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Establishing a time-scale for plant evolution

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Summary

• Plants have utterly transformed the planet, but testing hypotheses of causality requires a reliable time-scale for plant evolution. While clock methods have been extensively developed, less attention has been paid to the correct interpretation and appropriate implementation of fossil data.

• We constructed 17 calibrations, consisting of minimum constraints and soft maximum constraints, for divergences between model representatives of the major land plant lineages. Using a data set of seven plastid genes, we performed a cross-validation analysis to determine the consistency of the calibrations. Six molecular clock analyses were then conducted, one with the original calibrations, and others exploring the impact on divergence estimates of changing maxima at basal nodes, and prior probability densities within calibrations.

• Cross-validation highlighted Tracheophyta and Euphyllophyta calibrations as inconsistent, either because their soft maxima were overly conservative or because of undetected rate variation. Molecular clock analyses yielded estimates ranging from 568–815 million yr before present (Ma) for crown embryophytes and from 175–240 Ma for crown angiosperms.

• We reject both a post-Jurassic origin of angiosperms and a post-Cambrian origin of land plants. Our analyses also suggest that the establishment of the major embryophyte lineages occurred at a much slower tempo than suggested in most previous studies. These conclusions are entirely compatible with current palaeobotanical data, although not necessarily with their interpretation by palaeobotanists.

Introduction

There can be no doubt that plants have utterly transformed the planet, from their influence on weathering, soil formation and, hence, global biogeochemical cycles, to the creation of environments habitable by other organisms, with many of whom they have co-evolved. However, developing these general perceptions into precisely testable hypotheses requires, at the very least, an intrinsic time-scale so that land plant evolution can be calibrated with events in animal, fungal, biome, or biogeochemical evolution. Traditionally, the role of establishing an evolutionary timescale was the preserve of palaeobotanists reading the fossil record of plant evolutionary history, but over the past two decades, in particular, this role has been usurped entirely by the molecular clock. This has occurred because there is always a significant lag between the time of origin of a lineage and the age of its earliest recognizable fossil record, resulting from the delay in establishing diagnostic apomorphies, as well as the low probability of fossilization. The molecular clock overcomes these limitations by dating lineage divergence directly from molecular sequence data, with the fossil record providing the evidence on which to calibrate molecular distance across phylogenetic trees to geological time (Zuckerkandl & Pauling, 1965). Thus, the molecular clock meets a core aim of palaeontology and the fossil record remains entwined in achieving this aim. However, it could not be said that in practice there has been a happy relationship between molecular clocks and the fossil record. Although the two approaches are often in close enough accord, there are infamous instances of mismatch where cryptic histories of major evolutionary lineages must be inferred from molecular clock analyses, extending as much as twice as far back in geological time as the fossil record would otherwise indicate (Ramshaw *et al.*, 1972; Martin *et al.*, 1989, 1993; Wolfe *et al.*, 1989; Brandl *et al.*, 1992; Larouche *et al.*, 1995). These mismatches have been interpreted to reflect the delay between the origin of a lineage and its subsequent diversification (Cooper & Fortey, 1998); a

systematic bias in the molecular clock analyses (Rodriguez-Trelles *et al.*, 2002); and the inability of early strict clock methods to accommodate variation in rate predicted to occur in rapid radiations (Benton, 1999).

Molecular clock methodology has developed dramatically over the past two decades, in particular to accommodate rate variation (e.g. Takezaki *et al.*, 1995; Sanderson, 1997; Thorne *et al.*, 1998; Drummond *et al.*, 2006), and these developments have, to some extent, begun to ameliorate the discordance between molecular clock and palaeontological estimates of lineage divergence timing. Initial relaxed clock methods assumed heritability of evolutionary rates and so analyses in which they were applied (Heckman *et al.*, 2001; Wikström *et al.*, 2001) failed to accommodate known contrasts in life history traits and, therefore, rate shifts between lineages (Sanderson *et al.*, 2004). More recent attempts to obtain a time-scale for plant phylogeny have explored different methods of inference, and no longer require rate correlation. There has been a general trend towards better branch length estimation through denser taxon sampling, the implementation of fossil calibrations as minimum constraints, and the use of a greater number of calibrations, providing local corrections to rate estimation (e.g. Bell & Donoghue, 2005; Bell *et al.*, 2010; Magallón, 2010; Smith *et al.*, 2010). However, in the face of these methodological developments, little effort has been expended in addressing the problems inherent in the fossil calibration of molecular clock analyses (Bell & Donoghue, 2005). This is entirely surprising as, by definition, fossil calibration is the rate-determining step in molecular clock analyses and, furthermore, both theoretical and simulation studies have shown that greater improvements in the accuracy of molecular clock estimates can be expected from improvements in fossil calibration, not from additional sequence data (Yang & Rannala, 2006).

Problems with the nature, justification and implementation of calibrations

The paucity of concern over fossil calibration has now been overcome by a rich literature exploring the nature of the fossil record and the manner in which fossil data are best implemented, or at least controlled for, in molecular clock analyses. It is clear that fossil first occurrences are often a poor approximation for the time of lineage origin, and so these data should not be used to directly calibrate molecular clock analyses (Reisz & Muller, 2004; Benton & Donoghue, 2007). However, the relative quality of individual calibrations has been the subject of much discussion, as not all fossils that have been employed in molecular clock calibration are equally well constrained phylogenetically, in terms of their age assignment, or the degree to which they approximate the divergence event that they are used to date.

Phylogenetic classification is problematic as fossil taxa can lack the characteristics that diagnose living clades because the fossils are genuinely primitive, or because derived characteristics are simply not preserved in the fossil (Hennig, 1981). It is because of this equivocation that Jefferies (1979) and Hennig (1981) devised a taxonomic distinction between the crown group – the living clade – and the stem group – an assemblage of extinct taxa more closely related to this living clade than any other, plus additional fossil taxa whose membership of the crown was not clear because of incomplete preservation (Fig. 1). However, the stem concept is now widely perceived as being useful only for its evolutionary implications, and no longer as a means of constraining equivocation over the classification of an incompletely preserved fossil taxon (Donoghue, 2005), although the distinction between these two uses is merely a matter of interpretation (Donoghue & Purnell, 2009). In using fossils to calibrate the molecular clock, some authors have obviated this Gordian knot by arbitrarily, and somewhat ironically, allocating all such fossils to the crown (e.g. Magallón *et al.*, 1999), which is likely to

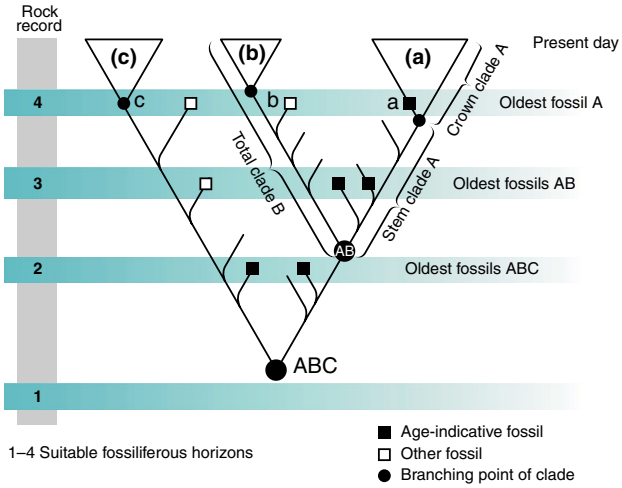

Fig. 1 Definitions of terms used in assigning fossils to clades. The crown clade consists of all living species and their most recent common ancestor, and this is preceded by a stem lineage of purely fossil forms that are closer to their crown clade than to another crown clade. The divergence or splitting point between a species in clade A and a species in clade B is the point AB. This is older than the points of origin of crown clades A and B (indicated as points a and b). Fossils may belong to a crown clade or to a stem lineage, and cladistic evidence should indicate which. The crown clade and the stem clade for a particular lineage are together referred to as the total clade. Therefore, if calibrating the divergence between crown clades a and b, this invariably means finding the oldest reliable fossil belonging to the total clade a and total clade b; the oldest of which will provide the hard minimum constraint for the divergence between the two (point AB). Four fossiliferous horizons are indicated, the source of all relevant fossils. Fossiliferous horizon 1 that contains no fossils assignable to the clade ABC marks a maximum constraint (soft bound) on the age of the clade. Fossiliferous horizon 2 marks a maximum constraint on the age of clade AB. Minimum constraints are indicated by the oldest fossils for ABC, AB and A.

overestimate the age of the clade (Wikström *et al.*, 2001) and is, thus, the worst possible interpretation of fossil data. The most conservative approach is to avoid trying to resolve equivocal interpretations of stem or crown group classifications and instead accept their classification to the total group, the sum of the stem and crown groups (Donoghue & Purnell, 2009). This is the most secure interpretation of the data and the most conservative in terms of its implications for molecular clock calibration as, at worst, calibrations established on such evidence can only underestimate lineage divergences. This is problematic when first occurrences of fossils are used as direct calibrations, but this is an inappropriate use of fossil data.

The age assignment of fossils used for molecular clock calibrations can be problematic because direct geochronological dating is rarely available and, instead, an absolute age is invariably established on the basis of correlations of the rock sequence in which the critical fossil was discovered to other geological localities where direct geochronological dates are available, or to fossil first or last occurrences that have been integrated in the International Union of Geological Sciences Geologic Timescale (Gradstein *et al.*, 2004). More often than not, it is necessary to correlate through a series of intermediate localities on the basis of similarity in sedimentary sequences (lithostratigraphy), fossil sequences (biostratigraphy), or variation in the magnetic polarity (magnetostratigraphy), isotopic composition (isotope stratigraphy), or even the colour (cyclostratigraphy) in rock successions. This daisy chain of inferences can lead to incalculable errors and, ultimately, to minimum and maximum age constraints on the fossil, each of which will have associated errors. Too often, the evidence on which the age interpretation is based is ignored entirely (or at least omitted) in the justification of calibrations that are then recycled in generations of analyses without considering how the stratigraphic correlations may have changed, for better or worse. It should be a basic requirement that, just as phylogenetic classification of fossil calibrations has to be justified, so does the age assignment, so that inferential steps can be reviewed and, if necessary, revised. For establishing minima, the minimum age interpretation of the fossil should be adopted; for maxima, the maximum age interpretation of the fossil should be adopted (Benton & Donoghue, 2007; Donoghue & Benton, 2007; Benton *et al.*, 2009). This conservative means of establishing a calibration by constraining rather than fixing the age of a node is preferable to calibrations that, although they potentially provide a closer approximation of the time of divergence, are poorly justified in their phylogenetic position and age (e.g. Sanderson, 2003; Magallón & Castillo, 2009).

Calibration consistency has been adopted widely as a means of assessing calibration quality and this is determined on the basis of the degree to which individual calibrations produce estimates that approximate other calibrations (Near & Sanderson, 2004). This approach has been extended further, as a basis for resolving equivocation over the phylogenetic position of fossils used in calibration (Rutschmann *et al.*, 2007; Ho & Phillips, 2009; Pyron, 2010). Both approaches assume that consistency is a positive quality to be sought in a set of calibrations; it is not. There is no reason to expect that the lag between lineage origin and first fossil occurrence will be consistent across any phylogenetic tree and, furthermore, consistent calibrations are redundant, by definition (Hugall *et al.*, 2007). As there is no reason to assume that the rate of molecular evolution is constant, it should be an expectation that any given set of calibrations will be inconsistent, reflecting the variable time lag to fossil first occurrence and providing improved local estimates of branch length. Rather than a justification for the outright rejection of inconsistent calibrations, there nevertheless remains a place for calibration consistency in identifying whether this reflects a biological signal or a systematic or geological artefact (e.g. Smith, 2007).

The best way to ensure the phylogenetic and temporal accuracy of fossil calibrations is to exclude from consideration any records that are equivocally constrained phylogenetically, to use the youngest unequivocal age interpretation of the fossil, and to employ these data as a minimum constraint on the timing of divergence rather than as a direct calibration (Donoghue & Benton, 2007). A consequence of employing fossil minima as constraints rather than as direct calibrations is that it places undue weight on the assumption of the age at the root, perhaps the least readily constrained assumption. A variety of approaches have been developed to overcome the limitations of fossil minima. These include attempts to estimate the probability of lineage divergence directly from phylogenetically and stratigraphically constrained fossil data (Foote *et al.*, 1999; Tavaré *et al.*, 2002; Marshall, 2008; Pyron, 2010; Wilkinson *et al.*, 2011). Direct estimation of the probability of divergence time from fossil data has great potential, particularly for establishing priors on Bayesian molecular clock analyses, but it can be assumption-laden (generation time, sampling effort, fossil preservation) and the requisite stratigraphic occurrence data are only readily available for groups with sporadic records. Alternatively, arbitrary mathematical functions have been advocated an employed to express vague notions, in terms of probability, of the relationship between fossil minima and the time of divergence (Hedges & Kumar, 2004; Drummond *et al.*, 2006; Ho, 2007; Benton & Donoghue, 2007; Donoghue & Benton, 2007; Benton *et al.*, 2009; Ho & Phillips, 2009; Bell *et al.*, 2010; Smith *et al.*, 2010). Like analyses of calibration consistency, these probability functions assume a homogeneous lag in time between divergence time and fossil first occurrences, for which there is no evidence (Inoue *et al.*, 2010), and none have material justification. Inoue *et al.* (2010) derived a simple flexible mathematical function that can be modified

to express and implement different hypotheses of prior probability on divergence time relative to fossil first occurrences. Finally, phylogenetic bracketing has been adopted as a basis for establishing maximum constraints (Reisz & Muller, 2004; Müller & Reisz, 2005; Benton & Donoghue, 2007; Donoghue & Benton, 2007; Fig. 1).

Establishing a maximum constraint on the timing of a lineage divergence is problematic, as the absence of fossil evidence for the existence of a lineage could be because of nonpreservation, or preservation in unexpected and therefore unsampled ecological or geographic contexts. However, the distribution of fossils is nonrandom (it could be readily modelled and so would be less of an obstacle were it random) as most organisms exhibit environmental controls on their distribution and so violations of occurrences predicted on this basis provide evidence of absence (Holland, 1995). It is possible to further constrain the interpretation of absence data using fossil records of sister lineages whose fossilization potential, ecology and biogeography are the same (Behrensmeyer *et al.*, 2000). Absence of fossil evidence of both this 'control lineage' and the lineage of interest cannot be interpreted as anything other than absence of evidence. Records of the control lineage in the absence of the lineage of interest provide evidence of its absence. However, given the nonlimiting nature of such inferences, constraints can be implemented as 'soft maxima' that do not preclude the possibility that divergence could have occurred before this time, but with diminishing probability (Yang & Rannala, 2006; Benton & Donoghue, 2007). Calibration constraints, established as hard minima and soft maxima, overcome concerns about the use of minimum constraints that substantially postdate lineage divergence and reduce the influence of the minimum constraint on divergence estimation (e.g. Bell & Donoghue, 2005; Smith *et al.*, 2010). Following these principles, the only poor calibrations are those minima that predate lineage divergence and maxima that postdate lineage divergence.

