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## Estimating the Phanerozoic history of the Ascomycota lineages: Combining fossil and molecular data



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### ABSTRACT

The phylum Ascomycota is by far the largest group in the fungal kingdom. Ecologically important mutualistic associations such as mycorrhizae and lichens have evolved in this group, which are regarded as key innovations that supported the evolution of land plants. Only a few attempts have been made to date the origin of Ascomycota lineages by using molecular clock methods, which is primarily due to the lack of satisfactory fossil calibration data. For this reason we have evaluated all of the oldest available ascomycete fossils from amber (Albian to Miocene) and chert (Devonian and Maastrichtian). The fossils represent five major ascomycete classes (Coniocybomyces, Dothideomyces, Eurotiomyces, Laboulbeniomyces, and Lecanoromyces). We have assembled a multi-gene data set (18SrDNA, 28SrDNA, RPB1 and RPB2) from a total of 145 taxa representing most groups of the Ascomycota and utilized fossil calibration points solely from within the ascomycetes to estimate divergence times of Ascomycota lineages with a Bayesian approach. Our results suggest an initial diversification of the Pezizomycotina in the Ordovician, followed by repeated splits of lineages throughout the Phanerozoic, and indicate that this continuous diversification was unaffected by mass extinctions. We suggest that the ecological diversity within each lineage ensured that at least some taxa of each group were able to survive global crises and rapidly recovered.

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### 1. Introduction

The Fungi constitute a major group of eukaryotic organisms (Hawksworth, 1991, 2001). They exhibit a broad variety of life-styles and morphologies ranging from single celled organisms to multi-cellular colonies which can be among the largest and possibly oldest organisms on earth (Brazee et al., 2012). Most aquatic and terrestrial ecosystems are occupied by a diverse range of fungal species. With over 64,000 described species in approximately 6400 genera, the phylum Ascomycota is by far the largest phylum in the fungal kingdom (Kirk et al., 2008; Blackwell, 2011). The autapomorphy of this group is a sack-like structure, the ascus, in which the sexual spores are produced. Species of the Ascomycota

are extremely variable in morphology and ecology. As degraders of persistent organic materials such as lignin and keratin, ascomycetes play an important role in nutrient cycling. Additionally, many ascomycetes participate in symbiotic associations including mycorrhizae and lichens.

Phylogenetic relationships among major groups of the Pezizomycotina have been the subject of many recent studies (e.g. Liu and Hall, 2004; Lutzoni et al., 2004; Spatafora et al., 2006; Schoch et al., 2009a; Miadlikowska et al., 2006; Hibbett et al., 2007; Ebersberger et al., 2012; Kumar et al., 2012; Morgenstern et al., 2012). Several attempts have also been made to date the origin and subsequent evolution of main fungal lineages by molecular clock methods (e. g. Heckman et al., 2001; Sanderson, 2003a; Berbee and Taylor, 1993, 2007; Taylor and Berbee, 2006; Padovan et al., 2005; Lücking et al., 2009, Berbee and Taylor, 2010; Gueidan et al., 2011; Floudas et al., 2012; Ohm et al., 2012,

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Amo de Paz et al., 2011, Prieto and Wedin, 2013). Fungi probably derived from aquatic ancestors and diverged at a relatively early stage during the evolution of the Eukaryota (e.g. Steenkamp et al., 2006; Liu et al., 2009; Lara et al., 2010). Their time of divergence, however, is still a matter of debate. Simon et al. (1993) were the first to apply a molecular clock to a fungal phylogeny. Subsequently, Heckman et al. (2001) estimated that Fungi had occupied terrestrial habitats for at least 1000 million years, an estimate which was revised by Sanderson (2003a). However, these studies did not consider substitution rate variation, a phenomenon now known to be common in many organism lineages. The existence of such variation in the fungal phylogeny was demonstrated by Berbee and Taylor (1993, 2010) and is a challenging problem, even under the assumption of relaxed clock models, which are able to accommodate variable substitution rates across individual groups and genes (e.g. Sanderson, 2003b; Drummond et al., 2006; Drummond and Rambaut, 2007). Considering the number of ascomycete species and the broad range of morphologies and life-forms they possess, substitution rate heterogeneity is likely to be quite drastic across their phylogeny, even at the class level (Lutzoni and Pagel, 1997; Woolfit and Bromham, 2003; Lumbsch et al., 2008). Besides improving analytical methods of molecular evolution, the integration of fossil evidence of individual fungal lineages would help to partly overcome this problem (Berbee and Taylor, 2010). Many other studies of molecular evolution showed the importance of constraining molecular clocks with fossil evidence (Benton et al., 2009; Hedman, 2010; Inoue et al., 2010; Magallon, 2010; Pyron, 2010; Wilkinson et al., 2011; Lukoschek et al., 2012; Sauquet et al., 2012). A crucial requirement for the use of fossils as minimum age constraints is their accurate placement to specific nodes in the phylogeny under study (Rutschmann et al., 2007; Marshall, 2008; Forest, 2009; Parham et al., 2012; Pyron, 2010; Dornburg et al., 2011). Reliable assignment of fossil taxa to modern phylogenies requires accurate information about their systematic position and age. In this regard, fossilized Fungi preserved in amber and chert are excellent material as they conserve even delicate microstructures regardless of their susceptibility to decay (Stankiewicz et al., 1998; Martínez-Delclós et al., 2004). This allows the precise assignment of fossil data to specific phylogenetic nodes.

In order to test the potential use for molecular evolution models of Fungi we have evaluated 13 extraordinarily well preserved and precisely dated fossil ascomycetes, which represent the oldest fossil representatives of their respective lineages (see Table 1). The fossil Fungi are preserved in amber from various deposits spanning an Albian to Miocene age (about 100–17 million years old) as well as in Devonian and Maastrichtian cherts (about 410 and 66.5 million years old, respectively).

Here we have assembled a multi-gene data set with a total of 145 modern taxa including representatives of most Ascomycota classes, and utilized five fossils of Pezizomycotina from amber and chert to estimate divergence times of the main classes. We have explicitly used fossil ascomycetes as minimum age constraints to avoid using secondary node calibrations (age estimates from previous studies). For comparison and to evaluate the influence of our internal node constraints, we also performed an analysis with identical parameter settings but with *Paleopyrenomycites* as the sole constraint for Pezizomycotina.

This is the first study that evaluates all available fossil ascomycetes that represent the oldest reliable evidence of respective extant lineages and discusses their suitability as minimum age constraints for molecular evolution studies. Five fossils were suitable for serving multiple calibration points within our dataset. Our results show that the integration of minimum age constraints in terminal groups of ascomycete classes significantly affects the estimated divergence times of both early branching nodes and

nodes of terminal groups of Ascomycota lineages by pushing them back in time. According to our results the diversification of the Pezizomycotina started in the Ordovician, followed by a continuous diversification throughout the Phanerozoic that was likely unaffected by mass extinctions.

## 2. Material and methods

### 2.1. Fossil ascomycetes from amber and chert

Specimens of all available fossil ascomycetes from amber and chert representing the oldest fossil evidence of their respective lineages (Table 1) were reinvestigated considering their potential use as minimum age constraints in molecular models, following the guidelines provided by Parham et al. (2012).

#### 2.1.1. Fossil Coniocybomycetes

A well-preserved specimen of *Chaenotheca* preserved in Baltic amber (50 – 35 Ma; Fig. 1h; Rikkinen, 2003) clearly belongs to the Coniocybaceae. Until recently, the phylogenetic position of this family remained enigmatic (Tibell, 2001; Tibell and Koffman, 2002). Prieto et al. (2013) proposed that this group of mazaediate fungi is an early diverging group in the inoperculate ascomycetes and defined new the class and order Coniocybomycetes, Coniocybales. Our data strongly support the findings of Prieto et al. (2013).

#### 2.1.2. Fossil Dothideomycetes

Several fossils from Mesozoic and Cenozoic amber deposits closely resemble extant species of the genus *Metacapnodium* (Metacapnodiaceae, Capnodiales; Schmidt et al., 2014). These Fungi belong to the sooty moulds, a term that is commonly used for an ecological group of saprophytic Fungi that live on the surfaces of living plants. Hyphae of *Metacapnodium* have a characteristic growth form with subglobose cells and gradually tapering apices. The oldest fossil representative of the Metacapnodiaceae is enclosed in Early Cretaceous Charentes amber from France dating about 100 Ma (Fig. 1b).

