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The polyphyletic nature of *Pleosporales*: an example from *Massariosphaeria* based on rDNA and RBP2 gene phylogenies

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ABSTRACT

Massariosphaeria is a loculoascomycetous fungus currently accommodated within the *Pleosporales*. However, based on morphology alone, it has been difficult to assess its familial position and its affinities to other fungi with bitunicate asci. In order to establish its evolutionary relationships, two regions of the rDNA (18S and 28S) and two regions of the RPB2 protein-coding gene were sequenced and analysed phylogenetically. Multigene phylogenies revealed that *Massariosphaeria* is not monophyletic and results are in disagreement with existing morphological-based classification schemes. Characters, such as ascomatal shape and ascospore morphology, have evolved more than once within the *Pleosporales*. The familial placement of several species is still obscure, except *M. grandispora*, which could be confidently assigned to the *Lophiostomaceae*. *M. typhicola* is closely related to *Trematosphaeria hydrela* (*Melanommataceae*), whereas *M. triseptata* is related to *Melanomma radicans* but shares close affinities to the *Sporormiaceae*. The placement of *M. roumeguerei* is still unresolved, and it does not appear to have any close evolutionary relationship to any known melanommataceous or pleosporaceous genera. Our molecular data also refute the monophyly of *Kirschsteiniothelia*, *Massarina*, *Melanomma*, and *Pleospora*, and support previous phylogenetic hypotheses that *Melanommataceae* is polyphyletic. There is a need for more phylogenetic (and taxonomic) studies within the *Pleosporales*, especially incorporation of more anamorphic taxa and type species.

Introduction

The genus *Massariosphaeria* (*Pleosporales*, *Dothideomycetes*) is characterised by a typical pleosporaceous morphology (Leuchtmann 1987), which include: black, bean-shaped or elongated, immersed (sometimes semi-immersed) ascomata; black and papillate ostioles; a thin-walled, smooth peridium (*textura prismatica*) with black to grayish cells; abundant, hyaline, septate hamathecium filaments; and bitunicate, cylindric-clavate asci. It is morphologically a relatively well-characterised genus,

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easily recognisable by the fusiform, multiseptate ascospores with relatively large thick walls, yellow to brown colour, some with transverse septum only, with the cell above the septum the largest, and surrounded by a prominent mucilaginous sheath (Tanaka & Harada 2004; Van 2005). All species have a tendency to form red pigments, on the host and especially in culture (Van 2005). The genus was first established as a section of *Leptosphaeria* (Müller 1950), but was later given generic status. This taxonomic arrangement has largely been accepted (Leuchtmann 1984; Huhndorf *et al.* 1990), although Barr (1989) included *Massariosphaeria* in *Chaetomastia*. Currently, there are about 28 accepted species *Index Fungorum*, June 2006 and both phragmosporous and dictyosporous taxa are included (Leuchtmann 1987; Shoemaker & Babcock 1989; Tanaka & Harada 2004).

Lophiostoma, Leptosphaeria, Melanomma, and *Trematosphaeria* are pleosporaceous genera with species that are morphologically similar to various *Massariosphaeria* species. This has resulted in taxonomic uncertainty with species being transferred from one genus to another (e.g. Shoemaker & Babcock 1989; Huhndorf *et al.* 1990; Tanaka & Harada 2003, 2004). The familial position of *Massariosphaeria* is still not well defined. This is partly because the circumscription of other families within the order *Pleosporales* is still unclear. *Massariosphaeria* is generally accepted to belong to the family *Lophiostomaceae* (Eriksson & Hawksworth 1991; Kirk *et al.* 2001). However, it has also been referred to the family *Dacampiaceae* by Barr (1992), no doubt because she included some species of *Massariosphaeria* in *Chaetomastia* (Barr 1989). Most of the known anamorphic *Massariosphaeria* species are aposphaeria-like, which is also the anamorph produced by *Melanomma* (*Melanommataceae*) (Kirk *et al.* 2001; Tanaka & Harada 2004).

