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## Molecular phylogeny of *Dictyosporium* and allied genera inferred from ribosomal DNA

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The relationships among mitosporic fungi with cheiroid conidia in the genera *Cheiromoniliophora*, *Dictyosporium*, *Kamatia* and *Pseudodictyosporium* were investigated using sequences of small ribosomal subunit (SSU), internal transcribed spacer regions (ITS) and the 5' portion of the large ribosomal subunit (LSU) of rDNA. Six new SSU sequences were determined and analysed together with related sequences from GenBank using parsimony and Bayesian analyses. A second alignment with 15 ITS and seven partial LSU sequence of rDNA for an expanded set of taxa was also analysed to determine the relationships among various species and genera. Results indicated that the "*Dictyosporium*" lineage of mitosporic fungi producing cheiroid conidia are closely related to one another and they form a strong monophyletic group in the *Pleosporales*. *Dictyosporium* appeared to be paraphyletic but its monophyly could not be ruled out. Most *Dictyosporium* species belonged in one of two lineages that correlated imperfectly with conidial shape and the number of conidial arms. The systematic positions of *Dictyosporium australiense* and *D. toruloides* were not fully resolved. The phylogenetic position of *Digitodesmium* was within *Dictyosporium*, although without statistical support. *Cheiromoniliophora elegans*, *Kamatia indica* and *Pseudodictyosporium wauense* were close relatives and formed a monophyletic clade divergent from species of *Dictyosporium*. Most morphological characters used to delineate genera and species correctly predicted phylogenetic relationships.

**Key words:** freshwater, hyphomycetes, lignicolous, *Pleosporales*, systematics

### Introduction

*Dictyosporium* is a mitosporic fungal genus that is commonly collected on decaying plant litter, soil and wood throughout the world (Ellis, 1971; Sutton, 1985; Goh *et al.*, 1999; Cai *et al.*, 2003a,b; Fryar *et al.*, 2004; Luo *et*

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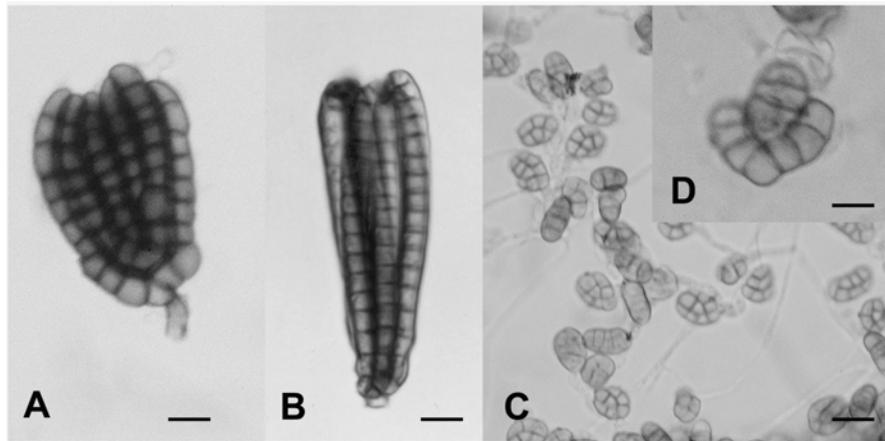
*al.*, 2004; Tsui *et al.*, 2004). It is characterised by multiseptate, cheiroid/digitate conidia borne on micronematous conidiophores with discrete and doliiform conidiogenous cells (Ellis, 1971; Goh *et al.*, 1999). Colonies on natural substrates often form compact sporodochia (Goh *et al.*, 1999). Currently over 20 species are accepted, and they are distinguished by morphological characters such as the number of conidial arms, the shape, colour and size of the conidia, and the presence/ absence of appendages (Goh *et al.*, 1999; Photita *et al.*, 2002; Cai *et al.*, 2003a,b). The genus has been considered homogeneous (Sutton, 1985), but depending on the species, conidia are either flattened or cylindrical (Fig. 1A, B) (Ellis, 1971; Goh *et al.*, 1999; Abrambarri *et al.*, 2001).