Over and above the quality of individual calibrations, there has been considerable discussion concerning the optimal number of calibrations required for a molecular clock analysis. This is of particular concern with relaxed clock analyses that allow for rates to vary across the tree. While there is a general consensus that, wherever possible, the greater the number of calibrations the better the estimate of branch length across the tree, in instances where fossils are used to calibrate the tree directly, it has been argued that calibrating all of the nodes will only lead to conclusions compatible with prior assumptions of clade age, obscuring information in the molecular data set (Hugall *et al.*, 2007). However, where trees are calibrated indirectly by sets of minimum and soft maximum constraints, so long as these are conservative interpretations of the phylogenetic and stratigraphic evidence, they will serve to correct branch length while allowing the molecular data to inform on clade age within these loose bounds.

We implemented these principles in deriving a suite of calibration constraints for plant evolution, chosen because they constrain divergences among the greatest wealth of molecular data which is of course provided by plants with sequenced genomes. These serve as exemplars for the quality of phylogenetic and geological data that we believe is required for molecular clock analyses. It is not intended that these represent the final word from the fossil record on evidence to constrain the timing of these divergences. Quite to the contrary, it is our view that this level of detail is required in establishing calibration constraints precisely because the database of fossils, their phylogenetic relations to and among living plants, the correlation between stratigraphic sections and the geochronological evidence all remain variables. As these variables change, their implications for calibrations are readily identifiable. We applied cross-validation techniques to these constraints to examine their congruence, and what this may imply regarding their veracity. We then conducted a preliminary clock analysis to determine the effects of maximum constraint choice in the base of the tree, and the impacts of different prior probabilities, on divergence times. The estimates from these analyses are then discussed in the context of previous studies to consider how differences in calibration may impact upon time-scales obtained.

Materials and Methods

Taxon sampling, phylogeny and molecular data

We provide minimum and maximum age constraints for 17 key divergences among the major land plant lineages following the procedures outlined in Benton & Donoghue (2007). We chose nodes that would be of interest to the study of evolution among plants in general and feature in many previous molecular clock studies (e.g. Heckman *et al.*, 2001; Wikström *et al.*, 2001; Soltis *et al.*, 2002; Sanderson, 2003; Schneider *et al.*, 2004; Magallón & Sanderson, 2005; Won & Renner, 2006; Moore *et al.*, 2007; Bell *et al.*, 2010; Smith *et al.*, 2010). The rooted topology used in this study is based on a consensus of the most commonly resolved and well-supported relationships featured in the recent literature (Qiu, 2008; Forest & Chase, 2009; Magallón, 2009; Magallón & Hilu, 2009; Renner, 2009). However, because we provide unequivocal minimum age constraints for each lineage, information presented here can assist in obtaining calibrations for studies considering any topological arrangement.

For the molecular clock analysis, we chose 18 taxa for which the complete nuclear and/or chloroplast genome sequences are available. The nucleotide sequences of seven protein-coding chloroplast genes (*atpB*, ATP synthase CF1

beta chain; *psaA*, photosystem I p700 chlorophyll A apoprotein A1; *psa B*, photosystem I p700 chlorophyll A apoprotein A2; *psbA*, photosystem II p680 reaction center D1 protein; *psbB*, photosystem II CP47 chlorophyll apoprotein; *rbcL*, ribulose-biphosphate carboxylase large chain; *rps4*, small subunit ribosomal protein S4) were selected because they have been sampled broadly and used widely in previous phylogenetic and divergence dating analyses (accession numbers are provided in Supporting Information Table S1). The final concatenated alignment contained 10 524 sites. The data were partitioned by codon and model selection was performed using MrModeltest 2.2 under the Akaike information criterion (Nylander *et al.*, 2004). The GTR + Γ model was selected for all three partitions.

Molecular clock analysis

Molecular clock analysis was performed using mcmctree (Yang & Rannala, 2006; Rannala & Yang, 2007; Inoue *et al.*, 2010), part of the paml 4 package (Yang, 2007), with branch lengths estimated in baseml. We implemented the GTR + Γ model with five gamma rate categories. Divergence times were estimated under the independent rates model, with the gamma prior on the overall substitution rate and the rate drift parameter specified with respective means and standard deviations *G* (0.035, 0.035) and *G* (0.05, 0.05). Two independent runs were performed, each consisting of 10 million iterations, discarding the first 2 500 000 generations as burn-in and sampling every 75, resulting in a total of 100 000 samples post burn-in.

Cross-validation and calibration consistency

We adopted the cross-validation approach of Near *et al.* (2005) to estimate the inconsistency between calibrations. In brief, this method measures the inconsistency between calibrations by running the analysis with each calibration independently and calculating the average difference ($\bar{D}_x$) and the sum of squared differences (SS) between the molecular estimates and the fossil age estimates for all remaining nodes. Because we implemented minimum and maximum constraints, rather than fixed calibrations, providing a time interval for each node during which it is reasonable to assume divergence has occurred, we expanded this approach and calculated $\bar{D}_x$ and SS by comparing molecular estimates with calibration spans, rather than fossil-based minimum estimates. Differences are expressed in terms of millions of years; mean estimates that are older than the maximum constraints are positive, while ages that younger than the minimum are negative, and anything that falls between the minimum and maximum bounds is equal to zero. Near *et al.* (2005) outlined an approach for identifying and removing the most inconsistent calibrations. First, the average squared deviation (*s*) of the difference between the molecular and fossil-based estimates is calculated when all calibrations are considered. Calibrations are then ranked in order and sequentially removed based on the magnitude of SS. Those with the largest SS values are removed first and *s* is recalculated. One tailed *F*-tests ($P < 0.05$) are used to test whether the removal of any calibrations would significantly reduce the value of *s*.

mcmctree always requires an upper bound, and so we applied a loose upper constraint at the root. However, we appreciate that there is a great deal of uncertainty regarding the timing of origin for the three most basal nodes in our tree (liverwort, moss and hornwort) which all share a maximum constraint. To examine the impact of this constraint and the prior applied at the root during cross-validation, we reran all of the above analyses considering two alternative limits: 509 Ma, representing the oldest description of Cambrian plant-like spores, and 1042 Ma, representing a sampled Precambrian locality yielding no plant-like spores.

Except for the prior on the root during cross-validation, a uniform distribution was used to define the uncertainty between the minimum and maximum bounds. The maximum bound was made soft by allowing 2.5% of the probability distribution to exceed the specified limit, as advocated by Yang & Rannala (2006) based on simulation experiments. For the cross-validation analysis the birth (λ), death (μ) and sampling (ρ) prior on times for uncalibrated nodes was specified as $\lambda = 1$, $\mu = 1$ and $\rho = 0$, which produces a uniform distribution.

Prior probability distributions We also explored the impact of altering the peak in prior probability between the minimum and maximum bounds, with the maximum constraint set to 1042 Ma. We modified the peak in prior probability between bounds by manipulating the location (*p*) and scale (*c*) parameters of the truncated Cauchy distribution (Inoue *et al.*, 2010) to set the peak at 110, 125, 150 and 175% beyond the fossil minima relative to the maxima.

To estimate divergence times among the major plant lineages, we included all 17 calibrations and used a uniform distribution between the hard minima and soft maxima to constrain node ages.

Results and Discussion

Calibrations

Seventeen sets of calibration constraints for the nodes in Fig. 2 are fully justified below, and the information is summarized in Table 1. Alternative angiosperm topologies and their calibrations are presented in Fig. 3.

Unnamed clade: *Arabidopsis–Populus* (node 17: minimum = 82.8 Ma; soft maximum = 127.2 Ma) This divergence represents the origin of core rosids and the splitting of total group Fabidae and Malvidae. Although the exact composition of Malvidae and Fabidae is in flux, the chosen

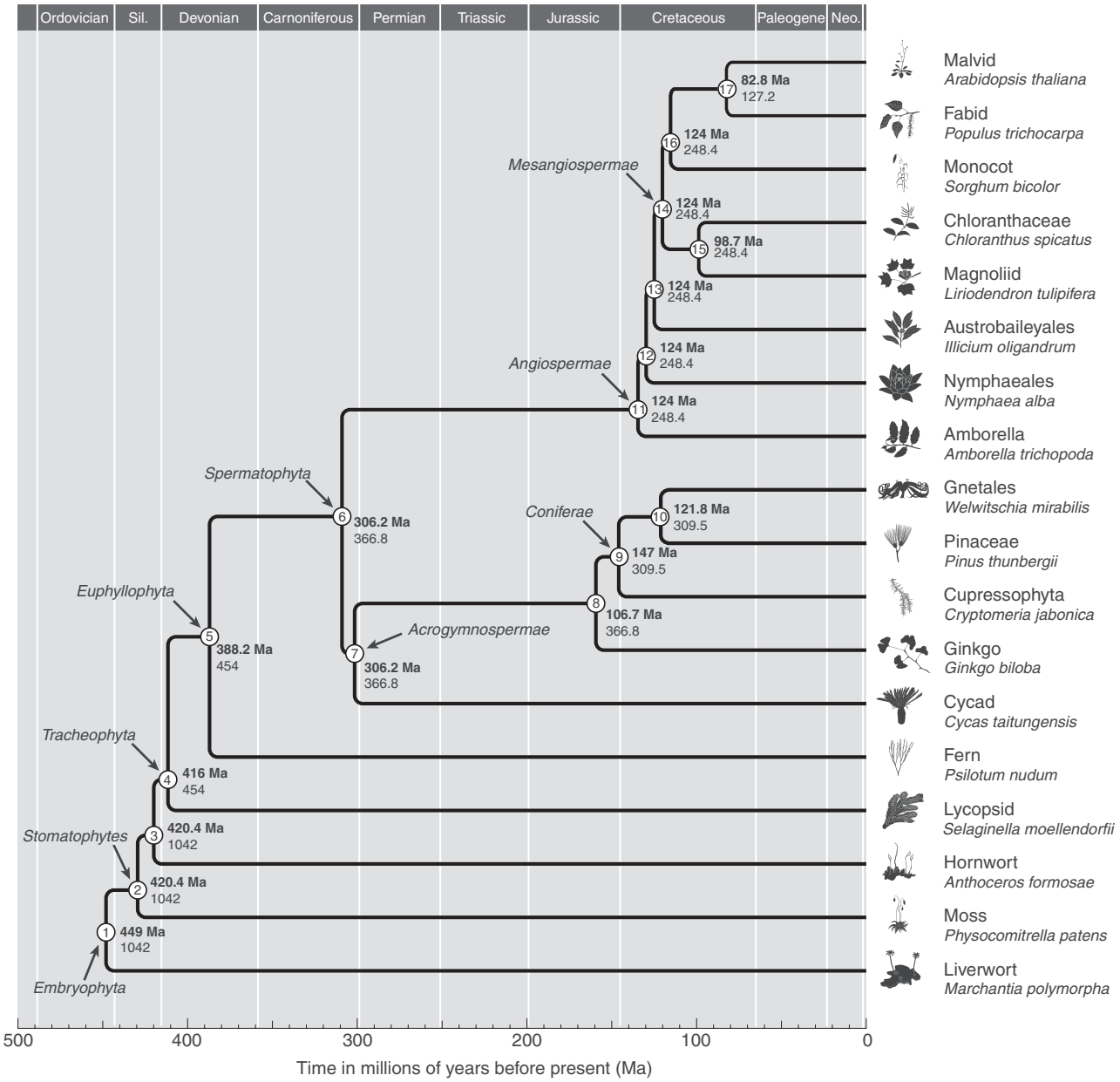

Fig. 2 A representative tree of relationships between model representatives of the major land plant lineages whose plastid or nuclear genomes have been fully sequenced. The topology is based upon a consensus of the most well-supported relationships as reviewed in recent literature (Qiu, 2008; Forest & Chase, 2009; Magallón, 2009; Magallón & Hilu, 2009; Renner, 2009). Calibrations are presented for all 17 nodes, consisting of a hard minimum constraint (bold) and a soft maximum constraint (not bold) for each. Justifications for these minima and maxima are provided in the text, and an overview in Table 1. The tree has been scaled to time on the basis of the minimum constraints.

minimum constraint is derived from fossils classified within Brassicales and Malphigiales, the assignment of which to Malvidae and Fabidae, respectively, is stable. Thus, the constraints are likely to be robust to future phylogenetic analyses.

The oldest potential evidence for core rosids is Normapolles pollen that first appears in the Cenomanian of Europe and North America (e.g. Pacltová, 1971; Doyle & Robbins, 1977). The precise systematic placement of this diverse pollen group (> 80 species; Pacltová, 1981) within eudicots is debated (e.g. Zavada & Dilcher, 1986; Batten, 1989; Sims *et al.*, 1999) and so, although there is good evidence for a relationship of some Normapolles species to crown Fagales (Friis *et al.*, 2006b), it is not unequivocal that all belong to core rosids. Thus, they do not provide sufficient evidence on which to establish a minimum constraint for the divergence of Fabidae and Malvidae.

Table 1 Summary of the calibrations derived in this paper

Node no.	Clade	Lineage 1	Lineage 2	Minimum providing fossil	Youngest date (Ma)	Minimum age constraint (Ma)	Evidence	Soft maximum providing fossil/ sediments	Oldest date (Ma)	Soft maximum age constraint (Ma)	Evidence
1	Embryophyta	Hepaticae total group	Stomatophyte total group	Trilete spores	449	449.0	Biostratigraphy	Torridon group sediment devoid of plant-like spores	994 ± 48	1042.0	Direct date
2	Stomatophytes	Musci total group	Anthocerotae + Tracheophyta total group	*Cooksonia*	422.9 ± 2.5	420.4	Biostratigraphy	,,	,,	,,	,,
3	Unnamed	Anthocerotae total group	Tracheophyta total group	,,	,,	,,	,,	,,	,,	,,	,,
4	Tracheophyta	Lycopsida total group	Euphyllophyta total group	*Zosterophyllum* sp.	418.7 ± 2.7	416.0	Biostratigraphy	Trilete spores	454	454.0	Biostratigraphy
5	Euphyllophyta	Monilophyta total group	Spermatophyta total group	*Ibyka* and *Rellimia*	388.2	388.2	Biostratigraphy	,,	,,	,,	,,
6	Spermatophyta	Angiospermae total group	Acrogymnospermae total group	*Cordaixylon iowensis*	307.2 ± 1.0	306.2	Biostratigraphy	Base of Vco zone which contains the first seeds	366.8	366.8	Biostratigraphy
7	Acrogymnospermae	Cycadophyta total group	*Ginkgo* + Coniferae total group	,,	,,	,,	,,		,,	,,	,,
8	Unnamed	*Ginkgo* total group	Coniferae total group	*Ginkgo ginkgoidia*	164.7 ± 4.0	160.7	Biostratigraphy	,,	,,	,,	,,
9	Unnamed	Gnetophyta + Pinaceae total group	Cupressophyta total group	*Araucaria mirabilis*	157 ± 10	147.0	Direct date	Sediments bearing *Cordaixylon iowensis*	309.5	309.5	Biostratigraphy
10	Unnamed	Gnetophyta total group	Pinaceae total group	*Liaoxia chenii*	122.1 ± 0.3	121.8	Direct date	,,	,,	,,	,,
11	Angiospermae	*Amborella* total group	Nymphaeales + Austrobaileyales + Mesangiospermae total group	Tricolpate pollen	125 ± 1.0	124.0	Magnetostratigraphy	Age of sediments below the oldest occurrence of angiosperm-like pollen which are devoid of such pollen	248.12 ± 0.28	248.4	Direct date
12	Unnamed	Nymphaeales total group	Austrobaileyales + Mesangiospermae total group	,,	,,	,,	,,		,,	,,	,,
13	Unnamed	Austrobaileyales total group	Mesangiospermae total group	,,	,,	,,	,,	,,	,,	,,	,,
14	Mesangiospermae	Chloranthaceae + Magnoliidae total group	Monocotyledoneae + Ceratophyllum + Eudicotyledoneae total group	,,	,,	,,	,,	,,	,,	,,	,,

Table 1 *Continued*

Node no.	Clade	Lineage 1	Lineage 2	Minimum providing fossil	Youngest date (Ma)	Minimum age constraint (Ma)	Evidence	Soft maximum providing fossil/ sediments	Oldest date (Ma)	Soft maximum age constraint (Ma)	Evidence
15	Unnamed	Magnoliidae total group	Chloranthaceae total group	*Endressinia brasiliana*	99.6 ± 0.9	98.7	Biostratigraphy	"	"	"	"
16	Unnamed	Monocotyledoneae total group	Ceratophyllum + Eudicotyledoneae total group	Tricolpate pollen	125 ± 1.0	124.0	Magnetostratigraphy	"	"	"	"
17	Unnamed	Malvidae total group	Fabidae total group	*Paleoclusia chevalieri* and *Dressiantha bicarpellata*	83.5 ± 0.7	82.8	Biostratigraphy	Oldest potential age of tricolpate pollen	127.2	127.2	Magnetostraigraphy

Minimum constraints are based on the youngest age interpretation on the geological formation containing the relevant fossil evidence. Maximum constraints are based on the oldest possible age of the sediments chosen. Justifications for choice of minima and maxima, along with their detailed age justifications, are provided in the text.

