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Fungal Biology Reviews

Opinion Article

Dating the molecular clock in fungi – how close are we?

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ARTICLE INFO

Article history:
Received 20 July 2009
Received in revised form
2 March 2010
Accepted 14 March 2010

Keywords:
Evolution
Fossil calibration
Fungi
Molecular clock
Phylogeny
Phylogeography
Rate variation

ABSTRACT

Integration of fungal evolution with the dates of plate tectonic movements, paleoecology, and the evolution of plants and animals requires a molecular clock. Imperfect though they may be, molecular clocks provide the means to convert molecular change into geological time. The relationships among clocks, phylogeography, fossils, and substitution rate variation, along with incorporation of uncertainty into clock estimates are the topics for this commentary. This commentary is timely because, for deeper divergences on the order of hundreds of millions of years, estimates of age of origin are benefiting from increasingly accurate organismal phylogenies and increasingly realistic models of molecular evolution. Taking advantage of Bayesian approaches permitting complex assumptions about node ages and molecular evolution, we used the program BEAST to apply a relaxed lognormal clock analysis to a data set comprising 50 loci for 26 taxa. In the resulting tree, branches associated with nodes calibrated by fossils showed more dramatic substitution rate variation than branches at nodes lacking calibration. As a logical extension of this result, we suspect that undetected rate variation in the uncalibrated parts of the tree is as dramatic as in the calibrated sections, underscoring the importance of fossil calibration. Fortunately, new and interesting fungal fossils are being discovered and we review some of the new discoveries that confirm the ancient origin of important taxa. To help evaluate which fossils might be useful for constraining the ages of nodes, we selected fossils thought to be early members of their clades and used ribosomal or protein-coding gene sequence substitution rates to calculate whether fossil age and expected lineage age coincide. Where ages of a fossil and the expected age of a lineage do coincide, the fossils will be particularly useful in constraining node ages in molecular clock analyses.

1. The negative evidence dilemma: the more ancient the age estimate, the lower the likelihood of finding contradictory evidence

Heckman et al. (2001) published age estimates for colonization of earth by fungal and plant lineages that shocked mycologists and botanists because they were about twice as old as the earliest fossil evidence of plants on land, e.g., Ascomycota and Basidiomycota diverged 1.2 billion years ago, and mosses diverged from vascular plants 680 million years ago. Yet Heckman *et al.* (2001) were the first to apply data from many loci per organism to questions of dating in fungi and, under the reasonable assumption that sampling many genes would average out gene-specific selective pressures, their efforts

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doi:10.1016/j.fbr.2010.03.001

resulted in a data set that kept good molecular time. Their analysis also produced the huge gap between dates consistent with the fossil record and dates consistent with the molecular data, which raised these important questions for the field. If the molecular dates were right, why does the fossil record lag hundreds of millions of years behind divergence times? If the molecular dates were wrong, what misunderstanding of molecular evolution could account for the skewing of divergence time estimates?

Fossils must be used to tie a molecular phylogeny to a geologic time scale, and linking fossils and molecular estimates is not without its problems. The ancient divergence times in the Heckman *et al.* (2001) paper illustrate a general difficulty in dating: The older the age estimate, the lower the likelihood that any conceivable geological or fossil evidence could ever contradict it. Maximum ages provided by the age of the earth (~4.6 Ba) or the development of an oxidizing atmosphere (~2 Ba) are too ancient to be of much use in limiting the age of origin of fungi. By their nature, fossils provide minimum ages for divergences and genetic lineages can be much older than even the oldest fossil representative (Fig. 1). The most useful fossils are those that represent very early members of their lineages. However, the odds of fossilization of the first members of a new group are surely small, as are the odds of finding and recognizing these fossils. Usually, the only way to assign a maximum age to an event involves setting some sort of probabilistic distribution for the likelihood of finding a fossil representing a clade, as discussed in the next section. Except for rare cases where a continuous fossil record documents a phenotypic change that is clearly associated with the emergence of a clade, e.g., the change in pollen morphology associated with the emergence of eudicots or the change in skeletal morphology associated with the divergence of birds and mammals, maximum ages for divergences will always be open to dispute.

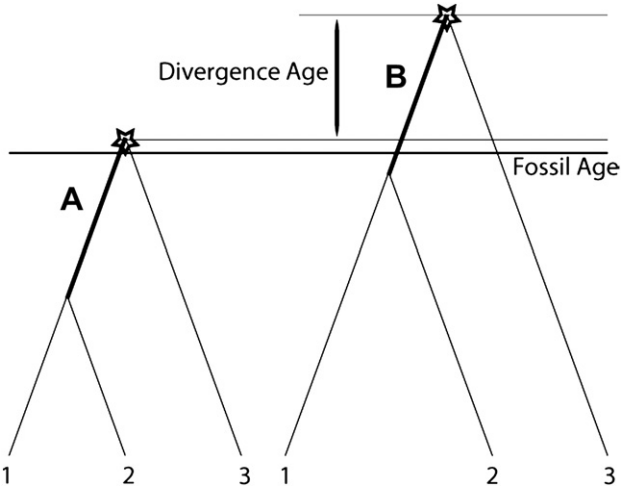

Fig. 1 – Relationship of a fossil assignable to a particular branch and the divergence (indicated by a star) leading to that branch. (A) If a fossil was formed shortly after a divergence, its geological age should agree with ages determined from divergences of DNA sequences from taxa 1 or 2 vs. taxon 3. (B) If a fossil was formed long after a divergence, its geological age should be significantly more recent than the node age estimated from nucleotide divergences between taxa 1 and 2 vs. taxon 3.

Given the paucity of fungal fossils, Heckman *et al.* (2001) calibrated their fungal phylogeny using a date of 1600 Ma for the divergence of fungi, animals and plants. This date had been inferred from a universal phylogeny of eukaryotes that used the bird/mammal split at about 310 Ma as the calibration point (Wang *et al.*, 1999). The assumption underlying the dates of Wang *et al.* (1999) and Heckman *et al.* (2001) was that the molecular clock ticks at the same rate throughout eukaryotes. Peterson *et al.* (2004) showed that it was possible to examine that assumption using multiple calibration points in a comparison of vertebrates and invertebrates to demonstrate that molecular clocks do not tick at a uniform rate. Peterson *et al.* (2004) also showed that Heckman *et al.* (2001), by picking a slowly evolving clade for calibration, arrived at very old ages for rapidly evolving lineages such as fungi and plants. Furthermore, analytical methods could not compensate for a lack of calibration points. Even with a phylogenetic method that allowed for substitution rate variation, when using the bird/mammal divergence as a single calibration, we found surprisingly old estimated ages for all lineages within fungi (Taylor and Berbee, 2006). Demonstrating the extent to which a single calibration point can control node ages, Lücking *et al.* (2009) rescaled previously published fungal phylogenies to the same fossil calibration, the 400 Ma fossil fungus *Paleopyrenomycites devonicus* (Taylor *et al.*, 2004, 2005; Taylor *et al.*, 1999). Even though the original phylogenies were made using different genes and different methods, after rescaling, they showed similar node ages (Lücking *et al.*, 2009). Consistent though those fungal node ages may be, their reliability will, however, be in doubt until rate variation within fungi is better modeled and until calibration of molecular dates is improved.

Rate variation is so clearly a feature of molecular evolution that most recent approaches to molecular clocks, as reviewed by Welch and Bromham (2005) and by Lepage *et al.* (2007), have been directed towards allowing rates to vary across different branches in a tree. Fungal rate variation was recognized early (Berbee and Taylor, 1993). More recently, rate variation has been investigated using likelihood methods designed to accommodate rate variation, that is, to relax a strict molecular clock (Taylor and Berbee, 2006). In the next sections we further investigate variation in substitution rates in fungi by using Bayesian methods as implemented in the program BEAST ver. 1.4.8 <http://beast.bio.ed.ac.uk/>; (Drummond and Rambaut, 2007). After describing briefly BEAST's Bayesian approach to rate variation and fossil calibration, we provide an example of the application of BEAST using a data set from our previous studies (Taylor and Berbee, 2006).

Bayesian methods require prior knowledge (prior probability distributions) to inform the analysis and influence the resulting posterior probability (Felsenstein, 2004). As they run, Bayesian programs including BEAST launch a succession of millions of Markov chain Monte Carlo (MCMC) generations. MCMC is a trial-and-error approach where selection usually favours replacement of less likely values (given the priors) with more likely values for evolutionary parameters. However, to explore a wide range of combinations of parameter values, occasional replacements by less likely values are

also permitted. With each generation, the likelihood (given the priors) of a slightly different parameter set is calculated. In running BEAST, likelihood increases with initial generations and then reaches a nearly stable plateau. The goal then is to establish posterior distributions of evolutionary parameter values in the form of samples from the generations after likelihood stabilizes. For example, a node age would be estimated as 350 Ma if that were the mean age from the sampled generations, and the confidence interval would extend from 300 to 400 Ma if 95 % of the estimated ages lay between these bounds. In this manner, at the end of the trial-and-error process, the frequencies of clades, node ages or rates for branches in the posterior distribution provide estimates of their posterior probabilities (Drummond and Rambaut, 2007).

2. Incorporating uncertainty about fossil calibrations into node age estimates: an example using the program BEAST and a Bayesian approach to a 50-gene data set

To explore the consequences of a Bayesian approach to dating nodes, we used BEAST to analyze a 50-gene data set consisting of amino acids inferred from codons found in DNA sequence for 26 taxa described earlier (Rokas *et al.*, 2005; Taylor and Berbee, 2006). Using BEAUTI (a program distributed with BEAST), we set priors for the analyses, and produced the necessary, correctly formatted XML input file for BEAST. Based on our earlier analysis (Taylor and Berbee, 2006), we used a Wagner model of amino acid substitution and a gamma site heterogeneity model with four rate categories as priors. BEAST analyses will not run if the likelihood of the combination of starting parameter values is too low. In order to run an analysis that included calibration points, we had to provide a sufficiently likely prior user tree. To create the tree, we ran BEAST without a calibration point for 500,000 generations. We then edited the XML file to include the resulting tree in Newick format (a standard format, used in PHYLIP etc., see <http://evolution.genetics.washington.edu/phylip/newicktree.html>), as a prior for further analysis. As recommended in BEAST's documentation, we used a Yule speciation process, which specifies a constant rate of species divergence.

