massarina*. It is becoming increasingly evident that there are *Massarina* species that should be transferred to other families, such as Lophiostomataceae (Liew, Aptroot & Hyde, 2002; Vijaykrishna, 2005). 18S rDNA sequence analyses have shown that *M. australiensis* K.D.Hyde and *M. bipolaris* K.D.Hyde should be transferred to the Lophiostomataceae, and that the family Massarinaceae should be circumscribed to include *Massarina*, *Keissleriella*, *Saccharicola*, *Helminthosporium*, and *Aquaticheirospora* for the time being. These two species are related to *Lophiostoma* species and, morphologically, they can only be distinguished on the basis of a slight difference in the ostiole. However, whether this character is phylogenetically significant in delineating the Lophiostomataceae and Massarinaceae is still obscure at present.

It appears that anamorphic characters have undergone convergent evolution and are phylogenetically insignificant in clarifying familial boundaries within the Pleosporales. Other anamorphic fungi, such as *Alternaria*, *Cladosporium*, *Curvularia*, *Fusicoccum*, *Coniothyrium*, *Pleurophomopsis*, *Stemphylium*, and *Titaea*, also have phylogenetic affinities to the Pleosporales and other bitunicate fungi (Guo *et al*., 2004; Lee, Kim & Jung, 2005; Schubert & Braun, 2005; Tanaka, Harada & Barr, 2005; Kirschner & Piepenbring, 2006; Phillips *et al*., 2006).

Molecular phylogenetics have helped to establish a number of anamorphic fungi to their teleomorphic counterparts, especially those which never produced any teleomorph in culture. For instance, Pinnoi *et al*. (2007) found that *Berkleasmium*, a hyphomycete characterized by sporodocia conidioma and solitary brown and muriform conidia with rounded ends, is related to other loculoascomycetes. The genus *Sporidesmium* and allies have been shown to be polyphyletic, and *Sporidesmiella fusiformis* W.P.Wu, *Sporidesmium tengii* W.P.Wu, *Sporidesmium obclavaturum* (Bubák & Syd.) Subram., *Sporidesmium australiense* M.B.Ellis, *Sporidesmium pachyanthicola* R.F.Castañeda & W.B.Kendr., *Neosporidesmium* sp., *Repetophragma ontariense* (Matsush.) W.P.Wu, and *Repetophragma goidanichii* (Rambelli) W.P.Wu have been shown to belong to the Dothideomycetes (Shenoy *et al*., 2006).

Aquaticheirospora lignicola is clearly morphologically dissimilar to all previously described anamorphs within the Pleosporales, and both morphological and molecular characterization provides sufficient evidence to support this anamorphic fungus as a new genus. Attempts were made to induce the production of the sexual stage in culture, but no teleomorphs were observed after 45 days.

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