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Ascorhombispora aquatica gen. et sp. nov. from a freshwater habitat in China, and its phylogenetic placement based on molecular data

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Abstract – *Ascorhombispora* is a new genus characterised by superficial, dark brown to black perithecial ascomata; bitunicate, swollen saccate asci; and dark brown, 3-septate trapezoid ascospores with a wide septum band. The 28S rDNA and 18S rDNA regions of this fungus were amplified and sequenced. Phylogenetic analysis was conducted in order to infer systematic placement of this fungus. Results show that *Ascorhombispora aquatica* nests within Pleosporales (Dothidiomycetes, Ascomycota), which corroborate with the morphological prediction.

Ascomycetes / lignicolous / fungi / morphology / Pleosporales / systematics / taxonomy

INTRODUCTION

Submerged woody substrata are essential components of freshwater ecosystems (Jacobson *et al.*, 1999; Pascoal *et al.*, 2005; Sakayaroj *et al.*, 2005). Wood-inhabiting fungi are important in freshwater ecosystems because they have the ability to decompose organic material and play an important role in nutrient cycling (Cai *et al.*, 2003; Wong *et al.*, 1998; Abdel-Raheem & Shearer, 2002; Fryar *et al.*, 2005; Vijaykrishna & Hyde, 2006). Although diverse taxonomic groups of fungi colonize and grow on submerged wood (Hyde & Goh, 1999; Tsui & Hyde, 2003; Vijaykrishna *et al.*, 2006), wood-inhabiting fungi in freshwater environments have received less attention in Mainland China, as compared to those in terrestrial environments.

During a continuous investigation of freshwater fungi from Southern China (Cai *et al.*, 2005a; 2006; Zhu *et al.*, 2005), we collected a pyrenomycete which superficially resembles *Caryospora* but possess significant morphological differences. Critical examination shows that these differences grant the establishment of a new genus.

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MATERIALS AND METHODS

Sample collection and morphology

Submerged substrata were collected by L. Cai from a small stream in a tropical forest in Jinghong, Xishuangbanna, Yunnan, China (N22°02′ E100°50′), and returned to the laboratory in zip lock plastic bags. The samples were processed and examined following the method described in Cai *et al.* (2003). Type specimens are deposited in HKU(M). Observations and photographs were made from materials mounted in water. The width of conidia were measured at the widest point. The range between minimum and maximum values for microscopic measurements is given. Mean values are in brackets with 'n' being the number of items measured. Single spore isolation of this fungus has been tried several times but remained unsuccessful.

DNA extraction, PCR and sequencing

DNA was extracted from herbarium specimen using E.Z.N.A Forensic DNA Extraction Kit (Omega product No: D3529-01/02). The ascomatal contents of new taxon were removed using fine forceps for extraction according to the manufacturer's protocols. Partial 28S rDNA and 18S rDNA were amplified using fungal specific primers LROR & LR5 and NS1 & NS4 (Cai *et al.*, 2005b; Hidayat *et al.*, 2006). PCR was carried out in 50 µl reaction volume containing 31.7 µl sterile water, 5 µl PCR buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTP, 1.5 µl of each primer of 10 µM, 0.3 µl of Taq Polymerase and 3 µl of DNA template. The PCR thermal cycle for two regions were similar, consisting of 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 50 s and elongation at 72°C for 1 min, with a final extension step of 72°C for 10 min. 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 product code: 27-9602-01). DNA sequencing was performed using the primers mentioned above in an Applied Biosystem 3730 DNA Analyzer at the Genome Research Centre, The University of Hong Kong.

Sequence alignment and phylogenetic analysis

For each fungal strain, sequences obtained from pair primers were aligned to obtain an assembled sequence using Bioedit. In total 2 datasets were analysed. They are 28S rDNA and 18S rDNA datasets. Novel sequences generated from this study were submitted to GenBank (NCBI EU196548 and NCBI EU196549). Sequences for each strain, together with reference sequences obtained from GenBank (Tab 1), were aligned using Clustal X. Alignment was manually adjusted to allow maximum alignment and minimise gaps (Cai *et al.*, 2005b; Promputtha *et al.*, 2005).