The Cenomanian 'Rose-Creek flowers' from the Dakota Formation of Nebraska (Basinger & Dilcher, 1984) potentially provide an alternative minimum constraint as they share similarities with the Rhamnaceae (Rosales) (Crepet *et al.*, 2004) within Fabidae. However, they lack diagnostic synapomorphies and have not been treated cladistically (Schonenberger & von Balthazar, 2006).

The oldest unequivocal representatives of total group Malvidae and Fabidae are *Dressiantha bicarpellata* (Capparales: Brassicales; Gandolfo *et al.*, 1998) and *Paleoclusia chevalieri* (Clusiaceae: Malphigiales; Crepet & Nixon, 1998). Both taxa have been included in phylogenetic analyses, placing them in the crown group of Malvidae and Fabidae, respectively, and both are derived from the same locality and stratigraphic formation, the South Amboy Fire Clay at Old Crossman Clay Pit (Sayreville, NJ, USA). This stratigraphic unit is assigned to the *Complexiopollis exigua-Santalacites minor* Zone (Christopher, 1979), which is the most basal of three zones within the *Sohlipollis* Taxon Range Zone (Christopher & Prowell, 2010). A minimum age can most reliably be established on the age of the overlying *Osculapollis vestibulus* Zone in Georgia and North Carolina which contains calcareous nanoplankton diagnostic of CC17 (Christopher & Prowell, 2010). The top of CC17 falls within the error for the top of the Santonian, at 83.5 Ma ± 0.7 Myr (Ogg *et al.*, 2004). Thus, we use 82.8 Ma as a minimum constraint for the divergence between the Fabidae and Malvidae total groups.

A soft maximum for the divergence of the Fabidae and Malvidae total groups can be established from the oldest records of tricolpate pollen, the oldest evidence of the Eudicotyledoneae + *Ceratophyllum* total group (see node 16). Because of the high preservation potential of pollen, their first appearance should precede the origin of core rosids. Therefore, 127.2 Ma, the maximum age interpretation of sediments containing tricolpate pollen (node 16), afford a soft maximum constraint for this divergence.

Unnamed clade: *Sorghum–Arabidopsis*, *Populus* (node 16: minimum = 124 Ma; soft maximum = 248.4 Ma) This divergence corresponds to the splitting of total group Monocotyledoneae from the total group of Eudicotyledoneae + *Ceratophyllum*. The oldest potential evidence for total group Monocotyledoneae is pollen records, the oldest possible records of which resemble *Liliacidites* from the Middle and Late Triassic (Cornet, 1989; Hochuli & Feist-Burkhardt, 2004), such as the *Crinopollis* group (Doyle & Hotton, 1991). However, the distinguishing characteristics of these and younger records are too subtle to justify minimum constraints (cf. Gandolfo *et al.*, 2000; Crepet *et al.*, 2004).

The oldest possible macrofossil remains of monocots are Araceae fossil sp. A and sp. B (Friis *et al.*, 2010) from the Portuguese Vila Verde 2 flora. The phylogenetic position of Araceae sp. A is equivocal (J. Doyle, pers. comm.), but

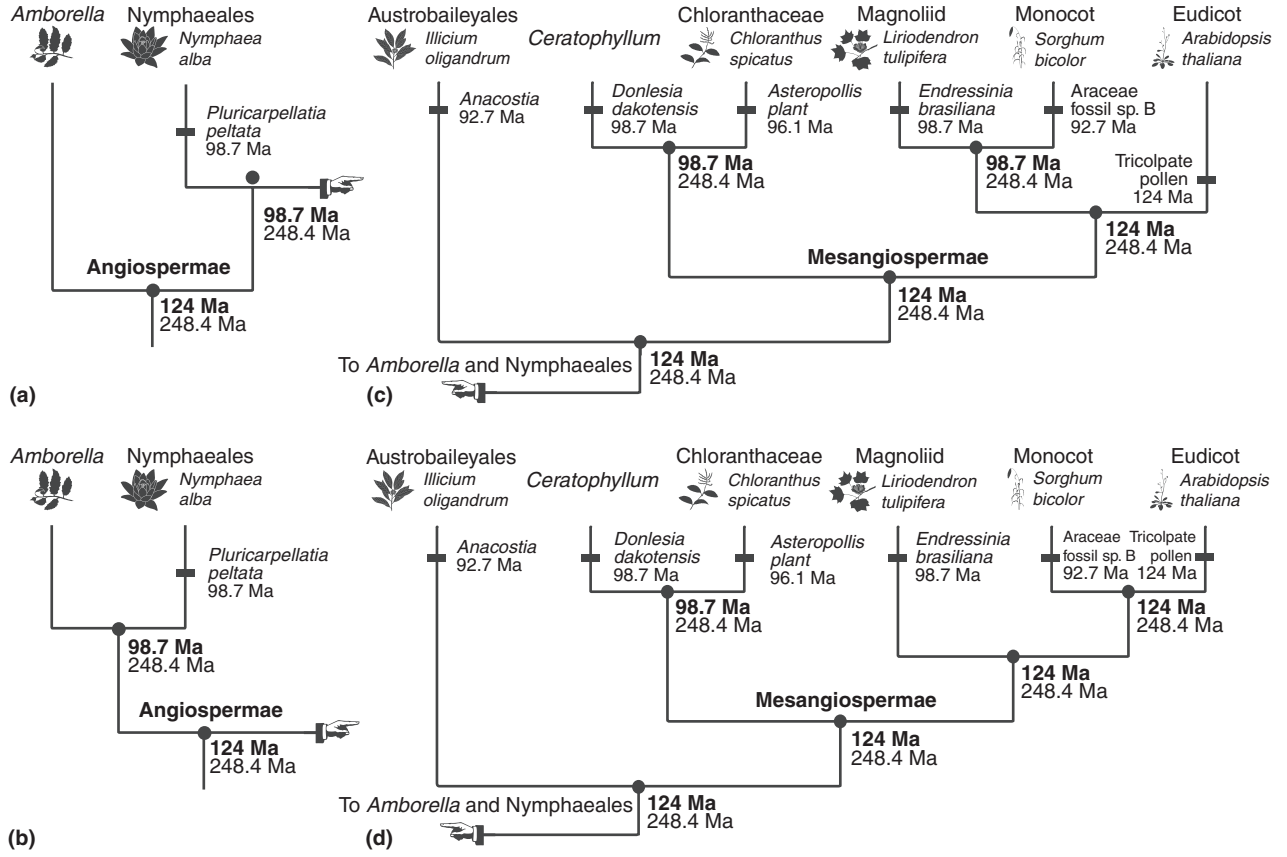

Fig. 3 Alternative topologies for angiosperms to those presented in Fig. 2, based upon (c) the morphological and three-gene analysis of Doyle & Endress (2010) and Endress & Doyle (2009), and (d) the four gene analysis of Qiu *et al.* (2010). Because of uncertainties regarding the basalmost branch in angiosperms, both alternatives are presented in (a) and (b). Calibrations are given for all nodes presented, consisting of a hard minimum constraint (bold) and a soft maximum constraint (not bold). Justification of these ages can be found in the text.

Araceae sp. B possesses three unequivocal synapomorphies of Araceae: a sessile flower with racemose inflorescence, absence of floral subtending bracts, and dimerous flowers (Endress & Doyle, 2009). Vila Verde 2 is considered part of the Figueira da Foz Formation and therefore has the same minimum age as the Vale de Água, Famalicão and Buarcos floras (see node 15). Thus, 92.7 Ma is the minimum age for total group Monocotyledoneae.

The oldest unequivocal evidence for total group *Ceratophyllum* is *Donlesia dakotensis* from the Dakota Formation (Dilcher & Wang, 2009), in sediments considered Late Albian (Brenner *et al.*, 2000; Gröcke *et al.*, 2006). From the synapomorphies of Ceratophyllum obtainable from data presented in Doyle & Endress (2010), the fossil possesses three synapomorphies: one carpel, dissected leaves, and dichotomous venation (H. Wang, pers. comm.). Therefore, the top of the Albian at 99.6 Ma ± 0.9 Myr (Ogg *et al.*, 2004) acts as a minimum constraint for total group *Ceratophyllum* in any topological scheme (Fig. 3).

Several fossils have been claimed to represent the oldest macrofossil evidence of crown Eudicotyledoneae. These include *Sinocarpus decussatus* (Leng & Friis, 2003, 2006), *Hyrcantha decussata* (Dilcher *et al.*, 2007) and *Leefructus mirus* (Sun *et al.*, 2011); all recovered from the Yixian Formation, of Liaoning Province, China. Unfortunately, none of these have been included in a cladistics analysis or demonstrated to possess any previously defined synapomorphies for the eudicot total group, crown group or any clade within the crown group, and thus none are currently suitable to provide an unequivocal minimum constraint for total group Eudicotyledoneae.

Therefore, the West Brothers Platanoid and *Sapindopsis* from Zone IIB of the Potomac group, both defined and cladistically treated in Doyle & Endress (2010), remain the oldest reliable evidence of crown and total group Eudicotyledoneae. An undoubtable minimum age for Zone IIB, and therefore these fossils, is 92.7 Ma (see node 15).

However, pollen evidence for the total group of *Ceratophyllum* + Eudicotyledoneae is older than the fossil evidence unequivocally assigned to the eudicot or *Ceratophyllum* total groups. Tricolpate pollen is widely accepted as a eudicot synapomorphy (Donoghue *et al.*, 1989; Doyle & Hotton, 1991; Chase *et al.*, 1993; Doyle & Endress, 2000; Judd & Olmstead, 2004; Doyle, 2005);

however, it is unclear whether this character has been lost in *Ceratophyllum* (which has inaperture pollen) or whether it was acquired after the divergence of *Ceratophyllum* and Eudicotyledoneae. This uncertainty indicates that tricolpate pollen in our topology can only calibrate a Monocotyledoneae–*Ceratophyllum* + Eudicotyledoneae divergence and not the total group *Ceratophyllum*–total group Eudicotyledoneae divergence or the Eudicotyledoneae crown group. It also cannot provide a maximum estimate for crown or total group Eudicotyledoneae, although it is commonly used as such (Bell *et al.*, 2005; Magallón & Castillo, 2009).

Although pollen with three apertures are present in *Illicium*, Schisandraceae and the *Eucommiidites* pollen genus (Erdtman, 1948), they can be distinguished readily from the eudicot condition. Tricolpates of *Illicium* and Schisandraceae are formed via Garside's rule rather than Fischer's rule (Huynh, 1976; Furness & Rudall, 2004), and are distinguished by their apertures which either are confluent or fuse near the poles (Doyle *et al.*, 1990). *Eucommiidites* pollen differ by showing bilateral symmetry (Couper, 1958), and have since been associated with pollen organs with inferred gymnospermous affinities (e.g. Pedersen *et al.*, 1989; Mendes *et al.*, 2010). The best-dated early record of Fischer's rule tricolpate pollen is from the Cowleaze Chine Member of the Vectis Formation of the Isle of Wight (Hughes & McDougall, 1990), which occurs within the M1n polarity chron at the top of the Barremian which is dated at 125 Ma ± 1.0 Myr (Ogg *et al.*, 2004). The maximum age of this stratigraphic unit is required for the soft maximum calibration constraint on node 17, the divergence of *Crucifer* from *Poplar*. This is established on the base of the M1n polarity chron, 127.2 Ma (Ogg *et al.*, 2004).

The tricolpate pollen records representing the *Ceratophyllum* + Eudicotyledoneae total group are substantially older than the earliest unequivocal records of Monocotyledoneae, and so provide a minimum constraint of 124 Ma for the divergence of Monocotyledoneae from the total group of Eudicotyledoneae + *Ceratophyllum*. A soft maximum of 248.4 Ma, based upon the oldest occurrence of angiosperm-like pollen in the Middle Triassic, is applied (see node 11).

