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Ranking higher taxa using divergence times: a case study in Dothideomycetes

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Abstract The current classification system for the recognition of taxonomic ranks among fungi, especially at high-ranking level, is subjective. With the development of molecular approaches and the availability of fossil calibration data, the use of divergence times as a universally standardized criterion for ranking taxa has now become possible. We can therefore date the origin of Ascomycota lineages by using molecular clock methods and establish the divergence times for the orders and families of Dothideomycetes. We chose Dothideomycetes, the largest class of the phylum Ascomycota, which contains 32 orders, to establish ages at which points orders have split; and *Pleosporales*, the largest order of Dothideomycetes with 55 families, to establish family divergence times. We have assembled a multi-gene data set (LSU, SSU, TEF1 and RPB2) from 391 taxa representing most family groups of Dothideomycetes and utilized fossil calibration points solely from within the ascomycetes and a Bayesian approach to establish divergence times of Dothideomycetes lineages. Two separated datasets were analysed: (i) 272 taxa representing 32 orders of Dothideomycetes were included for the order level analysis, and (ii) 191 taxa representing 55 families of *Pleosporales* were included for the family level analysis. Our results indicate that divergence times (crown age) for most orders (20 out of 32, or 63%) are between 100 and 220 Mya, while divergence times for most families (39 out of 55, or 71%) are between 20 and 100 Mya. We believe that divergence times can provide additional evidence to support establishment of higher level taxa, such as families, orders and classes. Taking advantage of this added approach, we can strive towards establishing a standardized taxonomic system both within and outside Fungi. In this study we found that molecular dating coupled with phylogenetic inferences provides no support for the taxonomic status of two currently recognized orders, namely *Bezerromycetales* and *Wiesneriomycetales* and these are treated as synonyms of

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Tubeufiales while *Asterotexiales* is treated as a synonym of *Asterinales*. In addition, we provide an updated phylogenetic assessment of Dothideomycetes previously published as the *Families of Dothideomycetes* in 2013 with a further ten orders and 35 families.

Introduction

Attempts to classify fungi at the higher levels have always been subjective (Liu et al. 2016; Hyde et al. 2017), especially when based solely on morphology and in some cases has resulted in unnecessary personalized conflicts in the literature. Mycologists have made attempts to establish more reliable ways to resolve taxa at the ordinal, familial and generic, as well as species levels (Liu et al. 2016; Jeewon and Hyde 2016; Divakar et al. 2017). Lumbsch and Huhndorf (2010) provided *Outline of Ascomycota*, which took into account molecular phylogenies, and this was followed by outlines of individual classes which also incorporated both morphology and phylogenetic data (e.g. *Families of Dothideomycetes*, Hyde et al. 2013; *Outline of Dothideomycetes*, Wijayawardene et al. 2014; *Outline of Sordariomycetes*, Maharachchikumbura et al. 2015; *Families of Sordariomycetes*, Maharachchikumbura et al. 2016). However, there have been many disagreements at various taxonomic levels, as the use of phylogenetic data may also be subjective and depends on the taxa analysed. For example, based on molecular data, Crous et al. (2017) accepted nine families in *Botryosphaeriales*, while Liu et al. (2016) suggested that this is excessive, as they considered that three of the monotypic families could in fact be treated as genera. This clearly indicates that assigning taxa to specific ranks can largely be subjected to personal interpretations. Therefore, further evidence is needed to try to resolve these problematic groups. Hyde et al. (2017) have reviewed the grouping and ranking of fungi and their classification, and we refer to their review in this paper and do not discuss this further.

The use of divergence times has recently been applied in fungi to support the ranking of taxa, particularly at the higher levels. Zhao et al. (2016) used divergence times to resolve subgenera and sections in the large genus *Agaricus*. Mapook et al. (2016) used it to support the introduction of the new family *Palawaniaceae*, while Phukhamsakda et al. (2016) provided additional support for the introduction of the family *Longipedicellataceae* with evidence from divergence times. Samarakoon et al. (2016) reassessed the *Xylariales* and reported that the previously discarded *Amphisphaeriales* evolved between 147 and 168 Mya and

should be reinstated as an order. This dating approach is relatively new and is only now being applied to provide additional evidence for ranking taxa (Pérez-Ortega et al. 2016; Zhao et al. 2016, 2017; Hyde et al. 2017; Divakar et al. 2017).

Dothideomycetes is the largest class of Ascomycota, with an estimated 19,000 species (Kirk et al. 2008). The class has a worldwide distribution, ranging from tropical rainforests, to temperate broad-leaved forests, and to deserts, and life modes that are saprobic, pathogenic or endophytic, and they can be found in terrestrial and aquatic habitats (Crous et al. 2006, Boehm et al. 2009; Schoch et al. 2009a; Suetrong et al. 2009; Tanaka et al. 2009; Zhang et al. 2009; Liu et al. 2011, 2012; Pérez-Ortega et al. 2014, 2016; Phookamsak et al. 2014; Jaklitsch et al. 2015; Raja et al. 2015; Tanaka et al. 2015; Bezerra et al. 2017). Despite some recent taxonomic controversies, Dothideomycetes is an ideal group to study and apply divergence time estimates for higher rankings for the following reasons: (1) a broad taxon sampling is possible; (2) classification and phylogeny have been well investigated (Boehm et al. 2009; Crous et al. 2009; Mugambi and Huhndorf 2009; Nelsen et al. 2009; Shearer et al. 2009; Schoch et al. 2009a; Liu et al. 2012; Hyde et al. 2013; Wijayawardene et al. 2014; Jaklitsch et al. 2015); (3) sufficient DNA sequence data are available from different gene regions to allow phylogenetic inferences to be made (Boehm et al. 2009; Crous et al. 2009; Mugambi and Huhndorf 2009; Nelsen et al. 2009; Shearer et al. 2009; Schoch et al. 2009a; Suetrong et al. 2009; Liu et al. 2012, 2014, 2015; Zhang et al. 2012; Ariyawansa et al. 2014, 2015; Boonmee et al. 2014, 2016; Jaklitsch et al. 2015; Raja et al. 2015; Tanaka et al. 2015); (4) morphological classification criteria are well understood (Crous et al. 2006, 2009; Mugambi and Huhndorf 2009; Nelsen et al. 2009; Shearer et al. 2009; Suetrong et al. 2009; Zhang et al. 2012; Hyde et al. 2013); (5) an increase in the number of species being described and establishment of new orders, families and genera that might be subjected to differences in perceptions (Boehm et al. 2009; Tanaka et al. 2009, 2015; Ariyawansa et al. 2014, 2015; Boonmee et al. 2014, 2016; Hongsanan et al. 2014; Liu et al. 2014, 2015; Pérez-Ortega et al. 2014, 2016; Phookamsak et al. 2014, 2015; Jaklitsch et al. 2015; Raja et al. 2015; Thambugala et al. 2015; Tian et al. 2015; Li et al. 2016; Hyde et al. 2016; Bezerra et al. 2017).

In this paper, we provide an updated multi-locus phylogeny of the class Dothideomycetes with sufficient number of representatives of each order and for the order *Pleosporales* representatives of its families to unravel evolutionary relationships and assess the reliability of molecular dating in assigning taxa to ordinal and familial ranks. In addition, interordinal and interfamilial phylogenetic relationships

were investigated to clarify taxonomic ambiguities. Analyses including a broad taxon sampling of the Dothideomycetes covering a wide range of taxa were performed to obtain a reliable time scale that can be used to establish ordinal and familial ranks. The applicability of using divergence time estimates in classification is discussed. We also discuss some cases where there are conflicts between phylogeny and divergence times.

Materials and methods

We use small subunits ribosomal RNA (SSU), large subunit ribosomal RNA (LSU), the translation elongation factor-1 alpha (TEF1) and the second largest subunit of RNA polymerase II (RPB2) were used as implemented in a previous study by Hyde et al. (2013). Sequences were obtained from GenBank following mostly previous publications (e.g. Schoch et al. 2006, 2009a; Suetrong et al. 2009; Zhang et al. 2012; Liu et al. 2012, 2014; Hyde et al. 2013, Pérez-Ortega et al. 2014, 2016; Jaklitsch et al. 2015; Raja et al. 2015; Tanaka et al. 2015; Tian et al. 2015; Bezerra et al. 2017) and these are listed in Supplementary Table 1.

Datasets for each gene (SSU, LSU, TEF1 and RPB2) were aligned separately with MAFFT version 6 (Katoh and Toh 2008) with subsequent manual adjustment in BioEdit 5.0.9 (Hall 1999). The software package jModeltest2.1.1 was used to select the best-fitting models of nucleotide substitution for each gene. The Bayesian information criterion supported the GTR + G + I model as the best fit for SSU, LSU and TEF1 and GTR + G for RPB2. Topological congruence of the four datasets was checked by visual comparison of phylogenetic trees obtained from maximum likelihood-based analysis with RAxML (Stamatakis et al. 2008), and a general time-reversible model (GTR) was applied with a discrete gamma distribution and four rate classes. Fifty thorough ML tree searches were done in RAxML v.7.2.7 under the same model. One thousand non-parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution. And all genes were subsequently combined in a super matrix using BioEdit 5.0.9. Bayesian analyses were performed by using Markov chain Monte Carlo (MCMC) sampling in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003, Zhaxybayeva and Gogarten 2002) to generate a reasonable starting tree for subsequent analyses of divergence date estimates in BEAST. Four simultaneous Markov chains were run for 10,000,000 generations and trees were sampled every 1000th generation, thus 10,000 trees were obtained. The suitable burn-in phases were determined by inspecting likelihoods and parameters in Tracer version 1.6 (Rambaut et al. 2013). Based on the tracer analysis, the first 1000

trees representing 10%, were discarded as the burn-in phase in the analysis. The remaining trees were used to calculate posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01).

Divergence time analyses were estimated using BEAST v1.8 (Drummond et al. 2012). Aligned sequence data were partitioned separately for each LSU, SSU, TEF1 and RPB2 data set, and loaded to prepare an XML file constructed with BEAUTI v1.8. Clock and substitution models were set to be unlinked (independently estimated for each gene partition), while the tree prior parameters were set to be linked across partitions (concatenation). An uncorrelated relaxed clock model (Drummond et al. 2006) with a log-normal distribution of rates for each gene estimate was used for the analyses. We used a Yule tree prior, which assumes a constant speciation rate per lineage, and a randomly generated starting tree. The tree prior was shared by all tree models; this consisted of a birth/death incomplete sampling tree prior and was used to model the speciation of nodes in the topology with uniform prior on probability of splits and extinctions. To ensure congruence we carried out the analyses three times for 100 million generations each, and sampling parameters every 10,000 generations. Tracer v.1.6 was used to check the effective sample sizes (ESS), and acceptable values were higher than 200. After removal of a proportion of each run as burn-in the remaining trees were combined in LogCombiner 1.8.0. Maximum clade creditability (MCC) tree was given by summarized data and was estimated in TreeAnnotator 1.8.0, and then visualized using FigTree (Rambaut 2009).

