



## Phylogenetic relationship and evolution of *Neodidymelliopsis* isolates collected from Iran

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### Abstract

Several isolates of the novel genus *Neodidymelliopsis* have been recently found in Iranian citrus orchards with severe dieback symptoms. *Neodidymelliopsis* belongs to Didymellaceae, an important family of Pleosporales, Dothideomycetes. None of the few studies on molecular dating of Ascomycetes have resolved the divergence time of genera in Didymellaceae. Motivated by this fact, we consider the reliable age of a fossil related to extant species of *Aigialus*, the estimated mean crown age of Dothideomycetes from other studies as a secondary calibration, also the second fossil which represent common ancestor of Capnodiales to calibrate the reconstructed tree. Our dating analysis is based on four genetic regions of 91 taxa from Capnodiales and Pleosporales, using BEAST analysis. The selected taxa of Pleosporales belong to Aigialaceae and Didymellaceae; including three newly discovered *Neodidymelliopsis* sp. isolates and one isolate of *Didymella* sp. from Iran. Our dating analyses suggest that Didymellaceae diverged from Aigialaceae in the Cretaceous, and initial divergence of Didymellaceae happened in the late Eocene followed by two divergences in the late Oligocene and several splits in the Miocene. Furthermore, the results suggest that the Iranian isolates of *Neodidymelliopsis* sp. and *Didymella* sp. diverged from other *Neodidymelliopsis* and *Didymella* isolates in the Pliocene and the late Miocene, respectively.

**Key words** – Didymellaceae – Divergence time – Fossil – Pleosporales – Secondary calibration

### Introduction

Coelomycetes are a form-class of fungi which produce their conidia and conidiophore within the cavity like pycnidia (globose to pyriform conidiomata from which the conidia arise throughout an apical opening) or sporocarp (de Gruyter et al. 2009, Chen et al. 2015). This form-class of fungi consists of numerous endophytic, pathogenic or saprobic fungi in terrestrial and marine ecosystems, or in plants and animals (Dai et al. 2014, Hyde et al. 2014, Wijayawardene et al. 2016).

Coelomycetous fungi has been recently assigned to different phylogenetic groups, e.g. Dothideomycetes, Leotiomyces and Sordariomyces (Wijayawardene et al. 2016, 2017). Dothideomycetes is well-known class of Ascomycota (Wijayawardene et al. 2017, 2018), while Pleosporales contains a quarter of the class (Kirk et al. 2008), with Didymellaceae as the largest family in this order. Didymellaceae encompasses more than 5,400 taxa in MycoBank (Crous et al. 2004, Crous & Groenewald 2017, Hashimoto et al. 2017). In the recent revision of Didymellaceae,

26 genera belong to this family have been reported (Chen et al. 2015, 2017, Valenzuela-Lopez et al. 2018). Members of this family are pathogenic on a wide range of host plants, which mainly cause leaf and stem lesions and some are of quarantine significance (Aveskamp et al. 2008, Boehm et al. 2009, Chen et al. 2017). Recently, some isolates of the novel genus *Neodidymelliopsis* and *Didymella* have been found on the citrus trees with severe dieback symptoms in Iran. This motivated our research to find out the divergence time of the selected isolates of Didymellaceae, particularly, isolates of *Neodidymelliopsis* sp. and *Didymella* collected from southern parts of Iran by comparing their nucleotide sequences. To achieve this aim, we adopted a molecular dating approach based on the molecular clock hypothesis (MCH), proposed by Zuckerkandl & Pauling (1965).

Molecular dating combines information from the fossils, recorded events, geological events and those achieved from data analyses to estimate the age of clades in a phylogenetic tree (Rutschmann 2006, Ho & Duchene 2014). It is also possible to use the estimated age of a node from previous studies to calibrate the molecular clock in a new study, as a secondary calibration (dos Reis et al. 2015). Recently, several molecular dating studies have tried to estimate the age of fungi based on the available fossils (Vijaykrishna et al. 2006, Beimforde et al. 2014, Hongsanan et al. 2016, Zhao et al. 2016, Hyde et al. 2017, Liu et al. 2017). However, lack of the reliable fossils is a limited factor in molecular dating studies, which could face even a bigger challenge when the microscopic structure of fungi is unknown (Prieto & Wedin 2013).

Here, we first constructed a phylogenetic tree based on four multi-gene datasets including the four sequenced isolates and some taxa of Didymellaceae obtained from the National Center for Biotechnology Information (NCBI) databases. We performed the dating analysis which updated the age of Dothideomycetes, Pleosporales and gave the first estimation of the divergence time of Didymellaceae genera.

## Materials & Methods

### Sample collection, DNA extraction and sequencing

We collected citrus samples with dieback, blight of vigorously growing shoots symptoms, from citrus orchards in three southern provinces of Iran (Kerman, Hormozgan and Khuzestan). Pathogen isolation and inoculum preparation were performed according to the methods described by Taylor & Hyde (2003). Pure cultures were obtained by single spore isolation methods (Chomnunti et al. 2014). Four isolates were deposited at Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Center in the Netherlands and Iranian Fungal Culture Collection (IRAN.C) at the Iranian Research Institute of Plant Protection (Supplementary table 1).

The genomic DNA was extracted using Doyle & Doyle (1987) protocol from the fungal mycelium produced in Potato Dextrose Agar (PDA) media at 25–27°C for 4 weeks. We amplified four genomic regions including partial large subunit nuclear rDNA (28S, LSU), internal transcribed spacer regions 1 & 2 and intervening 5.8S nrDNA (ITS), partial RNA polymerase II second largest subunit (RPB2) and partial beta-tubulin (TUB2) region using special primers (Table 1), as it was described by Chen et al. (2015). PCR products were visualized under UV light after electrophoresis in a 1.0 % (w/v) agarose gel containing 0.1 ug/mL ethidium bromide in 1 × TAE buffer. Sanger sequencing was performed by Macrogen Company (Seoul, Korea), and results were submitted to NCBI (Supplementary table 1).

### Sequence alignment and phylogenetic analysis

To determine the phylogenetic relationship of the selected Iranian isolates with the other available taxa, we considered LSU, RPB2, TUB2 and ITS genomic regions in all 26 accepted Didymellaceae genera, as mentioned by Valenzuela-Lopez et al. (2018). In addition, we used seven *Aigialus* and Capnodiales isolates; according to Beimforde et al. (2014), Phukhamsakda et al. (2016) as the out-group. The selected strains and their accession numbers were listed in Supplementary table 2. The genetic regions were separately aligned by MAFFT v.7 (Kato &

Standley 2013). We checked the alignment visually and adjusted it manually in Mesquite v.3.04 (Maddison & Maddison 2015). The alignments were concatenated by SequenceMatrix program (Vaidya et al. 2011). Linux version of IQ-tree tool v.1.6 (Nguyen et al. 2014) was used to reconstruct the phylogenetic trees and find the best substitution model. We assessed the reliability of the reconstructed branches by Bootstrap analyses on 1000 replicates, and visualized trees in FigTree v.1.4.2.

**Table 1** Primers used in this study.

Regions	Primer pairs	Reference
ITS	V9G/ITS4	White et al. (1990), de Hoog & Gerrits van den Ende (1998)
RPB2	RPB2-5F2/FRPB2-7cR	Liu et al. (1999), Sung et al. (2007)
LSU	LR0R/LR7	Vilgalys & Hester (1990), Rehner & Samuels (1994)
TUB2	Btub2Fd/Btub4Rd	Woudenberg et al. (2009)

### Node Calibrations

To calibrate the nodes, we took advantage of the age of the reliable fossils and the estimated mean crown age of Dothideomycetes. We compared three calibration scenarios, based on the minimum age of reliable fossils from the literature and the secondary calibration. The calibration based on the age of a node estimated in other study is referred to as secondary calibration (Ho & Duchene 2014, dos Reis et al. 2015). In all cases, we considered Pleosporales as a monophyletic group.

In scenario I, we used a fossil of *Margaretbarromyces dictyosporus* (Fossil I) which belongs to Pleosporales with the age of 35–55 Mya (Mindell et al. 2007, Berbee & Taylor 2010, Phukhamsakda et al. 2016). Since morphologically this fungus resembles *Aigialus* belonging to Aigialaceae, Pleosporales (Phukhamsakda et al. 2016), we assigned the estimated age of the fossil to the node of *Aigialus* cluster (AIG). To demonstrate the uncertainty of the fossil age we represented it by a lognormal distribution with an offset (minimum bound), because we did not have any information about the maximum bound of the age (Ho & Philips 2009), with the mean of TMRCA (the most recent common ancestor) of *Aigialus* = 35, SD = 3.5, offset = 34, giving 95% credibility interval (CI) of 58.