Phylogenetic analyses were performed by using PAUP* 4.0b10. Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) and weighted parsimony (WP) analyses were performed with gaps treated as missing data. WP analyses were performed with an estimated transition-transversion ration of 1.5:1. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions.

Table 1. Sequences used in the analyses, with their NCBI accession numbers.

GenBank No.	*Taxa*	*GenBank No.*	*Taxa*
[1]U46882	*Aniptodera chesapeakensis*	[1]AY787935	*Letendraea eurotioides*
[2]AF201453	*Aliquandostipite khaoyaiensis*	[1]AY016362	*Letendraea helminthicola*
[2]U46870	*Aniptodera chesapeakensis*	[1]AY154718	*Lewia infectoria*
[2]AY251122	*Anungitopsis amoena*	[2]AF164362	*Lophiostoma caulium*
[2]AY251102	*Batcheloromyces proteae*	[2]U42485	*Lophiostoma crenatum*
[1]AY016356	*Bimuria novae-zelandiae*	[1]U434791	*Melanomma radicans*
[1]AY016357	*Byssothecium circinans*	[1]AY004337	*Microxyphium citri*
[2]AY016339	*Byssothecium circinans*	[2]AY016340	*Microxyphium citri*
[1]AF163979	*Cochliobolus hawaiiensis*	[2]AF164370	*Montagnula opulenta*
[2]U42479	*Cochliobolus sativus*	[1]AY772016	*Munkovalsaria appendiculata*
[1]AF163990	*Curvularia inaequalis*	[1]AY016365	*Myriangium duriaei*
[1]AY853366	*Delitschia didyma*	[1]U43472	*Ophiobolus fulgidus*
[2]AF164354	*Delitschia winteri*	[2]AY642522	*Paraconiothyrium estuarinum*
[2]AY016341	*Delphinella strobiligena*	[1]AY544684	*Phaeosphaeria avenaria*
[1]DQ018102	*Dictyosporium digitatum*	[2]AF164372	*Phaeosphaeria nodorum*
[1]DQ018104	*Dictyosporium toruloides*	[1]AY004340	*Phaeotrichum benjaminii*
[1]DQ018103	*Digitodesmium bambusicola*	[2]L76624	*Phaeotrichum benjaminii*
[2]U42474	*Dothidea insculpta*	[1]AY004341	*Pleomassaria siparia*
[1]AY016360	*Dothidea ribesia*	[2]AF164373	*Pleomassaria siparia*
[1]AY544681	*Dothidea sambuci*	[2]U43466	*Pleospora betae*
[2]AY544722	*Dothidea sambuci*	[1]AF382386	*Pleospora herbarum var. herbarum*
[1]AY541492	*Farlowiella carmichaeliana*	[1]AY510376	*Preussia australis*
[2]AY251124	*Fusicladium convolvularum*	[1]AY510391	*Preussia minima*
[2]AY251125	*Fusicladium effusum*	[2]AF006726	*Scorias spongiosa*
[2]AY856920	*Helicoma monilipes*	[2]AF338393	*Spilocaea oleaginea*
[2]AY856925	*Helicoma olivaceum*	[2]U42478	*Sporormia lignicola*
[1]AY787932	*Helicomyces roseus*	[1]AY004342	*Stylodothis puccinioides*
[2]AF164358	*Hysterium pulicare*	[1]AY016369	*Trematosphaeria heterospora*
[2]AF164359	*Hysteropatella clavispora*	[2]AY016354	*Trematosphaeria heterospora*
[2]AF438182	*Jahnula australiensis*	[1]AY787939	*Thaxteriella helicoma*
[2]AF164360	*Keissleriella cladophila*	[2]AY856953	*Troposporella fumosa*
[1]AY849944	*Leptosphaeria calvescens*	[1]AF050290	*Venturia hanliniana*
[1]U43475	*Leptosphaeria doliolum*	[1]AY853401	*Westerdykella ornate*
[2]U04235	*Leptosphaeria microscopica*	[1]AY004343	*Westerdykella cylindrica*

1. 28S rDNA sequences.
2. 18S rDNA sequences.