Due to the limited and sporadic fossil records for fungi, it has been difficult to choose a reliable calibration point for the divergence time estimations of any fungal group. Previous molecular study on the porcini used the divergence between Basidiomycota and Ascomycota as a calibration (either 452 Mya according to Taylor and Berbee 2006 or 582 Mya according to Lücking et al. 2009), derived by different researchers using different methods but based on the same 400 million year old fossil, *Paleopyrenomycites devonicus*. The different estimates were due to the position of this fossil in different subphyla in Ascomycota since it became available and has been used in most fungal evolutionary studies (Heckman et al. 2001; Padovan et al. 2005; Taylor and Berbee 2006; Lücking et al. 2009). Lücking et al. (2009) provided a detailed discussion focusing on the placement of this fossil while recalibrating several earlier studies (Berbee and Taylor 1993; Doolittle et al. 1996; Redecker et al. 2000; Heckman et al. 2001; Padovan et al. 2005) by reassessing the systematic placement of *Paleopyrenomycites*. Several recent studies have widely used this fossil as the calibration point

(Gueidan et al. 2011; Prieto and Wedin 2013; Beimforde et al. 2014; Pérez-Ortega et al. 2016).

In this study, we used one calibration (A1): the divergence between Ascomycota and Basidiomycota, and a normal distribution was applied by setting the mean and the standard deviation to 582.5 and 50.15, respectively. To establish the influence of the calibration points on the results, a secondary calibration (A2) from the order *Capnodiales* with a constrained age of 100 Mya is used. The mean and the standard deviation were set to 100 and 150 respectively with representation from a fossil *Metacapnodiaceae* which is hypothesized to have given rise to the common ancestor of the order. All geological time intervals followed Gradstein and Ogg (2004). Mean node age, 95% highest posterior density (HPD) and posterior probability (pp) were mapped on the maximum clade credibility tree.

Results and discussion

Phylogenetic analyses

The combined LSU, SSU, TEF1 and RPB2 gene region data set consist of 391 taxa and 5099 unambiguously aligned sites, 1498 for the LSU, 1617 for the SSU, 856 for TEF1 and 1128 for the RPB2, with *Endogone pisiformis* (Zygomycota) as the outgroup taxon. Bayesian and ML analyses returned similar topologies with no significant conflicts. The maximum likelihood phylogenies obtained with RAxML (Figs. 1, 2) and BEAST analyses are generally congruent with results reported by other large scale phylogenies of Ascomycota e.g. James et al. (2006) and Schoch et al. (2009b), as well as the most recent broad scale phylogenetic studies of Dothideomycetes (Hyde et al. 2013; Hongsanan et al. 2015; Jaklitsch et al. 2015; Pérez-Ortega et al. 2016). The phylogenetic placement of Orbiliomycetes and Pezizomycetes support the statement that they are the two basal Pezizomycotina classes. Two sister clades were formed and named as the Eurotiomycetes-Lecanoromycetes and Leotiomycetes-Sordariomycetes clades. Phylogenetic relationships among the major groups of Dothideomycetes were recovered with high support and corroborate major classification schemes of previous studies (Schoch et al. 2009a; Zhang et al. 2012; Hyde et al. 2013; Jaklitsch et al. 2015). All accepted Dothideomycetes orders and *Pleosporales* families were monophyletic with the exception of *Asterinales* whose taxonomy is discussed later. The 364 strains of Dothideomycetes representing 32 orders, and 114 families segregate into two Dothideomycetes subclasses (Figs. 1, 2) as previously acknowledged based on the presence or absence of pseudoparaphyses (Schoch et al. 2006). The subclass

Fig. 1 The best scoring RAxML Dothideomycetes tree (compress ▶ overview tree) from 391 taxa based on a combined dataset of LSU, SSU, TEF1 and RPB2 sequences with all lineages collapsed to family level where possible. The tree was rooted with *Endogone pisiformis*

Pleosporomycetidae includes *Pleosporales*, *Mytilinidiales*, and *Hysteriales* and is similar to conclusions from previous publications (Schoch et al. 2009a; Boehm et al. 2009; Shearer et al. 2009; Suetrong et al. 2009; Hyde et al. 2013; Wijayawardene et al. 2014). The subclass Dothideomycetidae comprises *Capnodiales*, *Dothideales* and *Myriangiales* (Schoch et al. 2006, 2009a; Boehm et al. 2009; Zhang et al. 2009; Hyde et al. 2013). *Acrospermales*, *Dyfrolomycetales* and *Strigulales* formed a stable monotypic clade both in the RAxML phylogenetic trees (Figs. 1, 2) and MCC tree (Fig. 3). *Natipusillales* and *Zeloasperisporiales* also formed a stable monotypic clade even through their habitats are quite different; *Natipusillales* is a group of freshwater taxa while *Zeloasperisporiales* is a group of epiphytic taxa. Taxa of *Asterinales* are segregated into two different clades, *Asterinaceae* sensu stricto and *Asterinaceae* sensu lato, strains of *Asterinaceae* sensu stricto are sister to the *Asterotexiaceae* (*Asterotexiales*) and close to *Jahnulales*. The species of *Asterinaceae* sensu lato clustered together with *Cladoriellaceae* (*Cladoriellales*), and a new order is probably needed for this group.

An updated treatment of orders of Dothideomycetes and families of *Pleosporales* which takes into account divergence times is given in Table 1.

Changes in the classification of Dothideomycetes

We present an updated phylogeny of Dothideomycetes including all the accepted orders of which ten were recently established (Pérez-Ortega et al. 2014; Guatimosim et al. 2015; Hongsanan et al. 2015; Jaklitsch et al. 2015; Raja et al. 2015; Pérez-Ortega et al. 2016; Bezerra et al. 2017) since Hyde et al. (2013). The phylogeny presented herein supports 32 orders and 114 families and this includes the 22 orders and 64 families accepted in Hyde et al. (2013).

Divergence times estimates

In the divergence time analysis, although most parameters rapidly reached stationary, the first half of sampled trees (10,000 trees) were considered as part of the burn-in phase and further excluded. The 10,000 remaining trees were summarized in TreeAnnotator. The maximum clade credibility (MCC) tree with divergence estimates obtained through BEAST was topologically similar to those recovered by Bayesian and ML procedures regarding most of the major lineages within Dothideomycetes. The resulting
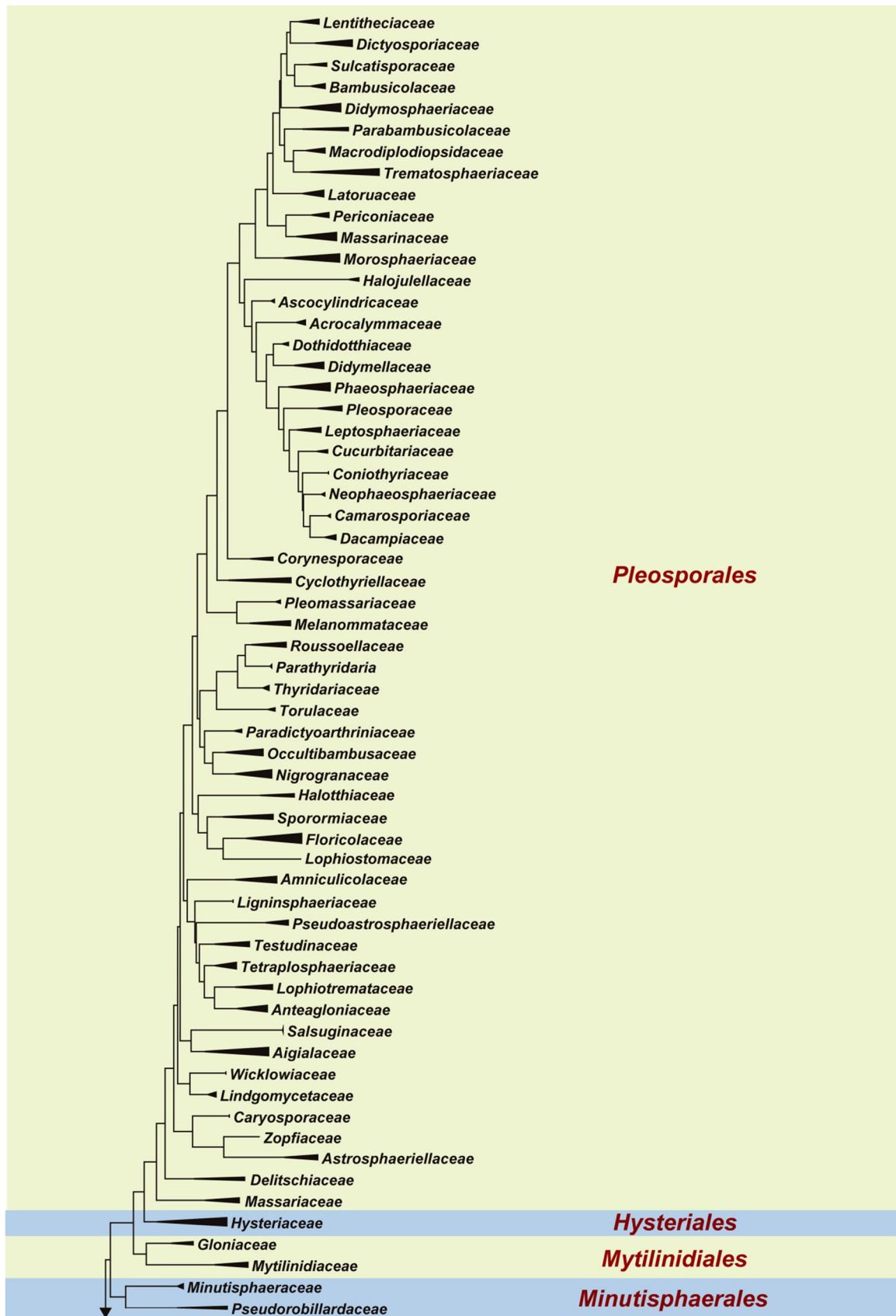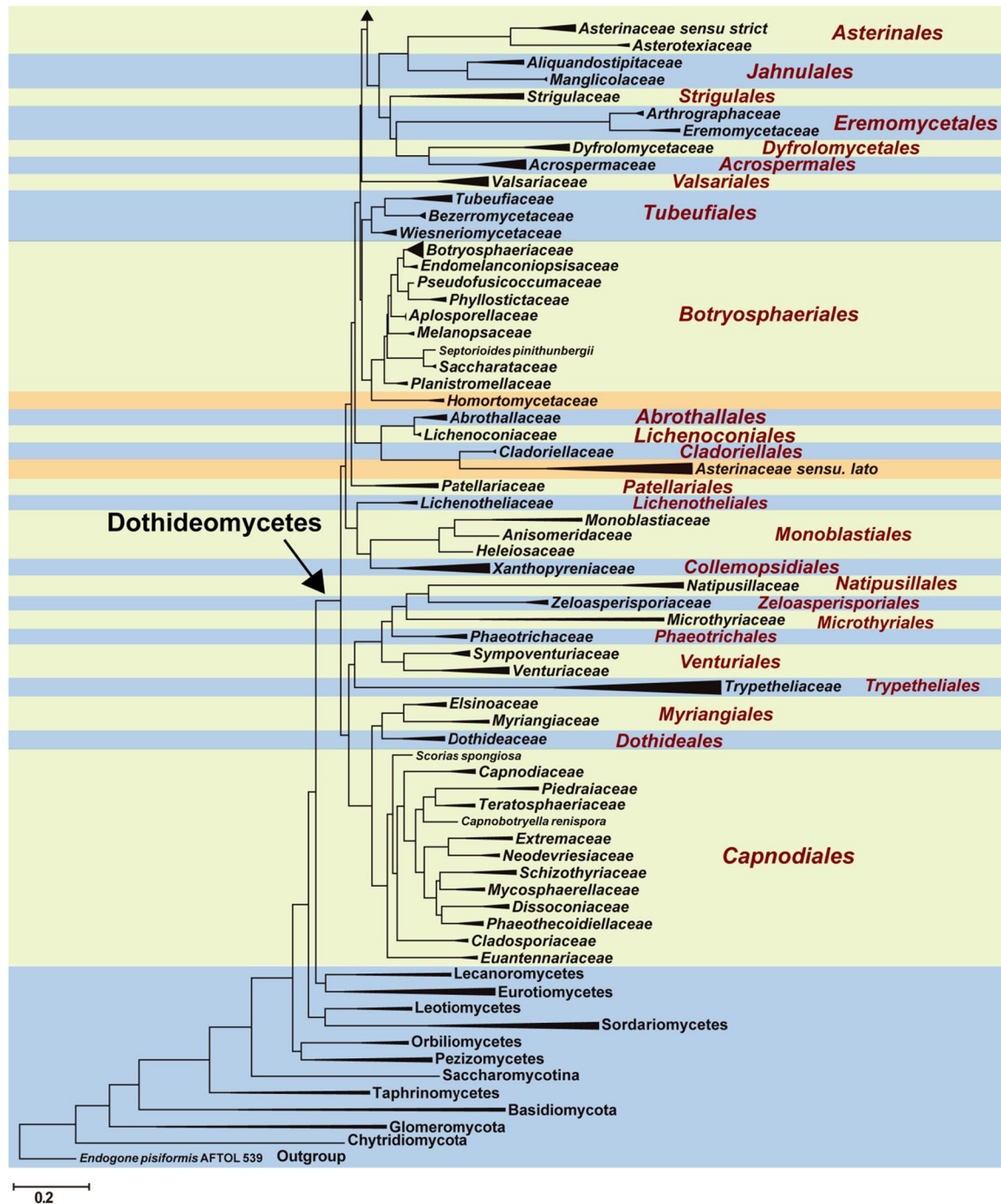
Fig. 1 continued

