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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

whether trees were significantly different. Trees were figured in TreeView (Page 1996). Other details are outlined in Jeewon et al. (2002, 2003). ML and Bayesian analyses (using models of nucleotide substitution derived from Model test) were also performed as outlined by Shenoy et al. (2006).

Results

Ribosomal DNA phylogenies

The 18S rDNA dataset comprises 60 taxa with 1022 characters (16 % parsimony informative). WP analyses yielded two trees that were identical in topology and not statistically different, based on KH tests. One of them is shown in Fig 1 (TL = 957.95, CI = 0.568, RI = 0.756, RC = 0.430, HI = 0.432). The trees generated in UP analyses were identical to those from WP but had slightly less nodal support. Phylogenies obtained show that *Massariosphaeria* spp. do not constitute a monophyletic lineage. *M. roumeguerei* constitutes an unresolved clade basal to *Lepidosphaeria nicotia, Neotestudina rosatii* and *Arthopyrenia salicis*. *M. grandispora* is a sister taxon to *Lophiostoma caulium, Trematosphaeria heterospora* and *Massarina bipolaris* with adequate support. *Massariosphaeria phaeospora,* the type species is basal to a clade consisting of *Kirschsteiniothelia elaterascus* and *Helicascus kanaloanus* with 65 % BS support (BS). *T. hydrela* and *M. typhicola* cluster together with high statistical confidence, but their relationship to other members of the *Pleosporales* was not statistically supported.

The 28S rDNA sequence data consisted of 70 taxa with 890 characters (27 % parsimony-informative characters). WP analyses yielded two trees (not significantly different), one of which is shown in Fig 2 [TL = 2412.07, CI = 0.359, RI = 0.655, RC = 0.235, HI = 0. 641]. Similar phylogenies were recovered with those from the 18S rDNA sequence data except for *M. triseptata*. This taxon clusters with *Melanomma radicans* and these two are nested in between the *Sporormiaceae* and *Lophiostomaceae* (with no BS). In contrast, in the 18S rDNA phylogenies, *M. triseptata* is nested between members of the *Sporormiaceae,* but this relationship was not resolved (all clades comprising *Eremodothis, Sporormia, Preussia,* and *Westerdykella* collapsed). *Massariosphaeria typhicola* is basal to *Lepidosphaeria, Lojkania, Neostestudina, Pleospora rubicunda,* and *Verruculina,* but this sister relationship did not receive any statistical confidence.

A combined 28S and 18S rDNA dataset was also analysed to check for any topological incongruence. This dataset consisted of 39 taxa and 1913 characters (18.3 % parsimony-informative) and our taxon sampling also included some newly released loculoascomycetous DNA sequences as used by Kruys et al. (2006) in their combined 28S and 18S rDNA dataset. Phylogenies obtained were almost identical to those obtained from those recovered from the 28S rDNA sequence data (results not shown).

RPB2 DNA phylogenies

The polyphyletic nature of *Massariosphaeria* was also strongly supported in the RPB2 sequence data analyses. Two separate datasets (A and B) were analysed for DNA sequences generated from the RPB2 gene region. Dataset A includes the first 1130 nucleotides of the RPB2 region amplified and sequenced with primer pairs RPB2-5f and RPB2-7cr, whereas dataset B (920 sites) includes another region of the same gene amplified and sequenced with primer pairs RPB2-7f and RPB2-11ar. WP analyses of dataset A, as well as dataset B, resulted in a single tree [TL = 8387.20, CI = 0.241, RI = 0.525, RC = 0.126, HI = 0.759 (dataset A); TL = 8888.0, CI = 0.275, RI = 0.541, RC = 0.148, HI = 0.725 (dataset B)] and depicted similar topologies with respects to the in-groups under study. Phylogenies from dataset A were essentially similar to those obtained from single gene rDNA datasets (results not shown). It should be noted that any slight difference in placement was only due to the unavailability of more sequence data from the *Pleosporales*. Both RPB2 gene regions were also combined but results are not discussed, as there were not enough taxa available to properly assess the relationship of *Massariosphaeria* species.