Maxtrees were unlimited, branches of zero length were collapsed and all parsimonious trees were saved. Clade stability was assessed in a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa. Kishino-Hasegawa test (KH Test) was performed in order to determine whether trees were significantly different. Trees were figured in Treeview (Cai *et al.*, 2005b; Hidayat *et al.*, 2006).

RESULTS

The LSU sequence consist of 526 nucleotides. Blast search in NCBI showed that this sequence is most similar to AY849944 *Leptosphaeria calvescens* (Pleosporales, Dothideomycetidae), AJ849362 *Phaeomyces kuwaitiensis* (Incertae sedis, Ascomycetes) and AY016357 *Byssothecium circinans* (Pleosporales, Dothideomycetidae). The 28S rDNA dataset consisted of 37 sequences. The final dataset comprised 590 characters after alignment. Unweighted parsimony resulted in 144 trees, while weighted parsimony yielded only 1 tree. KH test showed that these trees were not significantly different. The tree generated from weighted parsimony is shown in Fig 12.

The SSU sequence consisted of 947 nucleotides. Blast search in NCBI showed that this sequence is most comparable to AY 856953 *Troposporella fumosa* (Incertae sedis, Ascomycetes), AY 856920 *Helicoma monilipes* (Pleosporales, Ascomycetes) and AY251122 *Anungitopsis amoena* (Incertae sedis, Ascomycetes). The 18S rDNA dataset consisted of 41 sequences. The final dataset comprised 1050 characters after alignment. Unweighted parsimony resulted in 247 trees, while weighted parsimony yielded only 6 trees. KH test showed that these trees were not significantly different. One of the six trees generated from weighted parsimony is shown in Fig 13.

Phylogenetic analyses from 18S and 28S rDNA sequence provide additional information for its phylogenetic placement in Pleosporales, which corroborate the morphological data. However, the family level systematics of *Ascorhombispora* is still uncertain.

TAXONOMY

Ascorhombispora L. Cai & K.D. Hyde, gen. nov.

Ascomata perithecialis, superficialia, globosa vel subglobosa, coriacea, atro-brunnea vel nigra, ostiolata, solitaria vel gregaria, breviter papillata. Peridium pluribus stratis textura angulari compositum. Asci octospori, obpyriformis, late clavati vel saccati, pedicellati, bitunicati. Ascosporae 2-3-seriatae, late fusiformes vel rhomboideae, crassitunicatae, tunica gelatinosa praeditae, 3-septatae, non constricta, cellula interioris magnusae, trapezoideusae, atro-brunneae vel nigrae, verruculosae, cellula terminalis pusillusae, hemisphaerica, subhyalinae vel pallide brunneae, laevae.

Ascomata perithecioid, superficial, globose to subglobose, coriaceous, dark brown to black, ostiolate, solitary or gregarious, short papillate. Peridium relatively thin, *textura angularis* in longitudinal section, composed of 2 layers of angular cells. Asci 8-spored, obpyriform, broadly clavate to saccate, pedicellate,

bitunicate, apex rounded, persistent. Ascospores overlapping 2-3-seriate, broadly fusiform to rhombic, thick-walled, surrounded by gelatinous mucilaginous sheath, 3-euseptate, not constricted at septa, median septum wide, forming a darker band, central cells large, trapezoid, dark brown to black, verruculose, polar end cells small, hemispherical, subhyaline to light brown, smooth.

Etymology: *Ascorhombispora*, referring to the rhombic ascospores.