chronograms are shown in Figs. 3, 4 with bars representing 95% confidence intervals for each node. The mean dates for the divergence of Dothideomycetes recovered mostly agreed with reported estimates (Prieto and Wedin 2013; Beimforde et al. 2014, Hyde et al. 2017) and confidence intervals largely overlapped, and the age for the crown of Dothideomycetes is 366 (400–492) Mya.

Generally, two particular informative issues are discussed and indicated in the maximum clade credibility (MCC) tree; one is the stem node age and the other is crown node age. The crown group refers to a collection of species consisting of the living representatives of the collection together with their ancestors back to their most recent common ancestor as well as all of that ancestor's
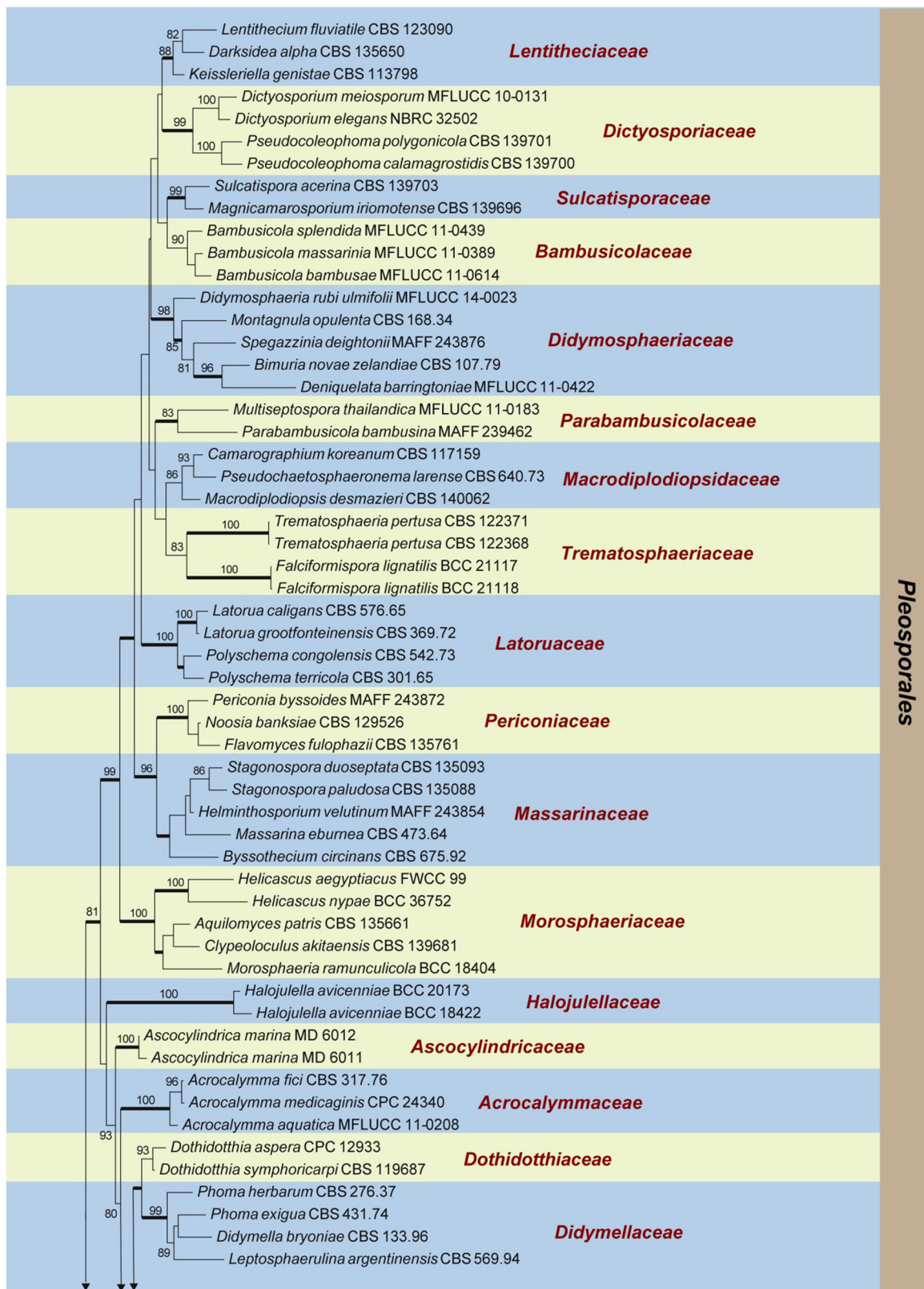
Fig. 2 The best scoring RAxML Dothideomycetes tree from 391 taxa based on a combined dataset of LSU, SSU, TEF1 and RPB2 sequences. Bootstrap support values for maximum likelihood (ML) >75% are given above the nodes; branches with Bayesian posterior probabilities (PP) above 0.95 are in bold. The original isolate numbers are given after the species names. The tree was rooted with *Endogone pisiformis*
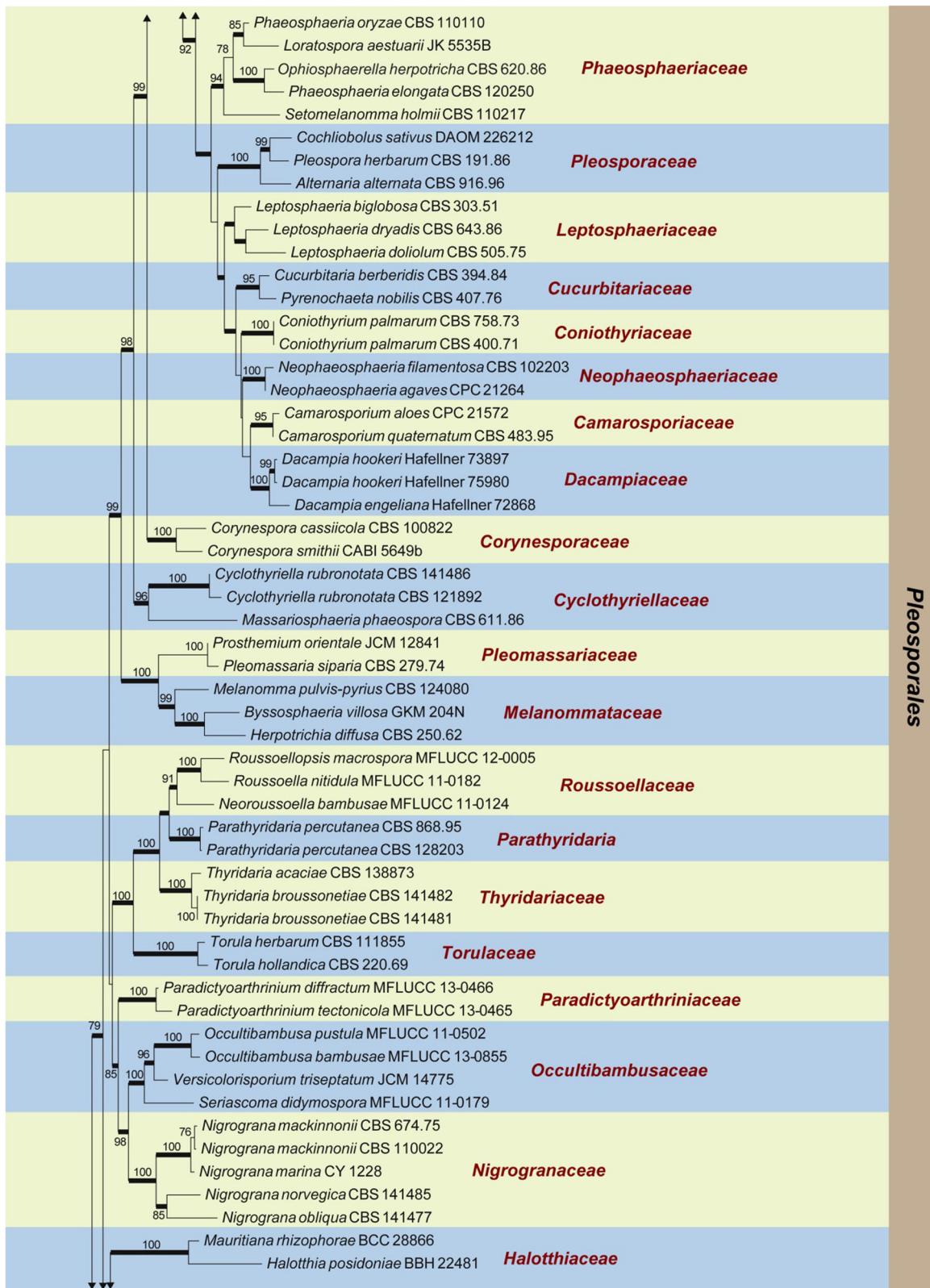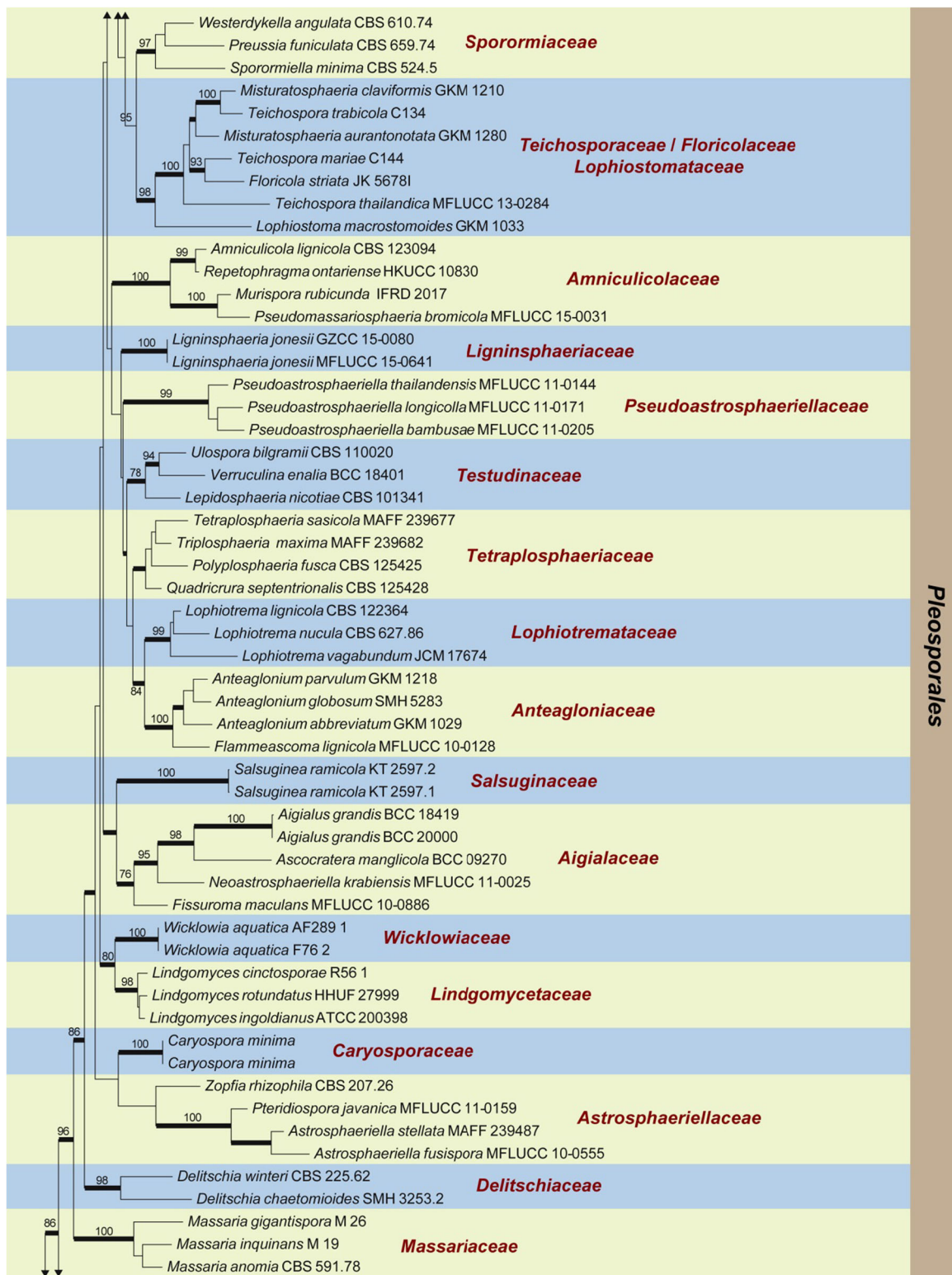
Fig. 2 continued