Distinctive conidiophores and a plethora of septate, mostly four-celled and slightly curved conidia are enclosed in a piece of Ethiopian amber (95–93 Ma; Fig. 1c; Schmidt et al., 2010a). The structures are very similar to those of the extant genus *Curvularia* (Pleosporaceae, Pleosporales) but could also represent a species of some other genus in the family (e.g. *Drechslera*, *Bipolaris*, *Exserohilum*). For this reason the authors did not assign the fossil to a modern genus and introduced the new fossil genus *Palaeocurvularia*.

The fossil parasite *Petropus brachyphylli* (Fig. 1d) was described from silicified conifer leaves (*Brachyphyllum patens*; Van der Ham and Van Konijnenburg-van Cittert, 2003) of late Maastrichtian chert (66.5 Ma) by Van der Ham and Dortangs (2005). *P. brachyphylli* is considered to be closely related to the extant *Phaeocryptopus*. *Phaeocryptopus* is very likely polyphyletic and either belongs in the Dothideales or Capnodiales (Zhang et al., 2011; Winton et al., 2007).

#### 2.1.3. Fossil Eurotiomycetes

*Aspergillus collembolorum* (Fig. 1f) is preserved in Eocene Baltic amber (50–35 Ma) and was described by Dörfelt and Schmidt (2005). The fossil includes numerous well preserved conidiophores very similar to those of modern species of the *Aspergillus flavus* group (Trichocomaceae, Eurotiales).

Rikkinen and Poinar (2000) described *Chaenothecopsis bitterfeldensis* from Bitterfeld amber (23 Ma; Fig. 1i). Two further specimens of the same genus were described from Eocene Baltic and Oligocene Bitterfeld amber dating back to 50–35 Ma and 23 Ma,

**Table 1**  
List of ascomycete fossils from amber and chert representing the oldest fossil evidence of their respective lineages, including assignment to extant relatives, repository, references for phylogenetic analyses and age. The fossils are arranged by their age (from old to young).

Fossil taxon	Level of assignment	Collection	Reference for fossil description	Material and age	Reference for stratigraphy
<i>Paleopyrenomycites devonicus</i> <sup>a</sup>	Pezizomycotina	PB 3411, W. Remy collection, Forschungsstelle für Paläobotanik, Westfälische Wilhelms-Universität, Münster	Taylor et al. (1999, 2005)	Devonian Rhynie Chert 410 Ma	Richardson (1967), Rice et al. (1995)
Metacapnodiaceae <sup>a</sup>	Metacapnodiaceae, Capnodiales	IGR.ARC-115.3b, Amber collection of Géosciences Rennes at the University Rennes 1	Schmidt et al. (2014)	Charentes amber 100 Ma	Néraudeau et al. (2002), Perrichot et al. (2010)
<i>Palaeocurvularia variabilis</i>	Pleosporaceae, Pleosporales	MB. Pb.2009/201, Museum für Naturkunde Berlin	Schmidt et al. (2010a,b)	Ethiopian amber 95–93 Ma	Schmidt et al. (2010b)
<i>Petropus brachyphylli</i>	Capnodiales or Dothideales	NHMM RD 265, Natuurhistorisch Museum Maastricht	Van der Ham and Dortangs (2005)	Limburg Chert, Netherlands 66.5 Ma	Jagt (1999)
<i>Anzia electra</i> <sup>a</sup>	<i>Anzia</i> , Parmeliaceae, Lecanorales	Oschin 5/0, collection of M. Oschin, Los Angeles	Rikkinen and Poinar (2002)	Baltic amber 50–35 Ma	Standke (2008)
<i>Aspergillus collemborum</i> <sup>a</sup>	<i>Aspergillus</i> , Trichocomaceae, Eurotiales	No. 805, collection of C & W Hoffeins, Hamburg	Dörfelt and Schmidt (2005)	Baltic amber 50–35 Ma	Standke (2008)
<i>Calicium</i> sp. <sup>a</sup>	<i>Calicium</i> , Caliciaceae, Lecanorales	GZG.BST.27296, Geoscientific Collections of the Georg August University, Göttingen (formerly Arnold collection 1294)	Rikkinen (2003)	Baltic amber 50–35 Ma	Standke (2008)
<i>Chaenotheca</i> sp.	<i>Chaenotheca</i> , Coniocybaceae, Coniocybales	GZG.BST.27297, Geoscientific Collections of the Georg August University, Göttingen (formerly Arnold collection 1285)	Rikkinen (2003)	Baltic amber 50–35 Ma	Standke (2008)
<i>Chaenothecopsis</i> sp.	<i>Chaenothecopsis</i> , Mycocaliciaceae, Mycocaliciales	GZG.BST.27286, Geoscientific Collections of the Georg August University, Göttingen	Tuovila et al. (2013)	Baltic amber 50–35 Ma	Standke (2008)
<i>Gonatobotryum piceae</i>	Pezizomycotina	No. F129/BB/F/CJW, collection of J. Wunderlich, Hirschberg an der Bergstraße	Dörfelt and Schmidt (2007)	Baltic amber 50–35 Ma	Standke (2008)
<i>Stigmatomyces succini</i>	<i>Stigmatomyces</i> , Laboulbeniaceae, Laboulbeniales	Zoologische Staatssammlung München	Rossi et al. (2005)	Bitterfeld amber 23 Ma	Blumenstengel et al. (1999), Dunlop (2010)
<i>Parmelia ambra</i> <i>Parmelia isidiiveteris</i>	Parmeliaceae, Lecanorales	AF9-17E and AF9-17B, amber collection of G.O. Poinar, Oregon State University	Rikkinen and Poinar (2000)	Dominican amber 17 Ma	Iturralde-Vinent and Mac Phee (1996), Iturralde-Vinent, 2001
<i>Phyllopsora dominicanus</i>	<i>Phyllopsora</i> , Ramaliaceae, Lecanorales	Poinar B 1–23, amber collection of G.O. Poinar, Oregon State University	Rikkinen and Poinar (2008)	Dominican amber 17 Ma	Iturralde-Vinent and Mac Phee (1996), Iturralde-Vinent (2001)

<sup>a</sup> The fossils used as minimum age constraints in this study as indicated in Fig 2.

respectively (Tuovila et al., 2013). All three fossils clearly belong to the order Mycocaliciales, which has usually been placed in the Eurotiomycetes (e.g. Schoch et al., 2009a).

#### 2.1.4. Fossil Laboulbeniomycetes

A well-preserved specimen of this highly specialized lineage was found in Bitterfeld amber (23 Ma; Rossi et al., 2005) and described as *Stigmatomyces succini* (Fig. 1k). The fossil fungus is attached to the thorax of a stalk-eyed fly (*Prospyracephala succini*, Diopsidae).

#### 2.1.5. Fossil Lecanoromycetes

Several specimens of *Anzia* (Rikkinen and Poinar, 2002) are preserved in Baltic amber dating back 50–35 Ma (Fig. 1e). Some of these fossils are morphologically identical to the extant species *A. japonica* which may be the closest living relative. The genus *Anzia* belongs to the Parmeliaceae (Lecanorales), the largest family of lichen forming Fungi and is morphologically very similar to the genus *Pannoparmelia* (Thell et al., 2012).

Poinar et al. (2000) described two species of *Parmelia* (*P. ambra* and *P. isidiiveteris*) from Dominican amber (17 Ma; Fig. 1l). Both fossils clearly belong to the family Parmeliaceae, but they cannot be assigned with certainty to any extant genus. However, neither of the two fossil species represents *Parmelia* sensu stricto.

