Fusoidispora aquatica: A new freshwater ascomycete from Hong Kong based on morphology and phylogeny inferred from rDNA gene sequences

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Vijaykrishna, D., R. Jeewon & Hyde K.D. (2005). *Fusoidispora aquatica:* A new ascomycete from Hong Kong based on morphology and phylogeny inferred from rDNA sequences. – Sydowia 57 (2): 267–280.

During a survey of freshwater ascomycetes in Hong Kong, an interesting ascomycete with fusoid ascospores that had mucilaginous pads at both apices was identified. The affinities of this taxon extend between a wide range of unitunicate ascomycetes (viz. Annulatascaceae, Magaporthaceae, Pleurotremaceae, Trichosphaeriaceae). In order to evaluate its familial placement and generic relatedness with other known ascomycetes, partial DNA sequences derived from the large subunit ribosomal DNA (28S rDNA) were analyzed. Results from the phylogenetic analyses indicate that the family Annulatascaceae is polyphyletic. The unidentified taxon has close affinities with the Annulatascaceae and Ophiostomataceae but does not belong in any existing genera. Thus a new genus and species, *Fusoidispora aquatica,* is established to accommodate it.

Keywords: Annulatascaceae, ascomycete, freshwater fungi, Ophiostomatales, phylogeny, 28S rDNA.

Woody substrates play an important ecological role in freshwater habitats. Submerged wood pieces are the sites of sexual reproduction for freshwater fungi. Such substrates support a diverse assemblage of teleomorphic reproductive bodies (Cai *et al.,* 2003; Jeewon *et al.,* 2003 a; Luo *et al.,* 2004; Tsui & Hyde 2004) and a number of new taxa have been discovered (Fallah & Shearer, 2001; Hyde & Goh, 1999). During a study of freshwater ascomycetes in tropical streams, an undescribed fungus was identified from submerged wood in Hong Kong. This taxon is characterised by having ascomata, which are partially immersed in the host substrate and ampulliform in shape. Asci are long-cylindrical and unitunicate in nature with a distinct, refractive, non-amyloid apical apparatus. Besides having long-fusoid, hyaline and 0–3 septate ascospores, they possess cap-like mucilaginous pads at both ends (Fig. 1).

Several freshwater fungi with similar morphological characteristics (large apical apparatus and ascospores with mucilaginous sheaths/pads) have been associated with the family Annulatascaceae