Type species. *Ascorhombispora aquatica* L. Cai & K.D. Hyde, sp. nov.

***Ascorhombispora aquatica* L. Cai & K.D. Hyde, sp. nov.** Figs 1-11

Ascomata 150-185 μm diam., 140-170 μm alta, perithecialis, superficialia, globosa vel subglobosa, coriacea, atro-brunnea vel nigra, ostiolata, solitaria vel gregaria, breviter papillata. Peridium 10-18 μm crassum, pluribus stratis textura angulari compositum. Filamenta interascalia non observabilis. Asci 100-198 × 72-102 μm, octospori, obpyriformis, late clavati vel saccati, pedicellati, bitunicati. Ascosporae 30.5-45 × 16-26.5 μm, 2-3-seriatae, late fusiformes vel rhomboidea, crassitunicatae, tunica gelatinosa praeditae, 3-septatae, non constricta, cellula interioris magnusae, trapezoideusae, 11-18 μm longe, atro-brunneae vel nigrae, verruculosae, cellula terminalis pusillusae, hemisphaerica, 3.5-4 μm longe, subhyalinae vel pallide brunneae, laevae.

Etymology: Meaning 'aquatic' in relation to the habitat of this species.

Ascomata 150-185 μm diam, 140-170 μm high, perithecioid, superficial, globose to subglobose, coriaceous, dark brown to black, ostiolate, solitary or gregarious, short papillate. Ostioles round, small, non-protruding. Peridium relatively thin, 10-18 μm wide, *textura angularis* in longitudinal section, composed of 2 layers of angular cells, outer later dark brown to black, relatively thick-walled, inner layer hyaline, relatively thin-walled. Interascal filaments not observed. Asci 100-198 × 72-102 μm ($\bar{x}$ = 186 × 88 μm, n = 15), 8-spored, obpyriform, broadly clavate to saccate, pedicellate, bitunicate, apex rounded, deliquescent. Ascospores 30.5-45 × 16-26.5 μm ($\bar{x}$ = 38.5 × 21 μm, n = 25), overlapping 2-3-seriate, broadly fusiform to rhombic, thick-walled, surrounded by gelatinous mucilaginous sheath, 3-euseptate, not constricted at septa, median septum wide, forming a darker band, central cells large, trapezoid, 11-18 μm long, dark brown to black, verruculose, polar end cells small, hemispherical, 3.5-4 μm long, subhyaline to light brown, smooth.

Habitat: Saprobic on submerged bamboo culms.