A dataset of 43 taxa combining the 28S rDNA + RPB2 (dataset A) was also informative. This dataset consisted of 2038 characters, but 412 had to be excluded (ambiguous alignment). WP, with 45 % parsimony-informative characters, yielded one tree [TL = 8558.09, CI = 0.338, RI = 0.529, RC = 0.179, HI = 0.662; Fig 3]. Clade support was moderate and results are generally concordant with phylogenetic placement of *Massariosphaeria* as determined by 28S rDNA. *Massariosphaeria* species fall into four distinct lineages. *M. triseptata* clusters with *Melanomma radicans* whereas *M. grandispora* fits within the *Lophiostomaceae* with strong statistical support. Conversely, a proper phylogenetic connection of *M. roumeguerei* and *M. typhicola* is still obscure as they constituent independent lineages without support. For all gene datasets, ML, NJ, and Bayesian analyses were also performed. ML and Bayesian based phylogenies were identical to those of MP and are not discussed here. Clades from NJ trees were not statistically strong although phylogenies were essentially similar (results not shown).

Discussion

The objective of this study was to assess the phylogenetic relationships of *Massariosphaeria* and establish its familial placement within the *Pleosporales*. Based on 18S rDNA phylogenies, Liew et al. (2000) found that *M. phaeospora* (the type species) was basal to other members of the *Massarinaceae* (as circumscribed by Eriksson & Hawksworth 2003). Inderbitzin et al. (2002) could not confidently assess the proper affinities of *M. phaeospora* as its phylogenetic placement was largely dependent upon the analytical methods used. They found that it formed an unresolved sister taxon relationship to other members of the *Pleosporales*. Phylogenies obtained here somewhat corroborate those of Liew et al. (2000) who suggested a close affinity to the *Massarinaceae*. Using a different taxon sampling, we found that the type species is basal to *Kirschsteiniothelia elaterascus* and *Helicascus kanaloanus* with moderate support. Although these two taxa have been referred to the *Pleosporales*, there is no agreement and conclusive evidence to justify an appropriate familial placement. In addition, *M. phaeospora* also appears to be closely related to other members of the *Massarinaceae* and other genera such as *Bimuria, Letendraea, Cucurbidothis,* and *Didymocrea*. These latter taxa have been referred to the *Melanommataceae*, *Cucurbitariaceae,* and *Zopfiaceae,*