Fig. 2 continued

descendants (WIKIPEDIA: https://en.wikipedia.org/wiki/Crown_group). The stem group refers to those taxa descended from the point where an ancestral taxon split into two sister groups to the point at which a further split gave rise to an extant crown group. Therefore, the stem age of any given groups of taxa is always older than the crown age
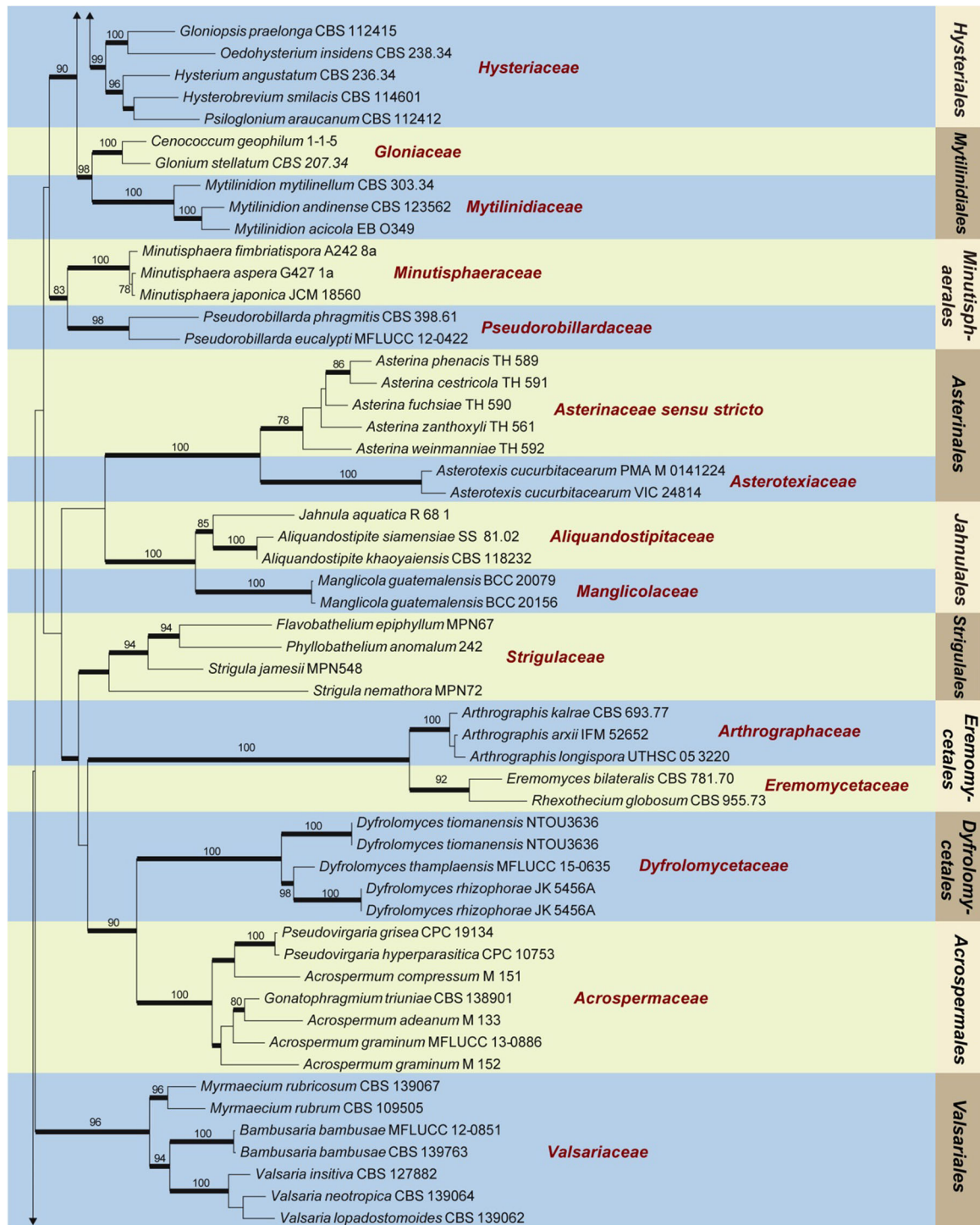
Fig. 2 continued

(Zhao et al. 2016). The species richness of the group, the net diversification rate, the timescale, and model setup can affect the length of branch between stem ancestor and crown clades (McPeek and Brown 2007; Zhao et al. 2016). As a case study, we use the crown age as priority with the stem age as further support, wherever the recommendations

are made. Both the crown age and stem age are given and considered in this study.