As noted at the outset, due to the vagaries of preservation, discovery and interpretation, few fossils can be expected to accurately date a divergence. BEAST and other Bayesian programs allow the user to incorporate uncertainty about calibration ages into date estimates. Soft bounds can be applied to specify the prior probability of different ages and the final posterior age distribution is based not only on the age priors but also on the molecular substitution rates. Hard bounds, on the other hand, limit the node age estimate by controlling the range of ages considered in the analysis. For the common case where the age of a fossil provides a minimum age but the true date for a divergence may be older than the earliest representative fossil (Fig. 1), BEAST allows the user to specify an exponential distribution with a hard bound representing a minimum age from a fossil and a soft bound representing the distribution of probabilities of even older ages. After initial experimentation, we used an exponential distribution with a minimum age of 300 Ma and a standard deviation of 30 Ma for the divergence of birds from mammals (Fig. 2A). With the soft bounds provided by a normal distribution, dates older or younger than a specified date are possible, but less probable (Fig. 2B). Reflecting uncertainty about the true minimum or maximum age of the root where the plants diverged from the opisthokonts (fungi + animals), we assigned the node age a prior normal distribution with a mean of 1700 million years and a standard deviation of 300 million years (Fig. 2B). For the fly/mosquito divergence, we used a normal prior with a mean age of 235 Ma (Peterson *et al.*, 2004) and a standard deviation of 24 Ma. Where a continuous fossil record exists, as with the eudicots divergence, hard bounds for the minimum and even maximum age can be specified. For the divergence of eudicots (*Arabidopsis*) from monocots (rice), we used

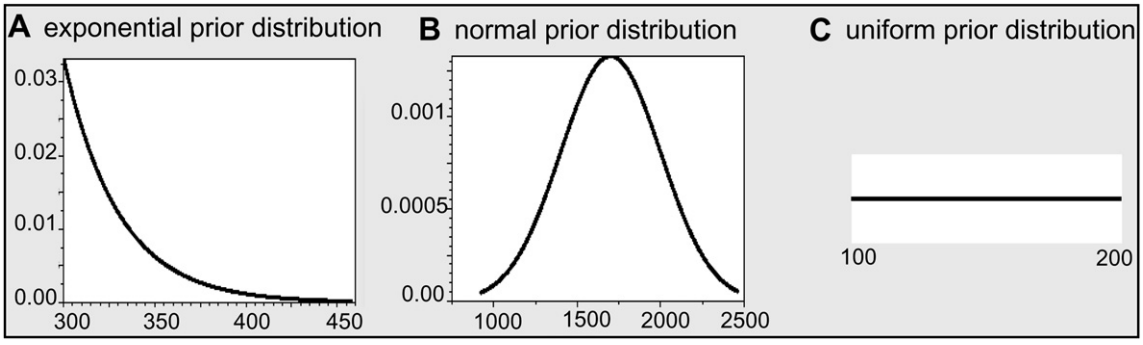

Fig. 2 – Alternative options for prior probability distributions on ages of nodes, given a fossil calibration point, as provided in BEAST software (Drummond and Rambaut, 2007). The *x*-axes represent age in millions of years; the *y*-axes in (A), (B), represent the instantaneous probability of the age. We used the exponential curve (A) to provide the prior probability for the age of divergence of bird from mammal. In this case, a 300 million-year old fossil provided a convincing lower limit for possible divergence time, and the tail of the curve is consistent with the possibility that the divergence could have taken place much earlier. The normal curve in (B) describes the prior probability for the age of the split of the plants from the animals plus fungi. The actual age is actively debated and this was reflected in our choice of a large (300 Ma) standard deviation about a prior mean of 1700 Ma. We used the uniform distribution (C) to constrain the age of origin of the eudicots between 100 and 200 Ma without specifying a particular date within the range.

a uniform prior with an upper hard bound of 200 Ma and a lower hard bound of 100 Ma (Fig. 2c).

At each generation, BEAST estimated the likelihood of the data, given the priors, the tree topology and other program parameters. Lacking the ability to run BEAST in parallel, this task was computationally intensive. Using three 2.66 GHz Intel Core 2 Duo Mac Os X ver. 10.5.6 computers with 2 GB of available RAM, the 15,713,400 completed generations that we analyzed came from four independent runs of the BEAST program; one 5 million generation run that was complete after more than 27 days and three other runs that were halted before completion. We collected one tree per 100 generations. As the criterion to recognize that the number of generations was sufficient, we looked for stable likelihood plateaus from independent runs using the graphing function in Tracer (a computer program supplied with BEAST), discarding as burnin the less likely first 5001 sampled generations per run. The mean likelihoods from the four runs were reassuringly similar, ranging from −ln 236,455 to 236,459. After discarding the burnin, 137,130 trees remained to be analyzed. The cumulative overall effective sample size (an estimate of the number of independent samples of the posterior distribution) was over 200, an acceptable level according to documentation in the BEAST program manual. The consensus of the 137,130 trees is shown in Fig. 3. The wide error bars for node ages represented the many equally likely solutions for age estimates in the posterior distributions, even under the relaxed clock model that allowed for substitution rate changes (Fig. 3).

Models of sequence evolution and soft bounds on fossil dates underestimate localized rate variation producing unreasonable node ages

The appeal of the Bayesian approach lies in its ability to provide a simultaneous probabilistic estimate of values for a highly complex set of parameters including tree topology and node ages, given the priors and given the data. However, weighing against these strengths, whether or not it is possible to select reasonable priors is a serious concern in Bayesian analysis (Felsenstein, 2004). Different priors for node ages had a striking effect on our results.

Given soft bounds as the priors for the age of eudicots (provided in preliminary experiments as an exponential distribution with a minimum age of 100 Ma or a normal distribution with a mean of 100 Ma) BEAST allowed the node date for the origin of eudicots to creep back in time to over 350 Ma, an unreasonable date well outside of the prior probable range (results not shown). Our large amount of sequence

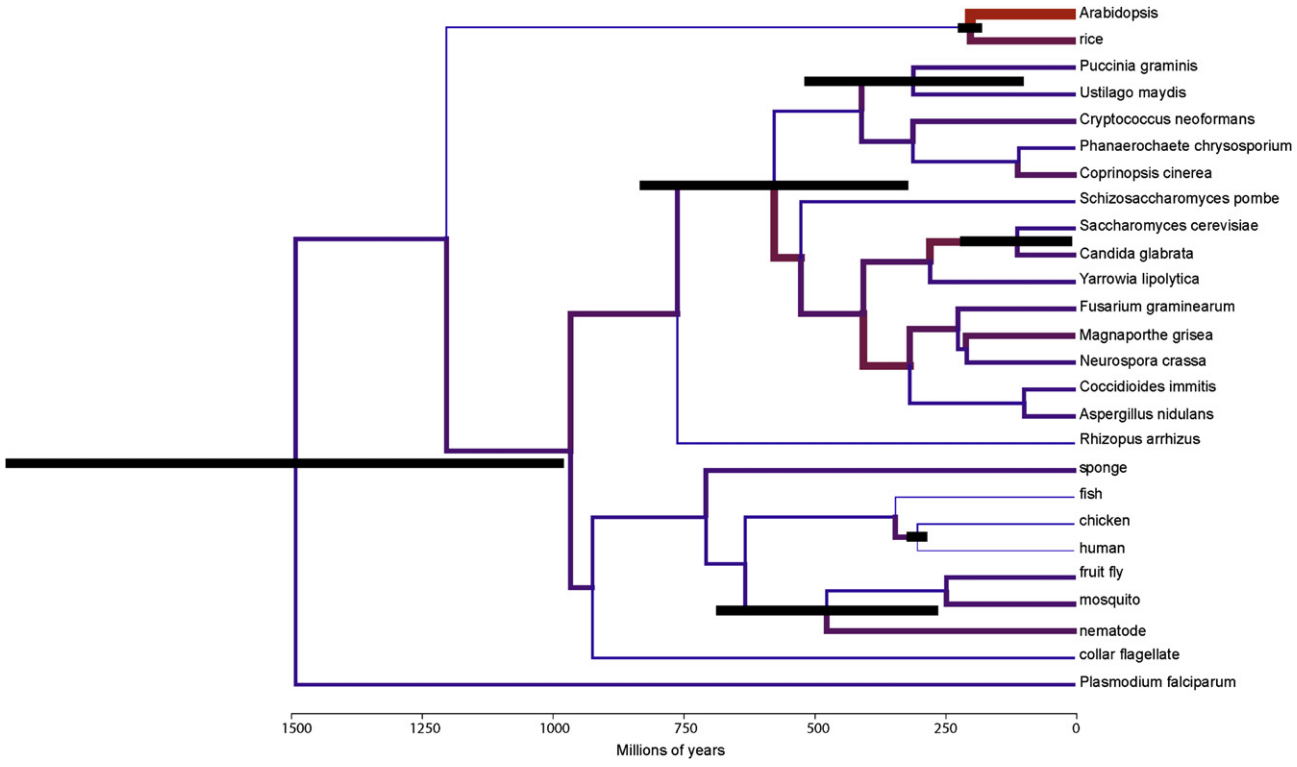

Fig. 3 – Amino acid substitution rates vary throughout plants, animals and fungi as highlighted by changes in branch thickness and colour throughout this phylogram. This tree was generated with the program BEAST, using four calibrations and rooted with the outgroup *Plasmodium falciparum*. Branch thickness is proportional to reconstructed substitution rates; colour indicates the rates from red for the highest substitution rates, through purple for intermediate and blue for the lowest rates. Only seven error bars (in black) are shown and each represents the 95 % posterior distribution for a node age. Omitted error bars were comparable in size to the five longest bars. Note that the exceptionally high rates from the node to *Arabidopsis* and rice, and exceptionally low rates from the node to the chicken and human, along with the narrow confidence intervals around both nodes. Narrow confidence intervals resulted from tension between substitution rates and fossil-based enforced hard bounds on the node dates, and they may be an artefact of the methods rather than an indication of high precision.

data per taxon along with the high substitution rates in the plants overwhelmed the information content of the fossil-based prior probability distribution. Imposing a hard bound in the form of a uniform prior of 100–200 Ma, the age estimate for the node was 200 Ma, at the limit of the bound interval with unusually high substitution rates on the descendant branches from the node (Fig. 3). The narrow confidence interval around the node (Fig. 3) reflected tension between the maximum 200 Ma age from fossil calibration and the low probability of a lesser age that resulted from the model of substitution rate variation. In the absence of an imposed minimum age, the age estimate of the mammal/bird split was an impossibly recent ~100 Ma (Taylor and Berbee, 2006). Forcing the mammal/bird node to a minimum age of 300 Ma (as documented through an excellent fossil record) resulted in exceptionally low substitution rates in the descendent lineages (Fig. 3). Here, the narrow confidence interval around the node reflected tension between the minimum 300 Ma age from fossil calibration and the improbability of an age this great, given the overall model of substitution rates. While these branches calibrated by hard bounds showed dramatic substitution rate variation, the uncalibrated parts of the tree may have had equally dramatic substitution rate shifts that could not be detected.

In the above examples as well as in some computer simulations (Yang and Rannala, 2006), narrow confidence intervals and dates near the limits of a hard bound indicated localized trouble rather than reliable precision in dating. Yang and Rannala (2006) (Fig. 4) showed that one bad calibration point at the root of a tree, in this case the assumption that the date range for a root with a hard bound was 3.5–4.5 when it actually was 1.0, gave ages for the root that were exactly 3.5 times too old. Even worse, at the end of analysis, the incorrect age estimates had narrow confidence intervals. Note that the analysis was precise due to the amount of sequence data, and that the most recent possible age, given the constraint, was chosen, but that it was the fossil calibration that determined the date, an inaccurate one in this case. Given a soft bound for the calibration, enough sequence data, and an adequate model of substitution, the estimate of the age of the node improved, although the apparent precision was still too high, given the true error.