In scenario II, we used two fossils *viz.* *M. dictyosporus* to calibrate AIG node and a Metacapnodiaceae fossil (~100–113 Mya, Schmidt et al. 2014) to calibrate the crown node of Capnodiiales (CAP). In this scenario, we used the same setting as scenario I for *M. dictyosporus* fossil, while used a lognormal distribution (mean = 100, SD = 4.5, offset = 99, CI = 120) for the Metacapnodiaceae fossil.

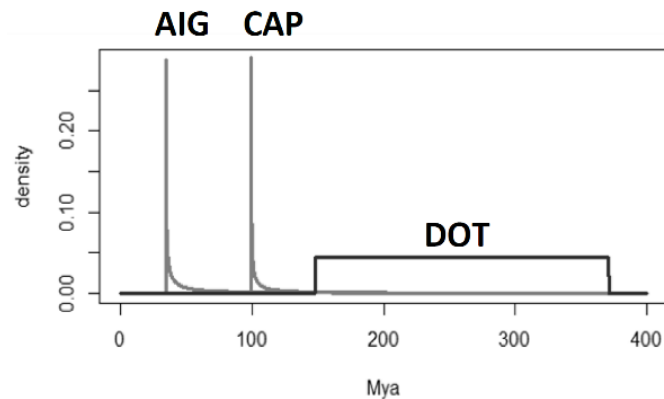
For scenario III, besides the age of two pre-mentioned fossils, we considered the estimated mean crown age of Dothideomycetes (107–459 Mya) on the root of the tree estimated by Gueidan et al. (2011), Prieto & Wedin (2013), Beimforde et al. (2014), Pérez-Ortega et al. (2016), Phukhamsakda et al. (2016) as a secondary calibration. We preferred a uniform distribution (with maximum of 457 and minimum of 107) for the secondary calibration, as simulation studies (Schenk 2016) reported that the normal prior distribution results in larger errors, compared to uniform distribution in the secondary calibrations. Fig. 1 illustrates the posterior probability density distribution of the three calibration points.

### Molecular dating

We used the BEAST v1.8.2 package (Drummond et al. 2012) for the molecular dating analysis. For this analysis, we used an uncorrelated lognormal relaxed clock model (UCLD) and the simplest model, Yule process (Drummond & Bouckaert 2014). GTR substitution model, 4 rate categories were used based on the suggestion from the model finder of IQ-tree. Since Gamma and invariable sites have a mutual effect (Drummond & Bouckaert 2014, Moran et al. 2015), applying both on a model is not biologically meaningful (Jia et al. 2014). Hence, we excluded invariable sites from the substitution model and set up the mean rate to the continuous-time Markov chains

model (CTMCs), recommended by Ferreira & Suchard (2008). We performed three MCMC analyses of 400 million generations, with sampling every 10000 steps. The results were evaluated by Tracer v1.6. The effective sample size (ESS) values of parameters were checked and improved to be more than 200 (Drummond & Bouckaert 2014). The resulted log and tree files were combined using LogCombiner1.8.0. Finally, we summarized the BEAST results by TreeAnnotator v1.4.7 with a burn-in of 10% and displayed them in FigTree v.1.4.2.

To compare different scenarios, we estimated the marginal likelihood (MLE) using path sampling (PS) and stepping stone sampling (SS), implemented in BEAST.



**Figure 1** – The plot posterior probability density distribution of used calibration points. The AIG, CAP and DOT curves show the probability of used calibration points assigned to MRCA of *Aigialus*, Capnodiales and Dothideomycetes nodes respectively.

## Results

### Phylogenetic analysis

The concatenated file produced by SequenceMatrix, adjusted in Mesquite, contained 91 taxa with 2,864 characters. Fig. 2 summarizes the results of our phylogenetic analyses; the four sequenced taxa have been marked with black diamonds. IR67 isolate clustered with *Didymella glomerata* CBS 528.66, while IR10, IR26 and IR14 isolates clustered with *Neodidymelliopsis longicolla* CBS 382.96 with high bootstrap supports (99-100).

### Molecular dating analysis

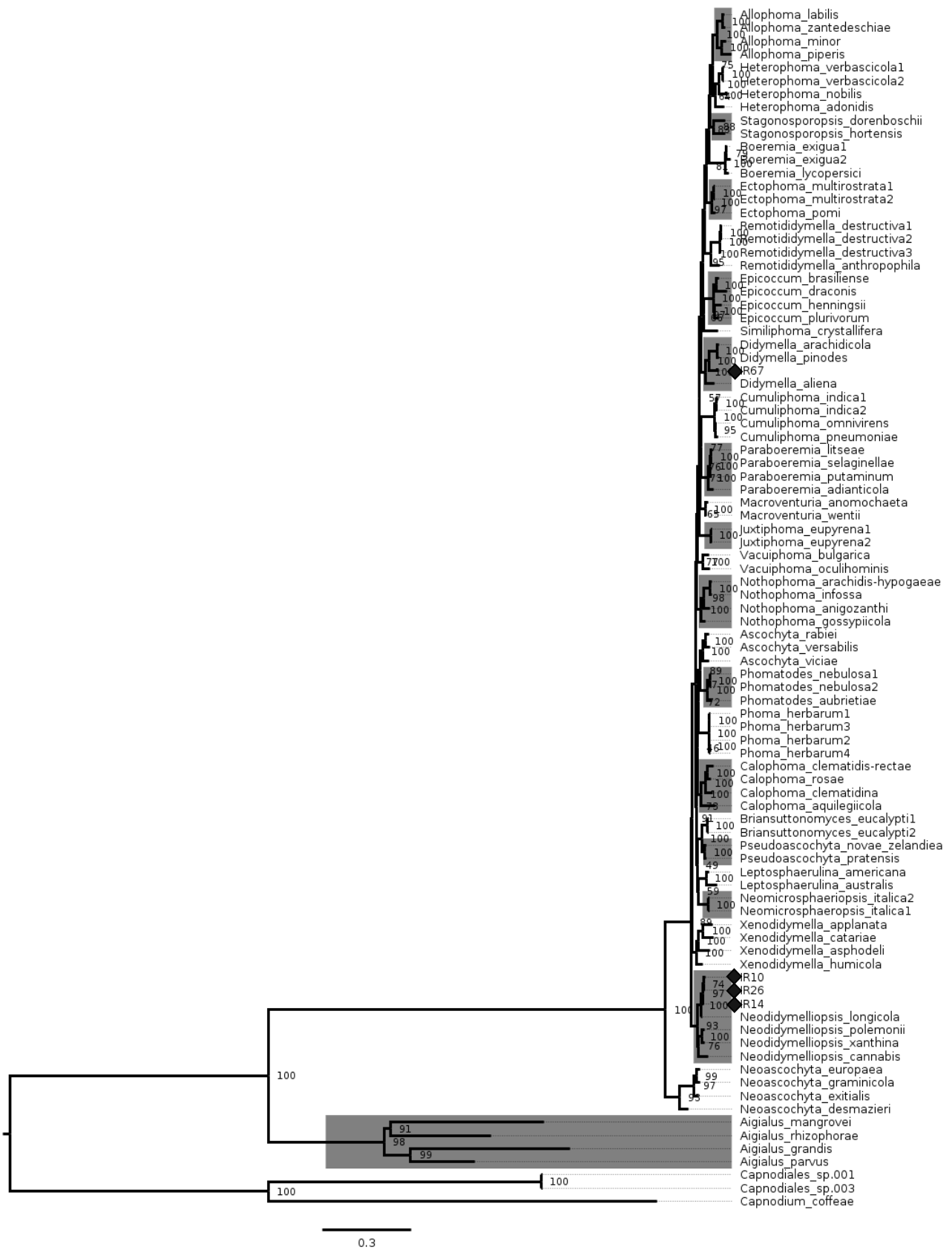
#### Comparing different scenarios

To compare different scenarios, we estimated the marginal likelihood (Table 2) which was slightly higher in scenario III, compared with the other scenarios.