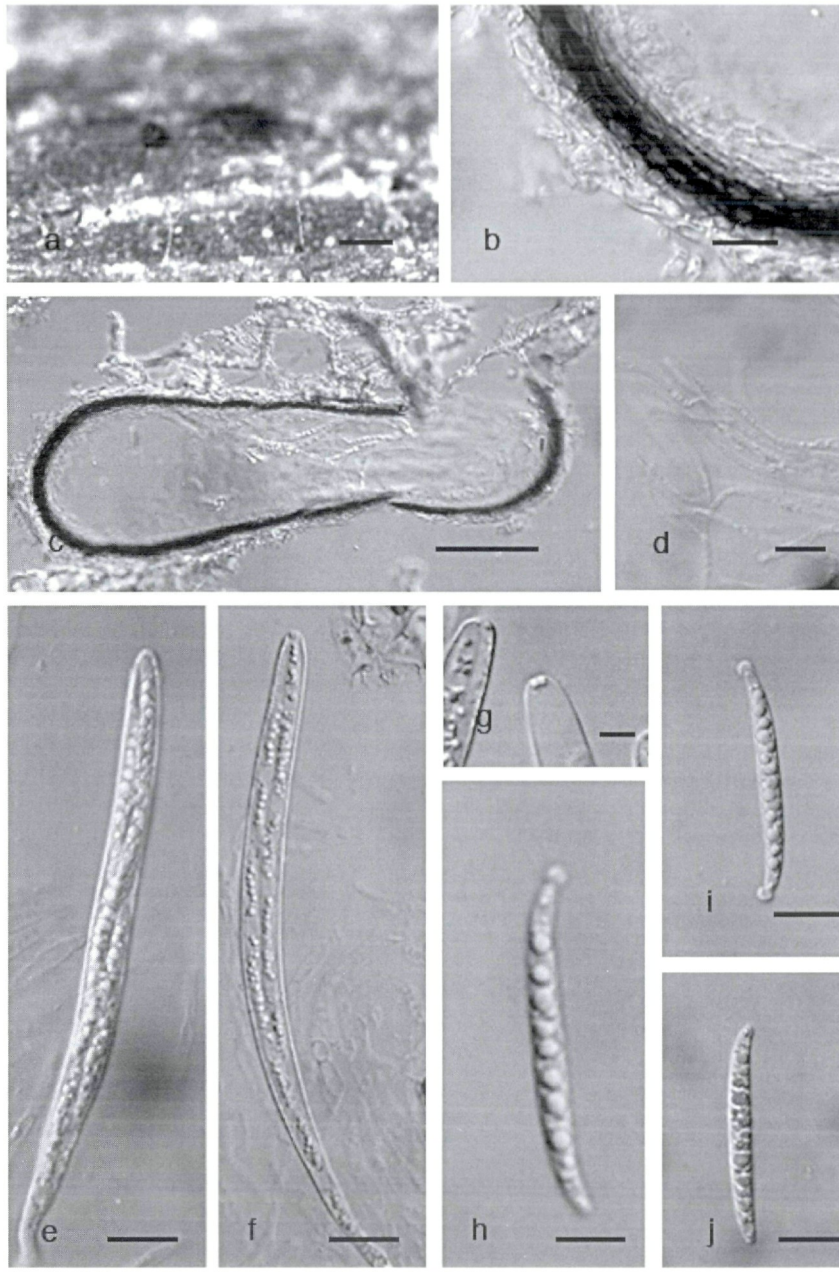

Fig. 1. a. Semi immersed ascomata as viewed on the substrate. b. Longitudinal median section through peridium. c. Longitudinal median section through ascoma. d. Paraphyses. e, f. Asci showing arrangement of ascospores. g. Apex of the asci showing apical ring. h, i, j. Ascospores with mucilaginous pads. Bars a = 100 µm, b = 20 µm, c = 30 µm, d–j = 10 µm.

(Ho & Hyde, 2000). There is ongoing debate regarding the classification of the Annulatascaceae and related genera (Campbell & Shearer, 2003; Raja *et al.*, 2003; Réblova & Winka, 2001; Réblova & Seifert, 2004).

Morphologically our new taxon has affinities towards members of the Annulatascaceae. However, due to the problems existing in the classification of the Annulatascaceae and the presence of overlapping characters with other groups of the Magnaporthaceae and Pleurotremaceae, it is hard to conclusively assign the new taxon to any existing taxonomic group. Therefore the objectives of this study were (i) to use morphology and phylogenetic analyses of molecular data to elucidate the taxonomy of this taxon, (ii) to assess the phylogenetic relationships of this taxon with morphologically similar genera and (iii) to test the monophyly of the family Annulatascaceae.

Materials and Methods

Morphology

Fully submerged woody substrata approximately 30 cm in length were collected from a small stream in the Tai Po Kau Forest in Hong Kong and returned to the laboratory in plastics bags. Substrates were incubated in plastic boxes on moist tissue paper and stored at room temperature (approx 22 °C) for about 30 d. The samples were examined from day one and at 7 days intervals thereafter. For microscopy, material was mounted in water and lactic acid. Vertical sections (8 µm) of fruiting bodies were cut with a Zeiss HM505E freezing microtome. Measurements were made from fresh material mounted in water. The widths of the asci and ascospores were measured at the widest point. Means ± standard errors are provided for ascus and ascospore sizes. Measurements for the asci and ascospores are provided as (min-)(mean-SD)-(mean+SD)(-max) × (min-)(mean-SD)-(mean+SD)(-max) µm and n is the number of measurements. Images were captured under differential interference microscopy (DIC) using an Olympus DX11 digital camera mounted on an Olympus BX50 microscope. Photographic plates were prepared in Adobe Photoshop V.8.0. Germination of ascospores were initiated on 1 % water agar (Choi *et al.*, 1999), however germinated ascospores failed to grow further on 2 % Potato Dextrose Agar. Fruit bodies on wood samples were dried and deposited at the Hong Kong University Mycology herbarium (HKU(M)).