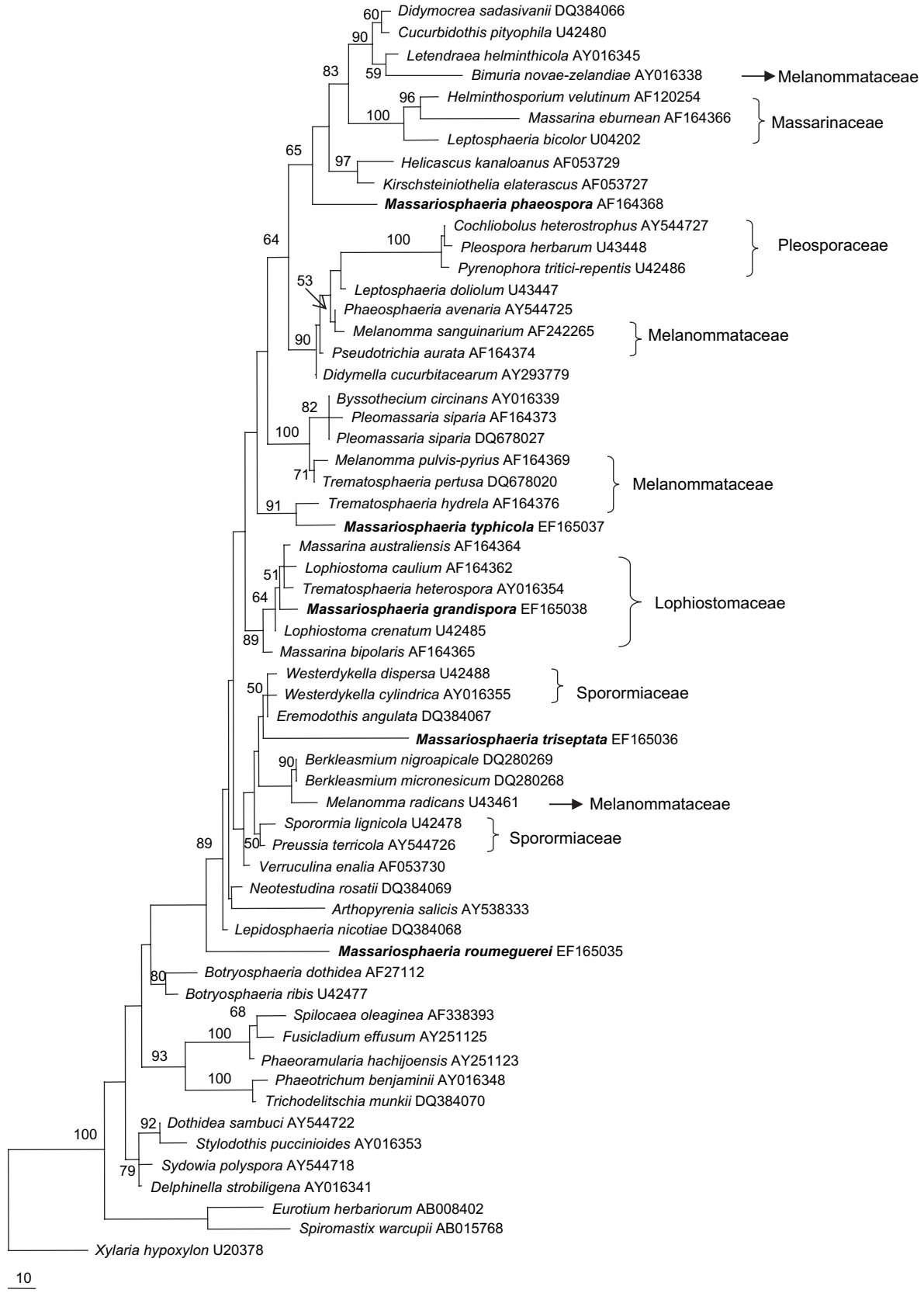

Fig 1 – Phylogram of one of two most parsimonious tree generated based on the 18S rDNA sequences. Data were analysed with random addition sequence, WP and treating gaps as missing characters. Values above the branches are parsimony BS (equal or above 50 %). The tree is rooted with *Xylaria hypoxylon*.