In the maximum clade credibility (MCC) trees in Figs. 3 and 4, the various ages of different orders and families are shown. The age for the crown of most Dothideomycetes orders (20 out of 32, or 63%) are between 100 to 220 Mya,
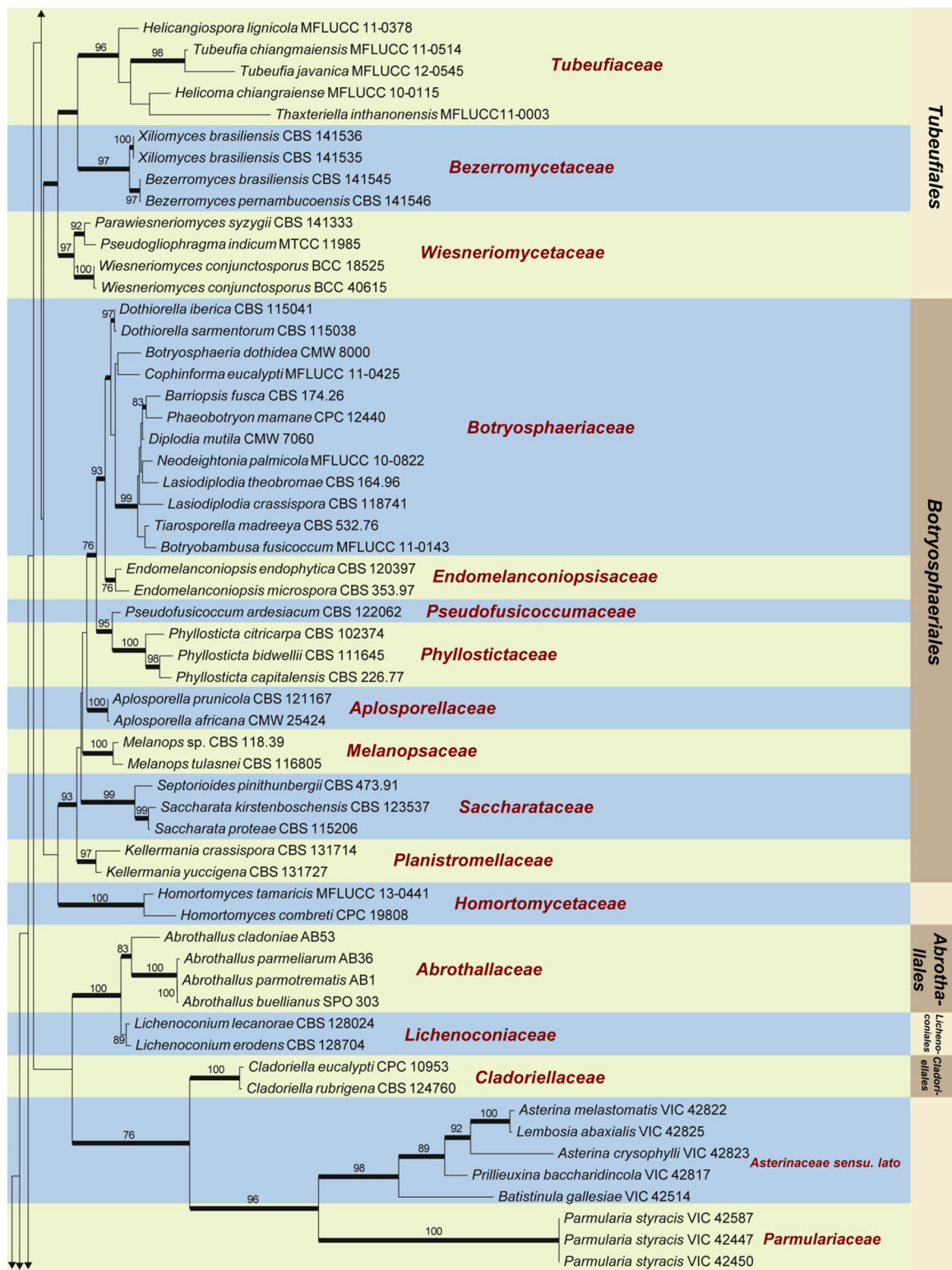
Fig. 2 continued

while it is younger for the orders *Abrothallales* (39 Mya), *Asterotexiales* (23 Mya), *Bezerromycetales* (14 Mya), *Cladoriellales* (6 Mya), *Lichenoconiales* (9 Mya), *Lichenotheliales* (46 Mya), *Wiesneriomycetales* (40 Mya) and *Zeloasperisporiales* (54 Mya), and the divergence times (stem age) of most orders are between 130 and
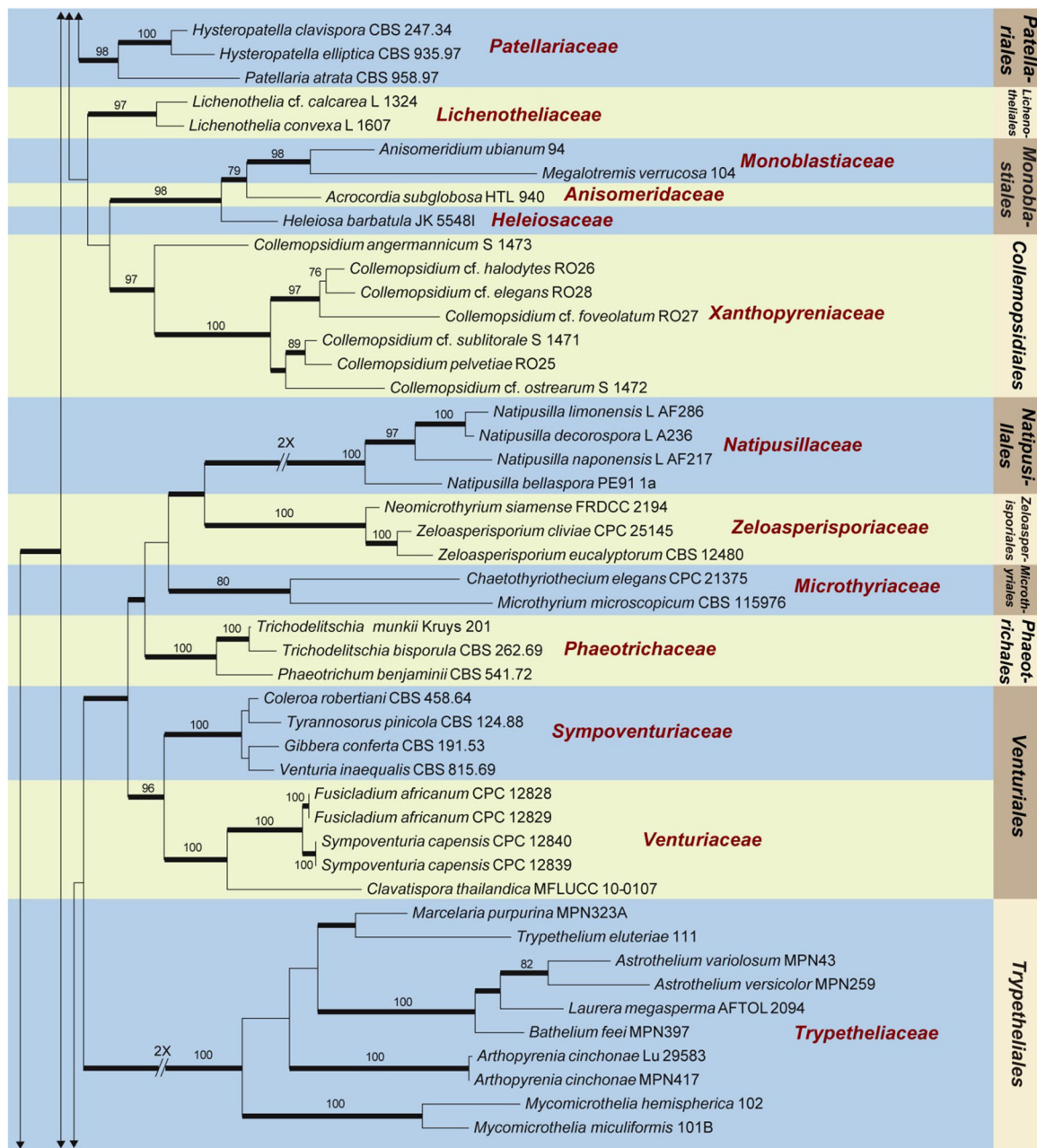
Fig. 2 continued

310 Mya. The detailed divergence time (stem versus crown age) of the orders are listed in Table 1. The age for the crown of most *Pleosporales* families (39 out of 55, or 71%) are between 20 and 100 Mya. The detailed divergence times (stem versus crown age) of the families of *Pleosporales* are listed in Table 2. Divergence times can therefore provide additional evidence for the status of new higher level taxa, or when such taxa e.g. families, orders and classes, are established. Below we recommend how to use divergence times to recognize subclasses, orders and families in the class Dothideomycetes. We suggest that

these times should be used as additional evidence for the introduction or acceptance of families, orders and subclasses.