DNA extraction, amplification and sequencing

DNA from the unidentified taxon was extracted directly from the ascomata growing on the substrate using a modified protocol of Walsh *et al.* (1991) and Hirata & Takamatsu (1996). Contents of the

ascomata were scooped out and added to eppendorf tubes containing 300 µL of 5 % Chelex solution and vortexed vigorously for 1 min. Samples were incubated at 100 °C for 10 min and then centrifuged at 13,000 rpm for 2 min. The supernatant was collected and heated at 100 °C for 10 min for the extraction of DNA and then stored at –20 °C. Part of the 28S rDNA was amplified by PCR with primers LROR (Bunyard *et al.*, 1994) and LR5 (Vilgalys & Hester, 1990) following the protocol of Jeewon *et al.* (2004). Amplified products were purified using a commercially available purification kit, according to the manufacturer's protocol (Amersham Biosciences, Catalog no. 27–9602–01). Sequencing reactions were carried out using the same primers as mentioned above using an Applied Biosystems 3730 DNA Analyzer at the Genome Research Centre, University of Hong Kong. The sequence of the unidentified taxon was visually checked and edited using the software program 4 Peaks version 1.5 (A. Griekspoor and Tom Groothuis, mekentosj.com).

Phylogenetic analyses

The sequence of the unidentified taxon was aligned with published sequences, downloaded from GenBank, using Clustal X 1.83 (Chenna *et al.*, 2003) with the default multiple alignment settings, followed by manual adjustment for optimisation using Se-Al v2.0a11 (Rambaut, 1996). The final alignment resulted in 14 ambiguously aligned regions of 199 characters.

Parsimony analyses were conducted under 3 different criteria. Equally weighted maximum-parsimony (MP1) and unequally weighted maximum-parsimony (MP2, MP3) were carried out using PAUP* 4b10 (Swofford, 2002). For the equally weighted analysis (MP1), the unambiguously aligned regions were unordered and weighted equally. For the MP2 analysis, the transition-transversion ratio for the unambiguously aligned regions was calculated using the "State changes and stasis" option under MacClade 4.0 (Madison & Madison, 2000). In the MP3 analysis, changes among the transitions, transversions and gaps were subjected to a symmetric step matrix generated with the program STMatrix version 2.1 (François Lutzoni & Stefan Zoller, Dept. of Biology, Duke University) as described in Miadlikowska *et al.* (2002). Out of the 14 ambiguously aligned regions, 10 regions were recoded using the software INAASE (Lutzoni *et al.*, 2000) and included in MP3 and the remaining 4 regions were excluded as they had more than 32 character states when they were recoded. Gaps were treated as fifth character for all step matrices. Trees were inferred using heuristic searches employing random starting trees with random stepwise addition of 1000 replicates. Tree-bisection-reconnection (TBR) branch swapping algorithm was used,

MULTREES option was in effect, and zero length branches were collapsed. Neighbour joining analyses were performed using a similar setting to MP3, with the exclusion of 14 ambiguously aligned regions. *Saccharomyces cerevesiae* (GenBank J01355) was used as outgroup taxon in all analyses.

To determine the best-fit model of evolution for the maximum-likelihood (ML) analyses, MODELTEST 3.06 (Posada & Crandall, 1998) was used. The ML tree was built in PAUP* 4b10 using a heuristic search, addition sequence set to "as is", with TBR branch swapping algorithm. The gamma model of site-rate variation was used with no enforcement of a molecular clock (Jeewon *et al.*, 2002; 2003b).