#### Divergence time

The constructed trees in all scenarios (Fig. 4, Supplementary figs 1, 2) were consistent to the best tree built by IQ-tree (Fig. 2). Most of the estimated mean of nodes are supported by a strong posterior probability. The crown and stem age of all nodes in scenario III are considered as the best scenario (see discussion, Table 3). Pleosporales diverged from Capnodiales at ~144.5 (107–202.9) Mya. Within Pleosporales, Didymellaceae diverged from Aigialaceae at ~86.7 (53.9–155.4) Mya. The mean age of the earliest split in Didymellaceae (*Neascochyta* from other genera) is ~35.7 (18.4–63.5) Mya. The newest split of *Briansuttonomyces* and *Pseudoascochyta* from other genera in Didymellaceae occurred at ~6.3 (2–13.2) Mya. Iranian isolates of *Neodidymelliopsis* sp. and *Didymella* sp. diverged from other *Neodidymelliopsis* and *Didymella* isolates at ~3.1 (0.9–6.9) and

~8.6 (3.5–16.8) Mya, respectively (Fig. 4). Time charts of the nodes based on the estimated crown and stem ages in this student can be seen in Supplementary tables 4, 5.

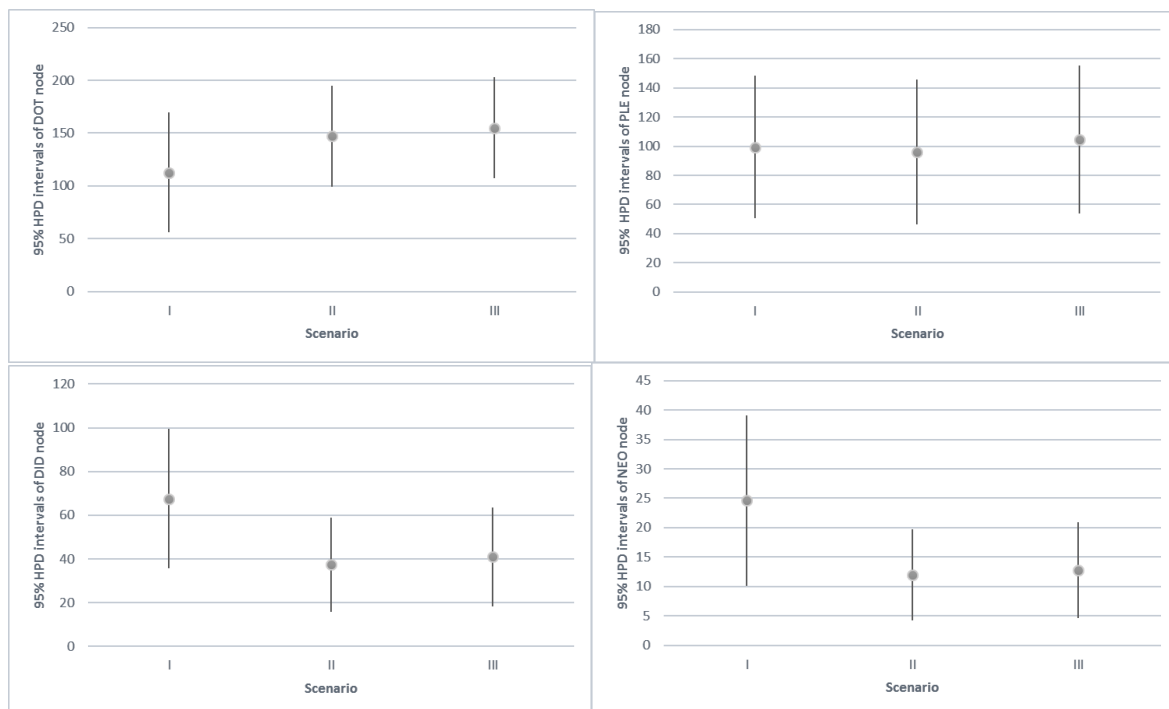


**Figure 2** – The result of phylogenetic analyses. Bootstrap values are shown next to the nodes and the Iranian isolates are marked with black diamonds.

**Table 2** The estimated marginal likelihood in different models.

Scenario	Log (Marginal likelihood) with SS	Log (Marginal likelihood) with PS
I	-26647.1	-26647.4
II	-26646.0	-26645.3
III	-26643.7	-26643.6

The comparison between the 95% HPD (Highest Posterior Density) of four main nodes, MRCA of Dothideomycetes (DOT), Didymellaceae (DID), Pleosporales (PLE) and *Neodidymelliopsis* (NEO) nodes showed that the intervals of the defined scenarios are overlapped (Fig. 3). In Table 3, estimated age of crown nodes and interval of 95% HPD are listed.



**Figure 3** – Comparison means and interval of estimated age for MRCA of Dothideomycetes (DOT), Didymellaceae (DID), Pleosporales (PLE) and *Neodidymelliopsis* (NEO).

**Table 3** The estimated divergence time of the crown nodes of Dothideomycetes in the three defined scenarios. Last column presents the estimated stem age in scenario III (the best scenario). Mean and 95% HPD intervals of each node have been presented in square brackets in millions of years (Mya).

Taxa (Node name)	Scenario I	Scenario II	Scenario III	
	(1 Fossil)	(2 Fossils)	(2 Fossils and 2 <sup>nd</sup> calibration)	
	Crown node	Crown node	Crown node	Stem node
Capnodiales (CAP)	44.4[17.6-82.5]	99[99-100.1]	99[99-100.1]	144.5[107-202.9]
Pleosporales (PLE)	92.4[50.5-148.1]	80.2[46.3-145.7]	86.7[53.9-155.4]	144.5[107-202.9]
<i>Aigialus</i> (AIG)	34.1[34-38]	34.1[34-59.4]	34.1[34-64.4]	86.7[53.9-155.4]
Didymellaceae (DID)	63[35.6-99.4]	32.5[16-59]	35.7[18.4-63.5]	86.7[53.9-155.4]
<i>Neodidymelliopsis</i> (NEO)	21.9[10.1-39.1]	10.1[4.3-19.7]	10.9[4.7-20.87]	27.3[15.9-45.8]
Iranian <i>Neodidymelliopsis</i> (IRneo)	3.3[1.2-6.3]	1.5[0.4-3.3]	1.6[0.5-3.5]	3.1[0.9-6.9]