Variation in nucleotide substitution rates is thought to correlate with the frequency of nuclear division. For example, in vertebrates in general (Ellegren, 2007) or in mammals in particular (Fontanillas *et al.*, 2007), substitution may be slow because the female germ line undergoes few mitotic divisions relative to generation time. If mitotic frequency correlates with overall substitution rate in fungi, where there is no separate germ line, then rate variation might be used to estimate life history parameters, such as, growth rate or the time between germination and reproduction for fungi living in substrates where direct observation is impossible.

3. Will new fossil finds help establish minimum clade ages?

In this next section, we offer predictions about which recent fossil finds are old enough to increase the precision of node age estimates. The broad fungal phylogenies that we have just discussed were produced with large data sets comprising 50 genes and our Bayesian analyses showed wide variation in evolutionary rates and statistical uncertainty about node ages.

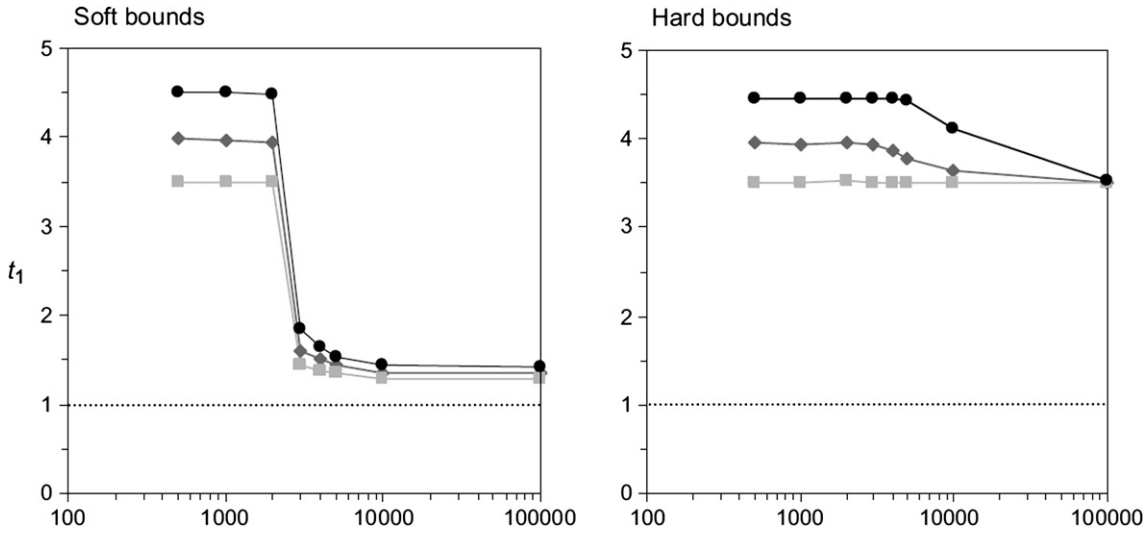

Fig. 4 – Comparison of soft bounds and hard bounds when using erroneous fossil divergence dates. This graph from a computer simulation by Yang and Rannala (2006) shows the posterior means and 95 % confidence interval where the 'y' axis represents age and the 'x' axis represents the amount of sequence data. In the simulation, the actual root date was 1.0 Ma, but the calibrating fossil was 'mistakenly' assigned a range of dates from 3.5 to 4.5 Ma. Left. Soft bounds permitted dates younger than 3.5 Ma, albeit with reduced probability, so that the combination of more data and three other good calibration points partially compensated for the erroneous root date. Right. Hard bounds prevented the use of younger dates for the erroneously dated fossil. Disturbingly, with more data, the confidence interval shrank to a point, greatly overestimating precision and underestimating error. Figure reprinted from Yang and Rannala (2006), fig. 6, p. 220, copyright (2006), with permission from Oxford University Press.

Most fungal lineages with early fossil records cannot yet be included in the broad analysis because they are represented by too few DNA sequences. For these key lineages, increasing the amount of sequence data may ultimately improve molecular dating.

To evaluate the ability of specific fossils to constrain minimum ages, we simplified matters by using the available conserved ribosomal SSU gene sequences for deep divergences. From our earlier experiments with molecular clocks and under our assumption that the divergence of animals from fungi was about 1 Ba, the average rate of substitution in the nuclear small subunit ribosomal genes was about 1–1.25 % per lineage per 100 Ma (Berbee and Taylor, 1993, 2001). Heckman *et al.* (2001) took the divergence of animals from fungi to be 1.6 Ba, which implied a slower rate of substitution, 0.78 %. Using our estimates of substitution in SSU per 100 Ma, a fossil is most likely to constrain the minimum age of a node if the ratio of its age to phylogenetic distance is about 100 million years for every 1.25 % substitution per lineage in the nuclear ribosomal small subunit gene. Because the rate estimates from Heckman *et al.* (2001) are lower, a fossil would have to be older relative to a node to constrain an age.

By any estimate, if the substitution percentage between sister taxa is much higher than 2 % per lineage per 100 Ma when calibrated by the fossil, the lineage is likely much older that the fossil (although it could also be evolving unusually quickly). We (i) arbitrarily selected two species with roughly average branch lengths that diverged from the node constrained by a specific fossil, (ii) estimated the percent substitution in SSU rDNA between the two species (iii) divided the pairwise distance by two for an estimate of the percent substitution per lineage and (iv) multiplied the per lineage percent substitution by the estimated SSU substitution rate per (100 Ma/1.25 % or 100 Ma/2 %) to estimate the time since divergence. We then compared the age of the fossil with the estimated time since divergence. For the estimates of pairwise distance, we used a Kimura two-parameter (K2P) model if the percent substitution was less than 5 %, and a general time reversible (GTR) model of substitution, with rate variation described by a gamma distribution using a likelihood estimate for a gamma parameter, if the percent substitution was over 5 %.

For recent nodes where change in the conserved SSU has been too slow to permit useful estimates, the more rapid substitutions at neutral sites in protein-coding genes served to test if a fossil was likely to provide a meaningful constraint. Neutral rates can be estimated from any protein-coding gene DNA sequence where a substitution does not change an amino acid because these synonymous nucleotide substitutions are thought to elude the forces of natural selection. Although factors such as codon bias can affect rates of synonymous substitution, they can be ignored for our rough estimates of fossil utility. Neutral rates of protein gene evolution in fungi, plants, animals and bacteria are typically between 1×10^{-8} and 10^{-9} substitutions per site per year (Kasuga *et al.*, 2002) and so we divided the per lineage percent substitution by 1×10^{-9} to estimate node age in years for comparisons with fossil age.

Fossil Glomeromycota

This group of fungi, generally accepted as the phylogenetic sister clade to the Ascomycota and Basidiomycota, consists of arbuscular mycorrhizal symbionts. The Glomeromycota have left excellent and ancient fossils. Redecker *et al.* (2000) described fossil spores from shallow marine sediments from the Ordovician, 455–460 Ma in age, that closely resemble Glomeromycota spores. Arbuscules, finely-branched Glomeromycota fungal hyphae within plant cells, were clearly preserved in cells of stems of *Aglaophyton*, a primitive land plant from 400 Ma Rhynie chert (Remy *et al.*, 1994) and from roots from the Triassic (250–199 Ma) (Stubblefield *et al.*, 1987). The pairwise difference between *Glomus eburneum* SSU sequence, GenBank accession number (GB) AM713431 and either a basidiomycete (*Agaricus bisporus* GB L36658) or an ascomycete (*Peziza gerardii* GB DQ646543) is about 19 %, or about 9.5 % per lineage (GTR model). These fossils are tremendously important and they provide some of the best and most readily interpreted evidence for the presence of their phylum. At a nucleotide substitution rate of 1.25 %/100 Ma, the oldest fossil at 460 Ma would be far younger than the divergence at 760 Ma. It is not until the substitution rate approaches 2 %/100 Ma that the divergence, at 475 Ma, would approach the fossil age. It seems likely that the Glomeromycota stem lineage arose earlier than suggested by the fossils and that it predated land plants. Whatever characters or features of habitat predisposed the ancient Glomeromycota towards symbiosis remain a mystery.

Ascomycota

Paleopyrenomycites devonicus (Taylor *et al.*, 2004, 2005; Taylor *et al.*, 1999) is a beautifully preserved fungal fossil consisting of perithecia immersed within stems of the Devonian plant *Asteroxylon mackiei*. The fossil is morphologically complex but, like the Devonian vascular plants that surround it, it does not fit comfortably within an extant taxonomic group. The fungus has an outer wall, interpreted as an outer perithecial wall, filaments in the perithecial neck, interpreted as periphyses, and asci containing variable numbers of spores and opening with what may be an operculum. Ascospores appear to be septate. This fossil is old enough to provide a meaningful constraint on molecular clock dates but ambiguity about its phylogenetic affinities make it difficult to know which node should be constrained (Taylor and Berbee, 2006). It is certainly an Ascomycota and provides a minimum age for the Ascomycota and Basidiomycota at 452 Ma (Taylor and Berbee, 2006) (Fig. 5). If, however, it could be considered a Pezizomycotina, it would push the divergence of Ascomycota and Basidiomycota back to 843 Ma, and if it were considered a Sordariomycete, back to 1.489 Ba (Taylor and Berbee, 2006) (Fig. 5). A conservative approach (minimizing the effect of the constraint) is to treat this fossil as a member of the stem lineage of the Ascomycota subphylum Pezizomycotina (Douzery *et al.*, 2004). This placement was endorsed by Lücking *et al.* (2009) who used the presence of an operculum to rule out *P. devonicus* being a Taphrinomycotina. Although it seems clear that the ascus tip in *P. devonicus* is modified (Taylor *et al.*, 2005, figs 22–24, 27–28), it may not be operculate in the sense of modern Pezizomycotina. Characters of