Branch support for all maximum-parsimony and neighbour joining analyses were assessed in bootstrap analyses with 1000 bootstrap replicates. Bayesian posterior probability (BPT) for the ML tree was calculated using MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). The same model of evolution used for ML analyses was implemented. One million generations were run for four Markov chains and sampled every 100th generation resulting in 10 000 trees. The first 2000 trees, which represented the burn-in phase of the analysis, were discarded, and the remaining 8000 trees were used to calculate the posterior probabilities in a consensus tree.

The tree topologies, derived under all the different criteria were compared using the Kishino Hasegawa test (Kishino & Hasegawa, 1989) as implemented in PAUP*. Trees were viewed using the software TREEVIEW version 1.6.6 (Page, 1996).

Results

Phylogenetic results

The final dataset for the 28S rDNA sequences contained 47 taxa of 883 characters (TREEBASE Accession no. S1323). Fourteen ambiguously aligned regions of 199 bp were excluded from MP1, MP2, ML and Bayesian analysis. Ten ambiguously aligned regions were included in MP3. Of the remaining 684 unambiguously aligned characters, 409 were constant, 103 parsimony uninformative and 172 parsimony informative in MP1. For the same dataset with a transition transversions ratio of 1.5:1 (MP2) the number of parsimony informative characters was 178. However, in MP3 the number of informative characters were increased to 192 for 694 characters (684 characters +10 recoded regions).

The equally weighted parsimony analysis (MP1) performed using 172 parsimony informative characters produced 4 equally most parsimonious trees (MPT) in 3 different tree islands. One of the trees

forming a single tree island is shown in Fig. 2. In Fig. 2 *Fusoidispora aquatica* is paraphyletic to clade A (*Ascitendus, Annulatascus, Pseudoproboscispora* and *Submersisphaeria*), clade B (*Aquaticola, Cryptadelphia, Rhamphoria)* (Fig. 2) and Ophiostomatales clade. In tree island 2 and 3, *Fusoidispora* formed a monophyletic group with *Cryptadelphia* spp. within the Annulatascaceae/Ophiostomatales clade (Fig. 2).

MP2 using 178 characters (with an estimated transition transversions ratio of 1.5 : 1) resulted in 8 equally MPT's, in one tree island. The 8 equally parsimonious trees were 1 step longer than those produced by MP1 and were not rejected by KH Test. ($P^* \geq 0.05$). MP3 performed with the inclusion of 10 recoded regions resulted in a single most parsimonious tree 4 steps longer than MP1. The phylogenetic position of *Fusoidispora* in MP2 and MP3, remained the same as in Fig. 2. Clade A consisting of *Annulatascus, Ascitendus, Submersisphaeria* and *Pseudoproboscispora* was maintained in all parsimony trees.

TrNeF model (Tamura & Nei, 1993) including estimation of invariable sites and assuming a discrete gamma distribution with six invariable sites was the best-fit maximum likelihood model estimated through MODELTEST. This model of evolution was used in the ML analysis, with other parameters set as default. The ML tree was identical to trees generated by both the equally weighted and unequally weighted analyses in terms of topology. In the ML analyses, *Fusoidispora* formed a monophyletic group with *Annulatascus biatriisporus* both within the Annulatascaceae/Ophiostomatales clade. The Bayesian analysis yielded a similar topology. The support, however, as estimated through Bayesian posterior probability, was higher than those obtained with bootstrapping (shown in Fig. 2). None of the analyses could give any branch support for the position of *F. aquatica*. In the MP2 and MP3 analyses *F. aquatica* is nested between Clade B of the Annulatascaceae and the Ophiostomatales clade (Fig. 2).