**Table 3** Continued.

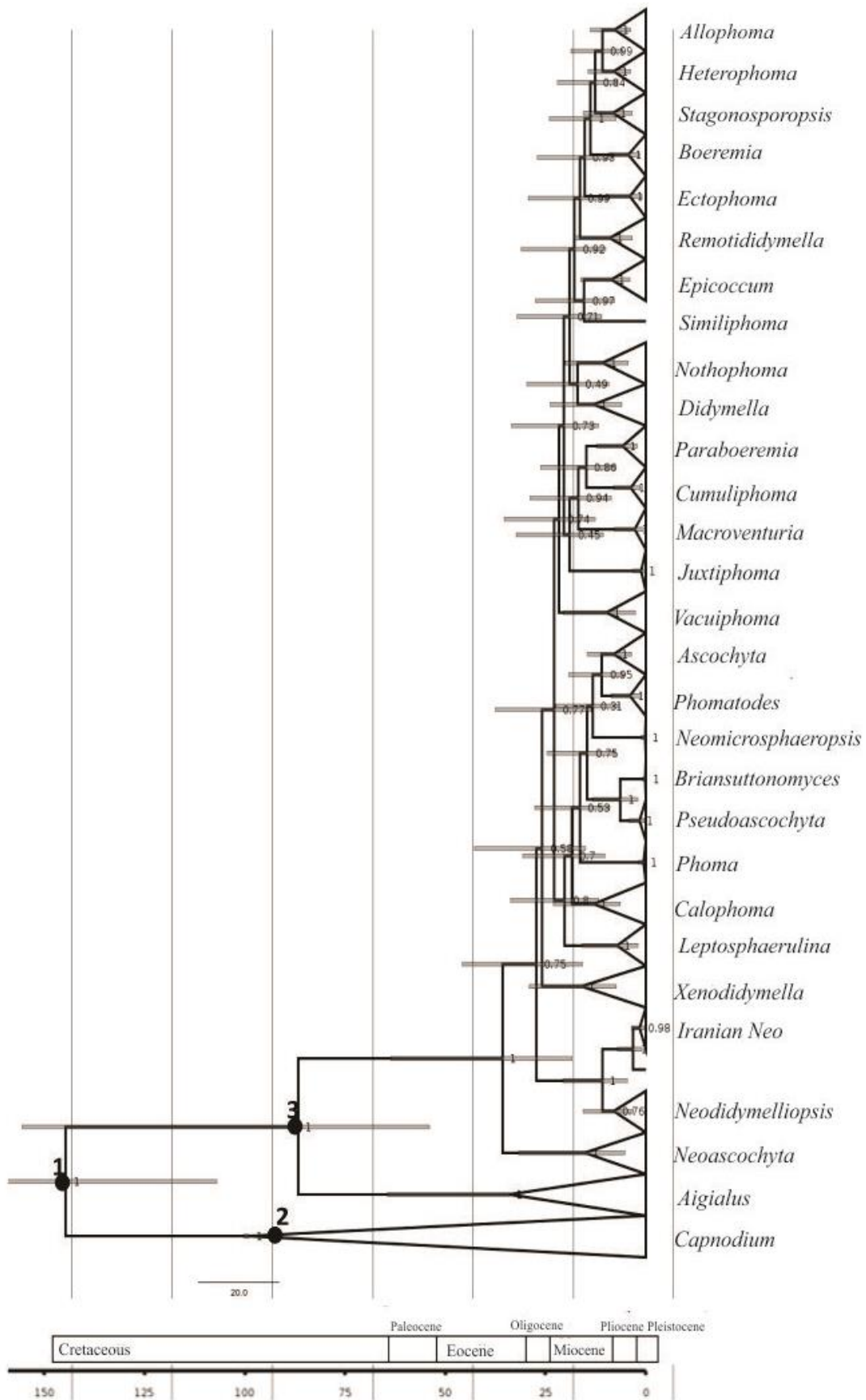
Taxa (Node name)	Scenario I	Scenario II	Scenario III	
	(1 Fossil)	(2 Fossils)	(2 Fossils and 2 <sup>nd</sup> calibration)	
	Crown node	Crown node	Crown node	Stem node
<i>Allophoma</i> (ALL)	14.2[7.2-23.3]	7.4[3.6-13.5]	7.7[3.8-13.8]	10.8[5.8-18.7]
<i>Heterophoma</i> (HET)	15.1[7.5-24.6]	7.7[3.6-13.9]	8.02[3.8-14.3]	10.8[5.8-18.7]
<i>Stagonosporopsis</i> (STA)	15.1[7-25.6]	8[3-15]	8.3[3.4-15.6]	12.6[6.2-22]
<i>Boeremia</i> (BOE)	9.1[3.9-16.5]	4.3[1.4-8.9]	4.5[1.6-9.3]	13.8[7.5-24]
<i>Ectophoma</i> (ECT)	8.3[2.9-16.9]	3.9[0.9-9.6]	4.1[1-9.9]	15.3[8.5-27]
<i>Remotididymella</i> (REM)	16.1[7.5-27.8]	8.7[3.1-17.3]	9.1[3.3-17.8]	16.5[9.3-29.2]
<i>Epicoccum</i> (EPI)	15.6[7.6-26.3]	8.3[3.7-15.6]	8.6[4-16.2]	15.4[7.9-27.5]
<i>Similiphoma</i> (SIM)	26.7[14.6-42.8]	15[7.6-26.9]	15.4[7.9-27.5]	16.5[9.3-29.2]
<i>Didymella</i> (DIM)	21.4[10.9-35.4]	12.6[5.8-23.2]	13.1[6.05-23.8]	17.1[9.3-29.6]
<i>Cumuliphoma</i> (CUM)	7.6[3.2-13.9]	3.6[1.2-7.6]	3.7[1.3-8]	14.9[7.5-26.2]
<i>Paraboeremia</i> (PAR)	11.5[4.9-21.3]	5.6[1.9-11.7]	5.8[2.1-12.1]	14.9[7.5-26.2]
<i>Macroventuria</i> (MAC)	5.4[1.5-11.6]	2.7[0.5-7.5]	2.9[0.5-7.8]	16.9[8.6-26.2]
<i>Juxtiphoma</i> (JUX)	2.4[0.6-5.3]	1.1[0.2-3.1]	1.1[0.2-3.3]	19[10.6-32.4]
<i>Vacuiphoma</i> (VAC)	16.6[6.2-31.1]	9.3[2.5-19.6]	9.9[2.7-20.4]	21.6[12.7-35.3]
<i>Nothophoma</i> (NOT)	21.5[9.9-38.5]	10.2[4.2-19.3]	10.7[4.4-20]	17.1[9.3-29.6]
<i>Ascochyta</i> (ASC)	14.7[6.9-25.3]	7.5[3.1-13.8]	8[3.5-14.5]	11[5.7-19.2]
<i>Phomatodes</i> (PHT)	7.7[2.7-14.9]	3.7[1-8.2]	3.9[1.1-8.6]	11[5.7-19.2]
<i>Phoma</i> (PHO)	1.2[0.3-2.8]	0.6[0.1-1.6]	0.6[0.1-1.7]	16.5[9-27.7]
<i>Calophoma</i> (CAL)	23.1[11.5-38.1]	12.3[5.6-21.8]	13.1[6.3-23.1]	18.5[10.2-30.7]
<i>Briansuttonomyces</i> (BRI)	0.1[0-0.9]	0.1[0-0.7]	0.1[0-0.7]	6.3[2-13.2]
<i>Pseudoascochyta</i> (PSE)	3.2[1-6.6]	1.5[0.3-3.9]	1.6[0.3-4.1]	6.3[2-13.2]
<i>Leptosphaerulina</i> (LEP)	14.3[5-27.6]	6.7[1.7-14.8]	7.2[2-15.9]	20.2[11.7-33.7]
<i>Neomicrosphaeropsis</i> (NEM)	0.6[0.04-2]	0.3[0.01-1.1]	0.3[0.01-1.1]	13.3[6.9-22.5]
<i>Xenodidymella</i> (XEN)	30.3[15.2-50.7]	14.7[6.7-27.4]	16[7.4-29.1]	25.8[15-42.7]
<i>Neosascochyta</i> (NEA)	31.2[13.8-54.6]	13.5[4-43.3]	15.2[5.3-31.7]	35.7[18.4-63.5]

## Discussion

In recent decade, there has been an increasing interest in molecular dating of species as an effective way of studying molecular evolution (Ho & Philips 2009, dos Reis et al. 2015). A few molecular dating studies have tried to estimate the divergence time of Ascomycetes and their orders (e.g. Beimforde et al. 2014, Hongsanan et al. 2016, Zhao et al. 2016, Hyde et al. 2017, Liu et al. 2017). In this study, we focused on divergence time of Didymellaceae genera for the first time, with added three *Neodidymelliopsis* and one *Didymella* isolates, collected from three provinces of Iran.

The phylogenetic tree indicated that Iranian *Didymella* isolate clustered with *D. glomerata* with a high bootstrap support, this result is also supported by morphological identification (data not published). Newly isolates of *Neodidymelliopsis* obtained in this study are closely related to *N. longicolla* with high bootstrap support. Morphological features of these new fungal isolates collected from citrus in southern Iran, confirmed that they share *Neodidymelliopsis* properties by having possessed pycnidial conidiomata and phialidic conidiogenesis, with hyaline, ampulliform, thin-walled conidiogenous cells, non or 1-septate, smooth-walled, hyaline to pale brown conidia. Characterization of conidiomata, conidia and conidiogenous cells of the isolates were in consistent with the description of *Neodidymelliopsis* reported by Chen et al. (2015). Since the main aim of this study was to focus on the molecular dating of these isolates, more morphological and pathological studies could be subject of further future works.

In the reconstructed phylogenetic tree (Fig. 1), the taxa related to Didymellaceae formed a sister group to the four selected *Aigialus* species within Pleosporales. The Capnodiales can be considered as an out-group, as they diverged from other taxa of Pleosporales. All the nodes of Didymellaceae, Aigialaceae, Pleosporales, and Capnodiales, where the main clusters have been



**Figure 4** – Dated phylogenetic tree of scenario III. The numbers next to the nodes indicate the posterior probability values. The gray horizontal bars show the 95% HPD intervals of the node ages. Nodes 1-3 are the calibration points (MRCA of Dothideomycetes, Capnodiales and *Aigialus* respectively).



branched are well-supported (Fig. 1). These results are congruence with the previous studies (Aveskamp et al. 2010, Hyde et al. 2016, Phukhamsakda et al. 2016, Chen et al. 2017, Valenzuela-Lopez et al. 2018). In molecular dating analyses, the means of estimated ages in scenario I (with one fossil) are lower than other scenarios, with widest interval of 95% HPD (Supplementary table 3). Although the intervals of 95% HPD and the estimated age of the nodes in scenario II and III are very similar, we consider scenario III as the best scenario due to the higher marginal likelihood.