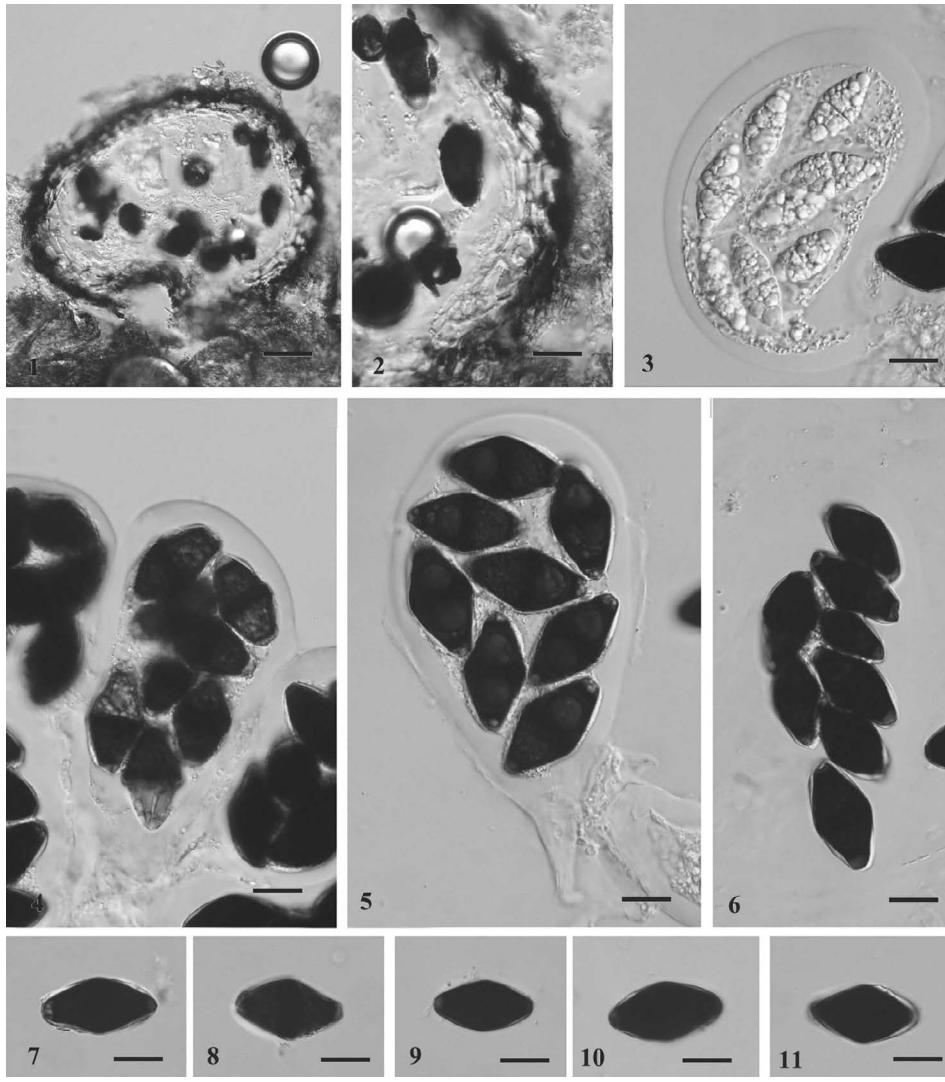
Known distribution: CHINA.

Anamorph: Unknown.

Holotype: CHINA, Yunnan, Jinghong, on submerged bamboo in a small forest stream, 26 January 2003, L. Cai, CAI-1H31 (HKU(M) 10859).

DISCUSSION

Ascorhombispora aquatica is unique among the genera of ascomycetes because of its unusual combination of morphological characters. These include: (i) superficial, coriaceous, non-stromatic ascomata; (ii) large, saccate asci; (iii) lack of interascal filaments; (iv) trapezoid (rhombic), 3-septate, dark brown to black ascospores with smaller end cells which are subhyaline to light brown. Genera of freshwater ascomycetes with species producing multiseptate brown ascospores include *Ascomauritiana* Ranghoo & K.D. Hyde, *Ascotaiwania* Sivan. & H.S. Chang,



Figs 1-11. *Ascorhombispora aquatica* from holotype. 1. Section of ascomata. 2. Peridium. 3. Young ascus. 4-6. Mature asci with ascospores. Note the deliquescent ascal wall in 6. Note the wide, dark band in the medium septum of ascospores in 4-5. 7-11. Ascospores. Note the mucilaginous sheath and paler end cells. Scale bars: 1 = 20 µm, 2-11 = 10 µm.

Caryospora De Not., *Paoayensis* Cabanela, Jeewon, & K.D. Hyde, *Savoryella* E.B.G. Jones & R.A. Eaton and *Zopfia* Rabenh. However, *Ascomauritiana*, *Ascotaiwania*, *Paoayensis* and *Savoryella* are different in having unitunicate asci (Ranghoo & Hyde, 1999; Sivanesan & Chang, 1992; Ho *et al.*, 1997; Chang *et al.*, 1998; Cabanela *et al.*, 2007).