Taxonomy

Fusoidispora D. Vijaykrishna, R. Jeewon & K.D. Hyde, **gen. nov.**

Ascomata perithecialia, immersa vel erumpentia, ampulliformia, atrobrunnea, coriacea, ostiolata, papillata, solitaria vel gregaria. Collum brevissimum, ascomata hospes ad libram incumbunt, collum sursum curvum, vulgo brunneum. Peridium multistratum, cellulis externis brunnis et intimis hyalinis textura angularis. Paraphyses hyphoideae, septatae, distales angustatae. Asci octospori, cylindrici, unitunicati, cum apparatu apicali, persisti nonamyloideo, refractivo, discoideo. Ascosporae imbricatae 1–2 seriatae, hyalinae, fusoideae vel falcatae, guttulatae, glabro-tunicatae, cum pulvino globoso mucilagino ad apices.

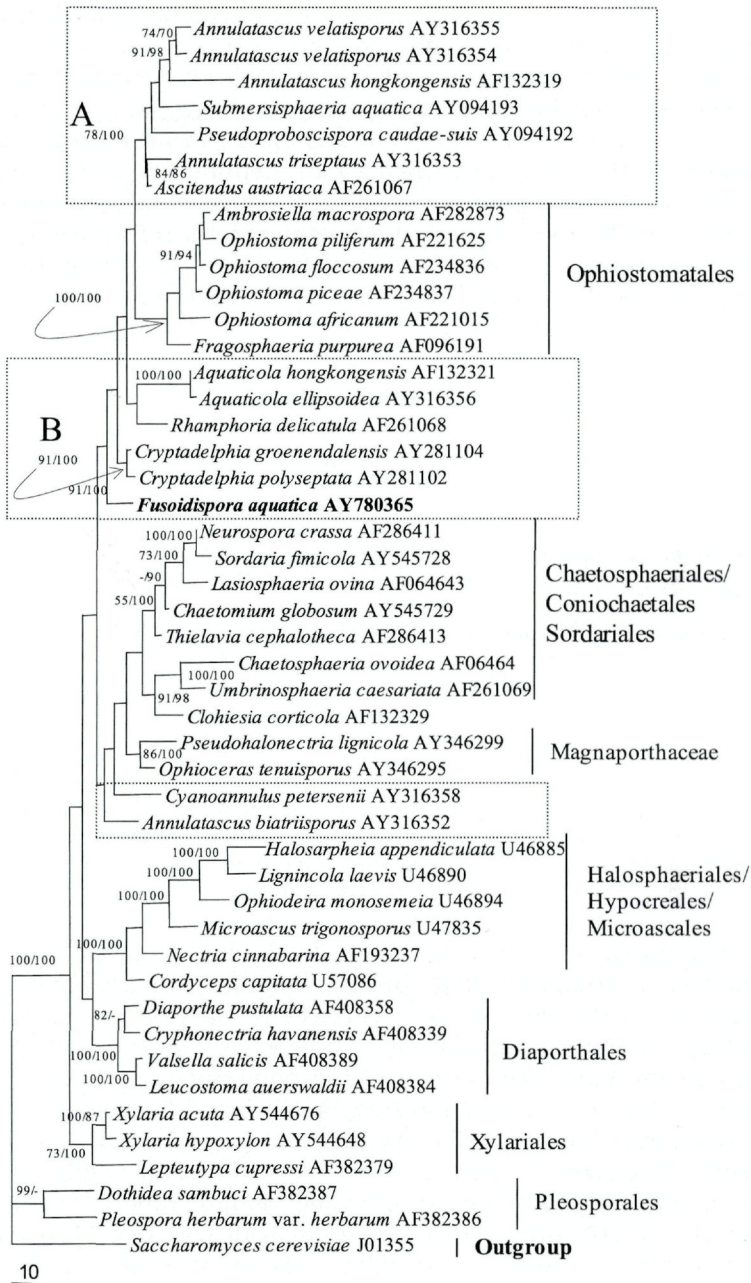

Fig. 2. Most parsimonious tree generated from unequally weighted parsimony analyses (MP1) of partial 28S rDNA sequences. Parsimony bootstrap and Bayesian posterior probability values > 50 % are given at the internodes. Members of Annulatascaceae are shown within dotted boxes.