Unnamed clade: *Chloranthus–Liriodendron* (node 15: minimum = 98.7 Ma; soft maximum = 248.4 Ma) This is the fundamental divergence between Magnoliidae and Chloranthaceae. The oldest possible record of total group Magnoliidae is pollen genus *Walkeripollis* from the Late Barremian or Early Aptian of Gabon (Doyle *et al.*, 1990), representative of Winteraceae (Doyle *et al.*, 1990; Doyle & Endress, 2010). However, this provides insufficient evidence on which to establish a hard minimum constraint. *Appomattoxia* fruits with adhering pollen of *Tucanopollis* and *Transitoripollis*, from the Puddledock flora of the Potomac group (Friis *et al.*, 1995), share a number of features with Piperales. Age determination in the Potomac group is based upon pollen zonation, the first established by Brenner (1963) and modified by subsequent workers (Doyle, 1969, 1992; Doyle & Robbins, 1977; Hughes, 1994; Hochuli *et al.*, 2006), resulting in the recognition of four zones: I, IIA, IIB and IIC. The Puddledock flora is assigned to Zone IIB (Christopher in Dischinger, 1987), which has been considered to be anywhere from Early to Late Albian in age (Doyle & Robbins, 1977; Doyle, 1992), with the potential for it to enter the Cenomanian, the age of Zone IIC above (Hochuli *et al.*, 2006). Because of the tentative nature of these pollen schemes (P. Hochuli, pers. comm.), we use the *Metoicoceras bergquisti* ammonite present in the Raritan Formation above (Cobban & Kennedy, 1990) to derive a minimum age for Zone IIB (and IIC). *Metoicoceras bergquisti* belongs to the *Metoicoceras mosbyense* zone (Cobban, 1983) which is overlain by the *Sciponoceras gracile* zone, the base of which is dated at 94.01 Ma (Ogg *et al.*, 2004). This falls within the error for the top of the Cenomanian, and so we use this date at 93.5 Ma ± 0.8 Myr, and thus 92.7 Ma as the minimum age of Zone IIB (and IIC). However, the relationship of *Appomattoxia* remains equivocal (Friis *et al.*, 2010) and at 92.7 Ma this material cannot be constrained as older than *Endressinia brasiliana* (Mohr & Bernardes-de-Oliveira, 2004), recovered from the Crato Formation of Brazil, which is unequivocally placed within Magnoliales (Doyle & Endress, 2010). The Crato Formation, part of the Santana Group (sensu Neumann & Cabrera, 1999), is considered to be Late Aptian–Early Albian on the basis of pollen (Batten, 2007) ostracod (Martill, 2007) and dinoflagellate (Heimhofer & Hochuli, 2010) biostratigraphy. Therefore, a minimum age for the Crato Formation can be derived from the top of the Albian (99.6 Ma ± 0.9 Myr; Ogg *et al.*, 2004), and is thus 98.7 Ma, and a maximum age from the base of the Aptian (125.0 Ma ± 1.0 Myr; Ogg *et al.*, 2004), and is thus 126 Ma.

The oldest possible records of total group Chloranthaceae are Barremian occurrences of the pollen genera '*Clavatipollenites*' (e.g. Couper, 1958; Kemp, 1968) and *Asteropollis* (e.g. Hughes *et al.*, 1979), followed by the Aptian appearance of *Pennipollis* (Hochuli & Kelts, 1980; Heimhofer *et al.*, 2007). The concept of '*Clavatipollenites*' is clouded by lack of clarity concerning the nature of the type specimens of the type species, *Clavatipollenites hughesii* Couper (1958; Hughes & McDougall, 1987), to the extent that all records are currently compromised as potential evidence for constraints on molecular clock analyses. *Asteropollis* and *Pennipollis* are known from pollen and mesofossil remains (Friis *et al.*, 1994, 1997, 1999) assigned to crown Chloranthaceae (Doyle *et al.*, 2003; Eklund *et al.*, 2004) and total group Chloranthaceae (Doyle *et al.*, 2008), respectively. There are older records of *Asteropollis* than *Pennipollis* and, together with its crown Chloranthaceae affinity, *Asteropollis* affords a more secure minimum constraint

on the origin of Chloranthaceae. *Asteropollis* and its associated mesofossil remains is known from the Catefica, Torres Vedras, Vale de Água, Famalicão and Buarcos floras of Portugal which have been considered of Barremian to Aptian age (Friis *et al.*, 1999). However, the stratigraphy is complex, and so reliable minimum ages for these floras can only be established from the first well-dated units above. Vale de Água, Famalicão and Buarcos are assigned to the Figueira da Foz Formation (Rocha *et al.*, 1981; Manuppella *et al.*, 2000; Dinis, 2001). A minimum age is provided by the marine sediments of the Costa d'Arnes Formation that overlie the Figueira de Foz formation. The units of this overlying marine succession were named from the lowest unit 'B' through to 'O' (Choffat, 1897, 1900). The oldest ammonites occur in unit C, in which *Calycoceras naviculare* is present (Callapez, 2003), indicative of the *C. naviculare* biozone, which falls within the error for the top of the Cenomanian (93.5 Ma ± 0.8 Myr; Ogg *et al.*, 2004), and thus 92.7 Ma defines a minimum age.

Catefica and Torres Vedras have been considered Late Barremian to Early Aptian in age (Friis *et al.*, 2006a, 2010). However, recent evidence suggests that they are considerably younger, within the 'Upper Almargem' Formation overlying a late Aptian to early Albian unconformity (Dinis *et al.*, 2008). Despite the uncertainty, an unequivocal minimum age is provided by the appearance of ostracod *Fossocytheridea merlensis* in the overlying Caneças Formation, an appearance attributable to the base of the Middle Cenomanian (Berthou, 1973, 1984), dated at 96.1 Ma (Ogg *et al.*, 2004).

Therefore, Catefica and Torres Vedras floras containing the *Asteropollis* plant provide a minimum constraint for total group Choranthaceae of 96.1 Ma. This is slightly younger than the minimum age for *E. brasiliana*, and thus 98.7 Ma is the minimum constraint for the splitting of the Magnoliidae and Chloranthaceae total groups. Sediments devoid of angiosperm-like pollen below their first occurrence in the Middle Triassic provide a soft maximum at 248.4 Ma (see node 11).

Mesangiospermae: *Chloranthus*, *Liriodendron–Sorghum*, *Arabidopsis*, *Populus* (node 14: minimum = 124 Ma; soft maximum = 248.4 Ma) The origin of Mesangiospermae or 'core angiosperms' represents the divergence of the Chloranthaceae + Magnoliidae total group from the Monocotyledoneae + *Ceratophyllum* + Eudicotyledoneae total group. The changing nature of angiosperm phylogeny has limited any discussion of fossils potentially assigned to the stem of the two lineages, as has the absence of synapomorphies for either crown group. Thus, suitable candidates for calibration of this node can only be sought from fossils already discussed in relation to the total groups of either Chloranthaceae, Magnoliidae, Monocotyledoneae and Ceratophyllum+Eudicotyledoneae (nodes 15 and 16). The minimum age of the Monocotyledoneae + *Ceratophyllum* + Eudicotyledoneae total group is provided by tricolpate pollen at 124 Ma (see node 16) and a minimum for the Chloranthaceae + Magnoliidae total group is *E. brasiliana* at 98.7 Ma (see node 15). Thus, 124 Ma provides a minimum constraint for Mesangiospermae. A soft maximum of 248.4 Ma, based upon the oldest occurrence of angiosperm-like pollen in the middle Triassic, is applied (see node 11).

Unnamed clade: *Illicium–Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 13: minimum = 124 Ma; soft maximum = 248.4 Ma) This marks the divergence of the Austrobaileyales and Mesangiospermae total groups. The earliest possible records of Austrobaileyales are seeds from the Aptian Drewry's Bluff clay balls in the Potomac group (Friis *et al.*, 1999, 2006a) and the Portuguese floras Famalicão and Torres Vedras (minimum ages of 92.7 and 96.1 Ma, respectively; see node 15). However, a nymphaealean affinity cannot be excluded. Younger seeds described as *Illiciospermum pusillum* from the Cenomanian–Turonian are distinct from nymphaealean seeds as a result of a raised section of the seed wall resembling the strophiole in extant *Illicium* (Frumin & Friis, 1999), and are thus more reliable. However, the crown Austrobaileyales affinity of macrofossil *Anacostia*, as evidenced by the presence of palisade exotesta, sclerotic mesotesta and an ascendent ovule position (Doyle *et al.*, 2008), negates reliance on *I. pusillum*. *Anacostia* is found in the Portuguese Buarcos, Famalicão, and Vale de Água floras, and North American deposits assigned to palynozone IIB of the Potomac group (Friis *et al.*, 1997). All localities have the same minimal age of 92.7 Ma (see node 15), and thus 92.7 Ma is a minimum for total group Austrobaileyales. However, all records of Austrobaileyales are younger than the oldest records of tricolpate pollen at 124 Ma (see node 16), indicative of total group Mesangiospermae. Thus, 124 Ma provides a minimum constraint for splitting of total group Austrobaileyales from total group Mesangiospermae. A soft maximum of 248.4 Ma, based upon the oldest occurrence of angiosperm-like pollen in the Middle Triassic, is applied (see node 11).

Unnamed clade: *Nymphaea – Illicium*, *Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 12: minimum = 124 Ma; soft maximum = 248.4 Ma) This divergence represents the splitting of the Nymphaeales total group from the total group formed by Austrobaileyales + Mesangiospermae. The oldest putative record of Nymphaeales is a leaf impression from the Early Jurassic of Utah that shows some similarity to extant *Nuphar polysepala* (Kirkland *et al.*, 2002). *Archaefructus*, from the Barremian-Aptian Yixian Formation (see node 10), has been assigned to total group Nymphaeales (Friis *et al.*, 2003; Doyle, 2008), but other phylogenetic analyses suggest that it is a stem Angiosperm (Sun *et al.*, 2002). Seeds with features of Nymphaeales and Illiaceae have been described from Drewry's Bluff clay balls, Virginia, and Portuguese fossil

floras at Famalicão and Torres Vedras (Friis *et al.*, 1999, 2006a). However, all of these records are too equivocal to use as the basis for a calibration constraint.

Pluricarpellatia peltata from the Crato Formation of Brazil (Mohr *et al.*, 2008), *Scutifolium jordanicum* from the Jarash Formation of Jordan (Taylor *et al.*, 2008) and *Monetianthus mirus* from Vale de Água, Portugal (Friis *et al.*, 2009) are all cladistically assigned to crown Nymphaeales (Pluricarpellatia in Taylor *et al.*, 2008). The Jarash Formation is minimally constrained to 95 Ma (96.1 Ma ± 1.1 Myr in Amireh *et al.*, 1998), whereas the Crato Formation and Vale de Água flora are minimally 98.7 and 92.7 Ma, respectively (see node 15 for age justification). Therefore, *Pluricarpellatia*, at 98.7 Ma, provides the oldest minimum constraint for the establishment of total group Nymphaeales, although this could change as the stratigraphies of the Jordanian and Portuguese sections improve. However, this is considerably younger than tricolpate pollen representing the Austrobaileyales + Mesangiospermae total group, and therefore 124 Ma (see node 16) provides a minimum constraint for Mesangiospermae. Sediments devoid of angiosperm-like pollen below their first report in the Middle Triassic can provide a soft maximum for this divergence, dated at 248.4 Ma (see node 11).

Angiospermae: *Amborella–Nymphaea*, *Illicium*, *Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 11: minimum = 124 Ma; soft maximum = 248.4 Ma) This divergence represents the origin of crown angiosperms (Angiospermae) and the splitting of the *Amborella* total group from the total group incorporating Nymphaeales, Austrobaileyales and Mesangiospermae. There is no unequivocal fossil record for total group *Amborella*, only a small staminate flower from the Catefica flora, Portugal (Friis *et al.*, 2000) with a stamen resembling *Amborella*, but resembling *Kadsura* (Austrobaileyales) in other respects. Therefore, tricolpate pollen at 124 Ma (see node 16) is the oldest unequivocal evidence of crown angiosperms because pollen grains from the Valanginian (Brenner & Bickoff, 1992; Brenner, 1996) and Hauterivian to Barremian (e.g. Hughes & McDougall, 1987; Hughes *et al.*, 1991) may not be restricted to the angiosperm crown.

A soft maximum on the origin of crown Angiospermae is informed by tenuous records of stem Angiospermae. The oldest possible angiosperm macroremains are *Furcula granulifera* (Harris, 1932) and *Sanmiguelia lewisii* (Cornet, 1986) from the Late Triassic of Greenland and North America, respectively. Both are problematic: the leaves of *F. granulifera* have bifurcated lamina and a forked midrib, both considered gymnosperm characters (Harris, 1932); *S. lewisii*, associated with pollen organ *Synangispadix tidwelii*, has been reinterpreted as a gymnosperm (Doyle & Donoghue, 1993). Almost all other pre-Cretaceous angiosperm records are of pollen that possess combinations of crown angiosperm synapomorphies: tectate exine, columellate exine, reticulate (perforate) exine and a lack of a distinct laminated endexine (e.g. Doyle, 2005; Zavada, 2007). Such pollen is first seen in the Permian and is present throughout the Triassic and Jurassic (e.g. Cornet, 1989; Hochuli *et al.*, 1989; Cornet & Habib, 1992; Hochuli & Feist-Burkhardt, 2004; Vasanthy *et al.*, 2004; Zavada, 2004; Zavialova, 2005). To inform a soft maximum for crown-Angiospermae we choose sediments devoid of angiosperm-like pollen below the oldest reports of pollen which possess synapomorphy combinations most similar to Cretaceous grains. These are pollen types C–F in Hochuli & Feist-Burkhardt (2004) from the Middle Triassic Snadd Formation, Norwegian Arctic. Type C, '*Retisulcites*', is also reported from slightly older sediments in the underlying Steinkobbe Formation, in assemblage K of Hochuli *et al.* (1989), considered Middle Anisian in age (Vigran *et al.*, 1998). A minimum age for the pollen can be derived from the top of the Anisian (238.8 Ma + 0.5/−0.2 Myr; Brack *et al.*, 2005), and is thus 238.6 Ma. A maximum age can be provided by the base of the Anisian, and, although a Global Boundary Stratotype Section and Point (GSSP) has yet to be defined, a direct date for the *Neopopanoceras haugi* ammonite biozone, the highest in the Olenkian stage, is 248.12 Ma ± 0.28 Myr (Ovtcharova *et al.*, 2006; Galfetti *et al.*, 2007). This provides a soft maximum of 248.4 Ma for crown Angiospermae.

Unnamed clade: *Welwitschia–Pinus* (node 10: minimum = 121.8 Ma; soft maximum = 309.5 Ma) This divergence represents the splitting of the 'gnepine' clade into Gnetales and Pinceae. The oldest possible records of total group Gnetales are ephedroid (ribbed) pollen grains that first appear in the Permian (Wilson, 1962; Osborn *et al.*, 1993; Wang, 2004) and *Palaeognetaleana auspicia* represented by cones associated with such pollen (Wang, 2004), the Late Triassic *Dechellyia gormani* (Ash, 1972) and Jurassic *Ephedrites sinensis* and *Ephedrites exhibens* (Wu *et al.*, 1986). All lack sufficient evidence for unequivocal placement within Gnetales. The next oldest records of Gnetales are from the Yixian Formation, Liaoning Province, China, including seeds and impressions of fragmented reproductive shoots of *Ephedra archaeorhytidosperma* (Yang *et al.*, 2005), and various compression-impression fossils, described as *Chaoyangia liangii* (Duan, 1998), *Gurvanella exquisita* (Sun *et al.*, 2001), *Liaoxia chenii* (Rydin *et al.*, 2006), *Siphonospermum simplex* (Rydin & Friis, 2010), *Ephedra hongtaoi* (Wang & Zheng, 2010) and *Gnetum*-like male spike strobiles (Guo *et al.*, 2009). Many of these are unequivocally assigned to total group Gnetales. *Liaoxia*, for example, possesses three unequivocal synapomorphies of Gnetales (Doyle & Donoghue, 1986; Doyle, 2006): multiple auxillary buds, opposite-decussate phyllotaxis, leaves linear with two veins, as well as compound male and female strobili and a terminal ovule on its stem (Rydin *et al.*, 2006). The Yixian Formation was originally considered

Jurassic but has since been dated to span 121.8–130.3 Ma (Chang *et al.*, 2009).