Cheiroid conidia are also known in other genera including *Cheiromoniliophora*, *Digitodesmium*, and *Pseudodictyosporium* (Fig. 1C, D) (Matsushima, 1971; Kirk, 1981; Tzean and Chen, 1990). The conidial arms of *Digitodesmium* become divergent at maturity (Kirk, 1981; Ho *et al.*, 2000), while those in *Dictyosporium* remain closely appressed (Sutton, 1985). Conidia of *Cheiromoniliophora*, *Kamatia* and *Pseudodictyosporium* resemble those of *Dictyosporium* in being cheiroid with non-divergent conidial arms (Rao and Subhedar, 1976; Tzean and Chen, 1990). They are distinguished from *Dictyosporium* by conidiogenesis and by the shape of the conidiogenous cells. The conidiophores in *Cheiromoniliophora*, *Kamatia* and *Pseudodictyosporium* are macronematous, well developed and irregularly branched, while those in *Dictyosporium* are micronematous and reduced. The paper aims to answer the following questions using sequence information from SSU, ITS (including 5.8S), and LSU of rDNA genes: (i) Is the genus *Dictyosporium* monophyletic? (ii) Are fungi that produce cheiroid/ digitate conidia closely related? (iii) Which fungi are the closest relatives of *Dictyosporium* and its allies?

## **Materials and methods**

### ***Fungal strains***

Cultures of type species and representative species of *Dictyosporium*, *Cheiromoniliophora*, *Kamatia* and *Pseudodictyosporium* were obtained from various culture collections (Table 1). We sampled as many species as possible, and included most of the available cultures from major public collections in the experiment. Cultures were maintained on potato dextrose agar and corn meal agar (CMA). The morphology of sporulating isolates was compared with species description (Ellis, 1971; Goh *et al.*, 1999) to confirm identities.



**Fig. 1.** Interference contrast micrographs of fungi with cheiroid conidia. **A.** Flattened conidia from *Dictyosporium elegans*. **B.** Cylindrical conidia from *Dictyosporium heptasporum*. **C.** Squash mount of *Cheiromoniliophora elegans*. **D.** Conidia of *Pseudodictyosporium wauense*. Scale bars: A, B = 13  $\mu\text{m}$ , C = 10  $\mu\text{m}$ , D = 5  $\mu\text{m}$ .

### ***DNA extraction, PCR amplification and sequencing***

Fungal mycelium was scraped off from Petri-dishes, and DNA was extracted as outlined by Jeewon *et al.* (2004) and Cai *et al.* (2005) or with a DNeasy® Plant Mini Kit (QIAGEN Inc., Mississauga, Ontario, Canada) according to the instructions supplied. The SSU was amplified with primers SL1 and CITS5. 12.5  $\mu\text{l}$  diluted DNA was amplified in a total volume of 25  $\mu\text{l}$  using puReTaq ready-to-go pcrbeads (Amersham Biosciences) with 10  $\mu\text{l}$  of  $\text{H}_2\text{O}$  and 1.25  $\mu\text{l}$  of each of the two primers (10  $\mu\text{mol}$ ). The amplification was done in a GeneAmp PCR system 9700 (Applied Biosystems) with the following program: 5 mins of denaturation at 95°C, and 40 cycles of: 95°C for 20 s, 52°C for 30 s, 72°C for 40 s, a final extension at 72°C for 7 min. Sequencing reactions were carried out using ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) with primers SL1, CITS5 (Inderbitzin *et al.*, 2001), NS2, NS4 (White *et al.*, 1990), NS19, MB1, and MB2 (sequences available through <http://plantbio.berkeley.edu/~bruns/primers.html#18s>) for SSU region. The ITS and LSU regions were amplified using an annealing temperature of 55°C for 35 cycles and sequenced with primers ITS1F, ITS4, CTB6 and TW13 (<http://plantbio.berkeley.edu/~bruns/primers.html#its>). The sequences were determined at the Nucleic Acid Protein Service unit at the University of British Columbia. Sequence fragments were subjected to the BLAST search engine at the National Center for Biotechnology Information to verify their identity if possible. Fragments were assembled using ABI PRISM® AutoAssembler™ v.1.4 (Applied Biosystems, Foster City, CA).