The superficial ascomata and the large brown ascospores of *Ascorhombispora aquatica* are similar to that found in species of *Caryospora.* The

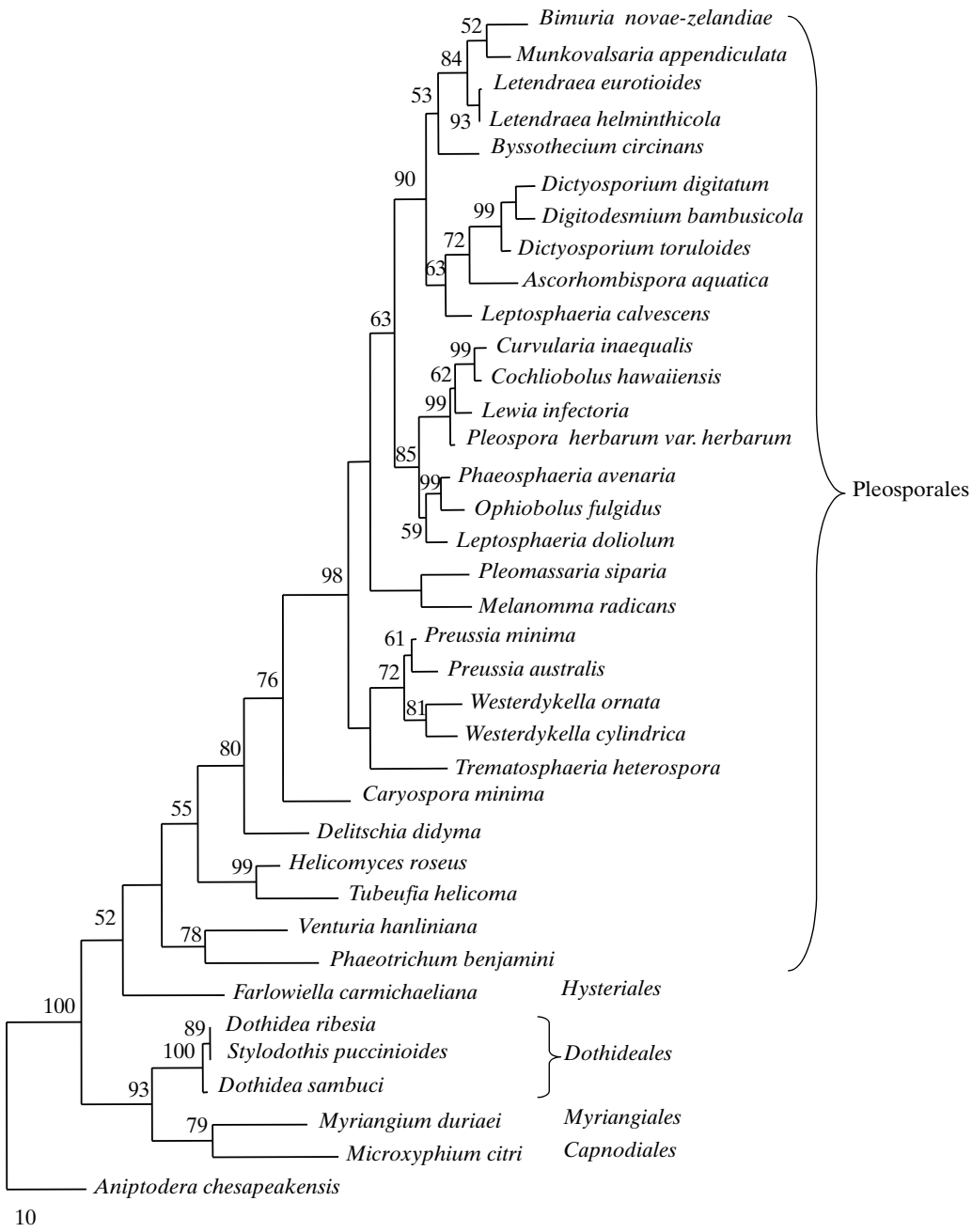


Fig 12. Phylogram of single tree generated from parsimony analysis based on 28S rDNA sequences. Data were analysed with random addition sequence, weighted parsimony and treating gaps as missing data. Bootstrap values ≥ 50% are shown above or below branches. The tree is rooted with *Aniptodera chesapeakensis*.