The oldest possible record of total group Pinaceae is *Compsostrobus neotericus*, represented by cones from the Late Triassic of North Carolina (Delevoryas & Hope, 1973, 1987), compared to Pinaceae by Miller (1988, 1999), although unequivocal characteristics of Pinaceae have yet to be identified in the fossils. *Pseudolarix erensis* from the Tsagaan-Tsav (= Tsagantsab = Tsagaantsav) Formation in East Gobi, Mongolia (Krassilov, 1982), the next oldest record, has been identified as a close relative of extant *Pseudolarix* based on the presence of brachioblasts with close annual rings, deltoid triangular cone scales, and semi-trullate and pointed seed wings (Gernandt *et al.*, 2008). Although direct dates have been obtained for the Tsagaan-Tsav (= Tsagantsab = Tsagaantsav) Formation (reviewed in Keller & Hendrix, 1997; Shuvalov, 2000), the exact stratigraphic position of *P. erensis* relative to dated sediments is difficult to determine. The youngest interpretation of the Tsagaan-Tsav would be from a radiometric date of 119 Ma ± 6 Myr (see Shuvalov, 2000) and thus 113 Ma acts as an unequivocal minimum constraint for total group Pinaceae.

Thus, the oldest reliable records of total group Pinaceae are younger than evidence of total group Gnetales found in the Yixian Formation at 121.8 Ma, and so this provides the minimum constraint for the divergence between the two. A soft maximum is based on phylogenetic bracketing and stratigraphic bounding. There are no records, however tenuous, of total group Pinaceae or total group Gnetales that are comparable in age to the oldest unequivocal record of Acrogymnospermae (crown Gymnosperms), *Cordaixylon iowensis* (see node 7). This affords a maximum constraint of 309.5 Ma.

Coniferae: *Cryptomeria–Welwitschia*, *Pinus* (node 9: minimum = 147 Ma; soft maximum = 309.5 Ma) This is the fundamental divergence of Coniferae into Cupressophyta and Gnetales + Pinaceae. Records considered too equivocal to provide a minimum constraint on the divergence of the gnepine crown group (node 10) are also too equivocal to constrain the timing of the origin of the gnepine total group. Thus, the oldest secure records of the crown group are also the oldest secure records for the gnepine total group. These occur within the Yixian Formation of Liaoning, China, the minimum age of which is 121.8 Ma (see node 10).

The oldest possible record of total group Cupressophyta is obscured by poor understanding of phenotypic character evolution, and thus it is not possible to resolve the phylogenetic position of fossil species that lie outside the crowns of the six extant families that comprise crown Cupressophyta (Araucariaceae, Cephalotaxaceae, Cupressaceae, Podocarpaceae, Scadopityaceae and Taxaceae). Triassic *Rissikia media* (Townrow, 1967) might represent the oldest possible record of total group Podocarpaceae and, in consequence, crown Cupressophyta and total group Cupressophyta. However, it lacks the Podocarpaceae diagnostic feature of one ovule per cone scale, instead possessing two (J. Doyle, pers. comm.). Other Triassic and Jurassic records (Florin, 1951; Yao *et al.*, 1997; Axsmith *et al.*, 1998) are similarly problematic. The timing of divergence of Cupressophyta will be far better constrained in light of a coherent framework of character evolution within the clade but, in the interim, the oldest secure record of total group Cupressophyta must be found among much younger records that fall within the crown of the one of the six families of Cupressophyta. The oldest of these is *Araucaria mirabilis*, represented by cones, from the Cerro Cuadrado petrified forest in La Matilde Formation of Patagonia, Argentina (Wieland, 1935; Calder, 1953; Stockey, 1975, 1978). These fossils possess a 'vascular plexus' at the ovule base, ovuliferous scale vascularization, two vascular strands to the conescale complex and an embryo with two cotyledons, all characters established to distinguish *Araucaria* section Bunya of the Araucariaceae (Wilde & Eames, 1948; Stockey, 1975), to which only extant *Araucaria bidwillii* belongs. The age of La Matilde Formation is poorly constrained as the stratigraphy is complex, although the volcanic deposits do allow radiometric dating. La Matilde Formation is overlain by volcanics dated to 157 Ma ± 10 Myr (Spalleti *et al.*, 1982), and thus the minimum constraint on the divergence of crown Cupressophyta, total group Cupressophyta and crown Coniferae is 147 Ma.

A soft maximum constraint on the divergence of crown Coniferae is provided by the oldest unequivocal record of crown gymnosperms (Acrogymnospermae) that does not co-occur with the oldest possible records of crown Coniferae. This is *C. iowensis* (see node 7), providing a soft maximum of 309.5 Ma.

Unnamed clade: *Ginkgo–Cryptomeria*, *Welwitschia*, *Pinus* (node 8: minimum = 160.7 Ma; soft maximum = 366.8 Ma) This represents the divergence of *Ginkgo* from Coniferae. The oldest possible records of total group Coniferae are leafy twigs superficially resembling lycopsids (Scott & Chaloner, 1983) and *Cordaites*-like stems (Galtier *et al.*, 1992) from Westphalian B and C (Scott & Chaloner, 1983; Galtier *et al.*, 1992) and, hence, they are not suitable for establishing constraints. The next oldest possible records are the Wachian conifers, considered coniferophytes, of which there are a number of well-described forms from the Pennsylvanian, such as *Emporia* (Mapes & Rothwell, 1984), *Thucydia* (Hernandez-Castillo *et al.*, 2001) and *Hanskerpia* (Rothwell *et al.*, 2005). Of these, *Emporia lockardii* (= *Lebachia lockardii*; Mapes & Rothwell, 2003), reported from the Late Pennsylvanian flora of Hamilton, Kansas (Mapes & Rothwell, 1984, 1988, 1991), has been resolved as total group Coniferae (Doyle, 1996, 2006, 2008; Hilton & Bateman, 2006). However, in other analyses *Emporia* is resolved as an outgroup of *Ginkgo* + Coniferae (Rothwell & Serbet, 1994; Rothwell *et al.*, 2009). Thus, there is no recourse other than *A. mirabilis*, the oldest

secure record of crown Coniferae (see node 7), as the oldest record of total group Coniferae.

The oldest possible records of total group *Ginkgo* is *Trichopitys*, represented by leaves and ovulate organs (Florin, 1949), and *Karkenia* sp., represented by ovulate organs associated with leaves of *Kerpia* (Naugolnykh, 1995, 2007). Unfortunately, their phylogenetic affinity is too equivocal to be of utility (Zimmermann, 1959; Meyen, 1984, 1987; Archangelsky & Cuneo, 1990; Zhou, 1997; Doweld, 2001). The next oldest possible records, *Toretzia* (upper Triassic: Stanislavsky, 1973), *Umaltolepis* (Lower Jurassic: Schweitzer & Kirchner, 1995) and *Yimaia* (Lower Jurassic: Schenk, 1867; or Middle Jurassic: Zhou & Zhang, 1988; Kirchner, 1992; Zhou & Zhang, 1992) have been proposed as close relatives of crown *Ginkgo* (Zhou, 1991, 1997). However, *Schmeissneria*, identified as an even closer relative of crown *Ginkgo*, potentially has no close relationship to total group *Ginkgo* (Wang *et al.*, 2007), and thus the phylogenetic position of *Toretzia*, *Umaltolepis* and *Yimaia*, which share one character less with *Ginkgo* than *Schmeissneria*, must be even more uncertain. Thus, to obtain a secure minimum constraint on the origin of total group *Ginkgo* there is no recourse but to use the oldest record of *Ginkgo*, *Ginkgo ginkgoidia* from the Fuglunda member, Mariedal Formation, SE Scania (Tralau, 1966), which possesses five of the seven characters diagnostic of crown *Ginkgo* (Yang *et al.*, 2008). A Bajocian age is often cited on palynological data (Tralau, 1966, 1968); however, the stratigraphic levels from which remains of *G. ginkgoidia* have been recovered are younger than those from which the palynological data were recovered. Foraminifera from the Fuglunder Member (Norling, 1972; Norling *et al.*, 1993; Guy-Ohlson & Norling, 1994) allow for an age interpretation spanning the Aalenian (175.6 Ma ± 2.0 Myr; Ogg, 2004) to the Bathonian (164.7 Ma ± 4.0 Myr; Ogg, 2004), providing a minimum constraint on the origin of total group *Ginkgo* at 160.7 Ma.

Of the two fundamental lineages, *Ginkgo* has the oldest secure record, placing a minimum constraint on their divergence at 160.7 Ma. The soft maximum must encompass possible records of conifers from the Westphalian B and C, and thus we advocate the same soft maximum as for crown gymnosperms and Spermatophyta, 366.8 Ma (see Spermatophyta, node 6).

Acrogymnospermae: *Cycas–Ginkgo*, *Cryptomeria*, *Welwitschia*, *Pinus* (node 7: minimum = 306.2 Ma; soft maximum = 366.8 Ma) This is the fundamental divergence of Acrogymnospermae (crown gymnosperms) into Cycadophyta and *Ginkgo* + Coniferae. The oldest possible records of Acrogymnospermae are cordaitean coniferophytes. Although cordaitean taxa have been considered outgroups of Acrogymnospermae (Rothwell & Serbet, 1994), there is a consensus of agreement between analyses that they are members of Acrogymnospermae (Doyle & Donoghue, 1992; Nixon *et al.*, 1994; Doyle, 1996, 2006, 2008; Hilton & Bateman, 2006). The oldest record occurs in the Namurian C Sprockhövel Formation (Phillips, 1980), but we rely instead on cordaitean taxa that are known from whole-plant reconstructions which have informed cladistic analyses, the oldest of which is *C. iowensis*, which occurs in the Laddsdale Coals (Cherokee Group, Desmoinesian Series) near What Cheer, Iowa (Trivett, 1992). The age interpretation of the Desmoinesian has varied, the most recent scheme equating the base of the Desmoinesian to the base of Westphalian D (Davydov *et al.*, 2004). The Laddsdale Coals are restricted to the lower to lower-middle part of the Desmoinesian Series on the basis of palynology (Ravn *et al.*, 1984; Peppers, 1996), and so they fall fully within the Westphalian D, the top of which corresponds to the top of the Moscovian, dated at 307.2 Ma ± 1.0 Myr (Davydov *et al.*, 2004; Heckel, 2008).

The oldest possible records of total group Cycadophyta are microstrobili (e.g. Zhu *et al.*, 1994) and megasporophylls (e.g. Zhu & Du, 1981; Gao & Thomas, 1989), of which *Crossozamia* is the least equivocal in terms of phylogenetic affinity (Gao & Thomas, 1989), resolved as total group Cyacadophyta (Brenner *et al.*, 2003; Hermsen *et al.*, 2006). However, this Lower Permian record is still significantly younger than *C. iowensis*, which provides a minimum constraint of 306.2 Ma on the fundamental divergence within Acrogymnospermae. A soft maximum is based upon the first appearance of seeds in the form of preovules which are attributable to the spermatophyte stem, the oldest interpretation of which is 366.8 Ma (see Spermatophyta, node 6).

Spermatophyta: *Cycas*, *Ginkgo*, *Cryptomeria*, *Welwitschia*, *Pinus–Amborella*, *Nymphaea*, *Illicium*, *Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 6: minimum = 306.2 Ma; soft maximum = 366.8 Ma) This is the fundamental divergence of Spermatophyta into Acrogymnospermae and Angiospermae. There is difficulty assigning taxa to either stem as only two morphological studies resolve this divergence (Doyle, 2006, 2008). In these studies no taxa were assigned to the Acrogymnosperm stem, and the composition of the Angiospermae stem varied significantly. The composition of the angiosperm stem fluctuates from one analysis to another, although Bennettitales and *Pentoxylon* are a consistent feature (Doyle, 2006, 2008; Hilton & Bateman, 2006; Rothwell *et al.*, 2009) and, of these, Upper Triassic records of Bennettitales (Harris, 1932; Crane, 1985) are the oldest. However, there are records of pollen at 238.6 Ma that exhibit combinations of synapomorphies seen in Cretaceous angiosperm pollen (see node 11). This is considerably younger than the oldest phylogenetically secure record of total group Acrogymnospermae, *C. iowensis*, which provides a minimum constraint on the divergence of

crown Spermatophyta into Acrogymnospermae and Angiospermae at 306.2 Ma (see Acrogymnospermae, node 7).

A soft maximum can be established with the first records of seeds in the form of preovules that satisfy the criteria of the seed habit, namely: (1) the possession of a single functional megaspore that is (2) enveloped in a nucellus (usually considered equivalent to the megasporangium), which is (3) surrounded (to some extent) by an integument or pre-integument and has (4) mechanisms enabling the capture of pollen before seed dispersal (Rothwell & Scheckler, 1988; Haig & Westoby, 1989). This habit arose on the stomatophyte stem in the form of various types of preovules which first enter the record in the Upper Fammenian (Late Devonian) VCo Spore Biozone (Prestianni, 2005), a well-documented example of which is *Elkinsia polymorpha* (Rothwell *et al.*, 1989); *E. polymorpha* has been recovered from the Hampshire Formation, West Virginia, from which the palynomorphs *Grandispora cornuta*, *Retispora macroreticulata*, *Retusotriletes phillipsii* and *Rugospora radiata* have been reported (Streel & Scheckler, 1990), which substantiate assignment to the VCo Biozone (Streel *et al.*, 1987). The VCo biozone spans 363.6–366.8 Ma (House & Gradstein, 2004) and thus provides a soft maximum constraint on the divergence of the Acrogymnospermae and Angiospermae total groups at 366.8 Ma.

Euphyllophyta: *Psilotum–Cycas*, *Ginkgo*, *Cryptomeria*, *Welwitschia*, *Pinus*, *Amborella*, *Nymphaea*, *Illicium*, *Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 5: minimum = 388.2 Ma; soft maximum = 454 Ma) This is the fundamental divergence of Euphyllophyta into Monilophyta and Spermatophyta. The oldest possible total group monilophyte is *Foozia*, which is reported from the Emsian Grès de Whépion Formation (Belgium), and is proposed to show anatomical similarities to cladoxyls (Gerrienne, 1992). However, the only synapomorphy of Monilophyta, mesarch protoxylem confined to lobes of xylem strand (Kenrick & Crane, 1997), has not been demonstrated in *Foozia* and, although *Foozia* possesses many of the features of Euphyllophytina (a node on the Euphyllophyta stem), it is unclear whether *Foozia* is a crown or stem euphyllophyte as it does not possess potential synapomorphies including megaphylls, monopodial root branching, and endogenous lateral roots (Schneider *et al.*, 2002). The next oldest possible monilophyte is *Ibyka* (Skog & Banks, 1973), recovered from the Manorkill Shale Member, a lateral equivalent of the Windom Member within the Moscow Formation of New York (Fisher *et al.*, 1962; Rickard, 1964). *Ibyka* exhibits mesearch protoxylem, a synapomorphy of crown Monilophyta (Kenrick & Crane, 1997). The Moscow Formation falls fully within the *Polygnathus ansatus* Biozone (Klapper, 1981; Kirchgasser, 2000), which forms part of the larger *varcus* Biozone (Ziegler *et al.*, 1976) which spans 390.7–388.2 Ma (House & Gradstein, 2004).