Recommendations for using divergence times to recognize subclasses, orders and families in the class Dothideomycetes

1. Subclasses should have evolved in the range of 235 and 250 Mya (crown age) and 260–322 Mya (stem age).

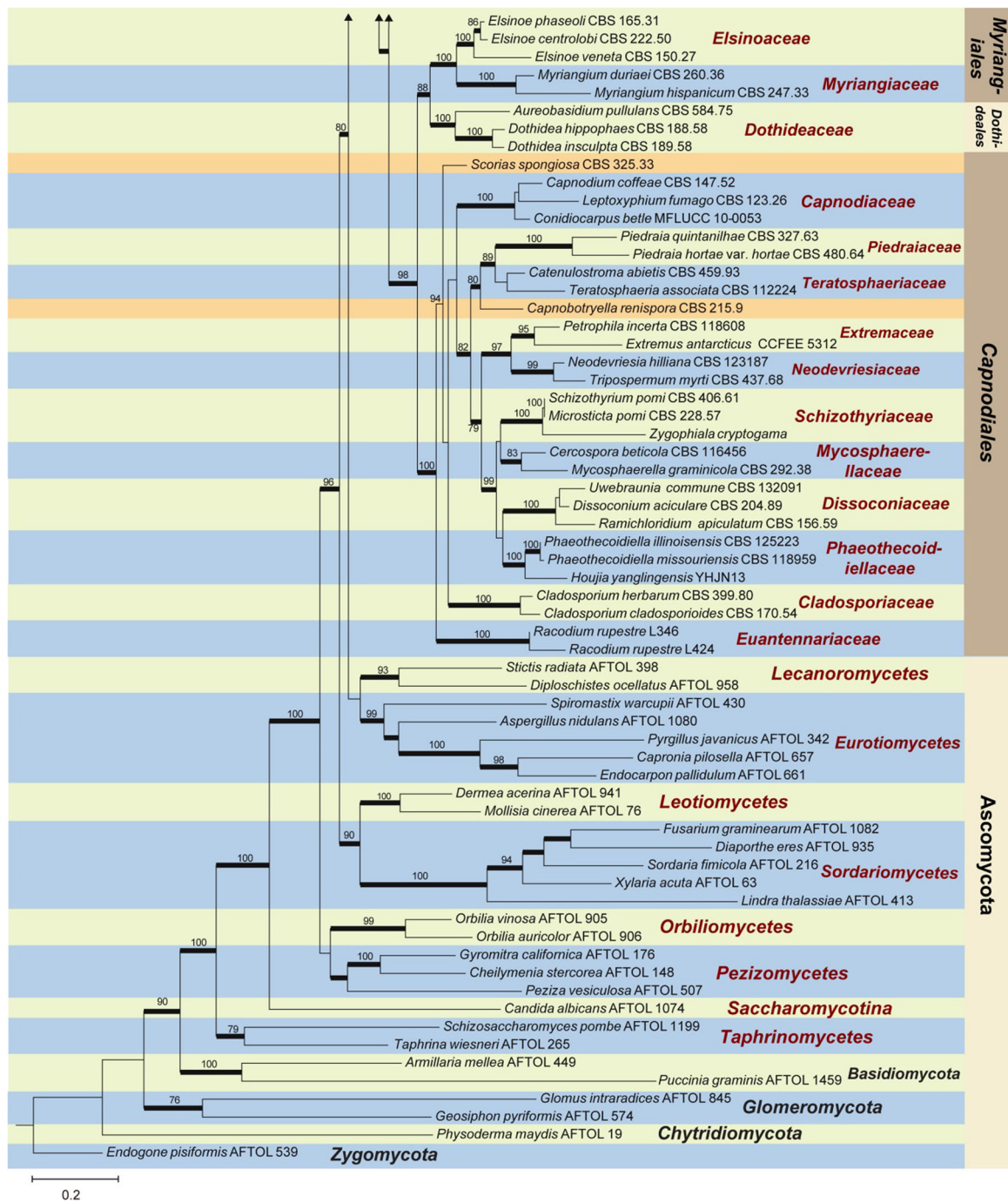**Fig. 2** continued

2. Order should have evolved in the range between 100 and 220 Mya (crown age) and 130 and 310 Mya (stem age).

3. Families should have evolved in the range between 20 and 100 Mya (crown age).

Taxonomic changes

In this section, we make changes to the higher ranking of Dothideomycetes based on our additional evidence from the maximum clade credibility (MCC) trees. We also

discuss cases where DNA sequence data can show how the present understanding of some families and orders may be incorrect.

Asterinales M.E. Barr ex D. Hawksw. & O.E. Erikss., Syst. Ascom. 5(1): 177 (1986)

Synonyms

Asterotexiales Firmino, O.L. Pereira & Crous [as 'Asterotexiales'], Persoonia 35: 238 (2015)

The family *Asterotexiaceae* was introduced for the monotypic genus *Asterotexis* with *A. cucurbitacearum* as the type species (Guatimosim et al. 2015), and the order *Asterotexiales* was established for this single family. Guatimosim et al. (2015) reported a close phylogenetic relationship between *Asterotexiales* and *Jahnulales*. In their analyses, however, they did not include taxa from *Asterinaceae* sensu stricto, but only *Asterinaceae* sensu lato (Hyde et al. 2016). There is confusion surrounding strains of *Asterinaceae* as taxa cluster into two distinct, but well-separated lineages. This discrepancy is thought to result from direct sequencing and thus more than one taxon being sequenced. Hyde et al. (2016) suggested that the group clustering near *Asterotexiaceae* were *Asterinaceae* species, while those clustering near *Parmulariaceae* were likely to be fungi associated with *Asterinaceae*. In our phylogenetic and BEAST analyses, we included taxa from *Asterinaceae* sensu stricto and sensu lato. *Asterotexiaceae* clustered together with strains from *Asterinaceae* sensu stricto and *Jahnulales* and is phylogenetically distinct from *Asterinaceae* sensu lato. We therefore treat *Asterotexiales* as a synonym of *Asterinales*. The age for the *Asterinales* (*Asterinaceae* sensu stricto and *Asterotexiales*) crown group is estimated at 133 Mya (stem age 227 Mya) which supports its status as an order.

Botryosphaeriales C.L. Schoch, Crous & Shoemaker., Mycologia 98: 1050 (2006)

Previous studies by Liu et al. (2016) on *Botryosphaeriales* reported that the order originated around 103 Mya in the Cretaceous period, and *Botryosphaeriaceae* (which includes *Pseudofusicoccumaceae* and *Endomelanconiopsisaceae*) and *Phyllostictaceae* lineages separated around 87 Mya. These dates coincide closely with the emergence of *Botryosphaeriales* (114 Mya) and divergence times of these same two families (72 Mya) as estimated in the present study thus supporting the status of *Botryosphaeriales* as an order and *Botryosphaeriaceae* and *Phyllostictaceae* as families. Liu et al. (2016) suggested that *Botryosphaeriaceae, Phyllostictaceae* and *Melanopsaceae* clearly warrant separate families, but they considered that separate families for *Aplosporellaceae*, *Planistromellaceae*, *Saccharataceae* and *Septorioideaceae* are difficult to justify. The crown ages for the *Botryosphaeriaceae*, *Phyllostictaceae*, *Planistromellaceae* and *Saccharataceae* groups estimated here are 44, 27, 25 and 28 Mya respectively (stem ages 52, 50, 85 and 114 Mya), which suggests that these constitute families. However, the divergence time of *Saccharataceae* and *Septorioidaceae* (28 Mya) point towards these being two genera in *Saccharataceae*. *Planistromellaceae* and *Melanopsaceae* diverged at 85 Mya and this confirms the recommendation of Liu et al. (2016) that they are two separate families. The present results indicate that the families *Aplosporellaceae*, *Endomelanconiopsisaceae*, *Saccharataceae* and *Septorioideaceae* may not represent familial ranks but instead should be considered as genera. However, the status of all families currently included in *Botryosphaeriales* remains unclear and should be re-evaluated in an in-depth study with greater taxon-sampling.

Floricolaceae Thambugala, Kaz. Tanaka & K.D. Hyde., Fungal Diversity 74: 244 (2015)

Thambugala et al. (2015) provided an account of *Lophiostomataceae* (family of *Pleosporales*) and related families and genera. Using a combined analysis of LSU, SSU, ITS and TEF1 sequences data of 109 taxa they introduced a new family *Floricolaceae* which formed a well-resolved distinct lineage. The 28 taxa used in the tree for this family formed nine distinct groups that were supported by morphology and molecular data. Thambugala et al. (2015) therefore introduced six genera in the family with *Floricola* as the type. Jaklitsch et al. (2016) however, introduced an epitype for the type species of *Teichospora* which is the type of *Teichosporaceae* and synonymized *Floricolaceae*, and they also synonymized all genera that Thambugala et al. (2015) had accepted under *Floricolaceae* and thus the family became monotypic. In our phylogeny (Fig. 2), the inclusion of only six strains, and some of the genera (e.g. *Asymmetrispora* and *Ramusculicola*) proposed by Thambugala et al. (2015), are well-supported. However, when we weigh in additional evidence from the maximum clade credibility (MCC) tree (Fig. 4), these strains appear to have evolved from 26 to 89 Mya (crown age), which is much older than the average for genera of Ascomycota or Basidiomycota (Hyde et al. 2017). Therefore, the use of these genera in *Floricolaceae* is justified here.

Tubeufiales Boonmee & K.D. Hyde., Fungal Diversity 68: 245 (2014)

Synonyms

Bezerromycetales J.D.P. Bezerra, C.M. Souza-Motta & Crous, Mycol Prog 16 (4): 301 (2017)

Wiesneriomycetales J.D.P. Bezerra, R.J.V. Oliveira, C.M. Souza-Motta, J.Z. Groenewald & Crous, Mycol Prog 16 (4): 305 (2017)
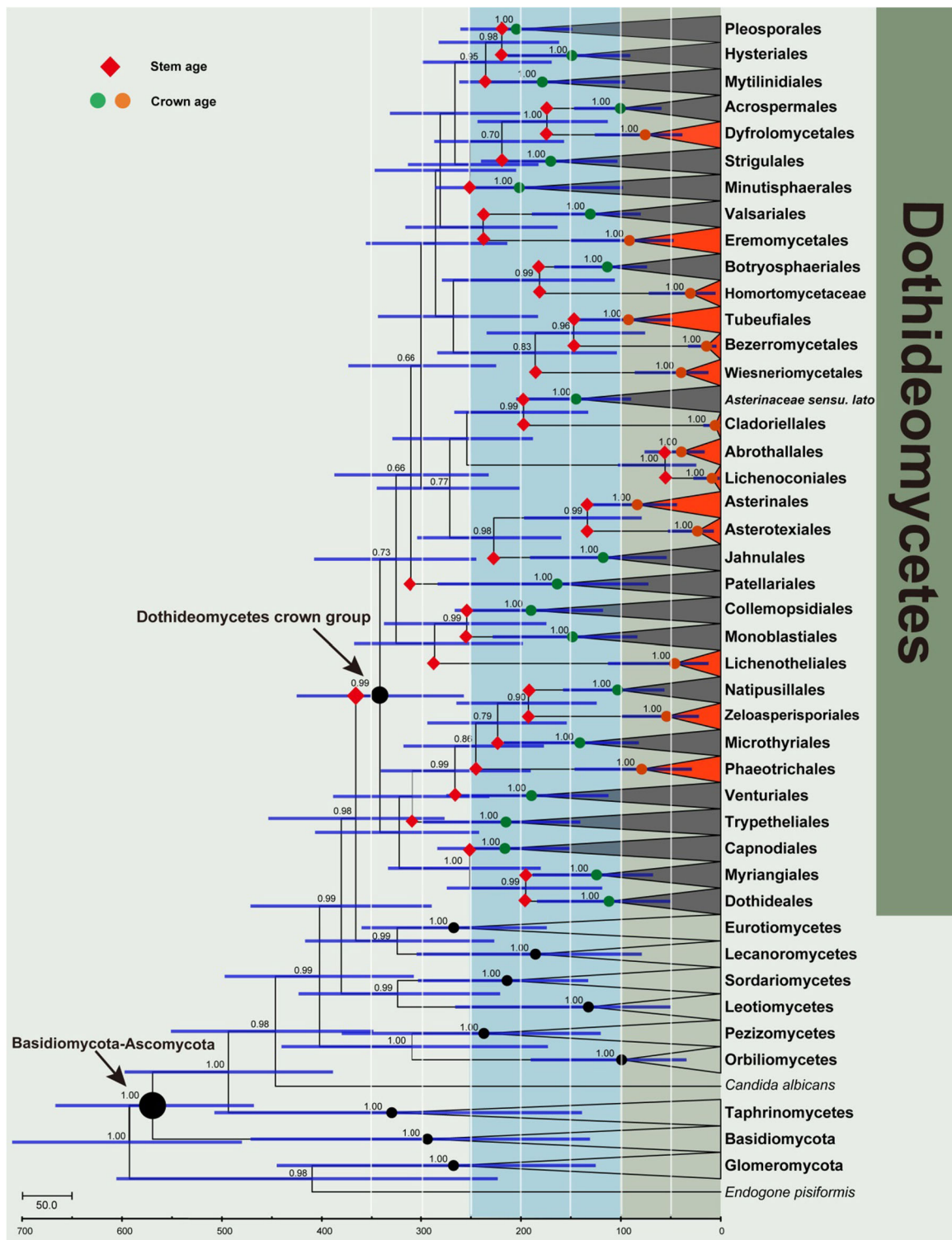
Fig. 3 Maximum clade credibility (MCC) tree with divergence times estimates for Dothideomycetes obtained from a Bayesian approach (BEAST) using single fossil (A1). *Numbers at nodes* indicate posterior probabilities (pp) for node support; bars correspond to the 95% highest posterior density (HPD) intervals. For estimated median age of nodes, see Table 1. The *circles in green* indicate the node ages agree with the recommendation made in the paper, and in *orange* indicate the opposite