Base on strains and parameters used in this study, we conclude that the earliest divergence in Didymellaceae is in the late Eocene when *Neascochyta* diverged from other genera in Didymellaceae, this followed by the separation of *Neodidymelliopsis* and *Xenodidymella* in the late Oligocene. Other 23 genera diverged from others repeatedly in the Miocene. We speculate that geological changes such as mountain uplift, climate changes and aridification in the Miocene which led to an expansion of plants, might have resulted in the emergence of plant associated fungi as in Didymellaceae genera (Aveskamp et al. 2008, Chen et al. 2015). Iranian *Neodidymelliopsis* sp. and *Didymella* sp. isolates diverged from other isolates in the Pliocene and the late Miocene, respectively (Supplementary table 5), before switching to more seasonal, drier and cooler climate (Amo De Paz et al. 2011).

Moreover, the crown ages of five genera of Didymellaceae including Iranian *Neodidymelliopsis* sp. are in the Pleistocene. This result suggests that the glaciation event of Pleistocene is not restrictive for these genera, as already proposed for some *Melanohalea* species (Ascomycetes) by Leavitt et al. (2012). Five genera including *Ascochyta*, *Neascochyta*, *Heterophoma*, *Phomatodes* and *Neomicrosphaeropsis* are specific to Fabaceae (Rosids), Poaceae (Monocots), Scrophulariaceae (Asterids), Brassicaceae (Rosids) and Tamaricaceae (Asterids), respectively. Among these five genera, *Neascochyta* is the oldest genus which is hosted by Monocots which are older than Asterids and Rosids (Barba-Montoya et al. 2018). The estimated ages of *Ascochyta* and *Phomatodes* chronologically corresponds to the age of their hosts *viz.* Fabaceae and Brassicaceae, estimated by Hohmann et al. (2015). This coincidence supports the co-evolution of Didymellaceae and their host plants proposed by Chen et al. (2017).

Furthermore, we compared the crown ages of *Aigialus*, Capnodiales and Didymellaceae with previous studies (Supplementary table 3). In agreement with Phukhamsakda et al. (2016), we dated *Aigialus* and Capnodiales to Eocene and Cretaceous, respectively. Similar to Prieto & Wedin (2013) study, we dated the Dothideomycetes crown group to the late Jurassic or early Cretaceous. The variation in the ages of the taxa in previous studies could be the consequence of using different fossils, models, sampling and characters. Additionally, previous studies have mostly used a controversial fossil of *Paleopyrenomycetes* to calibrate nodes of the tree (Prieto & Wedin 2013, Beimforde et al. 2014). Higher estimated ages in other studies can also be the result of considering exponential or normal distribution as the prior distribution for secondary calibration on the root node. In contrary, we prefer to use a uniform distribution, which based on Schenk (2016) simulation research. Further molecular dating studies in different locations and on various fungal taxa are needed to clarify this.

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## Supplementary Informations

**Supplementary table 1** Strains used in this study and their GeneBank accession numbers.

Isolate name	Strain number	Host	Province	GeneBank Accession Number			
				LSU	TUB	RPB2	ITS
IR26	CBS 142211; IRAN 2770C	<i>Citrus paradisi</i>	Khuzestan	KY35507 4	KY40779 0	KY386285	KY290226
IR14	CBS 142210;	<i>Citrus aurantium</i>	Kerman	KY35507 3	KY40778 9	KY386284	KY290225
IR10	CBS 142208; IRAN 2771C	<i>Citrus sinensis</i>	Kerman	KY35507 2	KY40778 8	KY386283	KY290224
IR67	CBS 142212 IRAN 2768C	<i>Citrus lemon</i>	Hormozgan	KY35507 5	KY40779 1	KY386286	KY290228

**Supplementary table 2** GeneBank, culture collection code and accession numbers of the isolates used in this study.

Species Name	Strain Number <sup>1</sup>	Host, Substrate	Country	GeneBank Accession Number			
				LSU	ITS	TUB	RPB2
<i>Aigialus grandis</i>	BCC 20000	Mangrove wood	Malaysia	GU4797 75	-	-	GU4798 14
<i>A. mangrovei</i>	BCC 33563	Mangrove wood	Thailand	GU4797 76	-	-	GU4798 15
<i>A. parvus</i>	BCC 18403	Mangrove wood	Malaysia	GU4797 78	-	-	GU4798 17
<i>A. rhizophorae</i>	BCC 33572	Mangrove wood	Thailand	GU4797 80	-	-	GU4798 19
<i>Allophoma labilis</i>	CBS 124.93	<i>Solanum lycopersicum</i>	The Netherlands	GU2380 91	GU2377 65	GU2376 19	KT3895 52
<i>A. minor</i>	CBS 325.82; FMR 14905	<i>Syzygium aromaticum</i>	Indonesia	GU2381 07	GU2378 31	GU2376 32	KT3895 53
<i>A. piperis</i>	CBS 268.93	<i>Peperomia pereskiifolia</i>	The Netherlands	GU2381 29	GU2378 16	GU2376 44	KT3895 54
<i>A. zantedeschiae</i>	CBS 131.93	<i>Calla</i> sp.	The Netherlands	GU2381 59	FJ42708 4	FJ42718 8	KT3895 57
<i>Ascochyta rabiei</i>	CBS 206.30	Unknown	Unknown	KT3896 95	KT3894 78	KT3897 72	KT3895 59
<i>A. versabilis</i>	CBS 876.97	<i>Silene</i> sp.	The Netherlands	GU2381 52	GU2379 09	GU2376 64	KT3895 61
<i>A. viciae</i>	CBS 451.68	<i>Vicia sepium</i>	The Netherlands	KT3897 01	KT3894 84	KT3897 78	KT3895 62
<i>Boeremia exigua1</i>	CBS 118.38	<i>Cheiranthus cheiri</i>	Denmark	KT3897 06	KT3894 89	KT3897 83	KT3895 82
<i>B. exigua2</i>	CBS 119.38	<i>Nicotiana tabacum</i>	Unknown	KT3897 07	KT3894 90	KT3897 84	KT3895 83
<i>B. lycopersici</i>	CBS 378.67	<i>Solanum lycopersicum</i>	The Netherlands	GU2379 50	GU2378 48	GU2375 12	KT3895 80
<i>Briansuttonomyces eucalypti</i>	CBS 114879; CPC 362	<i>Eucalyptus</i> sp.	South Africa	KU7285 19	KU7284 79	KU7285 95	-
<i>B. eucalypti</i>	CBS 11887; CPC 363	<i>Eucalyptus</i> sp.	South Africa	KU7285 20	KU7284 80	KU7285 96	-
<i>Calophoma aquilegiicola</i>	CBS 107.96	<i>Aconitum pyramidale</i>	The Netherlands	GU2380 41	GU2377 35	GU2375 81	KT3895 86
<i>C. clematidina</i>	CBS 102.66	<i>Clematis</i> sp.	UK	FJ51563 0	FJ42698 8	FJ42709 9	KT3895 87
<i>C. clematidis-rectae</i>	CBS 507.63	<i>Clematis</i> sp.	The Netherlands	FJ51564 7	FJ51560 6	FJ51562 4	KT3895 89
<i>C. rosae</i>	CGMCC 3.18347	<i>Rosa</i> sp.	China	KY7422 03	KY7420 49	KY7422 91	KY7421 35
<i>Capnodiales sp.001</i>	010301	<i>Dacrydium araucarioides</i>	New Caledonia	KF1579 91	-	-	-
<i>Capnodiales sp.003</i>	010302	<i>Epiphytic fungus</i>	New Caledonia	KF1579 92	-	-	-
<i>Capnodium coffeae</i>	CBS 147.52			DQ2478 00	-	-	DQ2477 88
<i>Cumuliphoma indica1</i>	CBS 654.77; FMR 15341	Unknown	India	GU2381 22	FJ42704 3	FJ42715 3	LT6232 61
<i>C.indica2</i>	CBS 991.95; FMR 15331	Soil	Papua New Guinea	GU2381 21	FJ42704 4	FJ42715 4	LT6232 62

Supplementary table 2 Continued.