A fossil specimen of the genus *Phyllopsora* (Ramalinaceae, Lecanorales) preserved in Dominican amber (17 Ma; Fig. 1m) was

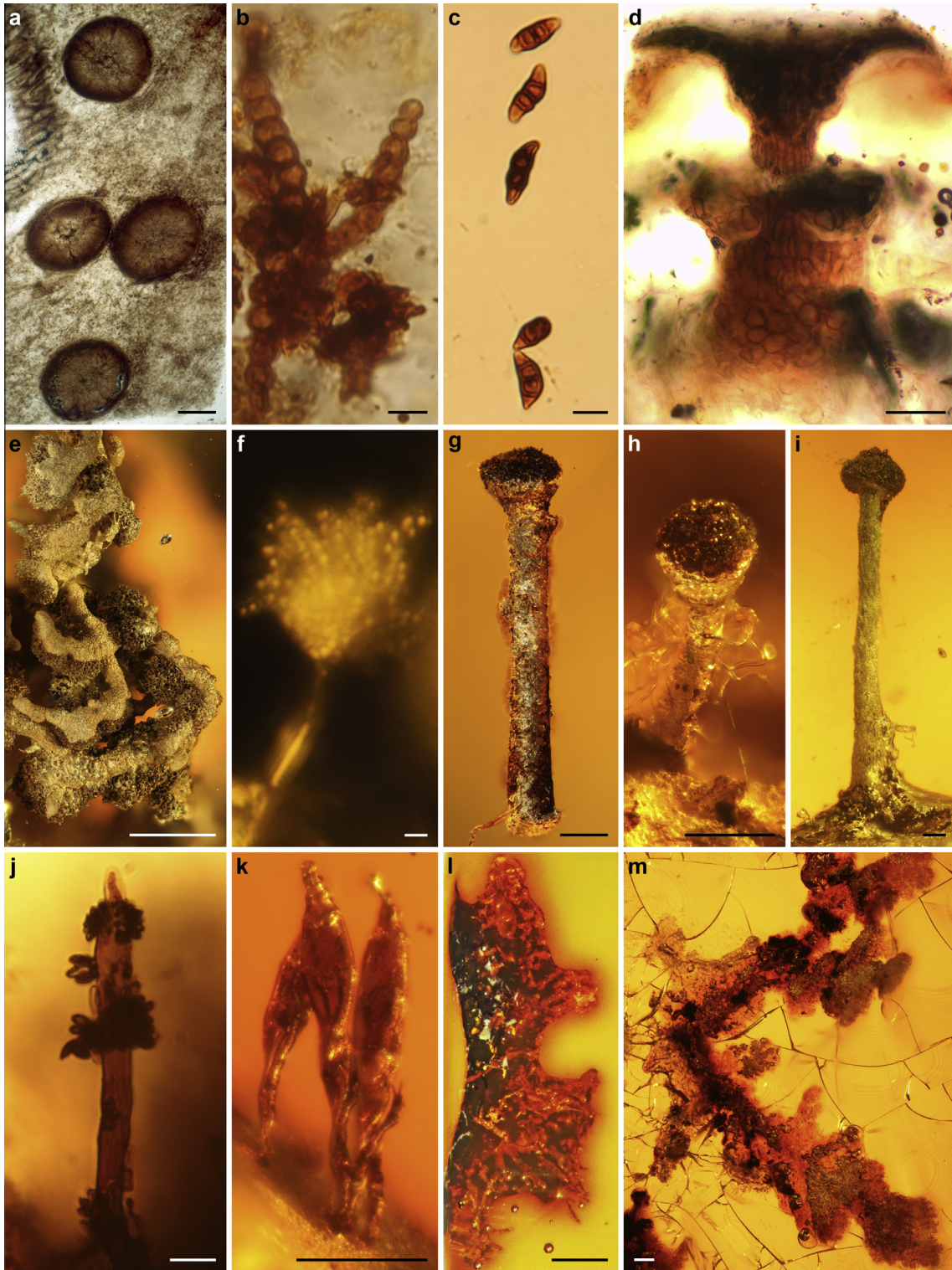
described as *P. dominicanus* by Rikkinen and Poinar (2008). The morphological features of *P. dominicanus* closely resemble those found in modern *Phyllopsora* species and are very similar to the recent *P. chlorophaea* for example.

A fossil representative of the genus *Calicium* (Rikkinen, 2003) is preserved in amber of the Baltic deposit dating back 50–35 Ma (Fig. 1g). Species of *Calicium* (Caliciaceae, Teloschistales) are typical “calicioid lichens”, a paraphyletic assemblage of Fungi sharing morphological similarities such as stalked fruiting bodies and a powdery spore mass called the mazaedium (Tibell, 1984).

#### 2.1.6. Fossil ascomycetes of groups with ambiguous systematic positions

*Gonatobotryum piceae* (Dörfelt and Schmidt, 2007) is enclosed in Baltic amber (50–35 Ma; Fig. 1j). The fossil specimen shows close similarities to modern *Gonatobotryum fuscum*, but developed different conidiophores and mature conidia. Teleomorphs are currently unknown for *Gonatobotryum* species (Arx, 1981).

*Paleopyrenomycites devonicus* (Fig. 1a) is by far the oldest evidence for ascomycetes. It is enclosed in Devonian Rhynie Chert dating back 410 million years (Taylor et al., 2005). *P. devonicus* was often assigned to Sordariomycetes, but its exact systematic position is disputed (Taylor et al., 2005; Eriksson, 2005; Padovan et al., 2005; Taylor and Berbee, 2006). An assignment to the Pezizomycetes seems also possible, since *Paleopyrenomycites* might have produced operculate asci (Lücking et al., 2009).



**Fig. 1.** Fossil ascomycetes from amber and chert representing the oldest known ancestors of respective lineages. (a) Perithecia of *Paleopyrenomycites devonicus* from Early Devonian (Pragian) Rhynie Chert. W. Remy collection PB 3411. Courtesy of Hans Kerp (University of Münster). (b) Moniliform hyphae of a Metacapnodiaceae representative from Early Cretaceous (Albian) Charentes amber. IGR.ARC-115.3b. (c) Conidia of *Palaeocurvularia variabilis* from Late Cretaceous (Cenomanian) Ethiopian amber. MB. Ph. 2009/200. (d) Hypostroma of *Petropus brachyphylli* from Maastrichtian chert from the Netherlands. NHMM RD 265. Courtesy of Raymond W. J. M. van der Ham (Naturalis Biodiversity Center, Leiden). (e) *Anzia electra* from Eocene Baltic amber. Hoffeins 950-1. (f) Sporulating conidiophore of *Aspergillus collembolorum* on a springtail from Eocene Baltic amber. Hoffeins 805. (g) Ascoma of *Calicium* sp. from Eocene Baltic amber. GZG.BST.27296. (h) Ascoma of *Chaenotheca* sp. on remnant bark in Eocene Baltic amber. GZG.BST.27297. (i) Ascoma of a resinicolous *Chaenothecopsis* sp. from Eocene Baltic amber. GZG.BST.27286. (j) Sporulating conidiophore of *Gonatobotryum piceae* on a conifer seedling from Eocene Baltic amber. Wunderlich F129. (k) Three thalli of *Stigmatomyces succini* on a dipteran from Oligocene Bitterfeld amber. Zoologische Staatssammlung München, *sine numero*. (l) *Parmelia ambra* from Miocene Dominican amber. Poinar AF9-17E. Courtesy of George O. Poinar, Jr. (Corvallis). (m) *Phyllopsora dominicanus* from Miocene Dominican amber. Poinar B 1-23. Scale bars: 10  $\mu$ m (b–d, f, and j), 100  $\mu$ m (a, g–i, k, and m), and 1 mm (e and l).

## 2.2. Taxon sampling for phylogenetic reconstruction and molecular work

For this study we used the small and large ribosomal subunit (nucSSU and nuLSU respectively) and RNA polymerase II protein coding genes RPB1 and RPB2 as implemented in a previous study by James et al. (2006). Sequences were obtained from cultured strains ordered from the CBS (Centraalbureau voor Schimmelcultures, Utrecht), JMRC (Jena Microbial Resource Collection), and from Genbank. Additional Fungi were collected from localities in Finland (2009) and New Caledonia (2011). The resulting taxon set consists of 145 species representing most classes of the Ascomycota. Accession numbers of all sequences are provided in Supplementary Table 1. For protein coding and ribosomal genes, we isolated DNA from fungal material using the Invisorb Spin Plant Mini Kit (Invitex, Berlin, Germany) and NucleoSpin©Plant DNA extraction kit (Macherey–Nagel) with the following modification to the manufacturer's protocol: some specimens were incubated up to 2 h to ensure the lysis of the ascocarps. PCR reactions were carried out with fungal specific primers: SSU ribosomal genes were amplified with the primers NS1, NS2, NS3, NS4 (White et al., 1990) and NS24 (Gargas and Taylor, 1992); LSU ribosomal genes were amplified with LR0 (Rehner and Samuels, 1994), LR3R (Moncalvo et al., 2000), LR5 and LR7 (Vilgalys and Hester, 1990). Genes coding for the RNA polymerase II were amplified with the primers RPB1-AFasc, RPB1-6R2asc, RPB1-DF2asc, RPB1-GIRasc and RPB1G2R (Hofstetter et al., 2007) for the largest subunit and fRPB2-5f, FRPB2-7cf, fRPB2-7cR, fRPB2-11aR and RPB2-11bR (Liu et al., 1999) for the second largest subunit. PCR reactions were performed according to the protocols listed in respective reference for mentioned primers. In case of melanin inhibiting the PCR, the DNA-templates were diluted up to 5000 fold sometimes with the addition of 200 ng/μl bovine serum albumin (BSA) (Kreider, 1996). PCR products were purified using PCRapace (Invitex, Berlin, Germany). All PCR products were sequenced in both directions with a MegaBACE 1000 automated sequencing machine and DYEnamic ET Primer DNA Sequencing Reagent (Amersham Biosciences, Little Chalfont, UK). All sequences were assembled and edited using Bioedit 5.0.9 (Hall, 1999) and Seaview 4 (Gouy et al., 2010).