The oldest possible record of the total group Spermatophyta is Lower Devonian *Pertica*, interpreted as a trimerophyte on the euphyllophyte stem by Banks (1975c) and only tentatively interpreted as a stem spermatophyte by Kenrick & Crane (1997) because of the paucity of anatomical information. The next oldest possible records are aneurophytalean progymnosperms, the oldest best known representative of which is *Rellimia thomsonii*, reported from the Panther Mountain Formation of New York (Bonamo, 1977). This formation is equivalent to the Ludlowville and Skaneateles formations (A. Bartholomew, pers. comm.) which lie below the Moscow Formation of New York (Bartholomew & Brett, 2007) but are still within the *varcus* Biozone presented in House & Gradstein (2004). Thus, although slightly older than *Ibyka*, its age cannot be constrained more accurately. Therefore, the top of the *varcus* Biozone at 388.2 Ma provides a minimum constraint on the divergence of Euphyllophyta into Monilophyta and Spermatophyta.

A soft maximum bound can be established on the oldest record trilete spores. It has been argued that true trilete spores, apparently distinguishable from cryptotrilete spores, are a synapomorphy of total group Tracheophyta (supplementary material of Steemans *et al.*, 2009). However, we question whether trilete spores are unequivocally restricted to total group Tracheophyta, a doubt expressed by others (Banks, 1975a, 1975b), as the trilete spores produced by bryophyte lineages are not universally cryptotrilete, and so not distinguishable from true trilete spores, especially those of hornworts (J. Doyle, pers. comm.), in which most extant taxa produce trilete spores. Therefore there is a good possibility trilete spores characterise the hornwort + tracheophyte crown clade (node 3). Nevertheless, there is no reason to doubt that trilete spores are restricted to crown Embryophyta, and thus their oldest record provides an uncontroversial soft maximum constraint for Euphyllophyta. The oldest trilete spores are from the Qusaiba-1 core from the Quasim Formation of northern Saudi Arabia (Steemans *et al.*, 2009). In a number of stratigraphic horizons within the core, the trilete spores co-occur with the chitinozoan *Acanthochitina barbata*, diagnostic of the *barbata* biozone within the Katian Stage (J. Verniers, pers. comm.). The very oldest records do not co-occur with *A. barbata* and so it is possible that they occur in the older *Tanuchitina fistulosa* biozone, though there are no records of *T. fistulosa* to confirm this. Therefore, these oldest records potentially fall within the lowest part of the *barbata* biozone, or at the transition between the *A. barbata* and *T. fistulosa* biozones (J. Verniers, pers. comm.). In either case, an unequivocal minimum age interpretation is afforded by the top of the *A. barbata* Biozone, dated at 449 Ma (Cooper & Sadler, 2004). The oldest stratigraphic records of trilete spores within the core co-occur with the chitinozoan *Armoricochitina nigerica*; originally considered restricted to the

Ashgill (Paris, 1990), its range is now known to extend into the Caradoc, to within the biozone characterized by *Fungochitina spinifera* (= *Fungochitina fungiformis*) (Paris *et al.*, 2007). The base of the *F. spinifera* zone falls within the *clingani* Biozone (*morrisi* Subzone) (Vandenbroucke *et al.*, 2008), the base of which is 454 Ma (Cooper & Sadler, 2004). Thus, 454 Ma is the maximum age of the first trilete spores and provides a soft maximum constraint for divergence of Euphyllophyta into Monilophyta and Spermatophyta.

Tracheophyta: *Selaginella–Psilotum*, *Ginkgo*, *Cryptomeria*, *Welwitschia*, *Pinus*, *Amborella*, *Nymphaea*, *Illicium*, *Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 4: minimum = 416 Ma; soft maximum = 454 Ma) This is the fundamental divergence of crown Tracheophyta (Eutracheophyta) into Lycopsida and Euphyllophyta. The oldest possible record of crown Tracheophyta is *Pinnatiramosus qianensis* from the Xiushan Formation, Guizhou Province, China (Geng, 1986; Cai *et al.*, 1996). It consists of a complex branching system and pitted tracheids; a crown Tracheophyte with uncertain placement. The *Pinnatiramosus*-bearing sediments are confidently assigned to the Telychian Stage of the upper Llandovery (early Silurian) on the basis of spore (Wang *et al.*, 1996; Wang & Ouyang, 1997) and brachiopod (Rong *et al.*, 1984; Wang *et al.*, 1996) biostratigraphy. However, *P. qianensis* has been reinterpreted as the rooting system of a Permian-age plant growing down from an unconformity surface through underlying lithified sediments of Silurian age (Edwards *et al.*, 2007). There are considerably older records of Tracheophyta than a Permian *P. qianensis.*

The oldest potential records of total group Lycopsida are *Cooksonia pertoni*, *Cooksonia hemisperica* and *Cooksonia cambrensis* from the Cloncannon Formation of County Tipperary, Ireland (Edwards & Feehan, 1980; Edwards *et al.*, 1983). These earliest occurrences are bracketed by marine sediments containing graptolites *Monograptus ludensis* and *Pristiograptus auctus* (also referred to as *Monograptus auctus* in Palmer, 1970) which are diagnostic of the *ludensis* Biozone (Zalasiewicz *et al.*, 2009). The top of the *ludensis* Biozone coincides with the Wenlock–Ludlow series boundary (422.9 Ma ± 2.5 Myr), providing a minimum age interpretation of 420.4 Ma. However, the assumption that these records of *Cooksonia* are assigned to crown Tracheophyta is established on characters preserved in younger records of the same species (Kenrick & Crane, 1997; Gonez & Gerrienne, 2010) and requires that the phylogenetic position of the younger records is secure. This can be considered with reference to the synapomorphies of crown Tracheophyta (decay-resistant tracheid cell wall, simple pitlets in the tracheid cell wall, and presence of a sterome; Kenrick & Crane, 1997). Only one crown Tracheophyte synapomorphy, sterome, has been demonstrated in one of the younger species, *C. pertoni* (Kenrick & Crane, 1997); and the veracity of this same character as a synapomorphy of crown Tracheophyta has been questioned (Cantino *et al.*, 2007); it is more likely to be a synapomorphy of total group Tracheophyta. Reinterpretation of *Cooksonia* as a total group tracheophyte is consistent with the presence of other unequivocal eutracheophyte symplesiomorphies or total group Tracheophyte synapomorphies, including multiple sporangia and differentially thickened water-conducting cells (tracheids) (Kenrick & Crane, 1997). Thus, at present, the earliest records of *Cooksonia* specimens can be unequivocally interpreted as members of total group Tracheophyta, but based on the available evidence it is not yet possible to discriminate whether they are stem or crown Tracheophyta.

The next oldest records of total group Lycopsida are undescribed zostrophylls (Tims & Chambers, 1984) and *Baragwanathia* from the 'Lower Plant Assemblage', Australia (Garratt, 1978) and *Zosterophyllum* sp. from Bathurst Island, Arctic Canada (Kotyk *et al.*, 2002). The zostrophylls of Tims & Chambers (1984) have not been described to the extent to which we can assess their assignment to crown Tracheophyta. *Baragwanathia* in Garratt (1978) is clearly a Eutracheophyte but uncertainties remain over the age of the 'Lower Plant Assemblage' from which it has been described. A number of concerns have been raised (Hueber, 1983, 1992) questioning the quality of graptolite preservation, a reliance on too few index species, an apparent 24 Myr stasis in the fauna, and the lack of a detailed description of the age-indicating graptolites. The issue regarding stasis has been addressed (Rickards, 2000) and, although further detailed descriptions of the graptolite fauna would be helpful, the information already provided regarding graptolite identification and the geology of the area (Garratt, 1978, 1981; Garratt & Rickards, 1984; Garratt *et al.*, 1984) is sufficient to place the assemblage in the Ludlow, even if criticisms regarding a Gorstian Stage (Lower Ludlow) age assignment are accepted (Hueber, 1992). Therefore, the age interpretations of the Ludlow fossils span 425.4–416 Ma (Melchin *et al.*, 2004).

The Bathurst Island *Zosterophyllum* sp. (Kotyk *et al.*, 2002) is unequivocally a zostrophyll given its possession of reniform sporangia, sporangia that dehisce along their distal margins, and laterally inserted sporangia (P. Kenrick, pers. comm.). All *Zosterophyllum* species are total group Lycopsida (Kenrick & Crane, 1997). The *Zosterophyllum* sp. on Bathurst Island co-occurs with conodont *Ozarkodina douroensis*, which is restricted to the Ludlow (as O. n. sp. B in Klapper & Murphy, 1974; Thorsteinsson, 1980; Uyeno, 1990; Mayr *et al.*, 2004). Thus, age interpretations can be derived from the top and bottom of the Ludlow, spanning 425.4–416 Ma.

The oldest potential total group Euphyllophyta is *Wutubulaka* from the Wutubulake Formation (Late Pridoli) of Xinjiang, China (Cai *et al.*, 1993; Yi *et al.*, 2007). Its systematic placement is tentative because it only

possesses a single synapomorphy of stem Euphyllophyta, pseudomonopodial branching (Kenrick & Crane, 1997). Thus, without further anatomical information to identify potential crown tracheophyte synapomorphies, it cannot provide a minimum for total group Euphyllophyta or crown Tracheophyta.

The next oldest candidates are *Eophyllophyton bellum* and *Psilophyton primitiuum* from the Posongchong Formation (Pragian) of Yunnan, China (Hao & Beck, 1993; Hao & Gensel, 1998). Younger representatives of both genera have been resolved as basal members of stem Euphyllophyta (Kenrick & Crane, 1997), and both Pragian fossils contain numerous synapomorphies of stem Euphyllophyta (Euphyllophytina; Kenrick & Crane, 1997), including pseudomonopodial branching, paired sporangia grouped into terminal trusses and dichotomous appendages. These fossils are the oldest unequivocal representatives of stem Euphyllophyta but they are significantly younger than the Bathurst Island *Zosterophyllum* sp. Thus, the minimum age for divergence of Tracheophyta into its fundamental lineages, Lycopsida and Euphyllophyta, is 416 Ma. A soft maximum constraint for Tracheophyta can be established at 454 Ma based on the oldest age interpretation of the oldest records of trilete spores (see Euphyllophyta, node 5).

Unnamed clade: *Anthoceros–Selaginella, Psilotum, Ginkgo, Cryptomeria, Welwitschia, Pinus, Amborella, Nymphaea, Illicium, Chloranthus, Liriodendron, Sorghum, Arabidopsis, Populus* (node 3: minimum = 420.4 Ma; soft maximum = 1024 Ma) This is the divergence of the Anthocerotae and Tracheophyta total groups. The oldest possible records for either total group are cuticle fragments and tubular structures from the Caradoc (Gray *et al.*, 1982) and Llanvirn (Vavrdová, 1984; but see Taylor & Wellman, 2009), respectively. Unfortunately, the oldest of this material that can be constrained phylogenetically (tracheids, stomata and cuticle with aligned epidermal cells) is contemporaneous with the first *Cooksonia*, or in younger sediments (Edwards, 2000; Wellman & Gray, 2000), and is therefore redundant for our purposes. Although various affinities have been suggested for older cuticles and tubes (smooth and banded), such as bryophyte (Kroken *et al.*, 1996; Graham & Gray, 2001; Graham *et al.*, 2004) or lichen (Taylor *et al.*, 1995, 1997), these are too equivocal to constrain the timing of clade divergence at this taxonomic level.

The oldest potential records of total group Anthocerotae are species of the Late Silurian to Early Devonian spore genus *Emphanisporites* that possess a zone of weakness at the spore equator ('pseudosuture'), a feature unique to hornworts among extant spores (Taylor *et al.*, 2011). The next oldest records are considerably younger, including Upper Cretaceous and Tertiary remains resembling extant *Notothylas* (Gupta, 1956; Nemejc & Pacltova, 1974; Chitaley & Yawalew, 1980) and Maastrichtian *Phaeoceros*-like spores (Jarzen, 1979). However, all of these reports are based upon either incomplete fossils that are not credible (Krassilov & Schuster, 1984) or equivocal spore data. The only unequivocal record is a complete plant from Oligocene Dominican amber (Frahm, 2005).

Katian (late Ordovician) trilete spores are the oldest possible evidence of total group Tracheophyta (Steemans *et al.*, 2009) but, as noted above (Euphyllophyta, node 5), it is not possible to preclude the possibility that trilete spores originated on the Anthocerotae + Tracheophyta stem, or even before. Thus, the oldest unequivocal record of total group Tracheophyta is *Cooksonia* from the *ludensis* biozone (see Tracheophyta, node 4). Thus, the minimum constraint on the divergence of Anthocerotae and Tracheophyta is 420.4 Ma. The earliest members of total group Tracheophyta (which would neither have possessed tracheids nor necessarily have been polysporangiate) would have had the same negligable fossilization potential of bryophyte grade plants, and thus it is a prior expectation that their oldest fossil record will be a poor approximation of the time of their origination. Thus, for the divergence of Anthocerotae and Tracheophyta, we advocate the same soft maximum constraint of 1024 Ma, as applied to Embryophyta (node 1), based on the age of sedimentary sequences that are older than all suggested hypotheses for land plant origin, sampled for spores with land plant affinities and demonstrated to be devoid of them.

Stomatophyta: *Physcomitrella–Anthoceros, Selaginella, Psilotum, Ginkgo, Cryptomeria, Welwitschia, Pinus, Amborella, Nymphaea, Illicium, Chloranthus, Liriodendron, Sorghum, Arabidopsis, Populus* (node 2: minimum = 420.4; soft maximum = 1024 Ma) This is the fundamental divergence of crown Stomatophyta into Musci and Anthocerotae + Tracheophyta. The oldest possible records of crown Stomatophyta are late Ordovician fragments of cuticle and tubular structures but their affinities are too equivocal to be of utility (see node 3). The fossil record of mosses is poor because of the lack of thickened tissue and their delicate nature (Lacey, 1969). It has been suggested that mosses may suffer disproportionally because they have an upright growth habit rather than the sprawling growth habit of other bryophytes (Krassilov & Schuster, 1984). The oldest possible records of total group Musci are Lower Devonian *Sporogonites* (Halle, 1916, 1936), Carboniferous *Muscites plumatusm* (Thomas, 1972; but see Rowe in Bateman *et al.*, 1998) and *Muscites polytrichaceus* (Renault & Zeiller, 1888), Permian (Neuburg, 1956, 1960; Smoot & Taylor, 1986) and Jurassic (Savicz-Ljubitzkaja & Abramov, 1959), but these are all too equivocal to provide a minimum constraint (Andrews, 1958; Smoot & Taylor, 1986). The oldest unequivocal record of total group *Musci* is *Eopolytrichum antiquum*, a member of the crown Musci family Polytrichaceae, from the Campanian (late

Cretaceous) of Georgia, USA (Konopka *et al.*, 1997; Hyvonen *et al.*, 1998; Koskinen & Hyvönen, 2004). The oldest records of total group Anthocerotae+Tracheophyta are considerably older. The oldest possible records are Katian (late Ordovician) trilete spores (see Euphyllophyta, node 5) and, unequivocally, *Cooksonia*, which provides a minimum constraint of 420.4 Ma for the divergence of crown Stomatophyta (see Tracheophyta, node 4). The soft maximum of 1024 Ma applied to Embryophyta is also appropriate here (see node 1).