### ***Phylogenetic analysis***

To investigate the phylogenetic placement of the fungi with cheiroid conidia, we created two sequence alignments. We aligned SSU sequences of 6 digitate mitosporic fungi and 28 taxa representing different families within *Pleosporales* retrieved from the GenBank in order to determine the phylogenetic position of the digitate fungi within the ascomycetes. *Dothidea hippophaeos* was designated as the outgroup. The alignment included the most similar sequences identified through BLAST search. A second alignment of ITS and partial LSU sequences included sequences from the 11 available species of *Dictyosporium*, as well as isolates representing four other cheiroid genera. Blast searches revealed no other closely related taxa. *Phoma herbarum* (AY293800, AY293788) and *Saccharicola bicolor* (as *Leptosphaeria bicolor* in GenBank) (U04203) were used as outgroups. Sequences were aligned with CLUSTAL X (Thompson *et al.*, 1997) (distributed by the authors, <ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>) and adjusted manually with Se-Al v.1 d1 (Rambaut, 1999). The alignment was analyzed using PAUP\* 4.0b10 software package (Swofford, 2003). Maximum parsimony (MP) was carried out using a heuristic search with 30 replicates, random sequence addition and TBR branch swapping. All characters were equally weighted and gaps were treated as missing data. Bootstrap support for the branches was based on 500 replicates with random taxon addition. Significance of differences in tree topologies was statistically evaluated by the Kishino-Hasegawa test (KHT) (Kishino and Hasegawa, 1989). Bayesian inference of phylogeny was calculated using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), with the general time reversible model of substitution assumed with among site variation described by a gamma distribution. Four simultaneous Markov chains were run from random starting trees for 1,000,000 generations and sampled every 100 generations (generating 10001 trees). The first 1000 trees were discarded as burn-in, hence inferences of posteriori probability were calculated from 9001 trees.

### **Results**

The SSU alignment for 34 taxa consisted of 1723 characters, of which 1471 were constant and 148 were parsimony informative. Forty-nine equally parsimonious trees were obtained (length = 455, CI = 0.655, RI = 0.779, RC = 0.51). Four *Dictyosporium* taxa constitute a the monophyletic sister group to *Pseudodictyosporium wauense* and *Cheiromoniliophora elegans*. These six species, *Dictyosporium* and allies formed a monophyletic clade with 100% bootstrap confidence that nested within the *Massarinaceae* in the *Pleosporales*