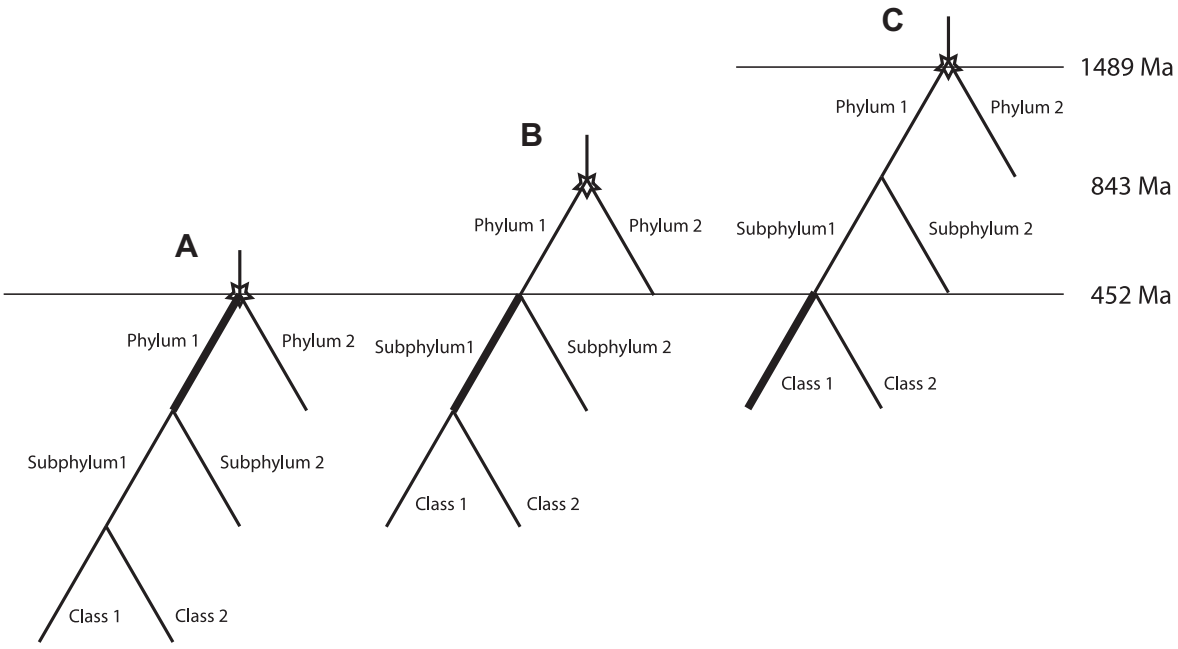

Fig. 5 – Minimum age and the identity of a fossil using the example of *Paleopyrenomycites*. The star indicates the divergence of Ascomycota and Basidiomycota. (A) If the fossil can be placed within a modern phylum (Ascomycota), but no further, the minimum age for the divergence of the phylum and its sister phylum would be 452 Ma. (B) If the fossil can be assigned to a subphylum (Pezizomycotina), then the divergence of the phyla would be pushed back to 843 Ma. (C) If the fossil can be assigned to a class (Sordariomycetes), then the divergence of the phyla would be pushed even further back to 1489 Ma.

Neolecta, an extant member of the earlier diverging Taphrinomycotina show that advanced morphological features including a well-developed fruiting body and ascus tip modification (Landvik *et al.*, 2003, figs 5–8) existed before the advent of the Pezizomycotina, making it possible that *P. devonicus* represents an earlier lineage.

Sung *et al.* (2008) described another astonishing ascomycetous fossil, *Paleoophiocordyceps coccophagus* from ~100 Ma Burmese amber (Fig. 6). The fungus, identified by the authors as an asexual representative of *Ophiocordyceps*, extends from the body of a dead scale insect in the form of two long synnemata covered with a lawn of conidium-producing phialides. Sung *et al.* (2008) used this fossil first to provide a minimum date for the age of the crown members of the Ophiocordycipitaceae and then to estimate that the Hypocreales as a whole were Jurassic in origin (~150–200 Ma). As an independent test of how close the fossil age was to the age of origin of the *Ophiocordyceps* crown clade, we arbitrarily picked *Ophiocordyceps aciularis* and *Elaphocordyceps capitatus*, two taxa that diverged at the basal node of the clade, from Sung *et al.*'s (2008) alignment (TreeBASE M3835). The SSU sequences for these two taxa were 3.2 % different, equivalent to 1.6 % per lineage (K2P model), and if the average substitution rate were 1.25 % per lineage per 100 Ma (Berbee and Taylor, 2001), their divergence would have been 128 million years ago. The percent substitution in three protein-coding regions, presumably mostly neutral, was 13.8 % or 6.9 % per lineage (GTR model). Assuming that the neutral substitution rate was, in line with expectations, about 1×10^{-9} substitutions per site per year (Kasuga *et al.*, 2002), the lineages would have separated from one another 69 million years ago. Based on these rough substitution rate calculations, which bracket the age of the fossil (69 Ma protein < 100 Ma fossil < 128 Ma SSU rDNA), *P. coccophagus* did indeed fossilize close to the time of origin of its lineage and this fossil provides an informative minimum age for its clade.

Lichens

Lichens are common in modern ecosystems and small thallus fragments often suffice for genus-level identification. Rikkinen and Poinar (2008) point out that several species in the Parmeliaceae are preserved in amber, including *Anzia* spp. from 35–40 Ma Baltic amber and *Parmelia* spp. found in Dominican amber dated variously at 15–45 Ma. *Anzia* is a distinctive and early-diverging genus in the Parmeliaceae (Arup *et al.*, 2007), and so these fossils give a minimum age estimate for the family of about 40 Ma. Unfortunately, the substitution levels were about 2.5 % in the SSU gene (K2P model), between *Menegazzia* GB AY584661 and *Parmelia* GB AF117985, lineages that originated from a basal node in the Parmeliaceae (Arup *et al.*, 2007). At 1.25 %/100 Ma, this node should be a minimum of 100 Ma and at 2.0 %, 62 Ma. Therefore, the 40 Ma fossils were probably formed well after the origin of their family. A more comprehensive molecular phylogeny for the Parmeliaceae would allow more useful calibration of internal nodes in the family. Once the phylogeny of *Anzia* was established, the 35–55 Ma Baltic amber specimens could establish a minimum age for the genus.

Other recently discovered fossils provide corroborative evidence about the existence of ecologically important groups

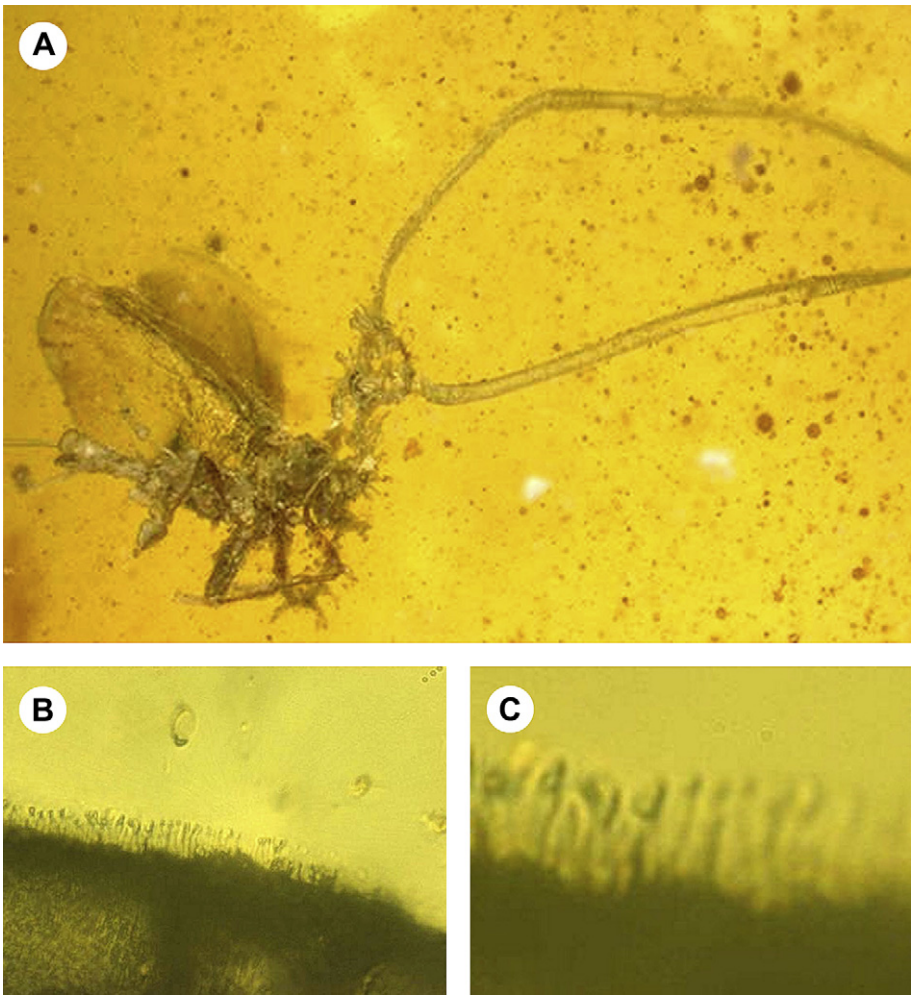

Fig. 6 – *Paleoophiocordyceps coccophagus*, the oldest known fungal parasite of an animal. (A) The fungus is producing synnemata (the thick, cord-like reproductive structures longer than the body of the insect) arising from a male scale insect (Albicoccidae) in Burmese amber. (B) Conidiogenous cells that are distributed in a hymenium-like layer. (C) Conidia and conidiogenous cells. Figure and slightly modified legend are reprinted from Sung *et al.* (2008), fig. 1, p. 496, copyright (2008) with permission from the author and from Elsevier.

of fungi, even though they may not be ancient enough to provide a minimum date for a node. Dothideomycetes have been recognized infrequently in the fossil record, and the oldest known fossil of the Pleosporales is Eocene in age, somewhere between 55 and 35 Ma (Fig. 7) (Mindell *et al.*, 2007). Given the ~7 % substitution in SSU rDNA (GTR evolutionary model) between a pair of taxa spanning the basal node in the order (*Pleospora herbarum* GB DQ767648 vs. *Farlowiella carmichaeliana* GB AY541482) (Schoch *et al.*, 2006), the Pleosporales clade is likely to be as much as four times older than the fossil, if rates of evolution were balanced over that time span (at 1.25 % substitution, 280 Ma, at 2 % substitution, 175 Ma).

Basidiomycota

Basidiomycota is the sister group to the Ascomycota and the two phyla must be equal in age. Hyphae with clamp connections are diagnostic of Basidiomycota and in modern ecosystems clamped hyphae permeate soil and organic matter. The oldest convincing basidiomycete fossils are of clamp connections from a Carboniferous coal ball (Pennsylvanian age, 299–318 Ma (Gradstein *et al.*, 2004)), which are far younger than even the minimum age of Ascomycota at 452 Ma. Finding older clamp connections, or finding a pattern towards increasing morphological diversity among hyphae with clamp connections could provide clues about the geographical distribution and diversity of these early-diverging basidiomycetes. Many modern fungi in Basidiomycota as well as some Ascomycota are ectomycorrhizal, forming dense hyphae in diagnostic patterns on roots. Hosts to ectomycorrhizal fungi include Pinaceae, a plant family with a fossil record dating back over 145 Ma (LePage, 2003). The earliest known fossil ectomycorrhiza is from the Eocene, 50 Ma (LePage *et al.*, 1997). Assuming that like their modern descendants, early Pinaceae were ectomycorrhizal, these ectomycorrhizal fungi originated much earlier than indicated by that first Eocene fossil.