Species Name	Strain Number <sup>1</sup>	Host, Substrate	Country	GeneBank Accession Number			
				LSU	ITS	TUB	RPB2
<i>C. omnivirens</i>	CBS 341.86; FMR 14915	<i>Phaseolus vulgaris</i>	Belgium	LT6232 14	FJ42704 2	FJ42715 2	LT6232 60
<i>C. pneumoniae</i>	CBS 142454; UTHSC: DL16-246; FMR 13739	Human respiratory tract	USA	LN9073 92	LT5929 25	LT5929 94	LT5930 63
<i>D. aliena</i>	CBS 379.93	<i>Berberis</i> sp.	TheNetherlands	GU2380 37	GU2378 51	GU2375 78	KP3304 16
<i>D. arachidicola</i>	CBS 333.75	<i>Arachis hypogaea</i>	South Africa	GU2379 96	GU2378 33	GU2375 54	KT3895 98
<i>D. glomerata</i>	CBS 528.66; PD 63/590	<i>Chrysanthemum</i> sp.	TheNetherlands	JX6811 05	FJ42701 3	FJ42712 4	GU3717 81
<i>Didymella pinodes</i>	CBS 525.77	<i>Pisum sativum</i>	Belgium	GU2380 23	GU2378 83	GU2375 72	KT3896 14
<i>Ectophoma multirostrata1</i>	CBS 110.79; FMR 15342	<i>Cucumis sativus</i>	TheNetherland	GU2381 10	FJ42703 0	FJ42714 0	LT6232 64
<i>E. multirostrata2</i>	CBS 274.60; FMR 15335	Soil	Maharashtra	GU2381 11	FJ42703 1	FJ42714 1	LT6232 65
<i>E. multirostrata3</i>	CBS 368.65; FMR 15336	Unknown	India	GU2381 12	FJ42703 3	FJ42714 3L	T62326 6
<i>E. pomi</i>	CBS 267.92; FMR 15346	<i>Coffea arabica</i>	India	GU2381 28	GU2378 14	GU2376 43	LT6232 63
<i>Epicoccum brasiliense</i>	CBS 120105; FMR 14907	<i>Amaranthus</i> sp.	Brazil	GU2380 49	GU2377 60	GU2375 88	KT3896 27
<i>E. draconis</i>	CBS 186.83; FMR14908	<i>Dracaena</i> sp.	Rwanda	GU2380 70	GU2377 95	GU2376 07	KT3896 28
<i>E. henningsii</i>	CBS 104.80	<i>Acaciamearnsi</i>	Kenya	GU2380 81	GU2377 31	GU2376 12	KT3896 29
<i>E. plurivorum</i>	CBS 558.81; FMR 14909	<i>Setaria</i> sp.	New Zealand	GU2381 32	GU2378 88	GU2376 47	KT3896 34
<i>Heterophoma adonidis</i>	CBS 114309	<i>Adonis vernalis</i>	Sweden	KT3897 24	KT3895 06	KT3898 03	KT3896 37
<i>H. nobilis</i>	CBS 507.91	<i>Dictamnus albus</i>	The Netherlands	GU2380 65	GU2378 77	GU2376 03	KT3896 38
<i>H. verbascicola1</i>	CGMCC 3.18364	<i>Verbascum thapsus</i>	China	KY7422 73	KY7421 19	KY7423 61	KY7421 87
<i>H. verbascicola2</i>	LC 8164	<i>Verbascum thapsus</i>	China	KY7422 74	KY7421 20	KY7423 62	KY7421 88
<i>Juxtiphoma eupyrena1</i>	CBS 374.91; FMR 15329	<i>Solanum tuberosum</i>	The Netherlands	GU2380 72	FJ42699 9	FJ42711 0	LT6232 68
<i>J. eupyrena2</i>	CBS 527.66; FMR 15337	Wheat field soil	Germany	GU2380 73	FJ42700 0	FJ42711 1	LT6232 69
<i>Leptosphaerulina americana</i>	CBS 213.55	<i>Trifolium pratense</i>	USA	GU2379 81	GU2377 99	GU2375 39	KT3896 41
<i>L. australis</i>	CBS 317.83	<i>Eugenia aromatica</i>	Indonesia	EU7541 66	GU2378 29	GU2375 40	GU3717 90
<i>Macroventuria anomochaeta</i>	CBS 525.71	<i>Decayed canvas</i>	South Africa	GU2379 84	GU2378 81	GU2375 44	GU4563 46
<i>M. wentii</i>	CBS 526.71	<i>Plant litter</i>	USA	GU2379 86	GU2378 84	GU2375 46	KT3896 42
<i>Neosascochyta desmazieri</i>	CBS 297.69	<i>Lolium perenne</i>	Germany	KT3897 26	KT3895 08	KT3898 07	KT3896 44

Supplementary table 2 Continued.

Species Name	Strain Number <sup>1</sup>	Host, Substrate	Country	GeneBank Accession Number			
				LSU	ITS	TUB	RPB2
<i>N. europaea</i>	CBS 820.84	<i>Hordeum vulgare</i>	Germany	KT3897 29	KT3895 11	KT3898 09	KT3896 46
<i>N. exitialis</i>	CBS 118.40	Unknown	Unknown	KT3897 32	KT3895 14	KT3898 12	KT3896 47
<i>N. graminicola</i>	CBS 301.69	<i>Lolium multiflorum</i>	Germany	KT3897 37	KT3895 19	KT3898 17	KT3896 50
<i>Neodidymelliopsis cannabis</i>	CBS 234.37	<i>Cannabis sativa</i>	Unknown	GU2379 61	GU2378 04	GU2375 23	KP3304 03
<i>N. longicolla</i>	CBS 38296	Soil in desert	Israel	KT3897 50	KT3895 32	KT3898 30	-
<i>N. polemonii</i>	CBS 109181	<i>Polemonium caeruleum</i>	The Netherlands	GU2381 33	GU2377 46	KT3898 28	KP3304 27
<i>N. xanthina</i>	CBS 383.68	<i>Delphinium</i> sp.	The Netherlands	GU2381 57	GU2378 55	KT3898 31	KP3304 31
<i>Neomicrosphaeriopsis italica</i>	MFLUCC 15-0485	<i>Tamarix</i> sp.	Italy	KU7298 54	KU9003 18	-	KU6748 20
<i>N. italica</i>	MFLUCC 15-0484	<i>Tamarix</i> sp.	Italy	KU7298 53	KU9003 19	KX4532 98	KU6955 39
<i>Nothophoma anigozanthi</i>	CBS 381.91; FMR 14914	<i>Anigozanthus maugleisii</i>	The Netherlands	GU2380 39	GU2378 52	GU2375 80	KT3896 55
<i>N. arachidis-hypogaeae</i>	CBS 125.93	<i>Arachis hypogaea</i>	India	GU2380 43	GU2377 71	GU2375 83	KT3896 56
<i>N. gossypicola</i>	CBS 377.67; FMR14912	<i>Gossypium</i> sp.	USA	GU2380 79	GU2378 45	GU2376 11	KT3896 58
<i>N. infossa</i>	CBS 123395	<i>Fraxinus pennsylvanica</i>	Argentina	GU2380 89	FJ42702 5	FJ42713 5	KT3896 59
<i>Paraboeremia adianticola</i>	CBS 187.83; FMR 15344	<i>Polystichum adiantiforme</i>	USA	GU2380 35	GU2377 96	GU2375 76	KP3304 01
<i>P. litseae</i>	CGMCC 3.18110	<i>Litsea</i> sp.	China	KX8290 37	KX8290 29	KX8290 53	KX8290 45
<i>P. putaminum</i>	CBS 130.69; FMR 15338	<i>Malus sylvestris</i>	Denmark	GU2381 38	GU2377 77	GU2376 52	LT6232 54
<i>P. selaginellae</i>	CBS122.93	<i>Selaginella</i> sp.	The Netherlands	GU2381 42	GU2377 62	GU2376 56	LT6232 55
<i>Phoma herbarum1</i>	CBS 377.92; IMI 21384	Human leg	UK	KT3897 56	KT3895 36	KT3898 37	KT3896 63
<i>P. herbarum2</i>	UTHSC:DL16-319; FMR 13812	<i>Humansuperficial tissue</i>	USA	LN9074 62	LT5929 55	LT5930 24	LT5930 24
<i>P. herbarum3</i>	CBS 502.91	<i>Nerium</i> sp.	The Netherlands	GU2380 82	GU2378 74	GU2376 13	KP3304 19
<i>P. herbarum4</i>	CBS 615.75; FMR 15340	<i>Rosa multiflora</i> cv. <i>Cathayensis</i>	The Netherlands	KF2517 15	FJ42702 2	KF2527 03	KP3304 20
<i>Phomatodes aubrietiae</i>	CBS 627.97	<i>Aubrietia</i> sp.	The Netherlands	GU2380 45	GU2378 95	GU2375 85	KT3896 65
<i>P. nebulosi</i>	CBS 100191	<i>Thlaspi arvense</i>	Poland	KP3304 46	KP3304 34	KP3303 90	KT3896 66
<i>P. nebulosi</i>	CBS 740.96	<i>Armoracia rusticana</i>	The Netherlands	KT3897 58	KT3895 40	KT3898 39	KT3896 67
<i>Pseudoascochyta novae-zelandiae</i>	CBS 141689; FMR 15110; ICMP 10493	<i>Cordyline australis</i>	New Zealand	LT5928 93	LT5928 92	LT5928 94	LT5928 95

Supplementary table 2 Continued.