Embryophyta: *Marchantia–Physcomitrella*, *Anthoceros*, *Selaginella*, *Psilotum*, *Ginkgo*, *Cryptomeria*, *Welwitschia*, *Pinus*, *Amborella*, *Nymphaea*, *Illicium*, *Chloranthus*, *Liriodendron*, *Sorghum*, *Arabidopsis*, *Populus* (node 1: minimum = 449 Ma; soft maximum = 1024 Ma) This represents the fundamental divergence of Embryophyta (crown land plants) into Hepaticae and Stomatophyta. The oldest possible record of crown Embryophyta is *Longfengshania*, from the Mesoproterozoic Little Dal Group of Canada and the Qingbaikou Group of North China (Du, 1982; Du & Tian, 1985; Hofmann, 1985; Xu, 2002), interpreted as a bryophyte by likening its thallus to a capsule and its stipe to a seta. However, this is just one of a number of interpretations of *Longfengshania* which is known from records through to the Ediacaran (latest Neoproterozoic; Tang *et al.*, 2007). Most reports favour the interpretation of these fossils as macroscopic algae (Du, 1982; Duan *et al.*, 1985; Xu, 2002; Tang *et al.*, 2007), with some directly questioning the bryophyte interpretation (Liu & Du, 1991). The next oldest possible record is another bryophyte-like fossil, *Parafunaria sinensis*, from the Early-Middle Cambrian Kaili Formation of Taijiang County, Guizhou Province, China (Yang *et al.*, 2004), the phylogenetic interpretation of which is just as equivocal (Conway Morris, 2006; Kenrick & Vinther, 2006).

The oldest possible records of embryophyte spores occur in the Bright Angel Shale, Rogersville shale and cores penetrating the Conasauga Group (Strother & Beck, 2000; Strother *et al.*, 2004). The very oldest records occur in the Bright Angel Shale which falls fully within the span of the *Albertella*, *Glossopluera* and *Ehmaniella* trilobite biozones (McKee & Resser, 1945; Resser, 1945), representing 509–507.2 Ma (Peng & Babcock, 2008). The spores exhibit two synapomorphies of Embryophyta: their permanent tetrad and dyad arrangements, and multilaminate sporoderm showing similarity to extant liverwort *Riccia* (Taylor, 2009). In the absence of corroborating evidence from mesofossils, it remains possible that these are convergent characteristics of a remote algal relative (Steemans & Wellman, 2003; Wellman, 2003). Although there appear to be substantial differences between these fossil spores and algal spores (Taylor & Strother, 2008), their phylogenetic affinity remains too equivocal to substantiate a calibration at this taxonomic level. The next oldest possible records are cryptospores, which consist of relatively thick-walled monads, dyads or tetrad spores with or without an ornamented or laevigate envelope, yet lacking a well-defined aperture (Richardson, 1996; Steemans, 2000; Wellman & Gray, 2000). The oldest record of cryptospores is tetrads recovered from the Zanjón Formation of Argentina, which are considered early to middle Dapingian in age on the basis of chitinozoan and acritarch biostratigraphy (Rubinstein *et al.*, 2010). Cryptospores can be accommodated within total group Embryophyta with confidence given similarities in wall composition and ultrastructure (Taylor, 1995), and *in situ* occurrences in Lower Devonian bryophyte-like plants (Edwards *et al.*, 1995) and Ordovician sporangia (Wellman *et al.*, 2003). However, it remains unclear whether they represent stem or crown Embryophyta.

The oldest records of total group Hepaticae are Middle Devonian (Hernick *et al.*, 2008) and Late Devonian (Hueber, 1961), and there are several reports from the Carboniferous (Krassilov & Schuster, 1984). Their phylogenetic classification relies on gross morphology, as the phenotypic synapomorphies of Hepaticae are limited to oil bodies and elaters, which are present neither in the fossils nor in many extant members of Hepaticae. However, trilete spores, for which considerable evidence supports a total group Tracheophyta or Anthocerotae + Tracheophyta affinity (see Euphyllophyta, node 5), substantiate a minimum constraint of 449 Ma on the divergence of crown Embryophyta into Hepaticae and Stomatophyta.

A soft maximum constraint for crown Embryophyta must encompass total group Embryophyta which might represent crown Embryophyta, including cryptospores (469.8 Ma) and Cambrian spores (509 Ma), but not *Parafunaria* and *Longfengshania*, whose claim on affinity to total group Embryophyta is extremely dubious. Nevertheless, there is good reason to believe that the earliest records of Embryophyta are a poor approximation of the time of origin of the clade, as characters that enhance fossilization potential (thickened cuticle and spore walls) were acquired within the crown group. Furthermore, there is a paucity of Ordovician, Cambrian and Neoproterozoic terrestrial sedimentary sequences in the geological record in which Embryophyte diversity could be sampled (cf. Smith & McGowan, 2007; McGowan & Smith, 2008). Although there are no direct records older than Cambrian spores, colonization of the land by plants modified biogeochemical cycles and we would anticipate evidence of this in physical and chemical proxies, such as the effect of vegetation on river dynamics (Cotter, 1978), nutrient runoff (Malkowski & Racki, 2009), weathering (Retallack, 1997) and the carbon cycle (Berner, 1997).

One potential proxy is the proposed positive relationship between the presence of terrestrial vegetation and the abundance of meandering rivers (Cotter, 1978; Davies & Gibling, 2010a,b), and thus the presence of point bar sedimentary

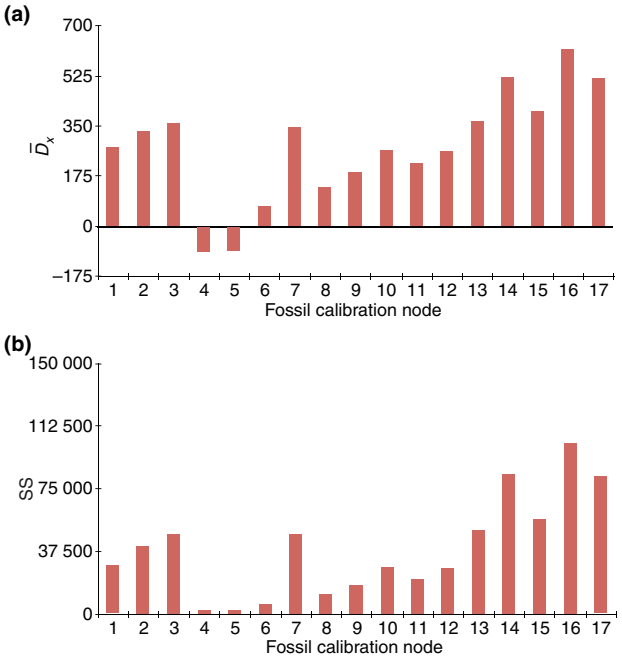

Fig. 4 When a given fossil calibration, implemented as a hard minimum and a soft maximum constraint, was used in isolation to estimate all other nodes in the tree, these estimated nodes were used to calculate (a) the average difference between the molecular estimates and their fossil constraint spans ($\bar{D}_x$), and (b) the sum of squared differences (SS) between the molecular estimates and their fossil constraint spans. As every calibration presented in this paper was subject to this process, $\bar{D}_x$ and SS values have been derived for every calibration, enabling comparison between them. $\bar{D}_x$ and SS values are years in millions.

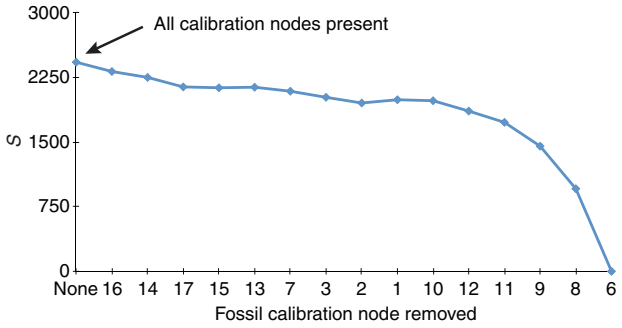

Fig. 5 Plot illustrating the effect of removing fossil calibrations on *s* (average squared deviation of the average difference between molecular and fossil estimates). Fossil calibration nodes were removed according to the magnitude of their sum of squared differences (SS) value (as in Fig. 4b), from largest to smallest. Open points would indicate that the removal of that specific fossil calibration resulted in a significant reduction in the variance of *s* according to a one-tailed *F*-test, yet no significant reductions were found. Fossil calibrations 5 and 4 are absent from the figure because they are the final two calibrations remaining.

sequences in the Precambrian could be used as evidence of a cryptic early episode of colonization of the land by plants. Davies & Gibling (2010a) argue that Precambrian point bar sequences are rare and equivocal (Eriksson *et al.*, 1998), and that river systems can be established in the absence of plants, as evidenced by the presence of fluvial systems on Titan and Mars (Jerolmack *et al.*, 2004; Wood, 2006; Lorenz *et al.*, 2008). Furthermore, the putative relationship between vegetation and meandering river systems only concerns rooted vegetation (Davies & Gibling, 2010a,b), a feature peculiar to crown Tracheophyta, such as herbacious lycpods and tree lycopods, rather than the smaller structures of stem Tracheophyta (Algeo & Scheckler, 1998). Finally, by their very nature, rooting systems have a high preservation potential and so it is entirely surprising that they are absent from Proterozoic point bar sequences.

Knauth & Kennedy (2009) describe decreases in $\delta^{13}C$ to Phanerozoic levels in *c.* 850 Ma carbonates which, in the absence of a clear geological driver with sufficient explanatory power, they argue are the consequence of an influx of terrestrial carbon enriched in ^{12}C by photosynthesizing communities. These conclusions have been criticized on the basis that the authors used incorrect assumptions concerning $\delta^{18}O$ and ocean temperature through time, and that they failed to consider the effect of post-depositional alterations $\delta^{13}C$ of the carbonates (Arthur, 2009). The presence of photosynthesizing communities does not require that they were crown Embryophyta. Nevertheless, the soft maximum constraint for Embryophyta must encompass this possibility and thus must antedate the c. 850 Ma shift in $\delta^{13}C$ identified by Knauth & Kennedy (2009). An arbitrary but objective and precise date can be obtained from Precambrian sediments of the Torridon Group, Scotland, which represent an environment in which embryophytes would be expected to have flourished were their lineage established. The sequences have been investigated palynologically but yield no evidence of spores or other remains with possible embryophyte affinities (Strother *et al.*, 2011). A direct date of 994 Ma ± 48 Myr was obtained from the Diabaig Formation of the Torridon group (Turnbull *et al.*, 1996), and thus 1042 Ma can provide a soft maximum constraint for crown Embryophyta.

Cross-validation of calibration constraints

To determine the consistency of the calibrations, we employed a modified version of the cross-validation method introduced by Near and colleagues (Near & Sanderson, 2004; Near *et al.*, 2005). In its original formulation, the cross-validation method explores the degree to which individual calibration points produce estimates that approximate the calibration points on other nodes. The average difference between the fossil calibrations and molecular estimates ($\bar{D}_x$) and the sum of these squared differences (SS) are used to quantify inconsistency. Our modification accommodates the changed nature of fossil calibrations that we advocate not as fixed calibrations but rather as minimum and maximum constraints.

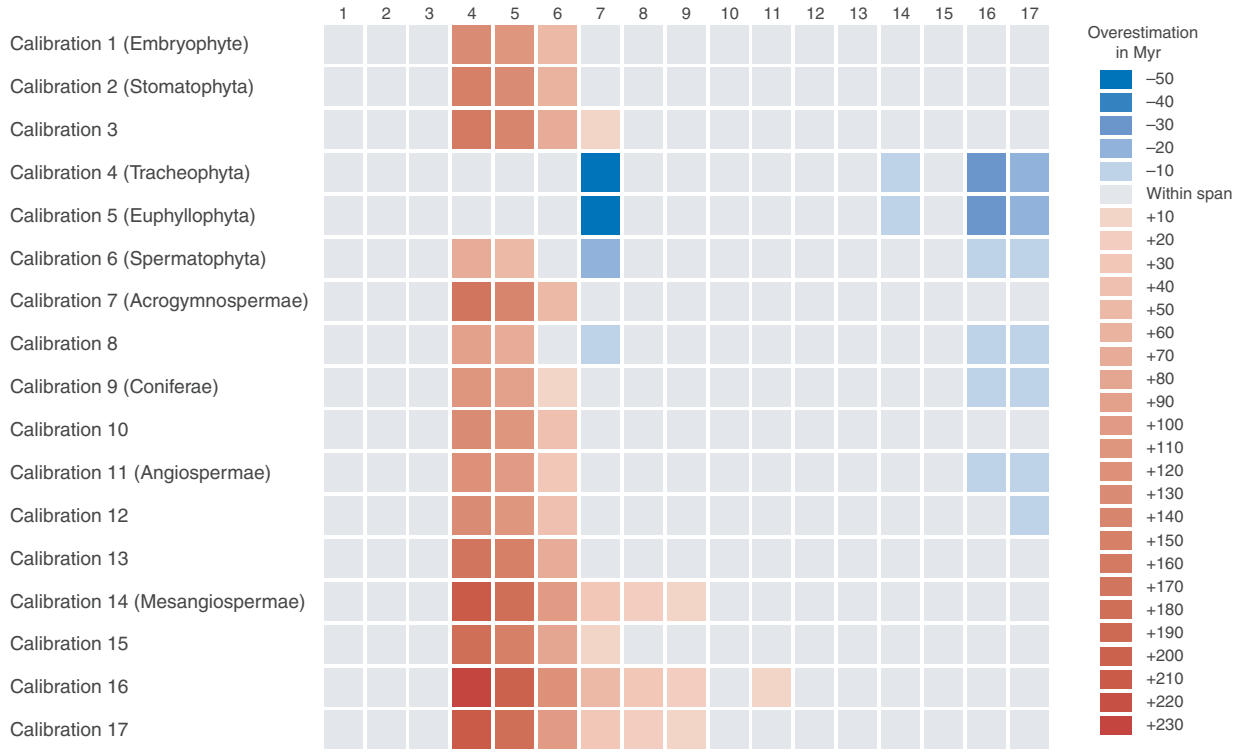

Fig. 6 A colour scaled representation of the differences between the molecular estimates and fossil constraint spans (Di) for every node which contributed to values of ($\bar{D}_x$) and sum of squared differences (SS) (Fig. 4) when each given fossil calibration is implemented in isolation as part of the cross-validation procedure. It indicates that, regardless of which calibration is used in isolation to estimate all other nodes in the tree, many tend to underestimate the calibration span for nodes 14, 16 and 17 in angiosperms, and heavily overestimate calibration spans for nodes 4 and 5; the Tracheophyta and Euphyllophyta nodes, respectively. Thus, the degree of overestimation of the Tracheophyta and Euphyllophyta is largely responsible for the patterns of $\bar{D}_x$ and SS observed in Fig. 4.