Ascomata perithecioid, immersed to erumpent, dark brown, ampulliform, lying horizontal to the host surface, coriaceous, ostiolate, papillate, solitary or gregarious. – Neck very short, neck at one end and curving upwards, usually dark brown. – Peridium multilayered with outer brown cells and inner hyaline cells of *textura angularis*. – Paraphyses hypha-like, septate, tapering slightly distally. – Asci 8-spored, cylindrical, pedicellate, unitunicate, apically rounded with a persistent, nonamyloid, refractive, discoid apical apparatus. – Ascospores overlapping 1–2 seriate, hyaline, fusoid to sickle-shaped, smooth-walled, septate, with globose mucilaginous pads at the apices.

Type species. – *Fusoidispora aquatica* D. Vijaykrishna, R. Jeewon & K. D. Hyde

Etymology. – Referring to the fusoid shape of the ascospore.

Fusoidispora aquatica D. Vijaykrishna, R. Jeewon & K.D. Hyde, **sp. nov.** – Figs. 1a–h.

Ascomata 115–215 µm longa, 180–300 µm diam. perithecialia, immersa vel erumpentia, ampulliformia, atrobrunnea, coriacea, ostiolata, papillata, solitaria vel gregaria, hospes ad libram incumbunt,. Collum brevissimum, ad 75 µm longum × 45 µm diam, sursum curvum, vulgo brunneum. Peridium multistratum, cellulis externis brunnis et intimis hyalinis textura angularis. Paraphyses ad 4.5 µm latae, hyphoideae, septatae, distales angustatae. Asci (152.0-)(163.8)-(173.65)(-178.0) × (8.5-)(9.2)-(10.4)(-11.0) µm n = 30, octospori, cylindrici, pedicellati 15–30 × 4–6 µm, unitunicati, cum apparatu apicali (ca 2 µm) persisti, nonamyloideo, refractivo, discoideo. Ascosporae (42.0-)(46.0)-(47.6)(-50.0) × (4.0-)(5.2)-(5.7)(-6.0) µm n = 30, imbricatae 1–2 seriatae, hyalinae, fusoideae vel falcatae, guttulatae, glabro-tunicatae, 0-(5) septatae, septa nonconstricta, cum pulvino globoso mucilagino ad apices.

Ascomata 115–215 µm long, 80–150 µm diam. perithecioid, immersed to erumpent, dark brown, ampulliform, ascomata lying horizontal to the host surface, coriaceous, dark brown, ostiolate, papillate, solitary or gregarious. – Neck very short, up to 75 µm long × 45 µm diam, neck at one end curving upwards, usually dark brown. – Peridium multilayered, outer layer formed of 3 rows of compressed brown cells and inner layer of hyaline cells of *textura angularis*. – Paraphyses up to 4.5 µm wide at the base, hypha-like, numerous, thin-walled, septate, tapering distally. – Asci (152.0-)(163.8)-(173.65)(-178.0) × (8.5-)(9.2)-(10.4)(-11.0) µm n = 30, 8-spored, cylindrical, pedicellate, 15–30 × 4–6 µm, unitunicate, apically rounded with a persistent, nonamyloid, refractive, discoid apical apparatus (ca 2 µm diam). – Ascospores (42.0-)(46.0)-(47.6)(-50.0) × (4.0-)(5.2)-(5.7)(-6.0) µm n = 30, overlapping 1–2 seriate, hyaline, fusoid to sickle shaped, guttulate, 0-(5)-septate, not con-

stricted at the septa, smooth-walled, with globose mucilaginous pads at the apices (Figs. 1, a–h).

Holotype. – CHINA, Hong Kong SAR, New Territories, Tai Po Kau Country Park, Tai Po Kau Forest Stream, on submerged wood, 26. 11. 2003, D. Vijaykrishna. (HKU(M) 17484), isotype in PDD (78746).

Anamorph. – Unknown.
Etymology. – *aquatica* – referring to the freshwater habitat.
Habitat. – Saprobic on submerged wood.
Distribution. – Hong Kong.