Species Name	Strain Number <sup>1</sup>	Host, Substrate	Country	GeneBank Accession Number			
				LSU	ITS	TUB	RPB2
<i>P. pratensis</i>	CBS 141688; FMR 14524	Soil	Spain	LT2231 31	LT2231 30	LT2231 32	LT2231 33
<i>Remotididymella anthropophila</i>	CBS 142462; UTHSC:DI16-278; FMR 13770	Human respiratory tract	USA	LN9074 21	LT5929 36	LT5930 05	LT5930 75
<i>R. destructiva1</i>	CBS 133.93; FMR 15349	<i>Solanum lycopersicon</i>	Guadeloupe	GU2380 64	GU2377 79	GU2376 02	LT6232 57
<i>R. destructiva2</i>	CBS 378.73; FMR 15328	<i>Lycopersicon esculentum</i>	Tonga	GU2380 63	GU2378 49	GU2376 01	LT6232 58
<i>R. destructiva3</i>	CBS 162.78; FMR 14906	<i>Lycopersicon esculentum</i>	The Netherlands	GU2380 62	GU2377 88	GU2376 00	LT6232 59
<i>Similiphoma crystallifera</i>	CBS 193.82; FMR 1534	<i>Chamaespartium sagittale</i>	Austria	GU2380 60	GU2377 97	GU2375 98	LT6232 67
<i>Stagonosporopsis dorenboschii</i>	CBS 426.90	<i>Physostegia virginiana</i>	The Netherlands	GU2381 85	GU2378 62	GU2376 90	KT3896 78
<i>S. hortensis</i>	CBS 572.85	<i>Phaseolus vulgaris</i>	The Netherlands	GU2381 99	GU2378 93	GU2377 04	KT3896 81
<i>Vacuiphoma bulgarica</i>	CBS 357.84; FMR 14917	<i>Trachystemon orientale</i>	Bulgaria	GU2380 50	GU2378 37	GU2375 89	LT6232 56
<i>V. oculihominis</i>	UTHSC:DI16-308; FMR 13801	Human superficial tissue	USA	LN9074 51	LT5929 54	LT5930 23	LT5930 93
<i>Xenodidymella applanata</i>	CBS 205.63	<i>Rubus idaeus</i>	The Netherlands	GU2379 98	GU2377 98	GU2375 56	KP3304 02
<i>X. asphodeli</i>	CBS 375.62	<i>Asphodelus albus</i>	France	KT3897 65	KT3895 49	KT3898 53	KT3896 89
<i>X. catariae</i>	CBS 102635	<i>Nepeta catenaria</i>	The Netherlands	GU2379 62	GU2377 27	GU2375 24	KP3304 04
<i>X. humicola</i>	CBS 220.85	<i>Franseria</i> sp.	USA	GU2380 86	GU2378 00	GU2376 17	KP3304 22

1 Abbreviation of culture collections: CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection, Beijing, China; CPC: Culture collection of Pedro Crous, housed at CBS; FMR, Facultatde Medicina, Universitat Rovirai Virgili, Reus, Spain; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakenham Lane, IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; U.K.; LC: Culture collection of Qian Chen, housed at CAS, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; PD: Plant Protection Service, Wageningen, the Netherlands; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA.

Supplementary table 3 The divergence time of the nodes in different study

Node	This study	Beimforde et al. (2014)	Prieto & Wedin (2013)	Gueidan et al. (2011)	Phukhamsakda et al. (2016)	Pérez-Ortega et al. (2016)
DOT	144.5 (107-202.9)	350 (273-459)	174 (107-204)	338	293 (213-371)	290
DID	35.7 (18.4-63.5)	-	-	-	-	-



### Supplementary table 3

Node	This study	Beimforde et al. (2014)	Prieto & Wedin (2013)	Gueidan et al. (2011)	Phukhamsakda et al. (2016)	Pérez-Ortega et al. (2016)
PLE	86.7 (53.9-155.4)	-	-	-	211 (153-277)	-
CAP	99 (99-100)	-	-	-	147 (102-202)	-
AIG	34.1 (34-64.4)	-	-	-	39 (35-49)	-

**Supplementary table 4** Time chart of the nodes based on the estimated crown age in the current study\*

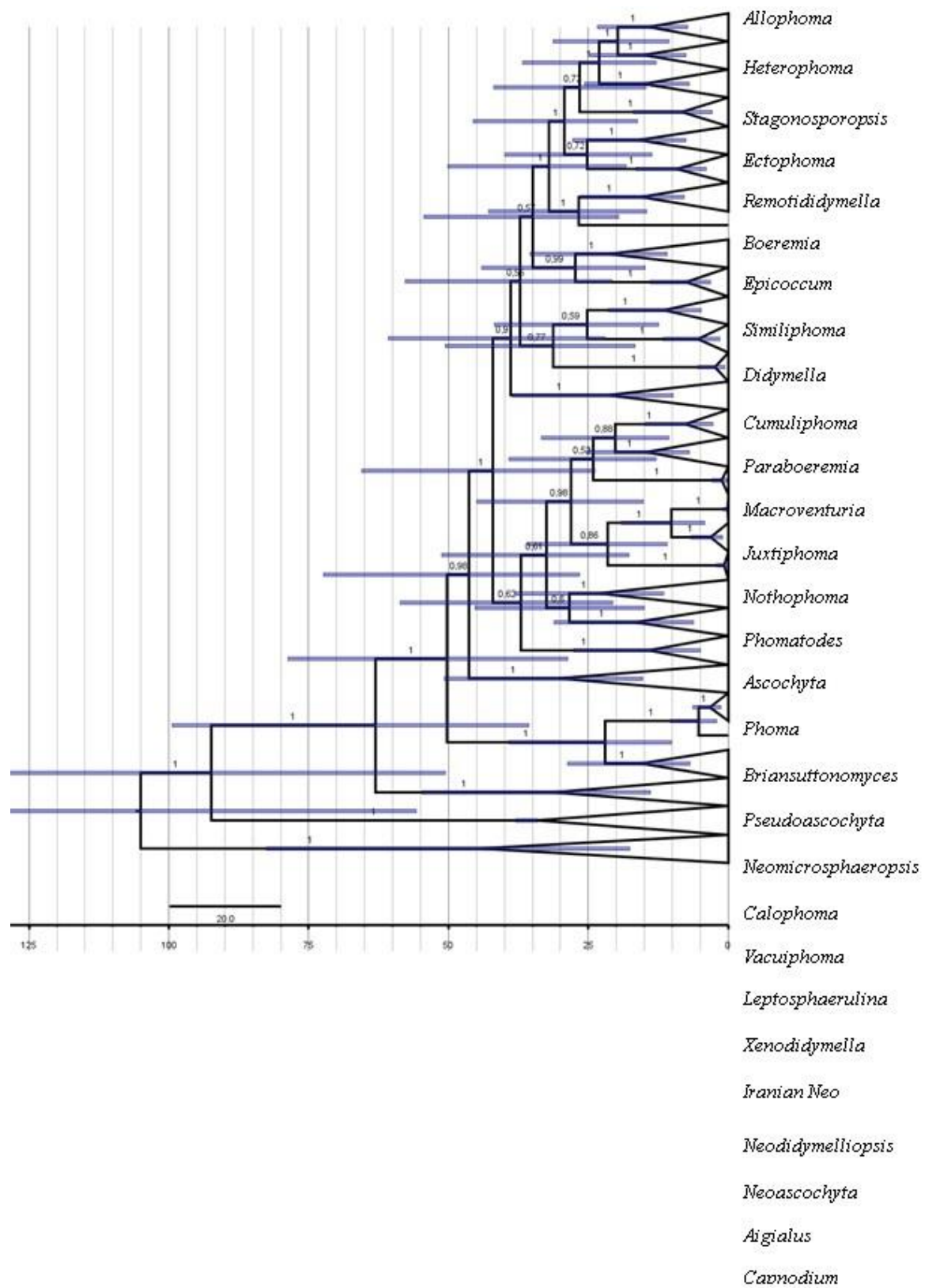
Period	Epoch	Stage	Age	Taxa	
Quaternary	Holocene		0-0.012		
		Pleistocene	Late	0.012-0.126	<i>Briansuttonomyces</i>
		Middle	0.126-1.8	<i>Neomicrosphaeropsis</i> ; <i>Phoma</i> ; <i>Juxtiphoma</i> ; Iranian <i>Neodidymelliopsis</i> ; <i>Pseudoascochyta</i>	
		Early	1.8-2.58	-	
	Miocene	Late		5.33-11.63	<i>Paraboeremia</i> ; <i>Leptosphaerulina</i> ; <i>Allophoma</i> ; <i>Ascochyta</i> ; <i>Heterophoma</i> ; <i>Stagonosporopsis</i> ; <i>Epicoccum</i> ; <i>Remotididymella</i> ; <i>Vacuiphoma</i> ; <i>Nothophoma</i> ; <i>Neodidymelliopsis</i> ;
			Middle	11.63-16	<i>Calophoma</i> ; <i>Didymella</i> ; <i>Neoascochyta</i> ; <i>Similiphoma</i> ; <b><i>Xenodidymella</i></b> ;
		Early	16-23.03	-	
Paleogene	Oligocene	Late	23.03-27.82	-	
		Early	27.82-33.9		
	Eocene	Late	33.9-37.8	<i>Aigialus</i> ; Didymellaceae	
		Middle	37.8-47.8		
	Early	47.8-56			
Paleocene		56-66			
Cretaceous			66-147.5	Pleosporales; Capnodiales; Dothideomycetes	