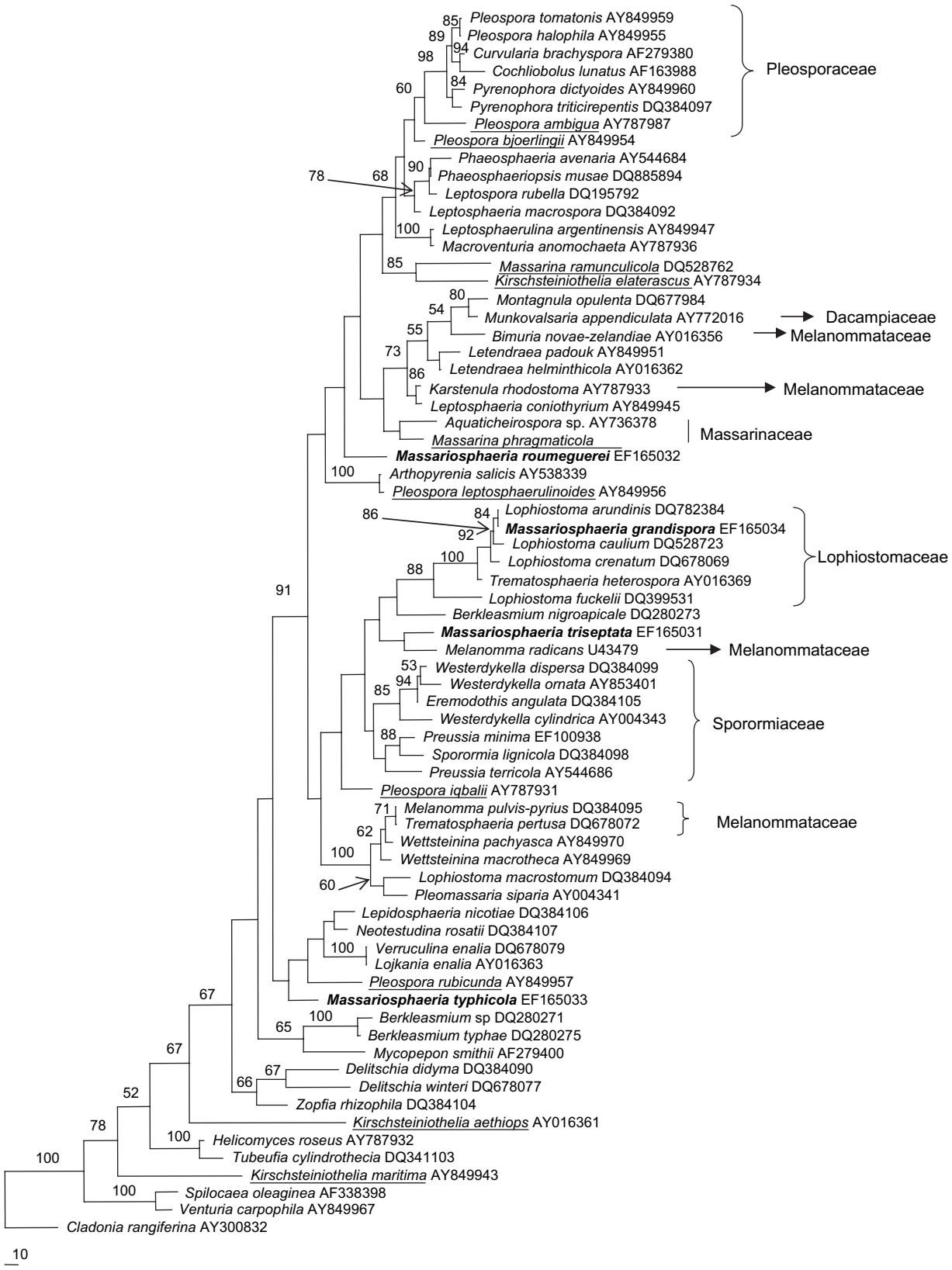

Fig 2 – Phylogram of one two most parsimonious tree based on 28S rDNA sequences. Data were analysed with random addition sequence, WP and treating gaps as missing state. Values above the branches are parsimony BS (equal or above 50 %). The tree is rooted with *Cladonia rangiferina*. Genera that are underlined are those that have been shown to be polyphyletic.