Discussion

The presence of mucilaginous pads at the apices of fusoid ascospores and long cylindrical asci with refractive apical rings are the most striking features of *Fusoidispora aquatica*. The mucilaginous pads seem to be lost sometime after release of the ascospores. This was observed repeatedly in squash preparations of the ascomata in water. The biological significance of these pads is unknown, but they may serve as an initial attachment of the ascospores to the substrate before germination (Hyde & Goh, 2003). Mucilaginous sheaths and pads are found in several freshwater ascomycetes (e.g. *Annulatascus velatisporus, Diluviocola capensis, Fluvatispora reticulata*) (Hyde & Goh, 2003).

The characters of *Fusoidispora aquatica* which include the ampulliform-shaped ascomata lying parallel to the host surface, and the presence of a short neck, which curves upwards, multiseptate ascospores is typical of Pleurotremaceae based on the type *Pleurotrema polysemum* (Barr, 1994; Eriksson, 1981). *P. polysemum* differs from *Fusoidispora* by lack of apical apparatus. *Saccardoella* (Ascomycetes *incertae sedis*), which is characteristic in having long cylindrical asci and multiseptate ascospores, has once been placed within the Pleurotremaceae. Recently, three new species with unitunicate asci and septate ascospores were described and assigned to *Saccardoella* by Fallah & Shearer (2001). However, *Saccardoella* is not considered an unitunicate genus and further examination of these three species has been suggested (Cai *et al.*, 2002; Fryar & Hyde, 2004). *Fusoidispora aquatica* could be confused with two of those recently described species. *S. horizontalis* differs from *Fusoidispora aquatica,* in having smaller ascospores with a distinct septa and lacking mucilaginous pads. *S. lacustris* is more similar to *Fusoidispora aquatica* in having septate ascospores with gelatinous sheaths at their apices, however, differs in the shape of the spores apex, hyaline to dark brown beaks, and larger fruitbodies.

The morphology of the asci and apical apparatus in *Fusoidipora aquatica* could be compared with members of the family Magnaporthaceae. The apical apparatus (Fig. 1g) is similar to species present within the family Magnaporthaceae, e.g. (*Ophioceras, Pseudohalonectria*). Magnaporthaceae contains taxa, which are parasitic or pathogenic on plants, and also sabrobic occurring on submerged wood (Shearer *et al.*, 1999). The freshwater taxa of this family are characterised by having a large globose to subglobose, deeply immersed ascoma, with a thick, long, brittle neck, and with long filiform ascospores. These characters, however, which are restricted to members of Magnaporthaceae, are absent in *Fusoidispora aquatica*. Therefore on morphological grounds our new taxon cannot be accommodated in this family. Phylogenies obtained also showed that the included members of the Magnaporthaceae are more closely related to other members of the Sordariales rather than *Fusoidispora aquatica*.

Fusoidispora aquatica could also be compared with recently described members of the Trichosphaeriales. This order, as circumscribed by Barr (1990), contains mainly terrestrial saprobic species, with dark perithecia. *Cryptadelphia* is a newly erected genus for *Brachysporium* anamorphs and is tentatively placed within the Trichosphaeriales (*Réblova* & Seifert, 2004). The morphological similarities between *Cryptadelphia* and *Fusoidispora* are cylindrical asci, with refractive apical apparatus, and hypha-like paraphyses. Morphologically, *Cryptadelphia* is more closely related to *Aquaticola*, in asci, ascospore and sterile tissue characteristics (*Réblova* & Seifert, 2004). Furthermore, *Fusoidispora aquatica* had the highest sequence similarity to *Cryptadelphia, Rhamphoria* and *Aquaticola*. Morphological characters and similarities in the 28S rDNA sequences suggest a close relationship between *Cryptadelphia*, *Fusoidispora*, *Rhamphoria*.