The Agaricomycetes, the main fungal clade containing ectomycorrhizae, may be even older than the most common

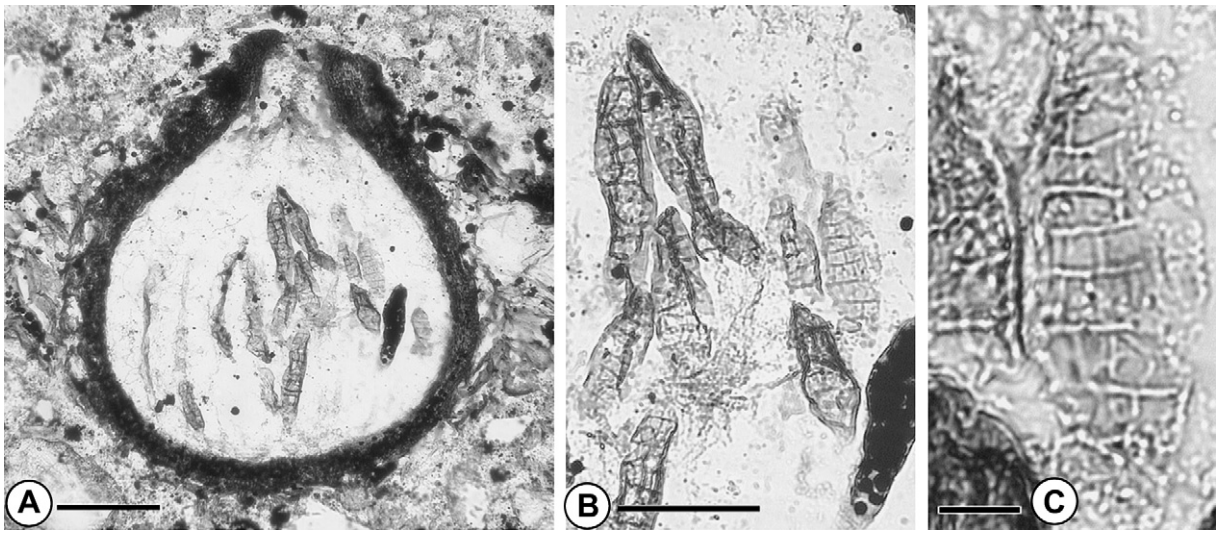

Fig. 7 – The oldest known fossil of the Pleosporales, 55–35 Ma, from permineralized Eocene fossils from Vancouver Island BC, Canada. Holotype of *Margaretbarromyces dictyosporus*. (A) Longitudinal section through ascoma, bar = 100 μm. (B) Longitudinal view of ascospores showing spore shape and septa. Bar = 50 μm. (C) Detail of ascospores with transverse and longitudinal septa. Bar = 10 μm. Photos reprinted from, and legend modified from Mindell *et al.* (2007), fig. 1, p. 682, copyright (2007), with permission from the author and from Elsevier.

host plants of ectomycorrhizal fungi, the Pinaceae and the rosids. To compare the relative ages of ectomycorrhizal fungi and their host plants, Hibbett and Matheny (2009) estimated plant and fungal node depths using BEAST. To go further in estimating the relative ages of ectomycorrhizal fungi and their host plants would require fossil calibration to capture the kind of lineage-specific rate variation evident in Fig. 3. However, in the absence of the fossil record that they would wish for, Hibbett and Matheny's (2009) analysis suggested that the ancestor of the Agaricomycetes must have been saprotrophic because the Agaricomycetes were more deeply branching than the lineages of their main ectomycorrhizal host plants.

Mushrooms are the most striking basidiomycetes in modern ecosystems. Each find of a fossil of one of these delicate and short-lived structures has been a cause for celebration. Hibbett *et al.* (1997) described *Archaeomarasmius*, a 90 Ma mid-Cretaceous fungus, which contributes to establishing a minimum age for the main clade of mushroom forming fungi, the Agaricales. *Marasmius rotula* (GB DQ113912) and *Amanita muscaria* (GB AF026631) are 4.7 % divergent in the SSU rDNA (K2P model), or 2.35 % per lineage so the age estimate of the group (117 Ma (@2.0 % substitution) or 188 Ma (@1.25 % substitution)) is likely older than the age of the fossil (90 Ma).

An intriguing 65–70 Ma fossil perhaps represents the earliest known member of the Geastrales, the earthstars and cannonball fungi (Krassilov and Makulbekov, 2003). Like modern earthstars, the fossil has an inner peridium with an apical ostiole. The inner peridium is seated on a lobed outer peridium. However, compared with modern earthstars, the expanse of tissue in the outer peridium is large relative to the surface area of the inner peridium. In contrast to modern earthstars, the fossil's peridium lobes are not star-like and they do not look as if they would fold into a sphere. Interpreting the fossil as a possible species in the *Sphaerobolus* and *Geastrum* clade (Hosaka *et al.*, 2006), the per lineage percent divergence of *Sphaerobolus stellatus* (GB AF026618) from *Geastrum saccatum* (GB AF026620) is 1.39 % in the SSU rDNA (K2P model). This means that the fossil age is in line with the rough substitution-based age of the group (111 Ma if 1.25 % substitution per year, 69 Ma if 2 % substitution per year). The fossil provides a potentially useful calibration point, with the caveat that its taxonomic identification is not completely secure.

Perennial woody polypores persist in nature far longer than mushrooms and might, therefore, fossilize more readily. Smith *et al.* (2004) described two fossil polypores from Vancouver Island, British Columbia, Canada. The older of the two fossils, *Quatsinoporites cranhamii* was from the Barremian stage in the early Cretaceous, from about 125–130 Ma (Fig. 8) (Smith *et al.*, 2004). Its regular pores and hyphal construction are certainly consistent with its identity as a polypore but more exact phylogenetic placement is difficult. The fossil has prominent cystidia, slightly rounded at their tips, projecting into the pores. This anatomy is consistent with the Hymenochaetaceae, where the fossil was classified by the authors. It is also anatomically similar to taxa such as *Phaeolus schweinitzii*, formerly considered part of Hymenochaetaceae but now usually placed in the Fomitopsidaceae. Representative species pairs spanning the relevant node such as the *Inonotus baumii* GB AY436630 (representing Hymenochaetaceae) and *Fomitopsis pinicola* GB AY705967 (representing Fomitopsidaceae) gave a pairwise difference of 3.2 %, or 1.6 % per lineage (K2P model), representing 128 Ma of evolution assuming 1.25 % per 100 million years, putting the age of origin of the lineage squarely within the range of ages assigned to the fossil.

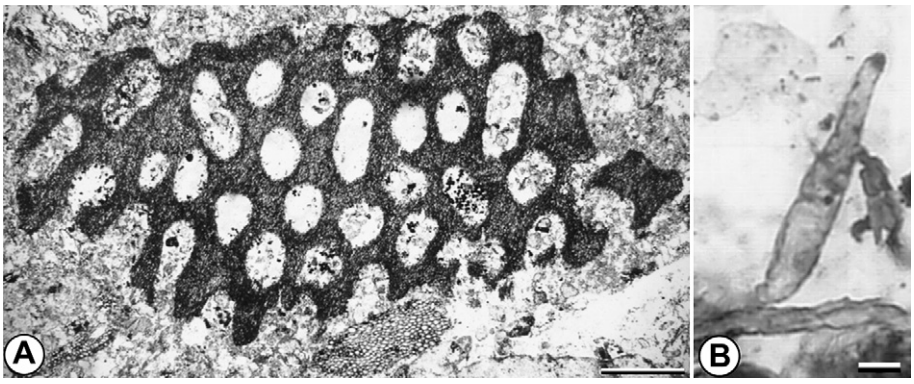

Fig. 8 – One of the earliest polypores, the 125–130 Ma old *Quatsinoporites cranhamii*. (A) Cross section through poroid hymenophore, bar = 0.5 cm. (B) Ampulliform cystidium with blunt apex and pitted walls, bar = 0.6 μm. Photos and legend reprinted from Smith *et al.* (2004), figs 1, 5, p. 182, copyright (2004), with permission from the author and from The Mycological Society of America.

Fungal affinities for other ancient and enigmatic fossils?

Butterfield (2005) raised the provocative possibility that 900 Ma-year old *Tappania* sp. (Fig. 9) was fungal. Hundreds of millions of years before multicellular plants and animals appeared in the fossil record, *Tappania* sp. accumulated in shallow marine seas. Its spherical cell bodies were somewhat over 100 μm in diameter and surrounded by a reticulum of anastomosing filaments (Fig. 9). Butterfield suggested that the anastomosing filaments around *Tappania* sp. were homologous with fungal hyphae. The ability of hyphae to fuse or anastomose, is, he proposed, a synapomorphy of the true fungi, shared among ascomycete croziers, coiled traps in nematode trapping fungi, and basidiomycete clamp connections. However, fungi are not alone in exhibiting cellular fusion and convergent examples of fusion include the pollen tube with the embryo sac in plants and of antheridia with oogonia in oomycetes. In extant fungi, a complex hyphal mesh like the one surrounding the *Tappania* sp. spores is usually associated with reproductive structures that are dispersed by terrestrial animals. Even among basal fungi (as long as they are not endoparasites) divergent hyphal-like outgrowths (rhizoids) develop for nutrient assimilation and for anchorage to a substrate. Such structures are missing from the *Tappania* fossils. *Tappania* is among the most ancient of known eukaryotic fossils and it is an interesting puzzle, but it may or may not be fungal and its uncertain relationships preclude its use as a calibration point.

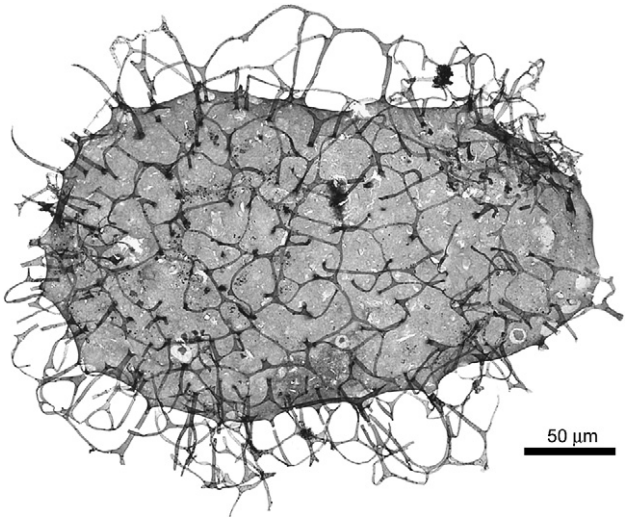

Fig. 9 – *Tappania* sp. from the Wynniatt Formation, Victoria Island, Northwest Territories, Canada. Specimen with multiply branched anastomosing processes with both transverse and oblique/intersecting septa. Reprinted from Butterfield (2005) with permission from the author and from the Paleontological Society.