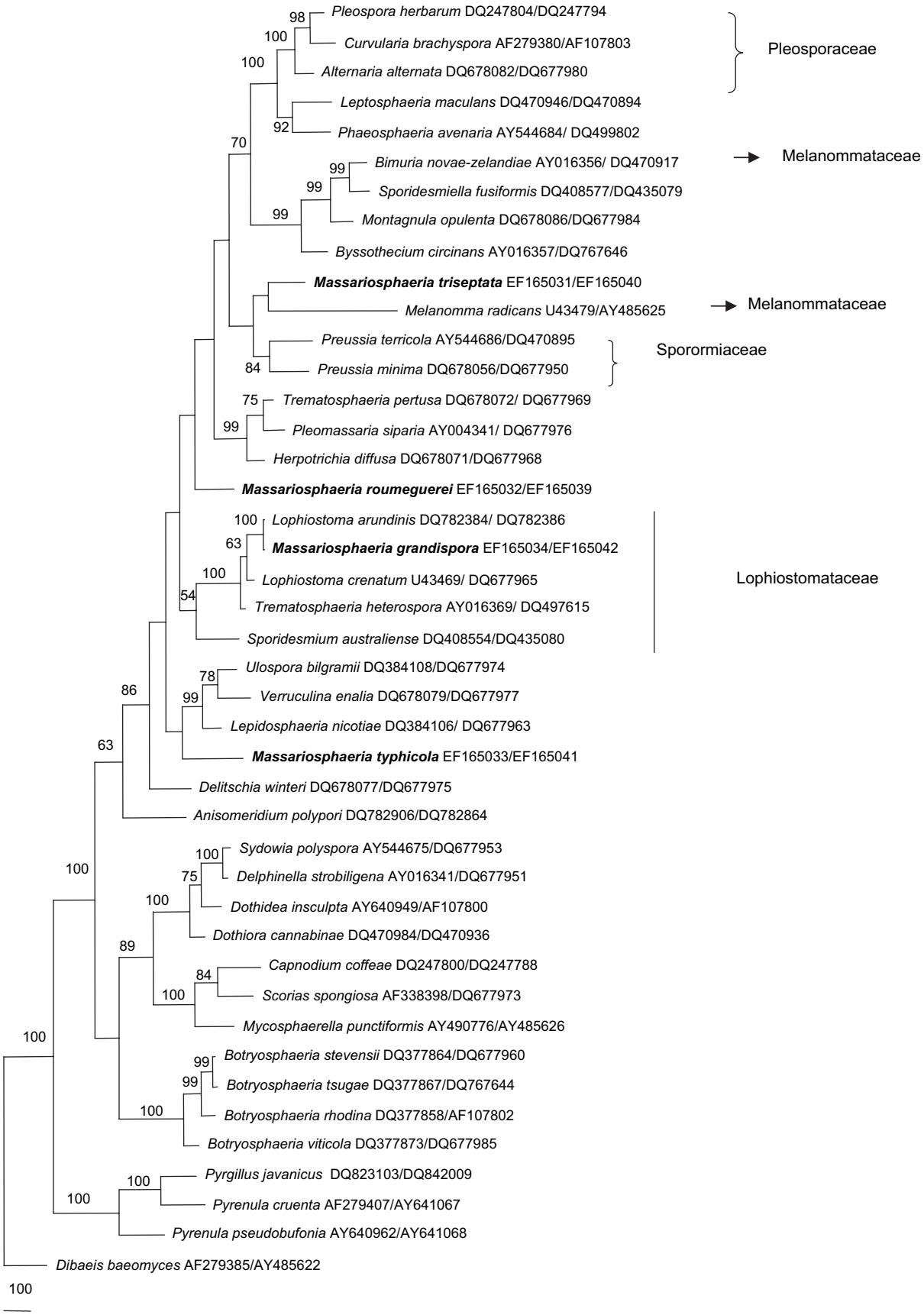

Fig 3 – Phylogram of the most parsimonious tree based on combined 28S nrDNA and RPB2 gene sequences. Data were analysed with random addition sequence,WP and treating gaps as missing state. Values above the branches are parsimony BS (equal or above 50 %). The tree is rooted with *Dibaeis baeomyces*.

respectively (e.g Lumbsch & Lindemuth 2001; Kruys *et al.* 2006), except *Letendraea*, which has been excluded from the *Tubeufiaceae* (Kodsueb *et al.* 2006b). Despite this, and given that the systematics of related genera are still in a transitional stage, it would be inappropriate to confidently accommodate the type species of *Massariosphaeria* in a particular loculoascomyceteous family. Barr (1989, 1992) considered that *Massariosphaeria* (and its type *M. phaeospora* transferred to *Chaetomastia*) should be classified in the *Dacampiaceae*. The type of *Chaetomastia* has tetrasporous asci and dark-brown 3-septate ascospores and differs in form from the species Crivelli (1983) placed in *Massariosphaeria*. Phylogenetic data in this study do not support this classification and no close affinities were found with taxa of the *Dacampiaceae* such as *Munkovalsaria* and *Polycoccum* (Fig 2). *Polycoccum* was not included in this study as we found that it was not related to any *Pleosporales* or *Dothideales*. However, the reliability of the 28S rDNA sequence of *P. vermicularium* deposited in GenBank (AY961601), needs to be verified. Parsimony analyses indicate that *Munkovalsaria appendiculata* is more related to *Montagnula opulenta* (*Montagnulaceae*) and *Bimuria novae-zelandiae* (*Melanommataceae*) and not *Massariosphaeria* species. The genus *Munkovalsaria,* with *M. donacina* as the type species, was established by Aptroot (1995) for bitunicate fungi with a black stroma that forms a clypeus around the ostiole; cylindrical asci with eight ascospores in one row (IKI negative); reddish to deep brown ascospores that are 1-euseptate, surrounded by a thin gelatinous sheath but without germ pore or slit. The *Dacampiaceae* are not very strongly characterized morphologically anyway, the main characters being the multilayered, rather soft ascoma walls and the relatively wide ostioles. It may turn out to be polyphyletic, but few sequences are available for any phylogenetic comparison.