## 2.3. Initial phylogenetic Analysis

Datasets for each gene (SSU, LSU, RPB1 and RPB2) were aligned separately using MAFFT version 6 (Kato and Toh, 2008) with subsequent manual adjustment to minimize the number of possible false homologies using Bioedit 5.0.9 (Hall, 1999) and Seaview 4 (Gouy et al., 2010). Unalignable regions and introns were excluded by using the mask function in Bioedit 5.0.9. Best fitting substitution model for each gene was chosen separately from seven substitution schemes included in the software package jModeltest 2.1.1 (Darriba et al., 2012), and models were chosen according to the Bayesian information criterion (BIC, Schwarz, 1978). The Bayesian information criterion supported the TrN + G model as the best fit for LSU, SYM + G for SSU, and GTR + G for RPB1 and RPB2. Topological congruence of the four datasets was assessed by visual comparison of phylogenetic trees obtained from maximum likelihood-based analysis with RaxML (Stamatakis et al., 2008), and all genes were subsequently combined in a super matrix using Bioedit 5.0.9. Bayesian analyses were carried out using Markov chain Monte Carlo (MCMC) in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) to generate a reasonable starting tree for subsequent analyses of divergence date estimates in BEAST. Analyses were run using four chains for 10 million generations each, sampling parameters every 1000th generation. All analyses were performed on the freely available computational resource CIPRES

(www.cipres.org). Average standard deviations of split frequency (ASDSF) lower than 0.01 were interpreted as indicative of independent MCMC convergence.

## 2.4. Fossil calibrations

The placement of the fossil *Paleopyrenomyces* (Fig. 1a) is challenging since its exact systematic position is not clear (Taylor et al., 2005; Lücking et al., 2009; Taylor and Berbee, 2006). The previously discussed possibilities for its placement include anywhere in the Pezizomycotina stem lineage, Pezizomycotina crown group, or members of the Pezizomycotina building operculate asci (Lücking et al., 2009). For our evolutionary model we adopted a conservative view and placed *Paleopyrenomyces* on the crown group of Pezizomycetes, thus assuming the common ancestor of all filamentous, sporocarp-producing Ascomycota (Pezizomycotina) to be at least 400 Ma. We decided to model the uncertainty of the group by applying a truncated normal distribution with an upper hard bound (truncation) set to 400 Ma, corresponding to the mean of the normal distribution with a standard deviation (SD) of 150, providing an upper 97.5% credibility interval (CI) of 700 Ma. This mode of calibration associates an increased uncertainty with the immediate upper bound, allowing a more generous interpretation of the age of a group compared to that of an exponential decay. The fossil *Anzia electra* (Fig. 1e; Rikkinen and Poinar, 2002) was used to calibrate the split between *Anzia* and other groups of parmelioid lichens here presented by *Canoparmelia* constraining the node to 35 Ma with a truncated normal distribution to model the uncertainty (mean = 35, SD = 50, CI = 135). Based on the fossil *Calicium* (Fig. 1g; Rikkinen et al., 2003) we constrained the common ancestor of *Calicium viride* and *C. salicium*, which are both morphologically indistinguishable from the fossil to 35 Ma (truncated normal distribution, mean = 35, SD = 50, CI = 135). Using the fossil *Aspergillus collemboforum* (Fig. 1f; Dörfelt and Schmidt, 2005) we constrained the common ancestor of *Aspergillus* to 35 Ma (truncated normal distribution, mean = 35, SD = 50, CI = 135). The fossil Metacapnodiaceae (Fig. 1b; Schmidt et al., 2014) gave rise to the hypothesis of the common ancestor of the order Capnodiales to be constrained to an age of 100 Ma (truncated normal distribution, mean = 100, SD = 150, CI = 400). All analyses of divergence time estimates using the above set of constraints were first run on empty alignments to check for cross prior influence, while constraining all calibrated nodes to monophyly.

## 2.5. Divergence time estimates

Subsequent divergence time analyses were carried out using BEAST 1.7.4 (Drummond et al., 2012). Separate partitions for each included gene were created with BEAUti 1.7.4 (BEAST package). To accommodate for rate heterogeneity across the branches of the tree (e. g. Berbee and Taylor, 2010) we used an uncorrelated relaxed clock model (Drummond et al., 2006) with a lognormal distribution of rates for each gene estimated during the analyses. A birth/death tree prior accommodating for incomplete sampling (Stadler, 2009) was used to model the speciation of nodes in the topology, with uniform priors on probability of splits and extinctions. To avoid using uninformative priors on the clock models we used vague priors on the substitution rates for each gene (exponential decays with mean 0.1 in units of substitutions per site per time unit). To ensure congruence we ran the analyses five times for 100 million generations each, sampling parameters every 25,000 generations, assessing convergence and sufficient chain mixing (Effective sample sizes > 200) using Tracer 1.5 (Rambaut and Drummond, 2009). After removal of a proportion of each run as burn-in the remaining trees were combined using LogCombiner (part of the BEAST-package), and summarized as maximum clade

credibility (MCC) trees in TreeAnnotator (part of the BEAST-package), and visualized using FigTree (Rambaut, 2006–9, <http://tree.bio.ed.ac.uk/software/figtree/>).

In order to evaluate the possible presence of constant diversification rate in the evolutionary history of the Ascomycetes, a Lineage-through-time (LTT) plot was constructed in Tracer 1.5 (Rambaut and Drummond, 2009) from the combined posterior distribution of sampled tree topologies (Fig 3). We also applied the Monte Carlo constant rates (MCCR) test (Pybus and Harvey, 2000) to further elucidate this hypothesis.

### 3. Results

#### 3.1. Topology of the Ascomycota phylogeny

With some exceptions the topologies resulting from the BEAST analyses (Figs. 2 and S1) are generally congruent with the results reported by James et al. (2006) and other large scale phylogenies of Ascomycota e.g. Schoch et al. (2009a,b). The placement of Pezizomycetes basal to Orbiliomycetes is consistent with e.g. Schoch et al. (2009a,b) but is opposite in James et al., 2006 and some recent papers (Ebersberger et al., 2012). Kumar et al. (2012) extensively discussed the phylogenetic placement of Orbiliomycetes with additional ultrastructural analyses that supported the basal state of Orbiliomycetes in the Pezizomycotina. However, molecular support for the node resolving the relationships between the two basal Pezizomycotina classes (Pezizomycetes and Orbiliomycetes) is low and topologies are unstable.

Geoglossomycetes build the sister group to the Lichinomycetes-Coniocybomycetes clade in our analyses. The genus *Leotia*, believed to be the most basal member of the Leotiomycetes, here groups together with the sister clade of Sordariomycetes with unanimous support (1.0 pp). Geoglossomycetes were shown to be a rather basal clade close to Pezizomycetes and Orbiliomycetes, and distinct from Leotiomycetes after transfer of a few genera previously otherwise assigned (Spatafora et al., 2006; Schoch et al., 2009a,b; Hustad et al., 2013). Leotiomycetes exclusive of most geoglossoid species are currently seen as sister taxon to both Sordariomycetes and Laboulbeniomyces (Schoch et al., 2009a,b). The placement of *Leotia lubrica* was problematic also in previous analyses (James et al., 2006) and could possibly be resolved with a higher taxon sampling of Leotiomycetes, which would however exceed the scope of this work.