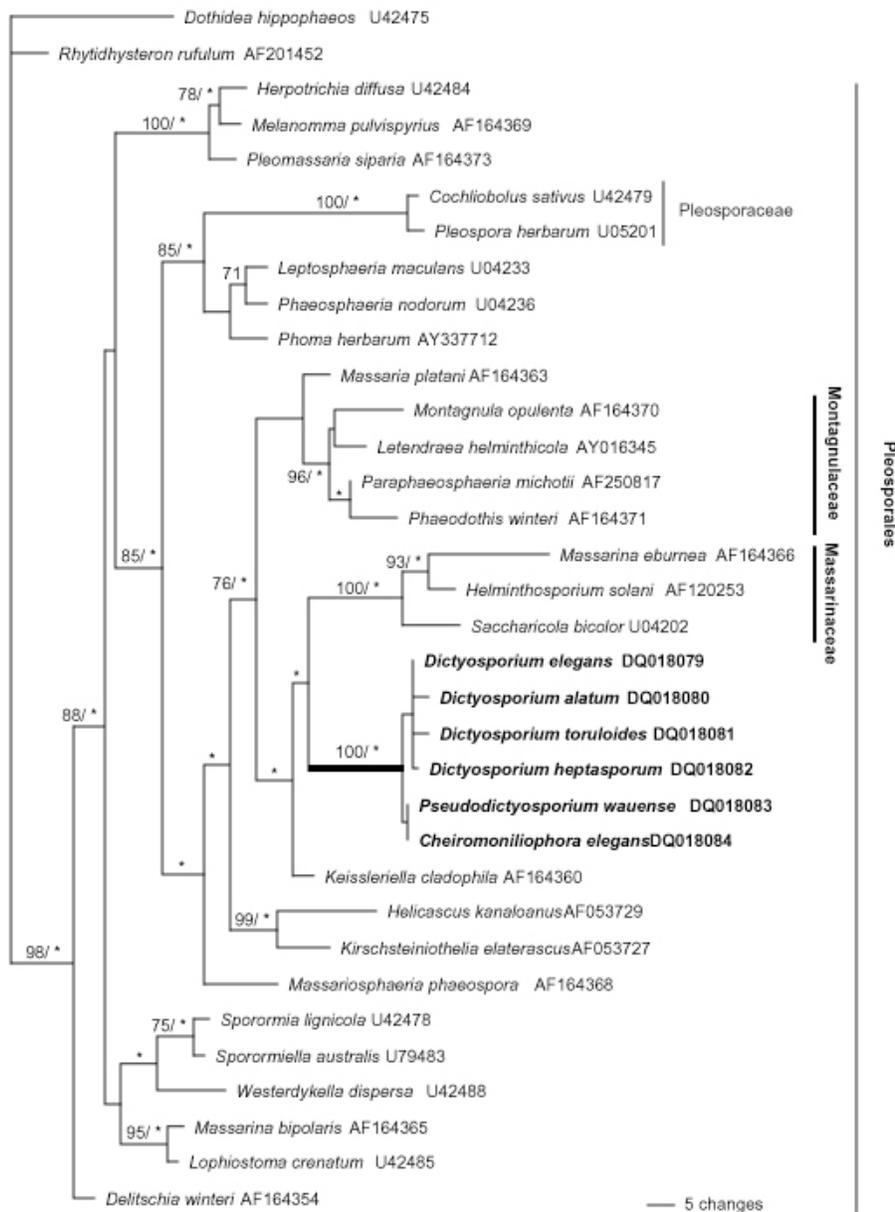
(Fig. 2). However, this sister relationship with *Massarina eburnea*, *Helminthosporium solani* and *Saccharicola bicolor* did not receive any bootstrap or Bayesian support. The next most closely related species was *Keissleriella cladophila* and then members of *Montagnulaceae* (Fig. 2). Both MP phylogenetic analysis and Bayesian analysis identified the same groups corresponding to the families of *Pleosporales* (Fig. 2).

The combined dataset of ITS and partial LSU for 17 taxa, including fifteen new ITS (593 bp) and seven new partial LSU (629 bp) sequences, was analysed by maximum parsimony, resulted in 2 trees (length = 632, CI = 0.736, RI = 0.695, RC = 0.512). Out of 1212 characters, 892 were constant, and 180 were parsimony informative. All the digitate fungi appear to form a monophyletic cluster with strong bootstrap support (Fig. 3). Of 11 species, *Dictyosporium* were divided into two well-supported clades (A & B). The phylogenetic positions of *Dictyosporium australiense* and *D. toruloides* were not fully resolved. *Digitodesmium bambusicola* was basal to other *Dictyosporium* taxa in clade B, but without bootstrap support. Together *Kamatia indica*, *Pseudodictyosporium wauense* and *Cheiromoniliophora elegans* formed a monophyletic group (Clade C) (Fig. 3). The consensus tree of Bayesian analysis identified the same groupings with a slightly different topology. Clade A and C formed a sister relationship without statistical support and *Dictyosporium toruloides* was basal to Clades A and C with 90% Bayesian probability (not illustrated).

To further test the support for paraphyly of the genus *Dictyosporium*, trees with topological constraints were generated (partial LSU sequences only). A tree forcing species of *Dictyosporium*, excluding *Digitodesmium bambusicola*, to form a single, monophyletic group was only 3 steps longer than the most parsimonious tree with constraints (data not shown). The monophyly of *Dictyosporium* cannot be completely ruled out.