And then there is *Prototaxites*. In the Devonian 400 Ma ago, at a time when land plants were less than a meter tall, *Prototaxites* grew to 1 m in diameter and 8 m tall, containing *ca*. 50 times the volume of the largest extant fungal fruiting bodies (Burdsall *et al.*, 1996). Constructed of filaments, *Prototaxites* fossils have been interpreted as vascular plants, basidiomycete fruiting structures, algae, or lichens (Hueber, 2001) and most recently, masses of rhizoids from rolled mats of liverwort thalli (Graham *et al.*, 2010). Intriguingly, the carbon isotope ratios of different fossils of *Prototaxites* show great variation and some specimens had levels of C^{13} enriched beyond levels expected in a photosynthetic organism (Boyce *et al.*, 2007). Boyce *et al.* (2007) suggested that *Prototaxites* might have been heterotrophic on plants or bacteria while Graham *et al.* (2010) interpreted them as mixotrophic assemblages of liverworts, fungi, and cyanobacteria. Missing from the picture are any organs of nutrient assimilation in *Prototaxites* fossils, leaving open to imagination whether the ancient living organisms photosynthesized or fed. Again, an interesting puzzle but too difficult to place systematically to serve as a calibration point for fungi, or any other terrestrial life forms.

4. Fungal phylogeography: a new synthesis of species, geography, and molecular evolution

Phylogeography, the study of geological ranges of lineages of organisms over time moves from history to science when repeated correlations of species distributions with global geological history suggest underlying testable evolutionary processes. Fungal species and their geographical ranges were initially broadly conceived. Because fungi disperse by spores and spores are easily carried over long distances, it was argued that barriers that stopped gene flow in plants and animals would not restrict travel of fungi. If the typical fungal species had a worldwide distribution, then a phylogeographic analysis would show fungal species to be restricted not by geographical barriers to dispersal, but their ability to adapt to local conditions (Moncalvo and Buchanan, 2008). Compared with numbers for other organisms, relatively few studies have focused on the phylogeography of fungi (Fig. 10, Beheregaray, 2008). To be fair, Beheregaray considered only papers that had the terms phylogeography or phylogeographic in the title, abstract or key words. Although this strategy enabled him to quickly search databases, it missed many fungal phylogeographic studies, for example, all those on the human pathogenic genera *Coccidioides*, *Aspergillus*, *Histoplasma* and *Paracoccidioides*. Study of fungal biogeography has focused on plant and animal pathogens but is also spreading to other fungi due to contributions of molecular data that help narrow species delimitation to reveal that many species, perhaps most, have restricted distributions (Taylor *et al.*, 2006).

Under closer examination, a few fungi like *Aspergillus fumigatus* have global distributions and show no endemism (Pringle *et al.*, 2005; Paoletti *et al.*, 2005; Rydholm *et al.*, 2006). More commonly, fungal species delimited using sequence data, especially multilocus data often turn out to have restricted ranges (Kasuga *et al.*, 2003; Koufopanou *et al.*, 1997; Moncalvo and Buchanan, 2008; Taylor *et al.*, 2006). As one example that surprised the community of fungal biologists, O'Donnell *et al.* (2000) showed that *Fusarium graminearum*, the cause of the economically important disease wheat scab, consisted of seven phylogenetic species. Only one of the seven was widely distributed across the northern hemisphere. Each of the remaining six was restricted to a single continent. From the phylogenetic pattern of distribution, Africa was the likely source continent and at least some of the speciation events were likely allopatric. Taylor *et al.* (2006) discussed other case studies where molecular data helped define species of *Neurospora*, *Lentinula*, *Saccharomyces* and *Schizophyllum*, revealing that, contrary to initial expectation, range restriction and not global distribution was the rule.

Narrower species definitions based on genetic isolation in nature, and not solely on morphology, are opening the door to questions involving times of range expansion and correlations between human activities, geological events and migration patterns. During the time period of a postdoctoral fellowship or graduate student degree program, an ambitious researcher can accomplish molecular species recognition for a good-sized group of species. Even though undescribed species may be unsampled and absent from consideration, the species that are included show distributions limited by oceans and mountains, or restricted by host or by elevation. Patterns of sequence divergence among narrowly circumscribed species indicate that fungi do traverse these barriers, but on time scales of hundreds of thousands or millions of years. The patterns of diversity do not usually reflect long, unchanging geographical association over hundreds of millions of years, as might be expected if patterns of plate tectonic movement were responsible for setting the patterns of fungal distribution in the modern world.

Where humans assist the migration of fungi, as with agricultural parasites, the patterns can develop in an order of magnitude shorter period, as recently shown in a coalescent study of *Rhynchosporium* (Zaffarano *et al.*, 2008). Whereas fungal long distance dispersal may have been slow, historically, it now can be quite rapid with tragic results as demonstrated by the fungal agents of pitch canker (*Fusarium circinatum*) (Ganley *et al.*, 2009; Gordon, 2006) or amphibian

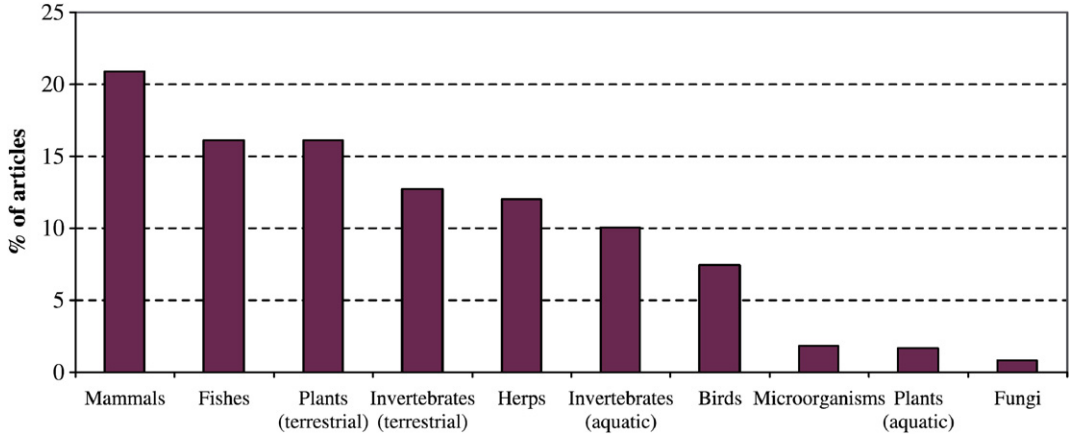

Fig. 10 – Fungi are trailing behind other taxonomic groups as subjects of articles published between 1987 and 2006 that include the term 'phylogeography'. The proportion of phylogeographical studies of fungi should increase as delimitation of fungal species improves and geographical ranges are revealed. Reprinted from Beheregaray (2008), fig. 5, p. 3760, with permission from Blackwell Publishing Ltd.

decline (*Batrachochytrium dendrobatides*) (Wake and Vredenburg, 2008).

Once well-delimited species and their distributions are in hand, extrapolation of calibrated rates of molecular substitution can be used to date older divergences or migrations (Fisher *et al.*, 2001; Kasuga *et al.*, 2003). A recent example of a study relating geography to molecular divergence is Jeandroz *et al.*'s (2008) analysis of the biogeography of truffles. The authors used estimates for SSU rDNA evolution from Padovan *et al.* (2005). Padovan *et al.* (2005) present two sets of ages; Jeandroz *et al.* (2008) used the more recent set. They concluded that nine independent dispersals were needed to explain the distribution of the genus. Starting with the split of *Peziza* from *Tuber* (529 Ma) for calibration, they extended this divergence time to the divergence times based on the SSU region for *Tuber* species, and then to divergence times for ITS regions and for beta-tubulin. As one example of their conclusions, they estimated the divergence of European *Tuber scruposum* from North American *Tuber whetstonense* at 18 Ma. Phylogenetic analysis of the species in *Tuber* suggested that the ancestor of this species pair was European. The low level of substitution suggested that *T. whetstonense* originated far more recently than the ~150 Ma opening of the North Atlantic, separating North America, Greenland, and Europe (Table 1). Although one might dispute their calibration of the origin of the genus at 529 Ma as being too old, a younger date would only increase the discrepancy between the origin of *T. whetstonense* and the opening of the North Atlantic, and

Table 1 – Events in the geological record and their correlations with fungal fossils

Geological period[a]	Event	Fungi
900 Ma Proterozoic Eon		Enigmatic fossil (fungus?) *Tappania* (Butterfield, 2005)
Tonian period		
460 Ma Ordovician		VA mycorrhizal spores (Redecker *et al.*, 2000)
430 Ma Silurian		Hyphae and conidia (Sherwood-Pike and Gray, 1985)
400 Ma Devonian	First whole fossilized land plants (Kendrick and Crane, 1997)	• VA mycorrhizae (Remy *et al.*, 1994; Taylor *et al.*, 1995) • Ascomycota (Taylor *et al.*, 1999) • Blastocladiomycota (Taylor *et al.*, 1994) • Enigmatic fossil (fungus?) *Protaxites* (Hueber, 2001)
310 Ma Carboniferous		Basidiomycota as fossilized clamp connections (Dennis, 1970)
250 Ma Permian–Triassic boundary	• Pangaea; continents united (Lomolino *et al.*, 2006) • Permian–Triassic extinction event	Fungal spore spike in fossil record (Visscher *et al.*, 1996).
150 Ma Jurassic	• Separation of Gondwanaland (Australia, Antarctica, South America, Africa…) from Laurasia (Europe, North America…), by the Tethys Sea (Lomolino *et al.*, 2006) • Separation of North America from Europe by Atlantic Ocean (Lomolino *et al.*, 2006)	
145-65 Ma Cretaceous	Rise of angiosperms and mammals	• Modern-appearing ascomycetes (Mindell *et al.*, 2007; Pirozynski, 1976; Sung *et al.*, 2008) • Mushrooms in amber (Hibbett *et al.*, 1995; Hibbett *et al.*, 1997) • Polypores (Smith *et al.*, 2004) • Possible Geastrales (Krassilov and Makulbekov, 2003)
100 Ma Cretaceous	Separation of Africa and South America by Atlantic Ocean (Lomolino *et al.*, 2006)	
75 Ma, Late-Cretaceous and intermittently afterwards until recent times	Asia and North America connected through Bering land bridge, allowing exchange of plant, animal, and perhaps fungal species between Asia and North America (Lomolino *et al.*, 2006)	Modern genera of ascomycetous epiphytes (Dilcher, 1965)
~50 Ma Paleogene period, Eocene epoch		Deeply diverging lichens representing Parmeliaceae (Lomolino *et al.*, 2006; Rikkinen and Poinar, 2008)

a Age as millions of years and geological period, unless otherwise specified (Gradstein *et al.*, 2008).

further strengthen their conclusion. A likely path for the ancestor of *T. whetstonense* would have been across the Bering land bridge.

The 18 Ma estimate for the divergence of the two *Tuber* species based on SSU divergence can be checked independently of the original calibration using an estimate of ITS substitution rates. *Tuber scruposum* and *T. whetstonense* differ (by gaps or by substitutions) at about 7.8 % of ITS sites or 3.9 % of sites/lineage. If we apply substitution rates in the ITS region of 1×10^{-8} (Kasuga *et al.* 2002), the two species diverged about 3.9 million years ago and if rates were 1×10^{-9} years, separation would have been 39 million years ago, dates that bracket the date (3.9 Ma < 18 Ma < 39 Ma) proposed by Jeandroz *et al.* (2008).