In our resulting single- and multiple-constrained trees Pezizomycetes constitutes the sister clade to remaining representatives of Pezizomycotina with strong support in both analyses (1.0 pp). The Orbiliomycetes build a sister group to Leotiomycetes, but in a position only indicated by low node support (0.88 vs. 0.91 pp). The Arthoniomycetes–Dothideomycetes-clade has unanimous support in both, single- and multi-constrained analyses (1.0 pp). The remaining representatives of the Pezizomycotina split in two clades. The first clade (0.68 vs. 1.0) consists of the Coniocybomycetes together with Lichinomycetes (1.0 vs. 0.99 pp), with the Geoglossomycetes as sister group (0.99 vs. 0.98 pp). The sister group to the Geoglossomycetes–Coniocybomycetes–Lichinomycetes clade is the Lecanoromycetes–Eurotiomycetes clade, in both analyses with unanimous support (1.0 pp). The second clade consists of the Leotiomycetes and Sordariomycetes grouping together with unanimous support in both analyses (1.0 pp).

#### 3.2. Divergence time estimations using five internal calibrations

Divergence time estimates using all five fossil calibration points are also shown in Fig. 2, with horizontal bars representing the 95% highest posterior density (HPD) intervals for each node.

Comparable results from both analyses (Figs. 2 and S1) are listed in Table 2. According to our data, the Ascomycota diverged from Basidiomycota in the Neoproterozoic, about 642 Ma (504–859 Ma, 95% HPD interval). The subphylum Pezizomycotina, containing all sporocarp forming members of the Ascomycota (except for *Neolecta* which resides in Taphrinomycotina and was excluded in our analysis as this group or taxon is still poorly understood), split from Saccharomycotina in the early Cambrian, around 537 Ma (443–695). The earliest split in the Pezizomycotina (Pezizomycetes from the remaining Pezizomycotina) occurred in the Ordovician, around 458 Ma (400–583). Within the Pezizomycotina, the Orbiliomycetes diverged in the Silurian, 430 Ma (353–554). Dothideomycetes + Arthoniomycetes diverged in the Late Devonian, 362 Ma (286–476) and this clade diverged from other Letiomyceta in the Early Devonian, around 397 Ma (330–521). Lichinomycetes split from Coniocybomycetes in the Permian, 274 Ma (197–379). Eurotiomycetes and Lecanoromycetes diverged in the early Carboniferous, 353 Ma (289–459). The earliest split in the Eurotiomycetes (Eurotiomycetes crown group) occurred around 336 Ma (273–437) and in the Lecanoromycetes 315 Ma (255–414).

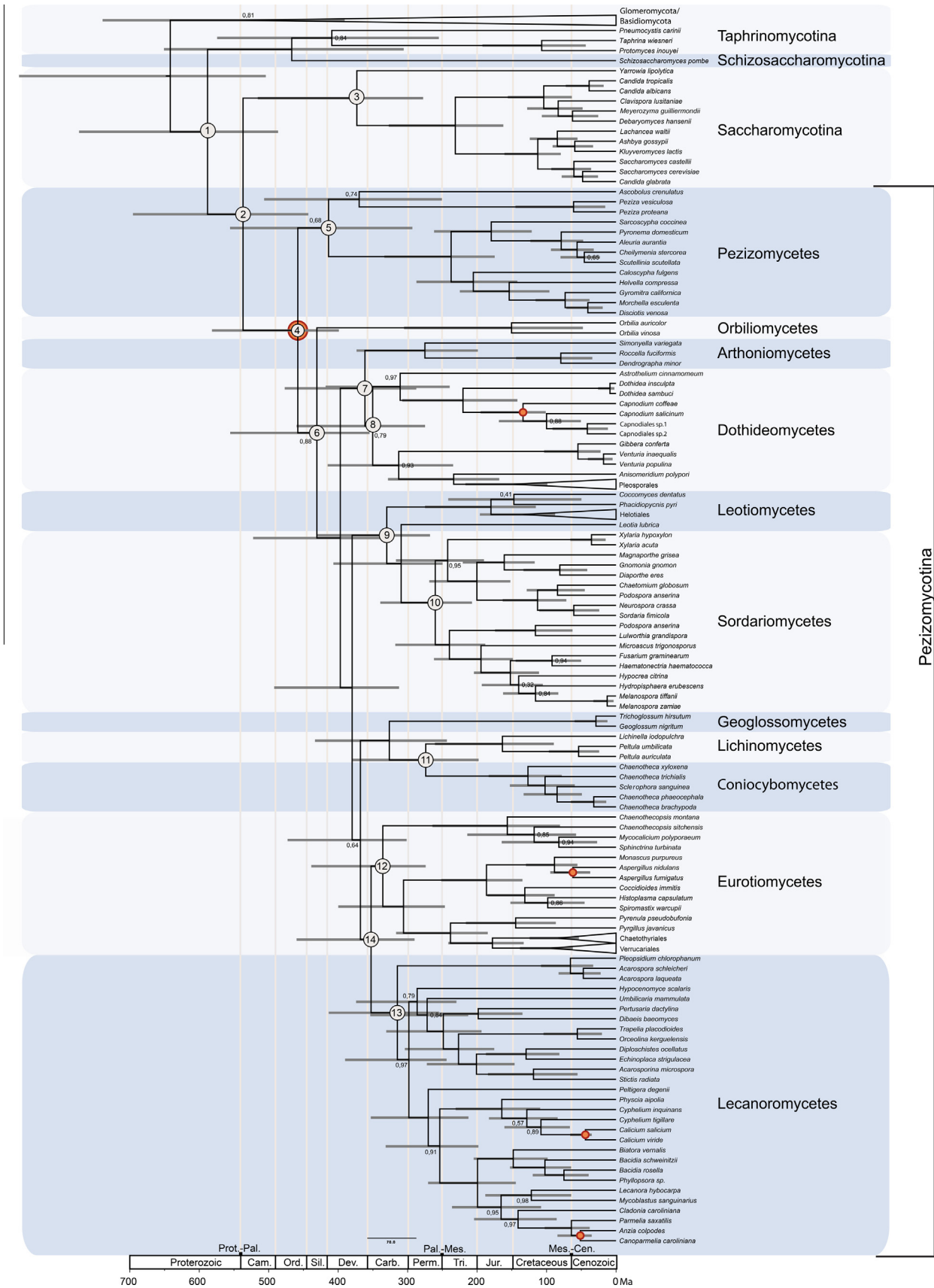
### 4. Discussion

#### 4.1. Systematic assignment of fossil Fungi

A crucial issue in molecular dating studies is the interpretation of morphological characters used to assign fossils to particular nodes in the phylogenies (Rutschmann et al., 2007; Marshall, 2008; Forest, 2009; Parham et al., 2012; Pyron, 2010; Dornburg et al., 2011; Feldberg et al., 2013). The use of morphological data to reconstruct the evolution of lineages through time can be limited due to homoplasy. Ascomycetes show many cases of parallel evolution in both vegetative and reproductive structures (e.g. Lumbsch, 2000; Schoch et al., 2009a). In this study we have only used fossils which we believe to represent extant families or genera (with the exception of *Paleopyrenomyces* which was assigned to Pezizomycotina). However, some level of uncertainty will always remain when working with fossil material. Besides *Paleopyrenomyces devonicus* we finalized our selection using fossils assigned to four extant taxa of ascomycetes (*Metacapnodiaceae*, *Anzia electra*, *Aspergillus collembolum* and *Calicium*; Table 1, Fig. 1) which provided minimum ages for the split of the lineage from its sister group. The remaining eight fossils (Fig. 1; Table 1) did not provide suitable minimum age constraints. This was mainly due to insufficient taxon sampling of our molecular data. While we could not use all of the 13 available fossils in our study, all of them are potentially of value for further studies in Fungi with denser taxon samplings, or focus on the evolution of individual groups of ascomycetes.

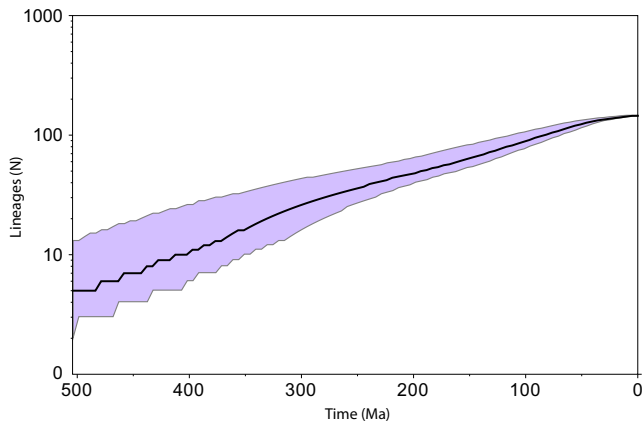
Gueidan et al. (2011) used *Palaeocurvularia* (Fig. 1c; Schmidt et al., 2010a,b) to constrain the split between Arthoniomycetes and Dothideomycetes. However, the fossil is represented by numerous conidia and related conidiophores, which resemble those produced by modern species of *Curvularia* but also those of *Bipolaris*, *Drechslera*, and *Exserohilum*. Since fragments of the possible teleomorph are poorly preserved, and the fossil conidia are more variable than those of any of the modern genera, Schmidt et al. (2010b) and Gueidan et al. (2011) avoided an assignment of the fossil to any modern family. In order to avoid a false assignment, we had to exclude *Palaeocurvularia* from our analyses.