## Discussion

For this study, we selected as many isolates as possible from species producing cheiroid/digitate conidia. These included 10 of the more than 20 total species of *Dictyosporium*, 1 of the 3 species of *Digitodesmium*, 1 of the 2 species of *Cheiromoniliophora*, and the type species of two monotypic genera *Kamatia* and *Pseudodictyosporium* (Kirk *et al.*, 2001). Mitosporic fungi producing cheiroid conidia form a distinct, well-supported, monophyletic lineage with close affinities to the *Massarinaceae* in the *Pleosporales* (Eriksson and Hawksworth, 2003). The closest relative to the "*Dictyosporium*" lineage are *Massarina eburnea* and *Saccharicola bicolor*, which are saprobes, which like "*Dictyosporium*" colonise dead wood. *Helminthosporium solani* is



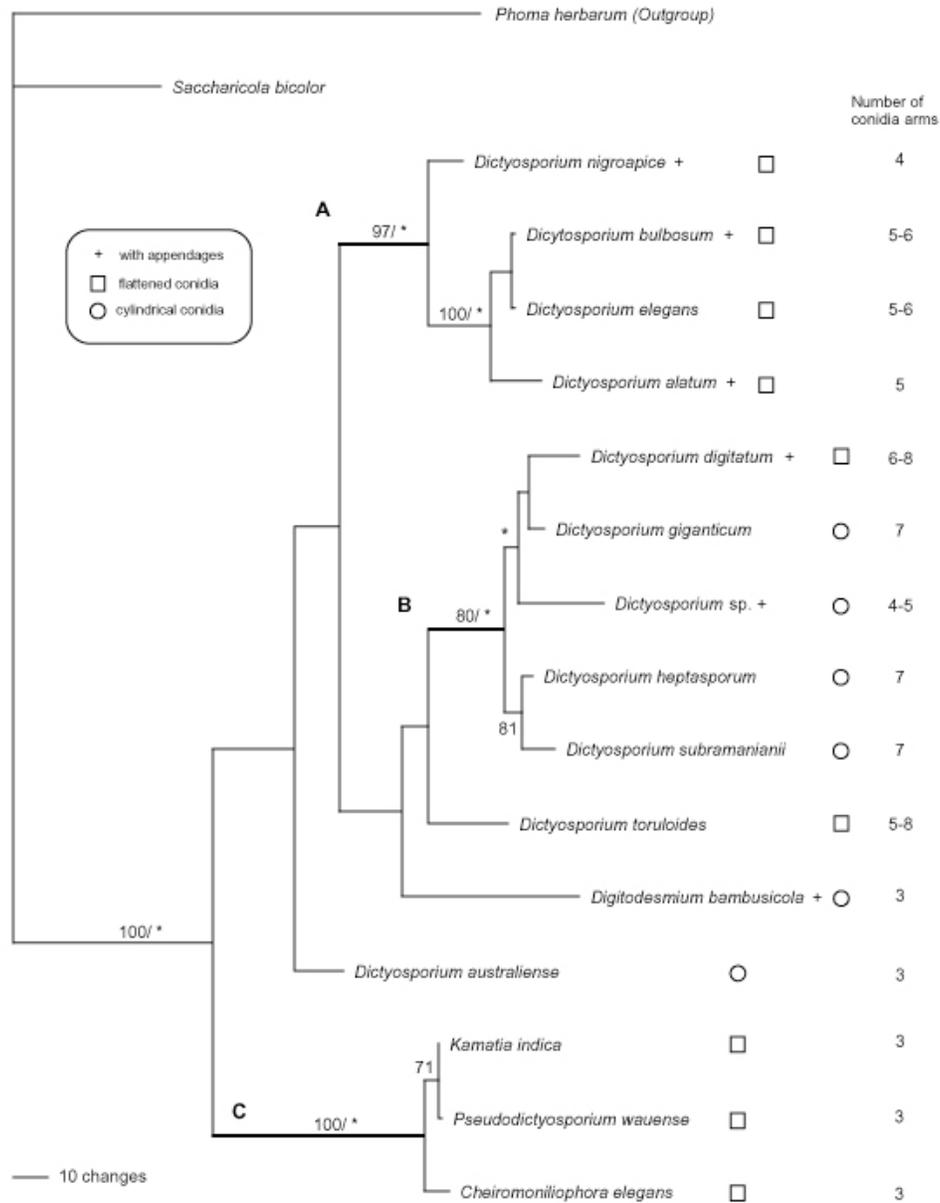
**Fig. 2.** One of the most parsimonious tree showing the relationships of fungi with cheiroid conidia with respect to other members of *Pleosporales*. *Dothidea hippophaeos* is the designated outgroup. Parsimony bootstrap percentages over 75% generated from 500 replicates are shown above the branches. Asterisks indicate branches with Bayesian posterior probabilities >95%.