Our studies provide some insights into the relationships of *M. typhicola*. However, in most phylogenies (except 18S rDNA), the association of this species with other taxa was not statistically supported and there are uncertainties with respects to its proper phylogenetic connections. 18S rDNA phylogeny (Fig 1) reveals a close relationship between *M. typhicola* and *Trematosphaeria hydrela* (*Melanommataceae*) with high statistical confidence. Although the latter is currently a melanommataceous loculoascomycete, its affinities with known bitunicate genera remains unclear, and any sister relationship lacks support. Similar findings were reported by Liew *et al.* (2000) and Inderbitzin *et al.* (2002). A close affinity between *M. typhicola* and *Trematosphaeria hydrela* is not surprising. It is probably significant that the ascomatal wall surface of *M. typhicola* described by Shoemaker & Babcock (1989) as deviating, *viz.* with radiating cells of *textura prismatica*. Similar structures can also be observed in *Trematosphaeria hydrela*. Due to unavailability of other DNA sequences from *T. hydrela* other gene phylogenies (in this case, 28S rDNA and RPB2) indicate that *M. typhicola* is an independent lineage basal to other fungi with bitunicate asci whose familial placement is still not fully resolved (Kruys *et al.* 2006). *M. typhicola* was considered to be a species of *Chaetomastia* (*Dacampiaceae*) by Barr (1989) but this familial placement could not be confirmed here.

Another particular feature in our gene phylogenies is the evolutionary relationships of *M. triseptata*. This taxon is nested in between the *Sporormiaceae* (Fig 1) and constitutes an unsupported sister relationship to *Berkleasmium* and *Melanomma radicans*. In contrast, 28S rDNA and combined 28S + RPB2 gene phylogenies (Figs 2 and 3, respectively) indicate a close association to *Melanomma radicans*. Although weakly supported, the later would make sense in light of morphological similarities, as the ascospores are not typical for *Massariosphaeria*, for instance because they are only three-septate. Although the 18S rDNA gene tree resulted in a slightly different topological arrangement, incongruence is not statistically supported. We believe that given the number of available taxa analysed and number of parsimony informative characters from the datasets, the 28S rDNA (Fig 2) and the combined 28S + RPB2 phylogenies (Fig 3) is more likely to represent the true phylogeny. Molecular data provide evidence to support that *M. roumeguerei* is not phylogenetically related to other melanommataceous genera. There were no topological conflicts, but phylogenies are incongruent with morphological-based classification schemes. We are unable to fully resolve these relationships, and the results obtained do not provide any insights into current species separation based on morphologies.

All gene phylogenies differ largely from the conventional classification system of *Massariosphaeria*, which recognizes that the genus belongs to the *Lophiostomaceae* (Eriksson & Hawksworth 1991; Kirk *et al.* 2001). Surprisingly only one species, *M. grandispora*, could be confidently assigned to the *Lophiostomaceae*. There is a close phylogenetic relationship between *M. grandispora, Lophiostoma caulium, L. crenatum, Massarina australianensis, M. bipolaris,* and *Trematosphaeria heterospora* (Fig 1). It should be pointed out here that *T. heterospora* has been referred to the *Melanommataceae* (Lumbsch & Lindemuth 2001) but in other papers in preparation, we found that it would be more appropriate to accommodate this taxon in the *Lophiostomaceae*, which also makes sense morphologically as it has the sideways compressed ostioles characteristic of most *Lophiostoma* species. Morphologically, *M. grandispora* possesses cellular pseudoparaphyses, ascospores that are 9–12-septate and hyaline with a wide sheath. It also has the sideways compressed ostioles characteristic of most *Lophiostoma* species. It has generally been classified as *Lophiotrema grandispora* (Shoemaker & Babcock 1989) but was reassigned to *Massariosphaeria* based on the presence of multitransseptate ascospores and ascomata that are composed of thin-walled pale brown cells around the ostiole (Tanaka & Harada 2003). Our results support the classification of *Massariosphaeria grandispora* in a genus of the *Lophiostomaceae*, possibly in *Lophiotrema*.