*Gonatobotryum piceae* (Fig. 1j; Dörfelt and Schmidt, 2007) was also excluded, because of the ambiguous phylogenetic position of this genus (Arx, 1981). The morphologically similar fossil *Gonatobotrys primigenia* (Caspary and Klebs, 1907) likely represents a



**Fig. 2.** Maximum clade credibility (MCC) tree with divergence times estimates for main groups of the Ascomycota obtained from a Bayesian approach (BEAST) using five fossil minimum age constraints. Numbers at nodes indicate posterior probabilities (pp) for node support. Bars correspond to the 95% highest posterior density (HPD) intervals. For estimated median ages of numbered nodes, see Table 2. Only node supports below 1.0 pp are shown. Assignments in the tree of the fossil minimum age constraints are marked with red circles. Geological periods are abbreviated as: Cam. = Cambrian, Ord. = Ordovician, Sil. = Silurian, Dev. = Devonian, Carb. = Carboniferous, Perm. = Permian, Tri = Triassic, Jur. = Jurassic.





**Fig. 3.** Lineage-through-time (LTT) plot constructed from combined posterior distribution of sampled tree topologies by using Tracer 1.5 (Rambaut and Drummond, 2009).

species of *Gonatobotryum* rather than *Gonatobotrys*. As the modern genus *Melanospora* (Ceratosmataceae, Sordariomycetes) is known as the teleomorph of *Gonatobotrys* (Vakili, 1989), this genus could potentially be used for calibration. A confident assignment of *Gonatobotrys primigenia* would require a re-investigation, however, this fossil which was part of the Künow collection of Berlin's Museum of Natural History is lost without any trace. Both fossils are well preserved and might serve as calibration constraints once their position within the ascomycetes has been clarified.

*Petropus brachyphylli* (Fig. 1d; Van der Ham and Dortangs, 2005) was not used in our calculation because of the uncertain taxonomic placement of the corresponding modern genus *Phaeocryptopus*, which is likely polyphyletic and either belongs to the Dothideales or Capnodiales (Zhang et al., 2011; Winton et al., 2007).

*Stigmatomyces succini* (Fig. 1k; Rossi et al., 2005) was also not used in our study although the fossil is well dated and confidently assigned to the genus *Stigmatomyces* (Laboulbeniomyces). Species of this ectoparasite class display distinct morphologies and their phylogenetic position has long been unclear, but Schoch et al. (2009a) have recently proposed a sister relationship of Laboulbeniomyces to Sordariomycetes. Primary analyses including sequences of the Laboulbeniomyces indicated the introduction of substantially long branches in resulting phylogenies (data not shown), and this class was therefore excluded from further

analyses to avoid introducing unnecessary bias into the branch length estimates.

The fossils *Parmelia ambra* (Fig. 11) and *P. isidiiveteris* (Poinar et al., 2000) cannot be assigned with confidence to particular genera within the foliose parmelioid lichens ("*Parmelia sensu lato*"). We were unable to use *Parmelia* because it would imply a constraint on the divergence of *Parmelia* and *Canoparmelia*, or *Anzia*, to a minimum of 17 Ma. As we had already used *Anzia electra* (50–35 Ma; Rikkinen and Poinar, 2002) to constrain the split of *Anzia* and *Canoparmelia*, an integration of the much younger fossil of parmelioid lichens would introduce redundancy.

Similar reasons led to the exclusion of *Phyllopsora dominicanus* (Fig. 1m; Rikkinen and Poinar, 2008) as an age constraint in our analysis. Our *Phyllopsora* sequences grouped together with *Bacidia* and constraining the divergence between these two genera to a minimum of only 17 Ma would not have been realistic (Printzen and Lumbsch, 2000; Rikkinen and Poinar, 2008).

The fossils *Chaenotheca* (Fig. 1h; Rikkinen, 2003), and *Chaenothecopsis* (Fig. 1i; Rikkinen and Poinar, 2000; Tuovila et al., 2013) were discarded from the analyses despite being of excellent quality. Initial tests for cross-prior influence on the age estimates of nodes indicated that the introduction of these constraints resulted in several other constraints showing bimodal posterior distributions. Finding the underlying cause of such phenomena can be difficult, but is possibly due to discordance between the fit of the tree prior and one or more node constraints. Removal of the mentioned fossils indicated substantial performance improvements across the tree, validating the decision for removal.

#### 4.2. The impact of internal node constraints on estimated divergence times of Pezizomycotina classes

Some studies have evaluated the variation resulting from different calibration strategies in fungal phylogenies (Taylor and Berbee, 2006; Lücking et al., 2009; Padovan et al., 2005), but none of them evaluated the impact of internal node constraints on models of fungal molecular evolution. Compared to the sole use of the Devonian *Paleopyrenomycites*, the use of four calibrations from Mesozoic and Cenozoic Pezizomycotina crown group fossils (of Dothideomycetes, Eurotiomycetes and Lecanoromycetes) in addition to the Devonian fossil resulted in older age estimates (Table 2). Using multiple age constraints only slightly affected the first split in the Pezizomycotina from 444 Ma (400–576, when using only *Paleopyrenomycites*) to 458 Ma (400–583) when using the four additional calibrations. All other Pezizomycotina have diverged

**Table 2**

Divergence time estimates of Ascomycota lineages obtained from Bayesian analysis using either *Paleopyrenomycites* as single calibration or together with 4 additional calibrations from amber (*Metacapnodiaceae*, *Anzia electra*, *Aspergillus collembolorum*, and *Calicium*; Table 1, Fig. 1). For each divergence, the median and the 95% Highest Posterior Density are provided. Divergence times are provided in millions of years (Ma). The node numbers correspond to numbers used in Fig. 2 to show their placement in the chronogram.

Nodes		One calibration		Five calibrations	
		Geological period	Time (Ma)	Geological period	Time (Ma)
1	Ascomycota crown group	–	–	Neoproterozoic	588 (487–773)
2	Pezizomycotina–Saccharomycotina	–	–	Cambrian	537 (443–695)
3	Saccharomycotina crown group	–	–	Devonian	373 (276–514)
4	Pezizomycotina crown group	Ordovician	444 (400–576)	Ordovician	458 (400–583)
5	Pezizomycetes crown group	Devonian	408 (262–543)	Devonian	413 (292–554)
6	Orbiliomycetes–other Pezizomycotina	Devonian	407 (328–534)	Silurian	430 (353–554)
7	Arthoniomycetes–Dothideomycetes	Carboniferous	335 (263–450)	Devonian	362 (286–476)
8	Dothideomycetes crown group	Carboniferous	321 (247–427)	Carboniferous	350 (273–459)
9	Leotiomycetes–Sordariomycetes	Permian	287 (234–388)	Carboniferous	309 (267–430)
10	Sordariomycetes crown group	Triassic	233 (182–316)	Permian	260 (207–339)
11	Lichinomycetes–Coniocybomycetes	Triassic	246 (164–355)	Permian	274 (197–379)
12	Eurotiomycetes crown group	Permian	282 (215–382)	Carboniferous	336 (273–437)
13	Lecanoromycetes crown group	Permian	260 (190–356)	Carboniferous	315 (255–414)
14	Eurotiomycetes–Lecanoromycetes	Carboniferous	327 (250–436)	Carboniferous	353 (289–459)

from the Orbiliomycetes in the Silurian 430 Ma (353–554), rather than in the Early Devonian some 407 Ma (328–534). Both analyses resulted in congruent relationships between Eurotiomycetes, Lecanoromycetes, Dothideomycetes and Lichinomycetes allowing a comparison of the divergence times of these Pezizomycotina classes; these divergence estimates are significantly older when using additional age constraints (Table 2).