\*The geological time scales were retrieved from the International Commission on Stratigraphy (ICS) website (www.stratigraphy.com)

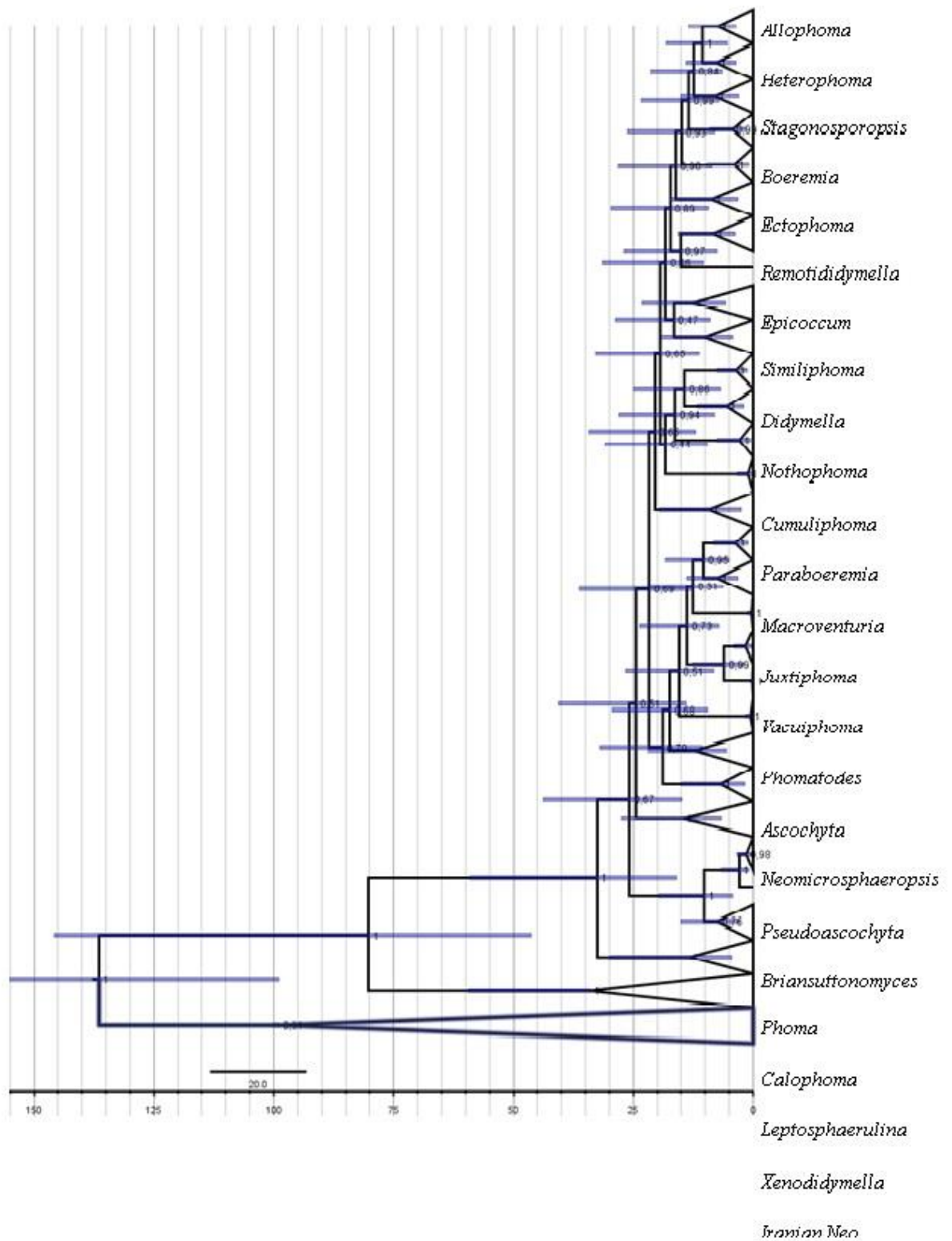
**Supplementary table 5** Time chart of the nodes based on the estimated stem age in the current study\*

<b>Period</b>	<b>Epoch</b>	<b>Stage</b>	<b>Age</b>	<b>Taxa</b>
Quaternary	Holocene		0-0.012	
	Pleistocene	Late	0.012-2.58	-
Neogene	Pliocene		2.58-5.33	Iranian <i>Neodidymelliopsis</i> - <i>Neodidymelliopsis</i>
		Miocene	Late	5.33-11.63
		Early	16-23.03	<i>Remotididymella</i> <i>Phoma</i> <i>Similiphoma</i> <i>Macroventuria</i> <i>Didymella</i> <i>Nothophoma</i> <i>Calophoma</i> <i>Juxtiphoma</i> <i>Leptosphaerulina</i> <i>Vacuiphoma</i>
Paleogene	Oligocene	Late	23.03-27.82	<i>Xenodidymella</i> <i>Neodidymelliopsis</i>
		Early	27.82-33.9	-
	Eocene	Late	33.9-37.8	<i>Neoascochyta</i>
		Middle	37.8-47.8	-
		Early	47.8-56	-
	Paleocene		56-66	
Cretaceous			66-147.5	<i>Aigialus</i> - <i>Didymellaceae</i> Pleosporales- <i>Capnodiales</i>

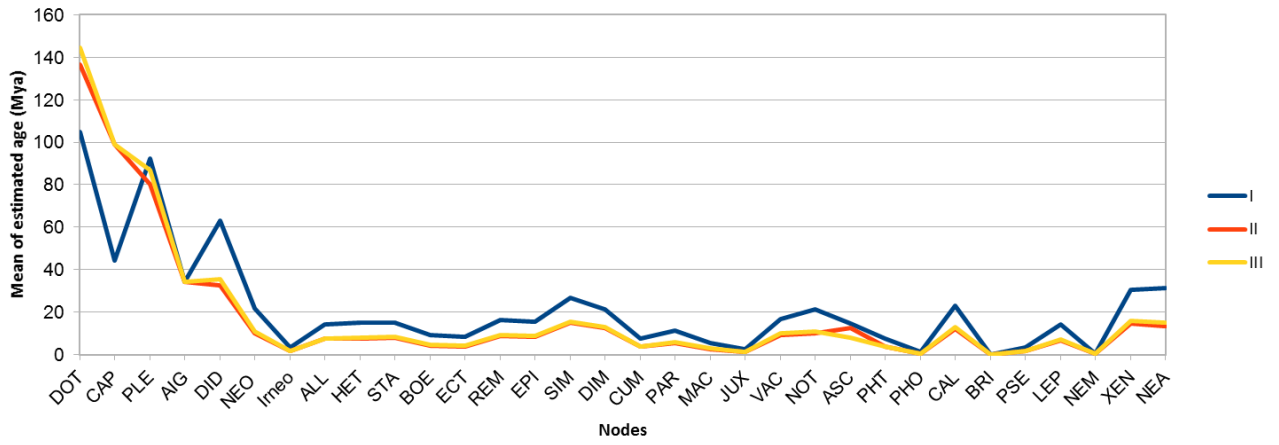
\*The geological time scales were retrieved from the International Commission on Stratigraphy (ICS) website (www.stratigraphy.com)



**Supplementary figure 1** – Dated phylogenetic tree of scenario I. The numbers next to the nodes indicate posterior probability values.



**Supplementary figure 2** – Dated phylogenetic tree of scenario II. The numbers next to the nodes indicate posterior probability values.



**Supplementary figure 3** – The means of estimated age of different nodes in scenario I (blue), II (red) and III (yellow)