The orders *Bezerromycetales* and *Wiesneriomycetales* were introduced by Bezerra et al. (2017) based on a phylogenetic study of endophytic fungi from the cactus

Tacinga inamoena in a Brazilian tropical dry forest. Both orders were established as monotypic with a single family, and one family (*Wiesneriomycetaceae*) was assigned to

Table 1 Dothideomycetes orders and included families based on updated phylogenetic tree and taking into account the divergence times (first two columns)

Order	Family	Divergence times (crown age)	Divergence times (stem age)
Abrothallales	*Abrothallaceae*	39 (16–76)	56 (24–102)
Acrospermales	*Acrospermaceae*	101 (59–146)	174 (113–243)
Asterinales	*Asterinaceae*	84 (43–132)	133 (79–196)
Asterotexiales	*Asterotexiaceae*	23 (7–52)	133 (79–196)
Bezerromycetales	*Bezerromycetaceae*	14 (4–32)	147 (75–234)
Botryosphaeriales	*Aplosporellaceae*	114 (73–166)	181 (106–279)
	Botryosphaeriaceae		
	Endomelanconiopsidaceae		
	Melanopsaceae		
	Planistromellaceae		
	Phyllostictaceae		
	Pseudofusicoccumaceae		
	Saccharataceae		
	Septorioideaceae		
Capnodiales	*Capnodiaceae*	216 (151–283)	251 (180–333)
	Cladosporiaceae		
	Cystocoleaceae		
	Dissoconiaceae		
	Extremaceae		
	Euantennariaceae		
	Metacapnodiaceae		
	Mycosphaerellaceae		
	Neodevriesiaceae		
	Phaeothecoidiellaceae		
	Piedraiaceae		
	Schizothyriaceae		
	Teratosphaeriaceae		
Cladoriellales	*Cladoriellaceae*	6 (0.5–17)	197 (132–266)
Collemopsidiales	*Xanthopyreniaceae*	190 (117–266)	254 (174–337)
Dothideales	*Dothideaceae*	112 (50–183)	195 (118–274)
Dyfrolomycetales	*Dyfrolomycetaceae*	76 (38–125)	174 (113–243)
Eremomycetales	*Arthrographaceae*	91 (47–150)	238 (163–316)
	Eremomycetaceae		
Hysteriales	*Hysteriaceae*	149 (90–213)	219 (161–282)
Jahnulales	*Aliquandostipitaceae*	118 (54–190)	227 (159–303)
	Manglicolaceae		
Lichenoconiales	*Lichenoconiaceae*	9 (1–27)	56 (24–102)
Lichenotheliales	*Lichenotheliaceae*	46 (12–112)	286 (198–367)
Microthyriales	*Microthyriaceae*	141 (84–217)	223 (154–293)
Minutisphaerales	*Minutisphaeraceae*	202 (97–286)	251 (182–313)
	Pseudorobillardaceae		
Monoblastiales	*Monoblastiaceae*	148 (83–228)	254 (174–337)
	Anisomeridaceae		
	Heleiosaceae		
Myriangiales	*Elsinoaceae*	124 (67–188)	195 (118–274)
	Myriangiaceae		
Mytilinidiales	*Gloniaceae*	178 (95–261)	235 (169–298)
	Mytilinidiaceae		

Table 1 continued

Order	Family	Divergence times (crown age)	Divergence times (stem age)
Natipusillales	*Natipusillaceae*	103 (56–157)	192 (124–264)
Patellariales	*Patellariaceae*	164 (72–283)	311 (244–407)
Phaeotrichales	*Phaeotrichaceae*	79 (18–146)	245 (177–318)
Pleosporales	*Acrocalymmaceae*	204 (148–260)	219 (161–282)
	Aigialaceae		
	Amniculicolaceae		
	Anteagloniaceae		
	Ascocylindricaceae		
	Astrosphaeriellaceae		
	Bambusicolaceae		
	Camarosporiaceae		
	Caryosporaceae		
	Coniothyriaceae		
	Corynesporaceae		
	Cucurbitariaceae		
	Cyclothyriellaceae		
	Dacampiaceae		
	Delitschiaceae		
	Dictyosporiaceae		
	Didymellaceae		
	Didymosphaeriaceae		
	Dothidotthiaceae		
	Halojulellaceae		
	Halotthiaceae		
	Latoruaceae		
	Lentitheciaceae		
	Leptosphaeriaceae		
	Ligninsphaeriaceae		
	Lindgomycetaceae		
	Lophiostomataceae		
	Lophiotremataceae		
	Macrodiplodiopsidaceae		
	Massariaceae		
	Massarinaceae		
	Melanommataceae		
	Morosphaeriaceae		
	Neophaeosphaeriaceae		
	Nigrogranaceae		
	Occultibambusaceae		
	Parabambusicolaceae		
	Paradictyoarthriniaceae		
	Periconiaceae		
	Phaeosphaeriaceae		
	Pleomassariaceae		
	Pleosporaceae		
	Pseudoastrosphaeriellaceae		
	Roussoellaceae		
	Salsuginaceae		

Table 1 continued

Order	Family	Divergence times (crown age)	Divergence times (stem age)
	Sporormiaceae		
	Sulcatisporaceae		
	Teichosporaceae		
	Testudinaceae		
	Tetraplosphaeriaceae		
	Thyridariaceae		
	Torulaceae		
	Trematosphaeriaceae		
	Wicklowiaceae		
	Zopfiaceae		
Strigulales	*Strigulaceae*	170 (103–239)	219 (156–286)
Trypetheliales	*Trypetheliaceae*	215 (140–298)	309 (231–388)
Tubeufiales	*Tubeufiaceae*	92 (48–148)	147 (75–234)
Valsariales	*Valsariaceae*	131 (79–189)	238 (163–316)
Venturiales	*Sympoventuriaceae*	189 (112–274)	266 (190–340)
	Venturiaceae		
Wiesneriomycetales	*Wiesneriomycetaceae*	40 (12–86)	186 (104–283)
Zeloasperisporiales	*Zeloasperisporiaceae*	54 (21–98)	192 (124–264)

The last two columns are the divergence time estimates of Dothideomycetes lineages (orders) obtained from a Bayesian approach (BEAST) using single calibration. For each divergence, the median and the 95% highest posterior density (HPD) are provided. Divergence times are provided in millions of years (Mya)

Tubeufiales. In our maximum clade credibility (MCC) tree (Fig. 3), the orders *Bezerromycetales* and *Wiesneriomycetales* are shown to have diverged recently with the age for their crown groups at 14 and 40 Mya respectively (stem ages 147 and 186 Mya) and it is more likely to be given familial rank which in line with our recommendations between 20 and 100 Mya (crown age). Our recommendation for orders is that they should be more than 100 Mya and this is not the case. Therefore, we synonymize *Bezerromycetales* and *Wiesneriomycetales* under *Tubeufiales*, which is the oldest name. The age for the crown of *Tubeufiales* is about 189 Mya (stem age 268 Mya), which supports its ordinal status.

Importance of accurately determining the fossil age

The justification of the age and phylogenetic position of the key fossils is an important step in calibrating a node in a divergence dating analysis and can be the core data for the application of divergence times for the classification. Fortunately, there are many studies that have led to credible calibrations and reliable divergence dates (Gueidan et al. 2011; Prieto and Wedin 2013; Beimforde et al. 2014; Pérez-Ortega et al. 2016). This provides us with the possibility to apply divergence times as further evidence to better resolve controversies in fungal classification.