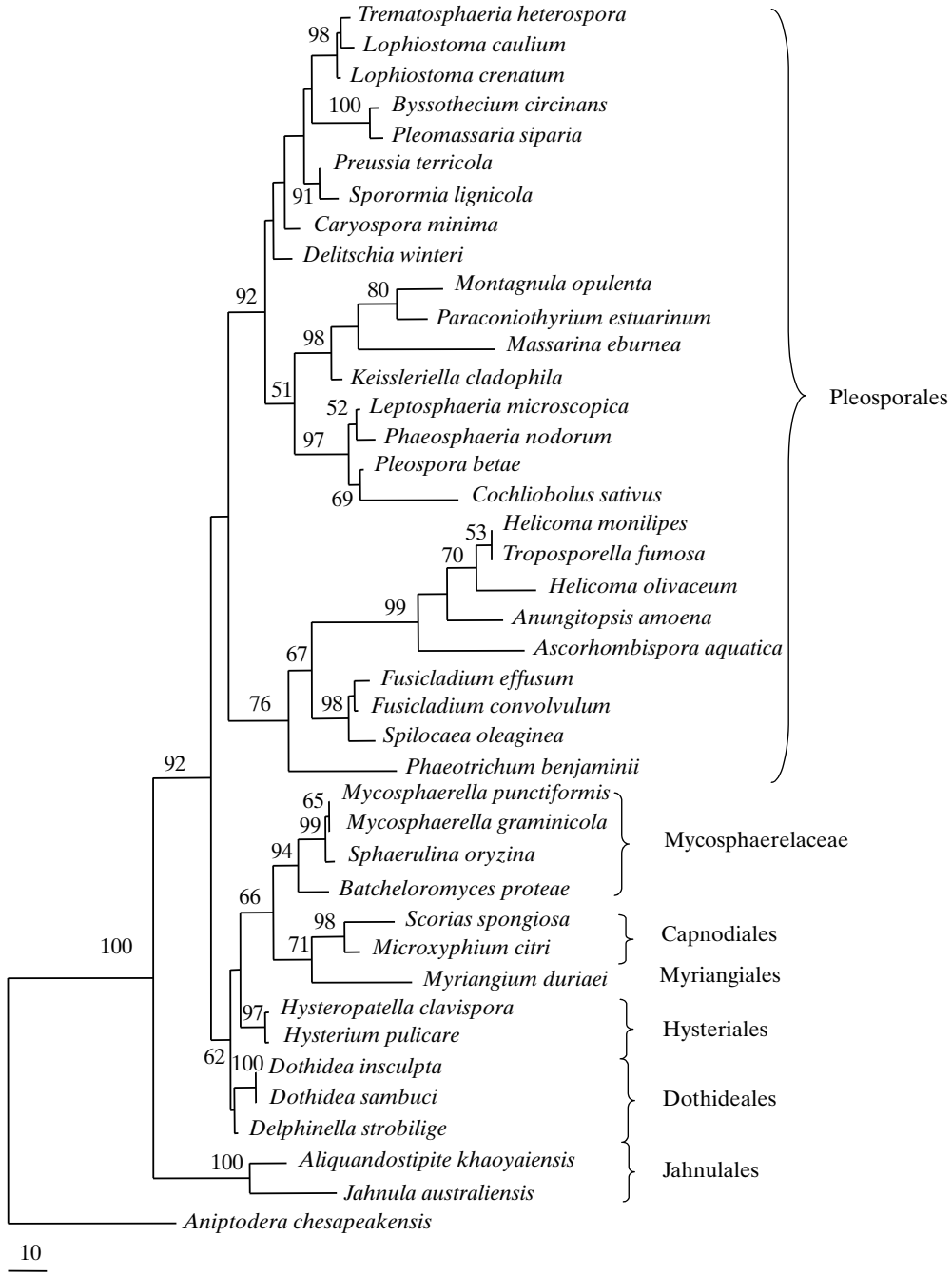


Fig 13. Phylogram of one of the 6 trees generated from parsimony analysis based on 18S rDNA sequences. Data were analysed with random addition sequence, weighted parsimony and treating gaps as missing data. Bootstrap values ≥ 50% are shown above or below branches. The tree is rooted with *Aniptodera chesapeakensis*.