Besides affecting the divergence times of early Ascomycota lineages, the integration of additional age constraints resulted in older age estimates of more recent ascomycete groups (terminal nodes). These effects are not only restricted to branches associated with fossil age constraints, although adjacent branches are slightly stronger affected, supporting the observations of Berbee and Taylor (2010).

Our results show that the use of fossil age constraints (even if relatively young) in terminal groups of ascomycetes significantly affects the estimated divergence times of both early branching nodes and terminal groups of Ascomycota lineages. This effect was also observed when using different BEAST parameters, e.g. unconstrained uniform probability distributions to model age uncertainties of groups associated with fossils (data not shown).

#### 4.3. Comparisons to previous studies

Compared to earlier studies our data indicate either much younger (Heckman et al., 2001) or much older (Berbee and Taylor, 1993) age estimates of Ascomycota lineages. Our results are generally more congruent with the estimates of recent studies (Padovan et al., 2005; Taylor and Berbee, 2006; Lücking et al., 2009; Gueidan et al., 2011; Prieto and Wedin, 2013) (Table 3). One likely explanation is that molecular clock methods have improved by developing relaxed molecular clock models, which allow for more flexible modeling of rate heterogeneity across phylogenetic trees (e. g. Sanderson, 2003b; Drummond et al., 2006, 2012). Additionally, more well resolved fungal phylogenies have recently been established (e. g. Spatafora et al., 2006; Schoch et al., 2009a; Miadlikowska et al., 2006; Hibbett et al., 2007; Ebersberger et al., 2012; Kumar et al., 2012; Morgenstern et al., 2012). Advances in both fields of research have enabled the establishment of increasingly realistic models of evolution for Fungi compared to earlier studies, resulting in different age estimates (e.g. Simon et al., 1993; Heckman et al., 2001).

Despite these improvements in methodology and data sampling, age estimates are not fully consistent across recent studies. Such discrepancies are likely to have various reasons such as inability to properly model evolutionary rates, parameter settings for the applied relaxed clock models, unequal taxon sampling, and choice of genes under study. Such differences make it difficult to compare inferred age estimates of individual studies. Another possible source for inconsistent age estimates in earlier studies is the assignment of the fossil *Paleopyrenomycites devonicus* (Taylor et al., 1999, 2005). This fossil constitutes a highly influential constraint, and since it became available has been used in all studies of fungal molecular evolution (Table 3). In early studies the fossil was interpreted as belonging to the Sordariomycetes (e.g., Heckman et al., 2001). More recent studies used *Paleopyrenomycites* to calibrate the Pezizomycetes crown or stem group (Taylor and Berbee, 2006; Lücking et al., 2009), the Ascomycota crown group (Taylor and Berbee, 2006), or as a constraint for the split between Leotiomyceta and other Pezizomycotina (Gueidan et al., 2011) due to the putative operculate ascus. However, the apparent operculate opening might also be a diagenetic phenomenon (Lücking et al., 2009). Lücking et al. (2009) provide a comprehensive discussion concerning the placement of this fossil while recalibrating several earlier studies (Berbee and Taylor, 1993; Simon et al., 1993; Doolittle et al., 1996; Redecker et al., 2000; Heckman et al., 2001; Padovan et al., 2005) by reassessing the systematic placement of *Paleopyrenomycites*. However, Taylor and Berbee (2006) convincingly showed the placement of this fossil at different positions in the Ascomycota tree (Ascomycota crown group, Pezizomycotina crown group, Sordariomycetes crown group) to have a dramatic effect on estimated ages of fungal lineages. Therefore our age estimates are best comparable to other studies using *Paleopyrenomycites* as constraint for the Pezizomycotina crown group (e.g., Taylor and Berbee, 2006; Lücking et al., 2009). Our resulting age estimates from the calibrations using *Paleopyrenomycites* as a sole constraint are overall consistent with the ages inferred by Lücking et al. (2009; Table 3) and those of Taylor and Berbee, 2006; calib. 2 and calib. 3 in Table 3).

Divergence times estimates obtained from our analysis that employed five internal calibration points correspond most closely to those of Gueidan et al. (2011), Prieto and Wedin (2013) and Lücking et al. (2009). Gueidan et al. (2011) used *Paleopyrenomycites* to calibrate the Pezizomycetes–Leotiomyceta split (which corresponds to the Pezizomycotina crown group with the excep-

**Table 3**  
Comparison of divergence time estimates from our analyses with previous studies (Heckman et al., 2001; Padovan et al., 2005; Taylor and Berbee, 2006; Lücking et al., 2009; Gueidan et al., 2011; Prieto and Wedin, 2013) in millions of years (Ma) which also used *Paleopyrenomycites* as fossil age constraints. For divergence times, only the medians are listed. Node numbers correspond to numbers used in Fig. 2 and Fig. S1 to show their placement in the chronogram. Triangle ( $\Delta$ ) marks the assignment of *Paleopyrenomycites*; psi ( $\psi$ ) indicates studies that also used external (non-ascomycotan or non-fungal) calibrations; phi ( $\Phi$ ) recalibration study.

Nodes	This study		Prieto & Wedin	Gueidan et al.	Lücking et al.	Taylor and Berbee	Padovan		Heckman et al.	
	Cali. 1 (1 fossil)	Cali. 2 (5 fossils)	(2013) (Scen. 4)	(2011) <sup><math>\psi</math></sup>	(2009) <sup><math>\Phi</math></sup>	(2006)	Cali. 1 <sup><math>\psi</math></sup>	Cali. 2 <sup><math>\psi</math></sup>	(2001) <sup><math>\psi</math></sup>	
1 Ascomycota crown	–	588	531	538	–	1316 745 652 400 <sup><math>\Delta</math></sup>	1148 724	1144		
2 Pezizomycotina–Saccharomycotina	–	537	–	–	400–520 <sup><math>\Delta</math></sup>	–	1072 657	1085		
3 Saccharomycotina crown	–	373	–	–	–	–	–	–	841	
4 Pezizomycotina crown	444 <sup><math>\Delta</math></sup>	458 <sup><math>\Delta</math></sup>	485 <sup><math>\Delta</math></sup>	455	320–400 <sup><math>\Delta</math></sup>	707 400 <sup><math>\Delta</math></sup> 400 <sup><math>\Delta</math></sup> 215	972 569	–		
5 Pezizomycetes crown	408	413	322	~310	–	–	~900	~500	–	
6 Orbiliomycetes–other Pezizomycotina	407	430	–	–	–	–	–	–	–	
7 Dothideomycetes–Arthoniomycetes	335	362	302	362	–	–	–	–	–	
8 Dothideomycetes crown	321	350	174	338	–	–	–	–	–	
9 Leotiomycetes–Sordariomycetes	287	309	247	~340	290–280	–	–	–	–	
10 Sordariomycetes crown	233	260	130	299	–	400 <sup><math>\Delta</math></sup> 226 211 122	653 <sup><math>\Delta</math></sup> 400 <sup><math>\Delta</math></sup>	400 <sup><math>\Delta</math></sup>	400 <sup><math>\Delta</math></sup>	
11 Lichinomycetes–Coniocybomycetes	246	274	267	–	–	–	–	–	–	
12 Eurotiomycetes crown	282	336	306	341	270–350	–	–	–	–	
13 Lecanoromycetes crown	260	315	305	322	280–330	–	–	816 453	–	
14 Eurotiomycetes–Lecanoromycetes	327	353	~320	371	–	–	–	–	–	

tion of Orbiliomycetes) and *Anzia electra* for calibrating the split between *Anzia* and *Canoparmelia*. Additionally, they also utilized a metacapnodiaceous fossil and *Palaeocurvularia* for the split of Dothideomycetes and Arthoniomycetes, together with several non-ascomycotan (Taylor et al., 1994; Redecker et al., 2000; Hibbett et al., 1995, 1997), and non-fungal (Crane et al., 1995; Douzery et al., 2004) calibration constraints. Compared to their studies, our data include more ascomycotan calibration points and no external constraints, which resulted in older ages for some of the ascomycete lineages.