Another recent species level study, this time from a Basidiomycota, shows how observation of range restrictions raise questions about timing of divergence of populations and about correlations between migration and speciation. Where the fly agaric *Amanita muscaria* had been considered one species with morphological variants, Geml *et al.* (2006, 2008) demonstrated from phylogenetic congruence patterns that it is composed of at least three cryptic species, clades I–III, all of which coexist in Alaska. The species cluster may have originated in the northwest of North America or in eastern Asia and then undergone contiguous range expansion (Fig. 11). Dating the divergence of Ustilaginomycetes and Agaricomycetes at 430 Ma, based on SSU rDNA evolution of *ca.* 1 % per 100 M years (Berbee and Taylor, 2001), Geml *et al.* (2006)

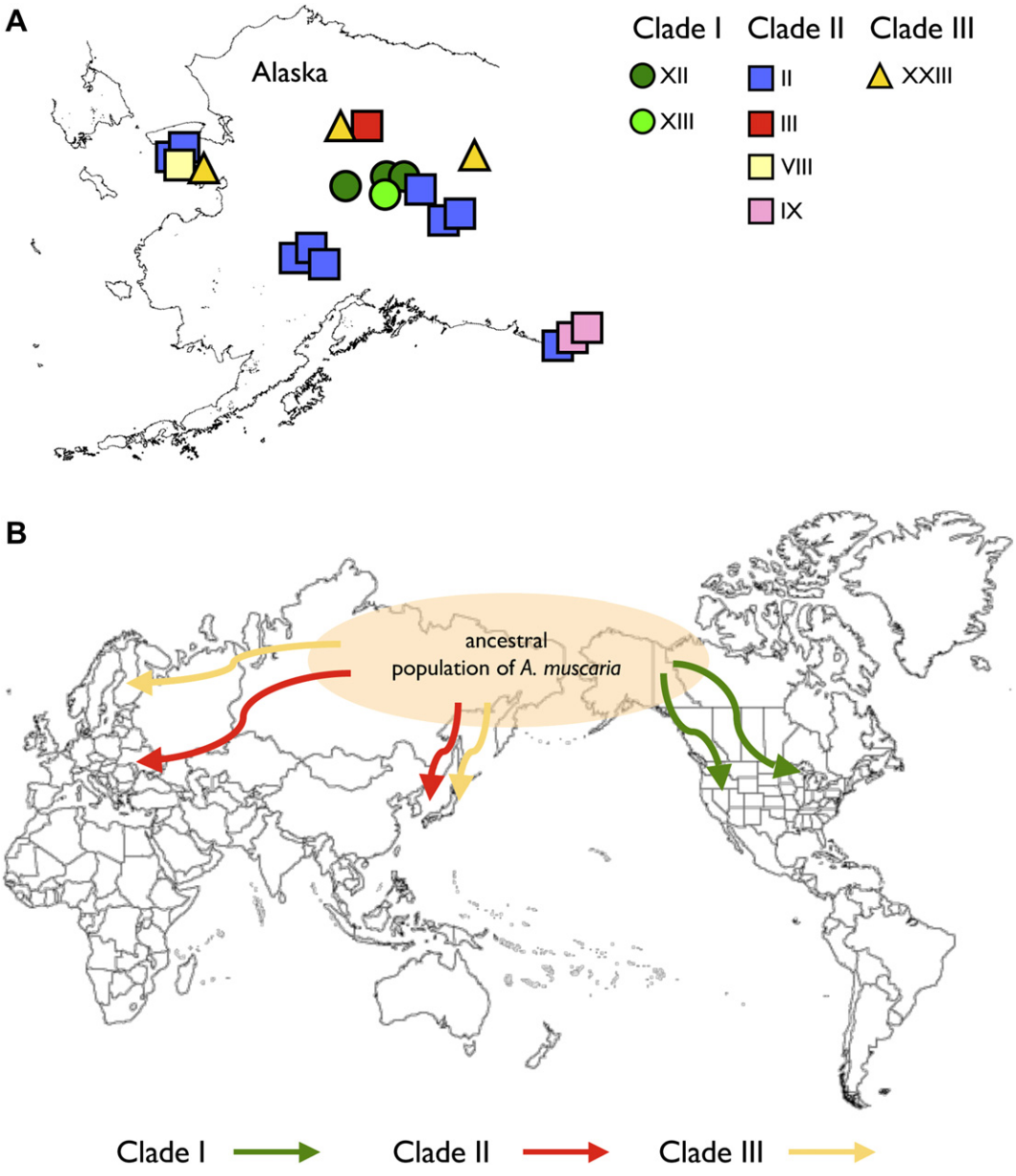

Fig. 11 – (A) Outline map of Alaska showing the geographic distribution of the sampled haplotypes of the three phylogenetic species. (B) Mercator world map showing the putative ancestral population and possible migration routes of the phylogenetic species. Reprinted from Geml *et al.* (2006), fig. 4, p. 236, with permission from the author and from Blackwell Publishing Ltd.

estimated divergence times between North American and Asian *A. muscaria* of 7.5 ±4.5 Ma, consistent with possible range fragmentation and allopatric speciation related to the opening of the Bering Strait, about 12 Ma ago, followed by range expansion.

5. Conclusion

Molecular clocks calibrated by fossils are the only available tools to estimate timing of evolutionary events in fossil-poor groups, such as fungi. Alas, fossil evidence remains scanty and substitution rates change chaotically from lineage to lineage, and together these two factors conspire to produce artefacts that skew divergence time estimates. Filling in gaps in the fossil record may be harder than developing analytical methods to better model patterns of molecular evolution. Then again, developing better analytical methods may not be easy, given that our analysis calls into question how well the current methods cope with the complex patterns of natural rate variation. Whatever the status of fossils and analysis, awareness of phylogeny, fossils and the clock is helping to align expectations for fungal evolution with expectations for plants and animals. Fungi were not fixed geographically and passive as continents wafted them apart, but instead fungal ranges changed more recently and dynamically through rare long distance dispersal. The same geographical barriers affecting the spread of plants and animal also limited the historical spread of fungi. Fungi are not simply ancient and unchanging, but have evolved just as dynamically as any other group of eukaryotes. We look forward to the development of a mutually corroborating body of fossil and phylogenetic evidence, leading not to a perfect clock, but to a better-characterized clock of known limitations.

Acknowledgments

Thanks to Will Iles, Jaclyn Dee, SeaRa Lim and to two anonymous reviewers for critical comments. Thanks also to J. Geml, who kindly provided the new colour version of Fig. 11. This research was supported in part by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada and a subcontract of NSF DEB 0732984 to M. Berbee, and grants NSF DEB 0516511 and NIH NIAID AI070891 to J. Taylor.

REFERENCES

Arup, U., Ekman, S., Grube, M., Mattsson, J.-E., Wedin, M., 2007. The sister group relation of Parmeliaceae (Lecanorales, Ascomycota). Mycologia 99, 42–49.

Beheregaray, L.B., 2008. Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. Molecular Ecology 17, 3754–3774.

Berbee, M.L., Taylor, J.W., 1993. Dating the evolutionary radiations of the true fungi. Canadian Journal of Botany 71, 1114–1127.

Berbee, M.L., Taylor, J.W., 2001. Fungal molecular evolution: gene trees and geologic time. In: McLaughlin, D., McLaughlin, E., Lemke, P. (Eds), The Mycota. Springer-Verlag, Berlin, pp. 229–245.

Boyce, C.K., Hotton, C.L., Fogel, M.L., Cody, G.D., Hazen, R.M., Knoll, A.H., Hueber, F.M., 2007. Devonian landscape heterogeneity recorded by a giant fungus. Geology 35, 399–402.

Burdsall, H.H., Volk, T.J., Ammirati, J.F., 1996. Bridgeoporus, a new genus to accommodate *Oxyporus nobilissimus* (Basidiomycotina, Polyporaceae). Mycotaxon 60, 387–395.

Butterfield, N.J., 2005. Probable proterozoic fungi. Paleobiology 31, 165–182.

Dennis, R.L., 1970. A Middle Pennsylvanian basidiomycete mycelium with clamp connections. Mycologia 62, 578–584.

Dilcher, D.L., 1965. Epiphyllous fungi from Eocene deposits in Western Tennessee, U.S.A. Palaeontographica Abteilung B 116, 1–54.

Douzery, E.J.P., Snell, E.A., Bapteste, E., Delsuc, F., Philippe, H., 2004. The timing of eukaryotic evolution: does a relaxed molecular clock reconcile proteins and fossils? Proceedings of the National Academy of Sciences of the United States of America 101, 15386–15391.

Drummond, A., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7, 214.

Ellegren, H., 2007. Characteristics, causes and evolutionary consequences of male-biased mutation. Proceedings of the Royal Society B-Biological Sciences 274, 1–10.

Felsenstein, J., 2004. Inferring Phylogenies. Sinauer, Sunderland, MA.

Fisher, M.C., Koenig, G.L., White, T.J., San-Blas, G., Negroni, R., Alvarez, I.G., Wanke, B., Taylor, J.W., 2001. Biogeographic range expansion into South America by *Coccidioides immitis* mirrors New World patterns of human migration. Proceedings of the National Academy of Sciences of the United States of America 98, 4558–4562.

Fontanillas, E., Welch, J.J., Thomas, J.A., Bromham, L., 2007. The influence of body size and net diversification rate on molecular evolution during the radiation of animal phyla. BMC Evolutionary Biology 7.

Ganley, R.J., Watt, M.S., Manning, L., Iturritxa, E., 2009. A global climatic risk assessment of pitch canker disease. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 39, 2246–2256.

Geml, J., Laursen, G.A., O'Neill, K., Nusbaum, H.C., Taylor, D.L., 2006. Beringian origins and cryptic speciation events in the fly agaric (*Amanita muscaria*). Molecular Ecology 15, 225–239.

Geml, J., Tulloss, R.E., Laursen, G.A., Sazanova, N.A., Taylor, D.L., 2008. Evidence for strong inter- and intracontinental phylogeographic structure in *Amanita muscaria*, a wind-dispersed ectomycorrhizal basidiomycete. Molecular Phylogenetics and Evolution 48, 694–701.

Gordon, T.R., 2006. Pitch canker disease of pines. Phytopathology 96, 657–659.