also close to the "*Dictyosporium*" lineage, but it produces long, cylindrical conidia and is a common plant pathogen (Olivier *et al.*, 2000). These mitosporic fungi have common features in the production of cheiroid/ digitate conidia with at least 3 rows of cells.

Based on the most parsimonious trees (MPT) from ITS and partial LSU sequences, *Dictyosporium* as currently circumscribed is paraphyletic and nine species are divided between two well-supported clades. Clade A includes *Dictyosporium elegans* (type species), and the conidia in Clade A are flattened and planate, while those in Clade B usually produces cylindrical conidia. The correlation is not perfect; *Dictyosporium digitatum* produces conidia flattened in one plane with 6-8 arms (Chen *et al.*, 1991) but groups with *D. giganticum*. *Dictyosporium toruloides* also produces flattened conidia but its phylogenetic position relative to the two groups was not resolved. *Dictyosporium australiense* produces conidia arranged in a triangular plane (Sutton, 1985) and its phylogenetic position was also unresolved. Species in clade A have 4-6 arms, while those in clade B have 6 or more, except *Dictyosporium* sp. A few *Dictyosporium* species have conidia bearing thin-walled appendages (Goh *et al.*, 1999). Results from the phylogenetic analysis indicated that the appendages may have arisen through evolutionary convergence (Fig. 3). Appendages are useful for the attachment of spores during dispersal, and are highly influenced by environmental factors.

*Digitodesmium* is separated from *Dictyosporium* based solely on its divergent conidial arms. Although the MPT from LSU and ITS data suggest that this separation may be artificial (Fig. 3), the nesting of *Digitodesmium* is without bootstrap support (Fig. 3). *Digitodesmium* currently consists of three species (Kirk, 1981; Cai *et al.*, 2003a), and our dataset suffered from incomplete taxon sampling. It seems desirable to maintain the current circumscription of both genera until additional taxa, particularly the type species, are included in the analysis to determine whether *Digitodesmium* should be a synonym to *Dictyosporium*.

*Cheiromoniliophora elegans*, *Kamatia indica* and *Pseudodictyosporium wauense* are closely related as they form a strong, monophyletic clade with 100% bootstrap support. They have flattened conidia with consistently three conidial arms borne on conspicuous conidiophores (Matsushima, 1971; Tzean and Chen, 1990). Unlike *Dictyosporium*, they do not produce sporodochia on natural substrates (Matsushima, 1971; Tzean and Chen, 1990). The Dictionary of fungi considered *Kamatia indica* a synonym of *Pseudodictyosporium wauense* (Hawksworth *et al.*, 1995). The synonymy is supported by *K. indica* and *P. wauense*'s nearly identical ITS sequences. Although *Cheiromoniliophora* differs from *Pseudodictyosporium* by its catenulate,



**Fig. 3.** One of the two parsimonious tree illustrating the phylogenetic relationships among *Dictyosporium* and related genera inferred from ITS and partial LSU obtained from PAUP (Tree length = 632 steps, CI = 0.736, RI = 0.695, RC = 0.512). Bootstrap confidence values (>70%) generated from 500 replicates are indicated on branches. Asterisks indicate branches with Bayesian posterior probabilities >95%.

spherical conidiogenous cells (Tzean and Chen, 1990; Casteñeda Ruíz *et al.*, 1997), these two genera are highly similar in conidial morphology. *Cheiromoniliophora* and *Pseudodictyosporium* are sister taxa and since their ITS sequence show only slight divergence, it may be unnecessary to retain *Cheiromoniliophora* as a distinct genus. In conclusion, our investigation provides evidence that *Dictyosporium* and its allies form a monophyletic clade in the *Massarinaceae*, united by shared morphological characters.

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