Several studies have shown that using multiple fossil constraints is one possible way to improve the accuracy of molecular dating (Graur and Martin 2004; Hedges and Kumar 2004; Rutschmann et al. 2007; Sauquet et al. 2012). However, the lack of well preserved and identifiable fungal fossils is a limiting factor in dating studies. Another approach is to use secondary calibrations obtained from previous studies (Prieto and Wedin 2013; Beimforde et al. 2014; Pérez-Ortega et al. 2016). In this study, we performed molecular dating analyses using a single relevant fossil, and a secondary calibration point. The estimates obtained based on different setups give similar results with an average ratio of 2.2 Mya (Order, Fig. 3) and 1.3 Mya (Family, Fig. 4) younger ages from analyses A1 and A2 (trees not shown) respectively. Both analyses are based on previous studies with a robust fossil record using five fossil calibration points (Beimforde et al. 2014; Pérez-Ortega et al. 2016). By comparing the inferred divergence dates with the widely accepted dates, we provide insight into using divergence times as additional evidence in higher ranking taxa (order and family) within the class Dothideomycetes and order *Pleosporales* as selected study groups. Although this could be seen as a circular argument, the use of divergence times can in reality serve as an additional criterion to justify fungal taxonomy conclusions and phylogeny.
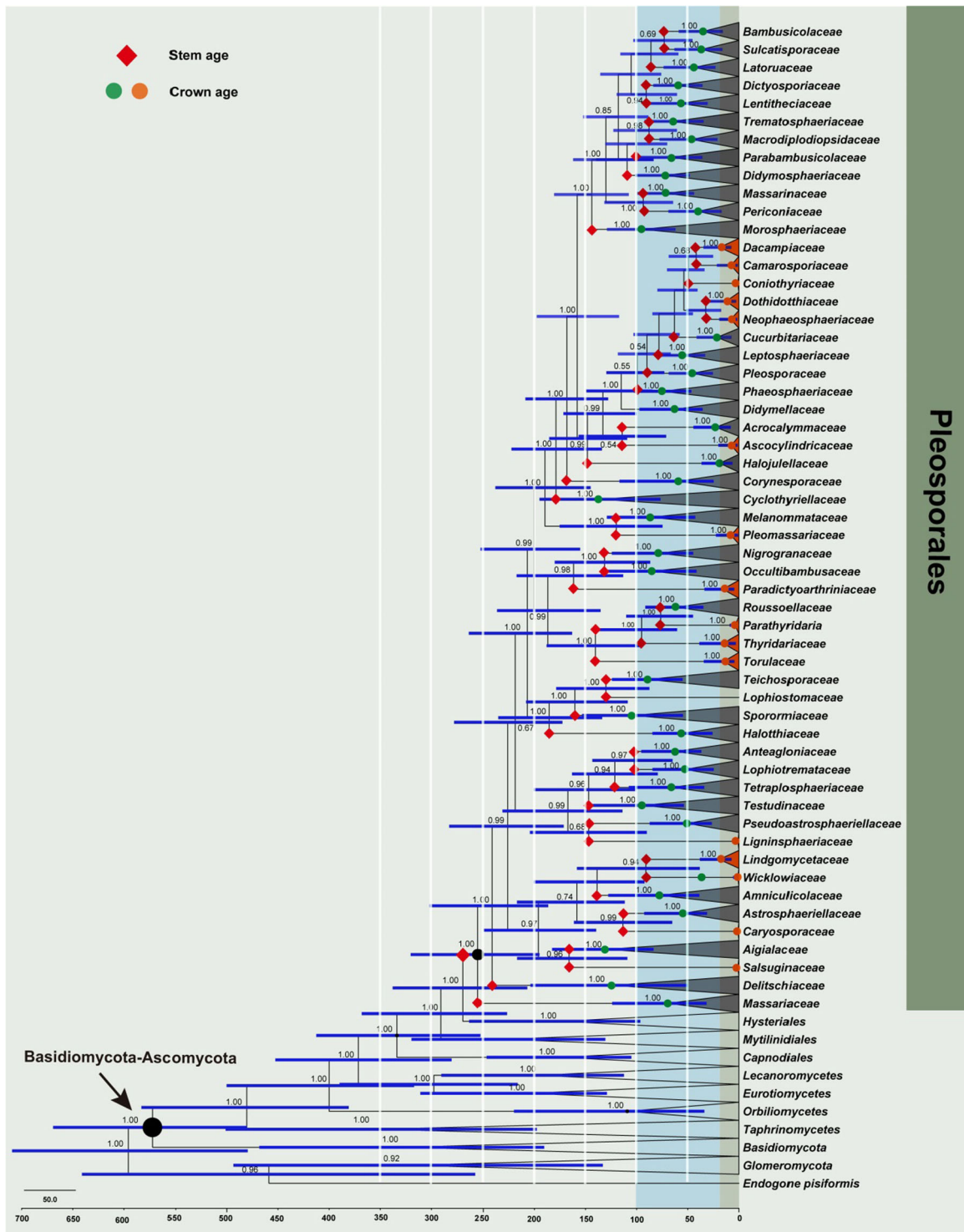
Fig. 4 Maximum clade credibility (MCC) tree with divergence times estimates for *Pleosporales* obtained from a Bayesian approach (BEAST) using a single fossil (A1). *Numbers at nodes* indicate posterior probabilities (pp) for node support; bars correspond to the 95% highest posterior density (HPD) intervals. For estimated median age of nodes, see Table 2. The *circles in green* indicate the node ages agree with the recommendation made in the paper, and in *orange* indicate the opposite

Table 2 Divergence time estimates of *Pleosporales* lineages (families) obtained from a Bayesian approach (BEAST) using single calibration

Family	Divergence times (crown age)	Divergence times (stem age)
Acrocalymmaceae	23 (8–44)	114 (71–156)
Aigialaceae	131 (83–182)	166 (83–182)
Amniculicolaceae	78 (38–127)	139 (92–200)
Anteagloniaceae	62 (36–95)	102 (64–143)
Ascocylindricaceae	8 (1–20)	114 (71–156)
Astrosphaeriellaceae	55 (31–92)	113 (65–161)
Bambusicolaceae	35 (15–58)	73 (45–103)
Camarosporiaceae	8 (1–21)	42 (25–68)
Caryosporaceae	2 (0–4)	113 (65–161)
Coniothyriaceae	2 (90–5)	50 (33–70)
Corynesporaceae	59 (24–116)	168 (127–208)
Cucurbitariaceae	21 (7–41)	63 (45–84)
Cyclothyriellaceae	138 (76–195)	179 (133–222)
Dacampiaceae	19 (7–34)	42 (25–68)
Delitschiaceae	124 (50–203)	241(186–301)
Dictyosporiaceae	60 (35–83)	91 (60–119)
Didymellaceae	63 (35–97)	115 (84–149)
Didymosphaeriaceae	72 (48–101)	109 (83–139)
Dothidotthiaceae	13 (2–32)	33 (17–51)
Halojulellaceae	19 (6–36)	148 (109–185)
Halotthiaceae	56 (22–109)	186 (133–135)
Latoruaceae	44 (23–73)	86 (59–115)
Lentitheciaceae	57 (30–92)	91 (60–119)
Leptosphaeriaceae	55 (32–81)	78 (57–103)
Ligninsphaeriaceae	2 (0–4)	147 (90–204)
Lindgomycetaceae	19 (7–38)	91 (38–158)
Lophiostomataceae	–	–
Lophiotremataceae	53 (24–84)	102 (64–143)
Macrodiplodiopsidaceae	46 (20–77)	88 (60–122)
Massariaceae	69 (31–123)	255 (195–320)
Massarinaceae	71 (43–99)	94 (64–131)
Melanommataceae	96 (42–129)	120 (74–175)
Morosphaeriaceae	95 (62–129)	143 (107–180)
Neophaeosphaeriaceae	8 (1–19)	33 (17–51)
Nigrogranaceae	79 (44–124)	131 (86–180)
Occultibambusaceae	85 (41–128)	131 (86–180)
Parabambusicolaceae	66 (35–101)	100 (70–130)
Paradictyoarthriniaceae	16 (4–33)	162 (113–217)
Periconiaceae	40 (17–68)	94 (64–131)
Phaeosphaeriaceae	75 (46–102)	99 (73–129)
Pleomassariaceae	8 (0.8–22)	120 (74–175)
Pleosporaceae	47 (25–68)	90 (66–118)
Pseudoastrosphaeriellaceae	50 (26–87)	147 (90–204)
Roussoellaceae	62 (34–91)	77 (44–110)
Salsuginaceae	2 (0–2)	166 (83–182)
Sporormiaceae	104 (54–153)	160 (108–208)
Sulcatisporaceae	37 (16–63)	73 (45–103)
Teichosporaceae	99 (55–124)	130 (87–178)
Testudinaceae	95 (53–142)	147 (100–200)

Table 2 continued

Family	Divergence times (crown age)	Divergence times (stem age)
Tetraplosphaeriaceae	66 (33–107)	121 (79–163)
Thyridariaceae	15 (3–38)	95 (60–138)
Torulaceae	15 (4–34)	140 (95–188)
Trematosphaeriaceae	65 (34–92)	88 (60–122)
Wicklowiaceae	2 (0–5)	91 (38–158)
Zopfiaceae	–	–

For each divergence, the median and the 95% highest posterior density (HPD) are provided. Divergence times are provided in millions of years (Mya)

Conclusions

We provide four examples where divergence times do not support the status of families and orders. Liu et al. (2016) also discussed the distinction of three genera as distinct families in *Botryosphaeriales* which was not supported in the MCC trees. These are examples where the distinctiveness of introduced higher taxa using phylogenies should be reinforced by further evidence e.g. divergence times.

Molecular clocks calibrated using fossils are important tools in estimating the timing of evolutionary events in fossil-poor groups. However, when fossil evidence is limited and there are considerable differences in substitution rates change between lineages, it is difficult to establish reliable divergence time estimates. The status of fossils and analysis, an awareness of the phylogeny, fossils and the clock will help to align expectations for fungal evolution. However, it will be many years before we can obtain natural classification for fungi. As more data and approaches become available and fossil and phylogenetic evidence becomes more reliable, we can obtain a better-characterized divergence dating with few limitations.

The major contribution of this paper is an updated phylogeny of the class Dothideomycetes to order level and the order *Pleosporales* to the family level. The addition of the maximum clade credibility (MCC) tree to support the phylogenetic conclusions show that some orders and some families are not supported and are therefore synonymized. A major conclusion is that any inference from phylogenetic trees depends on the taxa used. For example, Hyde et al. (2016) reported strain MFLUCC 15-1248 as a collection of *Neoacanthostigma septoconstrictum* as it had strong support in their phylogenetic tree. However, with the addition of ten extra strains in the genus, this strain clustered with a new species, *N. brownispora* with good support and the strain was renamed. Therefore, any interpretation of phylogenetic trees should be treated with caution as it depends entirely on the taxa chosen to build it. We predict that clade ages will improve the definitions (ranking) of higher taxa

and understanding of phylogenetic relationships. Given that the rates of molecular evolution vary with the molecular markers used (ribosomal versus protein coding ones), future studies can show how this can affect calibration as well as estimate evolutionary rates for specific genes and their impact on dating. The results obtained in phylogenetic and molecular clock studies are currently the best hypotheses using present methodologies and data; however, additional data, taxa and/or new methodologies, may result in modified conclusions.

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