Gradstein, F.M., Ogg, J.G., Smith, A.G., Agterberg, F.P., Bleeker, W., Cooper, R.A., Davydov, V., Gibbard, P., Hinnov, L., House, M.R., Lourens, L., Luterbacher, H.-P., McArthur, J., Melchin, M.J., Robb, L.J., Shergold, J., Villeneuve, M., Wardlaw, B.R., Ali, J., Brinkhuis, H., Hilgen, F.J., Hooker, J., Howarth, R.J., Knoll, A.H., Laskar, J., Monechi, S., Powell, J., Plumb, K.A., Raffi, I., Röhl, U., Sanfilippo, A., Schmitz, B., Schackleton, N.J., Shields, G.A., Strauss, H., Van Dam, J., van Kolfschoten, T., Veizer, J., Wilson, D., 2004. A Geologic Time Scale 2004. Geological Survey of Canada (Miscellaneous Report 86, 1 poster).

Gradstein, F.M., Ogg, J.G., van Kranendonk, M., 2008. On the geologic time scale 2008. Newsletters on Stratigraphy 43, 5–13.

Graham, L.E., Cook, M.E., Hanson, D.T., Pigg, K.B., Graham, J.M., 2010. Structural, physiological, and stable carbon isotopic evidence that the enigmatic Paleozoic fossil *Prototaxites* formed from rolled liverwort mats. American Journal of Botany 97, 268–275.

Heckman, D.S., Geiser, D.M., Eidell, B.R., Stauffer, R.L., Kardos, N.L., Hedges, S.B., 2001. Molecular evidence for the early colonization of land by fungi and plants. Science 293, 1129–1133.

Hibbett, D., Matheny, P.B., 2009. The relative ages of ectomycorrhizal mushrooms and their plant hosts estimated using Bayesian relaxed molecular clock analyses. BMC Biology 7, 13.

Hibbett, D.S., Grimaldi, D., Donoghue, M.J., 1995. Cretaceous mushrooms in amber. Nature 377, 487.

Hibbett, D.S., Grimaldi, D., Donoghue, M.J., 1997. Fossil mushrooms from Miocene and Cretaceous ambers and the evolution of Homobasidiomycetes. American Journal of Botany 84, 981–991.

Hosaka, K., Bates, S.T., Beever, R.E., Castellano, M.A., Colgan, W., Dominguez, L.S., Nouhra, E.R., Geml, J., Giachini, A.J., Kenney, S.R., Simpson, N.B., Spatafora, J.W., Trappe, J.M., 2006. Molecular phylogenetics of the gomphoid–phalloid fungi with an establishment of the new subclass Phallomycetidae and two new orders. Mycologia 98, 949–959.

Hueber, F.M., 2001. Rotted wood–alga–fungus: the history and life of *Prototaxites* Dawson 1859. Review of Palaeobotany and Palynology 116, 123–158.

Jeandroz, S., Murat, C., Wang, Y., Bonfante, P., Le Tacon, F., 2008. Molecular phylogeny and historical biogeography of the genus *Tuber*, the 'true truffles'. Journal of Biogeography 35, 815–829.

Kasuga, T., White, T.J., Taylor, J.W., 2002. Estimation of nucleotide substitution rates in eurotiomycete fungi. Molecular Biology and Evolution 19, 2318–2324.

Kasuga, T., White, T.J., Koenig, G., McEwen, J., Restrepo, A., Castaneda, E., da Silva Lacaz, C., Heins-Vaccari, E.M., De Freitas, R.S., Zancope-Oliveira, R.M., Qin, Z., Negroni, R., Carter, D.A., Mikami, Y., Tamura, M., Taylor, M.L., Miller, G.F., Poonwan, N., Taylor, J.W., 2003. Phylogeography of the fungal pathogen *Histoplasma capsulatum*. Molecular Ecology 12, 3383–3401.

Kendrick, P., Crane, P.R., 1997. The origin and early evolution of plants on land. Nature 389, 33–39.

Koufopanou, V., Burt, A., Taylor, J.W., 1997. Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. Proceedings of the National Academy of Sciences of the United States of America 94, 5478–5482.

Krassilov, V.A., Makulbekov, N.M., 2003. The first finding of Gasteromycetes in the Cretaceous of Mongolia. Paleontological Journal 37, 439–442.

Landvik, S., Schumacher, T.K., Eriksson, O.E., Moss, S.T., 2003. Morphology and ultrastructure of *Neolecta* species. Mycological Research 107, 1021–1031.

LePage, B.A., Currah, R.S., Stockey, R.A., Rothwell, G.W., 1997. Fossil ectomycorrhizae from the middle Eocene. American Journal of Botany 84, 410–412.

LePage, B.A., 2003. The evolution, biogeography and palaeoecology of the Pinaceae based on fossil and extant representatives. Acta Horticulturae 615, 29–52.

Lepage, T., Bryant, D., Philippe, H., Lartillot, N., 2007. A general comparison of relaxed molecular clock models. Molecular Biology and Evolution 24, 2669–2680.

Lomolino, M.V., Riddle, B.R., Brown, J.H., 2006. Biogeography, third ed. Sinauer Assoc., Sunderland MA.

Lücking, R., Huhndorf, S., Pfister, D.H., Plata, E.R., Lumbsch, H.T., 2009. Fungi evolved right on track. Mycologia 101, 810–822.

Mindell, R.A., Stockey, R.A., Beard, G., Currah, R.S., 2007. *Margaretbarromyces dictyosporus* gen. sp nov.: a permineralized corticolous ascomycete from the Eocene of Vancouver Island, British Columbia. Mycological Research 111, 680–684.

Moncalvo, J.M., Buchanan, P.K., 2008. Molecular evidence for long distance dispersal across the Southern Hemisphere in the Ganoderma applanatum–australe species complex (Basidiomycota). Mycological Research 112, 425–436.

O'Donnell, K., Kistler, H.C., Tacke, B.K., Casper, H.H., 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97, 7905–7910.

Padovan, A.C.B., Sanson, G.F.O., Brunstein, A., Briones, M.R.S., 2005. Fungi evolution revisited: application of the penalized likelihood method to a Bayesian fungal phylogeny provides a new perspective on phylogenetic relationships and divergence dates of ascomycota groups. Journal of Molecular Evolution 60, 726–735.

Paoletti, M., Rydholm, C., Schwier, E.U., Anderson, M.J., Szakacs, G., Lutzoni, F., Debeaupuis, J.-P., Latgé, J.-P., Denning, D.W., Dyer, P.S., 2005. Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. Current Biology 15, 1242–1248.

Peterson, K.J., Lyons, J.B., Nowak, K.S., Takacs, C.M., Wargo, M.J., McPeek, M.A., 2004. Estimating metazoan divergence times with a molecular clock. Proceedings of the National Academy of Sciences of the United States of America 101, 6536–6541.

Pirozynski, K.A., 1976. Fossil fungi. Annual Review of Phytopathology 14, 237–246.

Pringle, A., Baker, D.M., Platt, J.L., Wares, J.P., Latge, J.P., Taylor, J.W., 2005. Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. Evolution 59, 1886–1899.

Redecker, D., Kodner, R., Graham, L.E., 2000. Glomalean fungi from the Ordovician. Science 289, 1920–1921.

Remy, W., Taylor, T.N., Hass, H., Kerp, H., 1994. Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proceedings of the National Academy of Sciences USA 91, 11841–11843.

Rikkinen, J., Poinar Jr., G.O., 2008. A new species of *Phyllopsora* (Lecanorales, lichen-forming Ascomycota) from Dominican amber, with remarks on the fossil history of lichens. Journal of Experimental Botany 59, 1007–1011.

Rokas, A., Kruger, D., Carroll, S.B., 2005. Animal evolution and the molecular signature of radiations compressed in time. Science 310, 1933–1938.

Rydholm, C., Szakacs, G., Lutzoni, F., 2006. Low genetic variation and no detectable population structure in *Aspergillus fumigatus* compared to closely related *Neosartorya* species. Eukaryotic Cell 5, 650–657.

Schoch, C.L., Shoemaker, R.A., Seifert, K.A., Hambleton, S., Spatafora, J.W., Crous, P.W., 2006. A multigene phylogeny of the Dothideomycetes using four nuclear loci. Mycologia 98, 1041–1052.

Sherwood-Pike, M.A., Gray, J., 1985. Silurian fungal remains: probable records of the Class Ascomycetes. Lethaia 18, 1–20.

Smith, S.Y., Currah, R.S., Stockey, R.A., 2004. Cretaceous and Eocene poroid hymenophores from Vancouver Island, British Columbia. Mycologia 96, 180–186.

Stubblefield, S.P., Taylor, T.N., Trappe, J.M., 1987. Fossil mycorrhizae: a case for symbiosis. Science 237, 59–60.

Sung, G.-H., Poinar Jr., G.O., Spatafora, J.W., 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. Molecular Phylogenetics and Evolution 49, 495–502.

Taylor, J.W., Berbee, M.L., 2006. Dating divergences in the fungal tree of life: review and new analyses. Mycologia 98, 838–849.

Taylor, J.W., Turner, E., Townsend, J.P., Dettman, J.R., Jacobson, D., 2006. Eukaryotic microbes, species recognition and the geographic limits of species: examples

from the kingdom fungi. Philosophical Transactions of the Royal Society of London B Biological Sciences 361, 1947–1963.
Taylor, T.N., Remy, W., Hass, H., 1994. *Allomyces* in the Devonian. Nature 367, 601.
Taylor, T.N., Remy, W., Hass, H., Kerp, H., 1995. Fossil arbuscular mycorrhizae from the early Devonian. Mycologia 87, 560–573.
Taylor, T.N., Hass, T., Kerp, H., 1999. The oldest fossil ascomycetes. Nature 399, 648.
Taylor, T.N., Hass, H., Kerp, H., Krings, M., Hanlin, R.T., 2004. Perithecial ascomycetes from the 400 million year old Rhynie chert: an example of ancestral polymorphism. Mycologia 96, 1403–1419.
Taylor, T.N., Hass, H., Kerp, H., Krings, M., Hanlin, R.T., 2005. Perithecial ascomycetes from the 400 million year old Rhynie chert: an example of ancestral polymorphism (vol. 96, p. 1403, 2004). Mycologia 97, 269–285.
Visscher, H., Brinkhuis, H., Dilcher, D.L., Elsik, W.C., Eshet, Y., Looy, C.V., Rampino, M.R., Traverse, A., 1996. The terminal Paleozoic fungal event: evidence of terrestrial ecosystem destabilization and collapse. Proceedings of the National Academy of Sciences of the United States of America 93, 2155–2158.
Wake, D.B., Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences of the United States of America 105, 11466–11473.
Wang, D.Y.C., Kumar, S., Hedges, S.B., 1999. Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. Proceedings of the Royal Society of London Series B-Biological Sciences V266, 163–171.
Welch, J.J., Bromham, L., 2005. Molecular dating when rates vary. Trends in Ecology & Evolution 20, 320–327.
Yang, Z.H., Rannala, B., 2006. Bayesian estimation of species divergence times under a molecular clock using multiple fossil calibrations with soft bounds. Molecular Biology and Evolution 23 (1), 212–226.
Zaffarano, P.L., McDonald, B.A., Linde, C.C., 2008. Rapid speciation following recent host shifts in the plant pathogenic fungus *Rhynchosporium*. Evolution 62, 1418–1436.