The presence of *Fusoidispora aquatica* in an aquatic habitat coupled with the long cylindrical asci and ascospore mucilage, shows affinities towards the family Annulatascaceae (Ho & Hyde, 2000). Annulatascaceae was introduced by Wong, Hyde & Jones (1998) to accommodate *Annulatascus* and related genera from freshwater habitats. Species in this family have asci with massive apical rings (Ho & Hyde, 2000; Lee *et al.*, 2004) that probably facilitate strong ejection of ascospores in air and under water, and may be adapted for dispersal in freshwater, or in air when washed to the banks of rivers (Hyde & Goh, 2003). The ascospores in most species are equipped with sheaths or unique appendages that are characteristic of *F. aquatica* (Hyde *et al.*, 1998; 1999; Hyde & Goh, 2003). The ampulliform-shaped ascomata lying parallel to the host surface and leathery peridium can also be found in other members of the Annulatascaceae.

Annulatascaceae was believed to have affinities towards the Sordariales. However, studies on the 28S rDNA have provided various inputs into the phylogeny of this family. *Réblova'* & Winka (2001) and *Réblova* & Seifert (2004) showed that species of Annulatascaceae clustered with *Cryptadelphia* spp. (Trichosphaeriales) and *Rhamphoria delicatula* (Sordariomycetidae *incertae sedis*). However, Campbell & Shearer (2004) demonstrated that *Trichosphaeria pilosa* (the type genus of Trichosphaeriaceae) was phylogenetically distant from the Annulatascaceae and *Rhamphoria*. In another analysis, Huhndorf *et al.* (2004) showed that *Annulatascus* has phylogenetic affinities with the Ophiostomatales. Campbell & Shearer (2004) and Raja *et al.* (2003) showed that the Annulatascaceae, with the absence of Ophiostomatales in their datasets, was monophyletic.

Our results are congruent with earlier analyses (Raja *et al.*, 2003; Campbell & Shearer, 2004; Huhndorf *et al.*, 2004) that Annulatascaceae is not related to the Sordariales. However, with the incorporation of six species of Ophiostomatales in our dataset the family is shown to be polyphyletic. Annulatascaceae forms three distinct clades. Clade A consists of Annulatascaceae species that are characterised by having dark perithecial ascomata, long cylindrical asci with a massive refractive apical ring and form a bifurcating clade with the morphologically different Ophiostomatales. Clade B consists of species with shorter cylindrical to fusoid asci and a relatively smaller apical apparatus and also have close affinities with the Ophiostomatales. Clade C contains two other taxa, *Annulatascus biatriisporus* and *Cyanoannulus petersenii*, falling close to the Magnaporthaceae clade.

The relationship between Trichosphaeriales and Annulatascaceae is still not clear. The 28S rDNA sequences (AY590296, AY590297) for *Trichosphaeria pilosa* (type species of *Trichosphaeria* and the type genus of the family) (Campbell & Shearer, 2004) were not used in our analyses, because they had high similarity with *Cladosporium*, an anamorphic genus belonging to the Mycosphaerellaceae (bitunicate ascomycetes). We presume that, although the isolates were obtained in good faith from ATCC, they may have been contaminated or incorrectly identified at source (pers comm. J. Campbell).

Although the phylogeny of the Annulatascaceae is still obscure and in a transitional stage, we believe that *Fusoidipora aquatica* is best accommodated within the Annulatascaceae based on morphology. To clarify the relationship among the Annulatascaceae, Ophiostomatales, Pleurotremaceae and Trichosphaeriales further studies with a larger taxonomic sampling and additional genetic data is essential.

Acknowledgements

This research was funded by Hong Kong Research Grant Council no. HKU 7230/02M. The University of Hong Kong is acknowledged for supporting this research by providing D.V with a postgraduate studentship and R.J with a postdoctoral fellowship. Dr. J-M Moncalvo is thanked for teaching the phylogenetic analyses. Helen Leung and Heidi Kong are thanked for technical assistance. Justin Bahl, Lei Cai and three anonymous reviewers are thanked for their critical comments that helped improve this paper.

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(Manuscript accepted 16 June 2005)