saccate asci lacking interascal pseudoparaphyses, and the 3-septate, rhombic ascospores with the paler end cells are, however, morphological characters which exclude *A. aquatica* from *Caryospora.* In addition, *Caryospora* species form conical, distinctly carbonaceous ascomata on the host surface, which is also different from the ascomata of *Ascorhombispora* (Hawksworth, 1982). *Zopfia* is another bitunicate ascomycete that produces brown ascospores (Hawksworth and Booth, 1974). However, *Zopfia* have a series of morphological characters which differs from *A. aquatica*. The ascospores in *Zopfia* are 1-septate with the septum slightly or moderate constricted. Moreover, the asci in *Zopfia* are thick walled, 1-8-spored and are embedded in black, non-ostiolate, carbonaceous ascomata with pseudoparaphyses (Hawksworth & Booth, 1974).

The partial sequences of 28S rDNA (approx. 600 bp) and 18S rDNA (approx. 1000 bp) confirmed the taxonomic uniqueness of the new taxa, as no close relative was found. Phylogenetic analyses provided additional indications as to its phylogenetic position. In both 28S rDNA and 18S rDNA trees *Ascorhombispora aquatica* clustered with high bootstrap support in the Pleosporales. Analyses of unweighted parsimony and weighted parsimony were performed, which yielded mostly identical results. Morphologically, *Ascorhombispora aquatica* produces globose, black, perithecial ascomata with well-developed ostioles, and ascospores which are brown, septate and with gelatinous sheath. These characters also suggest a close relationship of this taxon to Pleosporales.

The 28S rDNA phylogeny shows that *Ascorhombispora aquatica* is nested in a clade with *Dictyosporium digitatum*, *Digitodesmium bambusicola*, *Dictyosporium toruloides* and *Leptosphaeria calvescens*. The first three anamorphic species have been recently concluded for their phylogenetic affinity with Pleosporales (Tsui *et al.* 2006), while *Leptosphaeria calvescens* is in Leptosphaeriaceae (Pleosporales). Similar molecular findings were obtained from phylogenies derived from the 18S rDNA dataset. In 18S rDNA tree, *Ascorhombispora aquatica* also groups with Pleosporales species, and is closely related to tubeufiaceous species. *Ascorhombispora aquatica* appear basal in the clade containing *Anungitopsis amoena, Helicoma monilipes, Helicoma olivaceum* and *Troposporella fumosa*. *Anungitopsis amoena* and *Troposporella fumosa* are anamorphic fungi which have not been known for their teleomorphic affinities, while *Helicoma monilipes* and *Helicoma olivaceum* are the anamorphic stage of Tubeufiaceae (Pleosporales). The family Tubeufiaceae, although not monophylic, is currently characterized by characterised by superficial, white and pallid to bright ascomata and filiform ascospores (Kodsueb *et al.* 2006). *Ascorhombispora aquatica*, although phylogenetically closely related to several Tubeufiaceae anamorphs, should not be placed in Tubeufiaceae since its ascomata are dark brown to black and ascospores are trapezoid.

Ascorhombispora aquatica does not show conclusive association with existing families in Pleosporales. This is not unexpected as *Ascorhombispora aquatica* showed distinct morphological characters as discussed above. The morphologically similar genus *Caryospora*, represented by *C. minima* in this analysis, does not cluster together in the trees, although they both belong in the Pleosporales.

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