Prieto and Wedin (2013) also utilized fossil age constraints solely from within the Ascomycota. With the exception of *Paleopyrenomyces* (Taylor et al., 1999, 2005) and the fossil *Calicium* (Rikkinen, 2003) they utilized a different set of additional age constraints. Prieto and Wedin (2013) relied on the fossils *Alectoria succina* (Mägdefrau, 1957) and *Parmelia ambra* (Rikkinen and Poinar, 2000), which were excluded in our study because of insufficient information for assigning these fossils to a corresponding modern genus (compare Section 4.1). The fossil *Alectoria* is only poorly pictured in a short paper by Mägdefrau (1957) and is not available for re-evaluation. It was part of the private collection of A. Scheele (Allgäu, Germany) but has seemingly been lost forever. We strongly object to including doubtful fossils, which could introduce erroneous information and result in highly biased age estimates. In contrast we utilized the fossil *Anzia electra* (Rikkinen and Poinar, 2002), which reveals much better preservation. In contrast to Prieto and Wedin (2013) we avoided integrating the fossils *Chaenotheca* (Rikkinen, 2003) and *Chaenothecopsis* (Tuovila et al., 2013) as they caused cross-prior influence strongly affecting other node age estimates (compare Section 4.1). Additionally, Prieto and Wedin (2013) did not include the influential metacapnodiacean fossils described by Rikkinen et al. (2003) and Schmidt et al. (2014) and the extraordinarily well preserved *Aspergillus collembolorum* (Dörfelt and Schmidt, 2005).

However, the general congruence of recent studies using comparable parameter settings indicates an increase in convergence of age estimates. Our results indicate that further inclusions of reliable fossil constraints are likely to lead to even more accurate estimated ages of individual lineages.

#### 4.4. Reconstruction of the evolutionary history of ascomycete lineages

According to our results (Fig. 2), all Pezizomycotina classes originated in the Phanerozoic, while the main diversification began in the Ordovician with the divergence of Pezizomycetes (the earliest branching class of Pezizomycotina) from the remaining Pezizomycotina. Based on the results of our study it is impossible to draw any certain conclusions on the assumption of constant diversification rates in the Ascomycota. The LTT plot (Fig. 3), suggest a slight declining trend in the number of accumulated ancestral lineages, but whether this is best explained by extinction events or by insufficient taxon sampling cannot be determined. The MCC test supports these indications (results not shown) though the number of replicates to achieve significant results was insufficient.

It has been assumed that the marine and freshwater ascomycetes evolved from ancestors that occupied terrestrial habitats (Spatafora et al., 1998; Vijaykrishna et al., 2006). Around 530 marine fungal species are known, 424 of which occur in various orders of the Pezizomycotina (mostly members of Halosphaeriales, Spatafora et al., 1998; Jones et al., 2009). Additionally, 511 freshwater Fungi are known in three Pezizomycotina classes: Leotiomycetes, Dothideomycetes and Sordariomycetes (Shearer, 2001; Cai et al., 2003). Because marine Fungi occur in many distinct Ascomycota lineages the question of a marine origin of the Pezizomycotina is still disputed (e.g. Jones et al., 2009). However,

a marine origin is not well supported in recent literature, e.g. it is still inconsistent with the ancestral reconstruction in Schoch et al. (2009a) and other recent studies (e.g. Sakayaroj, 2005; Schoch et al., 2006).

The majority of the Pezizomycetes are terrestrial and live saprotrophically in soil. They typically build apothecia with operculate asci and it has been proposed that all other ascomata forms and spore release mechanisms have evolved from this type of sporocarp with active spore release. According to our results the Pezizomycetes diverged from other Pezizomycotina during the Ordovician. Since microbial mats including Fungi were already present in the Proterozoic and fungal-like hyphae are known from this period (Butterfield, 2005), a Neoproterozoic origin of the Ascomycota and an Ordovician origin of the Pezizomycotina is conceivable. Our results suggest that the Leotiomycota (Pezizomycotina excluding Pezizomycetes and Orbiliomycetes) split from basal Pezizomycotina in the Silurian and subsequently diverged during the Devonian. This supports a coevolutionary scenario of major land plant lineages and major Pezizomycotina lineages in the early Paleozoic. During the Devonian, main lineages of vascular plants (except, e.g. angiosperms) appeared, and the terrestrial vegetation changed from small plants in the Early Devonian to the progymnosperm forests of the Late Devonian (Meyer-Berthaud et al., 2010). This entailed the development of soils and distinct root systems, which may have onset the formation of new ecological niches of ascomycetes. Parasitic Pezizomycotina species may have evolved in aquatic or terrestrial Devonian habitats, for example together with vascular plants, algae or arthropods.

A recently discovered fossil lichen described by Honegger et al. (2013) appears to confirm the presence of lichen-forming fungi since the Early Devonian (415 Ma). Our results further indicate an initial diversification of Lecanoromycetes in the late Carboniferous, which proceeded continuously, apparently unaffected by mass extinction events and major global climatic changes. This scenario correlates with the global development of forest ecosystems since the Carboniferous. Beyond this it is difficult to relate the development of distinct Pezizomycotina classes to the evolution of other organisms (plants and/or animals) since almost all classes (with the except for Orbiliomycetes and Laboulbeniomycetes) comprise a broad range of different life forms such as parasitic, lichen-forming and other symbiotic and saprophytic forms. According to our results Lichinomycetes and Coniocybomycetes appeared subsequently in the Permian, 274 Ma (197–379).

According to our results, the origins of many Lecanoromycetes genera reach back to the Late Jurassic. *Biatora*, for instance, seems to represent an old lineage, which diverged from the *Bacidia-Phyllopsora* clade some 148 million years ago (Fig. 2). *Biatora* and *Phyllopsora* are closely related and share similar habitat preferences, but are strictly allopatric, with *Phyllopsora* being restricted to tropical habitats and *Biatora* to temperate and cool regions of the Northern Hemisphere. Our results largely correlate with an assumed divergence of these genera about 140–170 Ma due to expansion of the Tethys ocean separating Laurasia from Gondwana (Printzen and Lumbsch, 2000).

If we follow extant fungal lineages backwards in time we inevitably arrive at the question of how old genera could be. According to our data, most genera originated in the Mesozoic with some, like *Hypocenomyces* or *Peltigera* (Lecanoromycetes) extending back to the Permian period. As the Ascomycota represents a vast group (~64,000 species), our data set represents only a fraction of all Ascomycota species and does not allow precise interpretations of the appearances of particular genera. Additionally, we must assume that the vast majority of Phanerozoic species is extinct and thus cannot be considered in molecular analyses.

#### 4.5. Conclusions and outlook

Ambers and cherts have the potential to preserve delicate structures with extraordinary quality. In this way fossil inclusions can sometimes be determined to genus level, allowing the precise assignment of the fossils to recent phylogenies. Here we evaluate all available fossil ascomycetes representing the oldest reliable evidence of the respective extant lineages and discuss their suitability as minimum age constraints for molecular evolution studies. We used fossil species from amber that are assignable to three Pezizomycotina classes in order to constrain a molecular clock for a multi-gene Ascomycota phylogeny. This is one of the first studies to evaluate the impact of internal node constraints on models of molecular evolution for the Ascomycota. Comparison of analyses performed using multiple-fossil calibration points vs. analyses using only a sole minimum age constraint (*Paleopyrenomycites*) show that the use of fossil age constraints (even if relatively young) in terminal groups of three Pezizomycotina classes significantly affects the estimated divergence times of basal nodes and nodes of terminal groups of all Ascomycota lineages. Our estimated divergence times were exclusively based on internal age constraints (either one or five) but largely agree with estimates in recent studies (Taylor and Berbee, 2006; Lücking et al., 2009; Gueidan et al., 2011; Prieto and Wedin, 2013).

According to our results (Fig. 2) the diversification of the Pezizomycotina started in the Ordovician, proceeded continuously throughout the Phanerozoic, and was largely unaffected by mass extinction events. Lineages of extant ascomycetes typically possess a variety of different life forms in each lineage. Classes or even families of ascomycetes may comprise both specialist and generalist species. We suggest that the diverse ecological strategies present in ascomycete lineages allowed at least some members to survive major extinction events. Such a scenario has already been suggested to explain the phenomenon that many species but only a relatively low number of genera of vascular plants became extinct at the Cretaceous-Paleogene boundary (Upchurch et al., 2007). Furthermore, fungal spores are likely to survive unfavorable environmental conditions at times of extinction events (e.g. Sussman and Douthit, 1973).

Further phylogenetic studies, increased taxon sampling, and the integration of a more comprehensive fossil record will generate more reliable chronograms that will prove the hypothesis of a constant diversification of ascomycotan Fungi during the Phanerozoic. An ongoing screening of newly discovered ambers (Schmidt et al., 2006, 2010a) is accumulating determinable fossils of Fungi (including lichens) which are likely to further improve models of molecular evolution for fungal phylogenies, especially for individual groups of Fungi.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jmpev.2014.04.024>.

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