On morphological grounds, it has been very difficult to predict the familial placement, and to date, only one species of *Massariosphaeria* (*M. phaeosphaeria*, the type species), has had its 18S rDNA partially analysed (Liew *et al.* 2000). It is thus unknown whether the genus is mono- or polyphyletic. This issue needs to be addressed as there have been a few reports on polyphyly of some pleosporaceous genera. (e.g. Kodsueb *et al.* 2006a; Liew *et al.* 2002). This paper is a continuity of several taxonomic studies on the *Pleosporales* where we have targeted important dothideomycetous genera, such as *Leptosphaerulina, Letendraea, Pleospora, Tubeufia, Pyrenophora*, and *Wettsteinina* (Kodsueb *et al.* 2006a,b; Pinnoi *et al.* 2007). The present work had three objectives: (1) to determine whether *Massariosphaeria* represents a natural group; (2) to verify the familial placement of *Massariosphaeria;* and (3) to discuss phylogenetic findings with respect to morphological-based classification schemes.

Materials and methods

DNA extraction, amplification and sequencing

Cultures of fungi used in this study were obtained from the Centraalbureau voor Schimmelcultures (*Massariosphaeria grandispora* = CBS613.86; *M. roumeguerei* = CBS612.86; *M. triseptata* = CBS614.86; *M. typhicola* = CBS609.86). Isolates were grown on potato-dextrose agar (PDA) and malt-extract agar (MEA) for two to four weeks and total genomic DNA was extracted from mycelia following the protocols as outlined by (Cai *et al.* 2005, 2006a,b). GenBank accession numbers are as follows and shown on the trees: 18S rDNA (*Massariosphaeria grandispora* = EF165038; *M. roumeguerei* = EF165035; *M. triseptata* = EF165036; *M. typhicola* = EF165037); 28S rDNA (*Massariosphaeria grandispora* = EF165034; *M. roumeguerei* = EF165032; *M. triseptata* = EF165031; *M. typhicola* = EF165033); RPB2 (*Massariosphaeria grandispora* = EF165034/EF165042; *M. roumeguerei* = EF165032/EF165039; *M. triseptata* = EF165031/EF165040; *M. typhicola* = EF165033/EF165041).

DNA amplification was performed by PCR. For partial 18S and 28S rDNA amplification, NS1 & NS4 and LROR & LR5 primers (White *et al.* 1990; Vilgalys & Hester 1990) were used. Two separate regions of the RPB2 gene were amplified with primer pairs RPB2-5f & RPB2-7cr and RPB2-7f & RPB2-11ar (Liu *et al.* 1999). All amplification reactions were performed in a 50 μl reaction volume as follows: 1 × PCR buffer, 0.2 mM dNTP, 0.3 μM of each primer; 1.5 mM $MgCl_2$, 0.8 units Taq Polymerase and 5–10 ng DNA. PCR thermal cycle parameters for partial 18S and 28S rDNA amplification was as follows: 94 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s and elongation at 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The parameters for the PCR thermal cycle of the partial RPB2 gene amplification consisted of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min and elongation at 72 °C for 1.5 min, with a final extension step of 72 °C for 10 min (Liu *et al.* 1999). PCR products were purified using minicolumns, purification resin, and buffer according to the manufacturer's protocols (Amersham, Foster, CA). DNA sequencing was performed using the primers mentioned above in an Applied Biosystem 3730 DNA analyser.

Sequence alignment and phylogenetic analyses

Sequences were aligned in Clustal X (Thompson *et al.* 1997) and Bioedit (Hall 1999). Four single gene datasets were analysed (18S rDNA, 28S rDNA, RPB2 5f-7cr, RPB2 7f-11ar) and two combined ones (RPB2 5f-7cr + RPB2 7f-11ar and RPB2 5f-7cr + 28S rDNA). Phylogenetic analyses were performed in PAUP 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) and weighted parsimony (WP) analyses were performed. Gaps were treated as missing data and fifth character to increase the probability of finding the most parsimonious tree/s but only gapmode = missing was used in the final analyses. WP analyses were also performed using a symmetric step matrix generated with the program STMatrix v2.2, by which the relative frequencies of nucleotide substitutions were calculated and converted into costs of changes (Francois Lutzoni & Stefan Zoller, Department of Biology, Duke University, Durham, NC). Trees were inferred using the heuristic search option with 1 K random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple parsimonious trees were saved. Descriptive tree statistics [Tree Length (TL), CI, RI, RC, HI] were calculated for trees generated under different optimality criteria. Clade stability was assessed in BS analyses with 1 K replicates, each with ten replicates of random stepwise addition of taxa. Kishino–Hasegawa (KH) tests (Kishino & Hasewaga 1989) were performed in order to determine