Of special interest was the sister group relationship of *M. triseptata* and *Melanomma radicans* to *Berkleasmium micronesmium* and *B. nigroapicale* (Figs 1 and 2). The latter are hyphomycetous species, which have been shown to belong to the *Pleosporales*, in particular to the family *Sporormiaceae* (Pinnoi *et al.* 2007). Despite a close relationship between these taxa, there is little chance for a close genetic connection between them, as *M. triseptata* is known to produce mostly *Aposphaeria*-like anamorphs (Leuchtmann 1984; Tanaka & Harada 2004). *Berkleasmium* is a rather small group of hyphomycetes that includes fungi characterised by macronematous conidiophores that are mostly unbranched and closely packed in a sporodochium, while spores are solitary, brown, muriform, clavate or oblong, with rounded ends or irregular, and often have a protruding hilum (Bussaban *et al.* 2003;Ellis 1971; Zhao & Liu, in press). No teleomorphs are known for *Berkleasmium,* but phylogenies have indicated a close affinity of

B. nigroapicale to *Westerdykella cylindrica* and *Sporormia australis*, which are both currently in the family *Sporormiaceae* (Pinnoi *et al.* 2007). Similar results are obtained here but a close relationship between *Berkleasmium, Melanomma radicans,* and *M. triseptata* is unexpected. However, any relationship between them is possibly largely dependent upon taxon sampling. There are simply inadequate DNA sequences available from known databases to further elucidate the phylogeny of *M. triseptata*. Conversely, *Aposphaeria* is a coelomycetous genus with globose conidomata, conidiophores that are cylindrical to lageniform and formed all around the locular cavity and conidia that are ellipsoid, hyaline, and aseptate (Leuchtmann 1984). The latter has been suggested to represent anamorphs of *Melanomma*, but in this study, no phylogenetic associations were observed between *Massariosphaeria* species and *Melanomma* species such as *Melanomma pulvispyrius* and *Melanomma sanguinarium* (Fig 2). Therefore, any proper phylogenetic conclusions regarding relationships of *Aposphaeria, Massariosphaeria* and *Melanomma* should be interpreted with caution and future studies should incorporate more taxa, including the anamorphic types. We would also advocate the use of alternative phlylogenetic markers, if possible, as either rDNA or RPB2 DNA sequence data reported lack of phylogenetic signal to fully resolve evolutionary relatedness.

Molecular data indicate that the morphologically well-characterised genus *Massariosphaeria* is polyphyletic. Further members of the *Melanommataceae* are interspersed in different clades among the *Pleosporales*. Classification among many loculoascomycete taxa has always been subjected to controversy because of overlapping morphological characters used to delimit genera and species. The polyphyletic nature of *Massariosphaeria* supports previous speculation that ascomatal and ascospore morphologies have undergone convergent evolution among the *Pleosporales* (e.g. Berbee 1996; Kodsueb *et al.* 2007; Lumbsch & Lindemuth 2001; Schoch *et al.* 2006). A number of loculoascomycetes genera such as *Kirschsteiniothelia, Massarina*, *Melanomma, Pleospora,* and *Trematosphaeria* included in this study are shown to be polyphyletic. Therefore, a similar evolutionary scenario for *Massariosphaeria* is not surprising. The familial placement and phylogenetic relationships of *Massariosphaeria* taxa studied were also not fully resolved, but there is sufficient evidence to justify its placement within the *Pleosporales*. However, its familial position is still partially resolved and precarious because of: (1) inadequate taxa available for analytical comparison; (2) its affinities to other taxa whose placement are obscure and presumably polyphyletic; and (3) lack of statistical support to conclusively determine any appropriate phylogenetic connection.

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