The results of the cross-validation analysis show that on average the fossil constraints of most nodes yield overestimates, while the constraints on two nodes (Tracheophyta and Euphyllophyta) yield underestimates (Fig. 4). We then calculated the average squared deviation of the average difference between molecular and fossil estimates (s) based upon all 17 calibrations. One-tailed F-tests were used to determine whether a significant decline in s would result from the sequential removal of the calibrations, starting with calibrations that produced the largest SS values, working towards calibrations producing the smallest (Fig. 4b), until only two calibrations remained. We found no significant reductions in s after the sequential removal of any calibrations (Fig. 5); here, s takes into account both the minimum and maximum constraints and most estimates lie between these limits, so a large decline in s is not expected.

Using an alternative strategy to examine consistency, we directly assessed the relationship between the node used for calibration and the resulting molecular estimates at other nodes (Fig. 6). Further scrutiny of the cross-validation results revealed that Tracheophyta and Euphyllophyta were the most inadequately estimated nodes; molecular estimates grossly exceed the maximum constraints when any other node is used for calibration (Fig. 6). All calibrations (with the exception of the Tracheophyta and Euphyllophyta calibrations themselves) poorly estimate these nodes, and so it appears that the degree of overestimation at Tracheophyta and Euphyllophyta determines the patterns of $\bar{D}_x$ and SS seen in Fig. 4. We consider the Tracheophyta and Euphyllophyta calibrations to be inconsistent because these nodes greatly affect the patterns of over- and underestimation in cross-validation analyses (Fig. 6), and because only these nodes yield underestimates of other constraints on average (Fig. 4a).

In conclusion, the cross-validation analysis suggests that the soft maxima applied to the divergence of crown Tracheophyta and crown Euphyllophyta may be underestimates or, alternatively, that a degree of rate variation has gone undetected. In either instance, rather than advocating the exclusion of these fossil constraints, it is our view that they require further scrutiny. Both Tracheophyta and Euphyllophyta rely on the oldest age interpretation of the oldest records of trilete spores to establish their soft maximum constraints. While there are records of candidate stem embryophytes and, possibly, stem Stomatophyta from the Cambrian (Strother & Beck, 2000; Strother *et al.*, 2004; Taylor & Strother, 2008, 2009), there are no records older than the earliest trilete spores that could be shoe-horned into Tracheophyta to extend the soft maximum constraint further

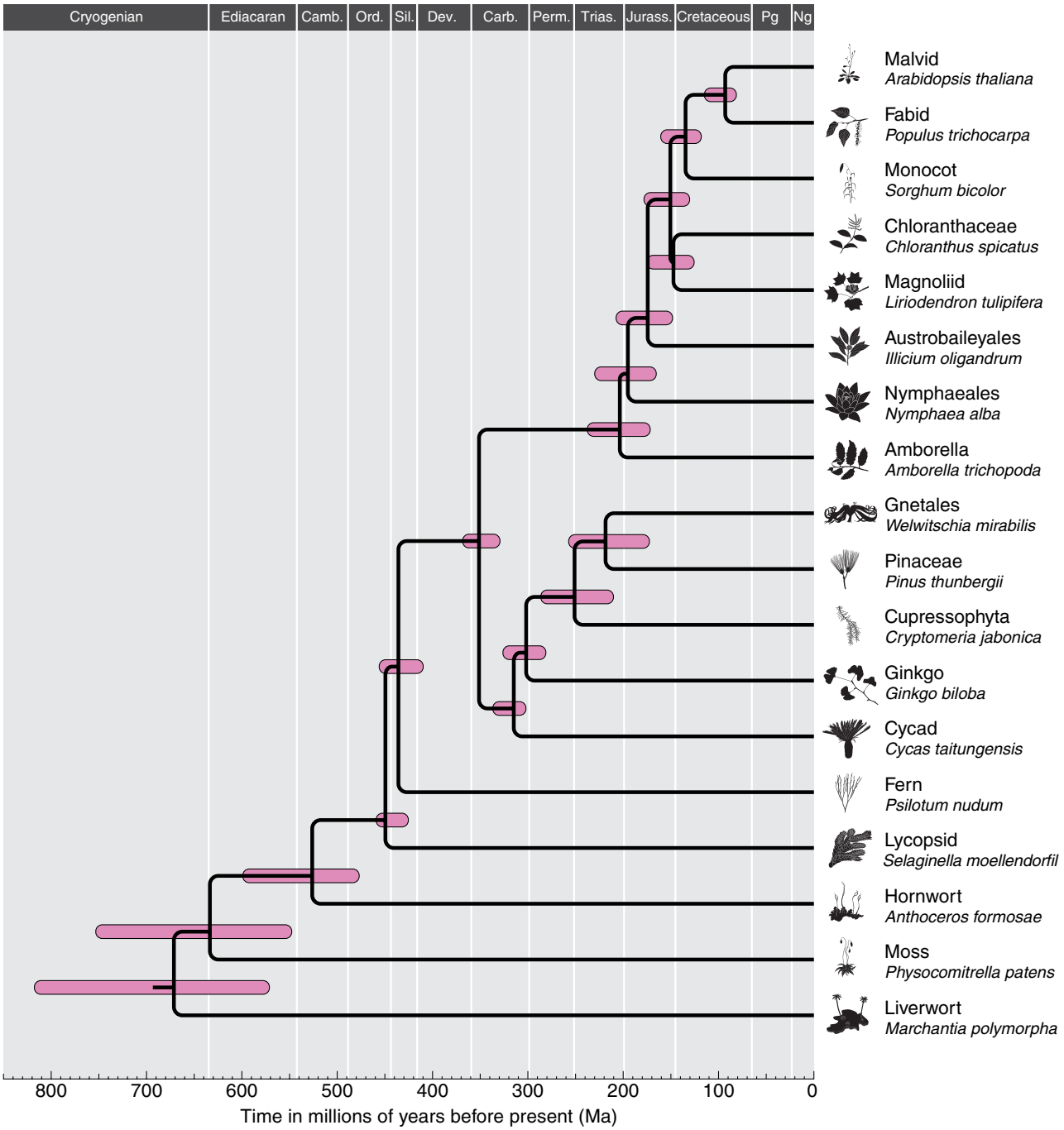

Fig. 7 Chronogram for land plant evolution when all 17 calibrations derived in this paper were implemented with a uniform prior between the minimum and maximum constraints of each. Each node represents the mean divergence time estimate and their associated 95% credibility intervals. This chronogram and all other clock analyses performed (Table 2) reject both a post-Jurassic origin for Angiospermae and a post-Cambrian origin for Embryophyta. They also suggest a more gradual establishment of the major embryophyte lineages than suggested in previous molecular clock studies.

back in geological time. However, there is a paucity of Ordovician, Cambrian and Ediacaran terrestrial rock sequences from which such fossils could be recovered, and thus the consistency of the earliest appearance of Tracheophyte fossils in stratigraphic sections around the world may well be an artefact of the rock record (Inoue *et al.*, 2010).

Molecular clock analysis

Although the core purpose of this study was to establish fossil constraints for molecular clock analyses of plant evolution, it would be remiss of us to fail to explore the implications of implementing these constraints, in the man-

Table 2 Divergence time estimates using all 17 calibrations derived and manipulations to the basalMax and the prior probability densities within each calibration

		509 Ma basalMax		1042 Ma basalMax									
					110% prior peak		125% prior peak		150% prior peak		175% prior peak		
Node	Clade	Uniform prior		Uniform prior	Specified prior	Posterior	Specified prior	Posterior	Specified prior	Posterior	Specified prior	Posterior	
1	Embryophyta	**505**	489–513	**670**	568–815	508	**590**	515–704	597	**628**	554–743	746	**733**	604–863	894	**803**	607–999
2		**499**	481–510	**632**	548–750	483	**555**	499–637	576	**592**	536–672	731	**694**	578–774	887	**761**	581–909
3		**460**	441–479	**524**	475–596	483	**477**	446–517	576	**504**	460–565	731	**555**	482–657	887	**593**	491–789
4	Tracheophyta	**429**	416–449	**446**	425–456	420	**421**	416–434	426	**427**	418–445	435	**438**	429–467	445	**457**	441–650
5	Euphyllophyta	**417**	396–439	**434**	410–452	395	**401**	390–420	405	**409**	398–427	421	**424**	413–439	438	**447**	431–618
6	Spermatophyta	**346**	326–366	**351**	330–368	312	**332**	316–352	321	**334**	320–356	337	**340**	331–363	352	**357**	347–409
7	Acrogymnospermae	**315**	306–334	**316**	306–337	312	**310**	306–317	321	**313**	306–324	337	**313**	306–335	352	**328**	313–352
8		**303**	282–324	**304**	283–327	181	**292**	258–309	212	**293**	256–314	264	**292**	265–318	315	**316**	303–333
9	Coniferae	**251**	209–285	**252**	212–286	163	**203**	160–261	188	**208**	182–254	228	**235**	220–259	269	**272**	261–288
10		**218**	171–258	**219**	174–259	141	**170**	133–231	169	**177**	153–220	216	**211**	188–227	263	**252**	222–266
11	Angiospermae	**198**	170–231	**205**	175–240	136	**170**	151–195	155	**175**	159–198	186	**202**	187–226	217	**229**	216–257
12		**191**	164–224	**197**	169–231	136	**164**	146–187	155	**169**	155–191	186	**196**	182–217	217	**223**	211–248
13		**172**	149–201	**177**	152–208	136	**149**	137–167	155	**156**	145–172	186	**184**	169–199	217	**211**	192–231
14	Mesangiospermae	**148**	132–201	**152**	133–179	136	**135**	128–145	155	**142**	132–154	186	**168**	149–182	217	**192**	167–216
15		**145**	127–201	**149**	128–176	114	**130**	115–142	136	**138**	127–151	174	**165**	146–179	211	**189**	163–213
16		**134**	124–201	**137**	124–161	136	**128**	124–135	155	**131**	124–144	186	**152**	133–170	217	**173**	148–202
17		**93**	83–201	**94**	83–115	87	**88**	83–96	94	**93**	84–101	105	**104**	92–170	116	**116**	107–122

Values in bold represent mean estimates, followed by 95% credibility intervals. 'Specified prior' refers to ages corresponding to the specified mode between the minimum and maximum constraints for each node.

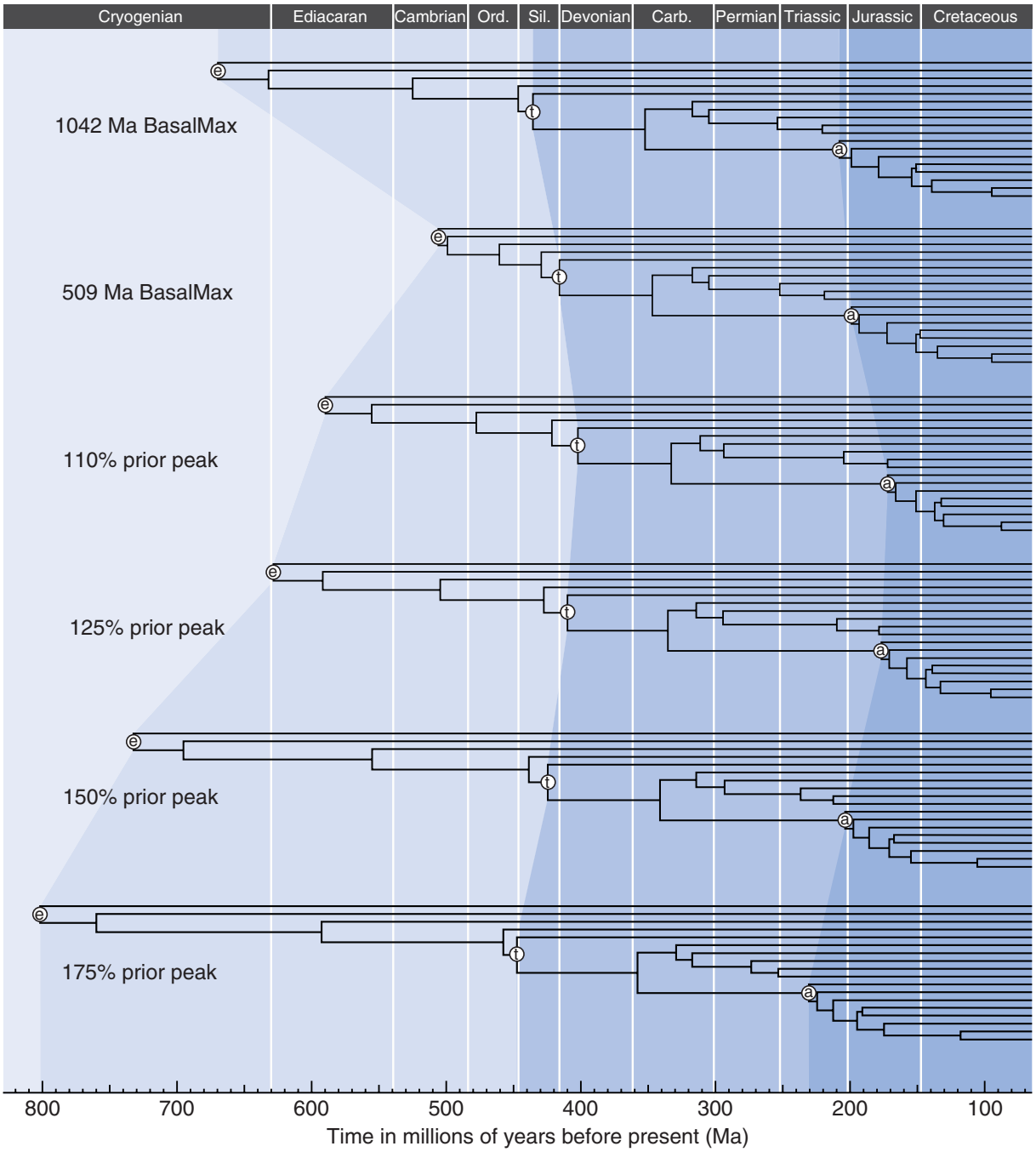

Fig. 8 Chronograms for the six molecular clock analyses conducted. 1042 Ma BasalMax refers to the analysis employing all calibration constraints unaltered, whereas 509 BasalMax indicates a change of the maximum constraint on nodes 1, 2 and 3 to 509 Ma. Both clock analyses employed a uniform prior. The remaining four clocks represent permutations to the position of the peak in prior probability density between our original calibration constraints, to 110, 120, 150 and 175% between the minimum and the maximum constraints. The crown embryophyte (e), crown tracheophyte (t) and crown angiosperm (a) nodes are labelled, with connective shading linking these respective nodes between analyses.

ner that we advocate, for the time-scale of plant evolution. Divergence estimates were based on our data set of seven genes, implementing all of the 17 sets of fossil constraints.

In our initial analysis we implemented a uniform prior spanning the interval between minimum and maximum constraints, representing 97.5% of the probability of the timing of divergence, with a 2.5% tail extending from the maximum constraint. The mean molecular estimates from this analysis (Table 2) suggest that major lineages of embryophytes (nodes 1–6) became established over a 319-Myr

period ranging from the Cryogenian through to the Devonian (Fig. 7). Estimates also indicate that the gymnosperm and angiosperm crown groups originated in the Carboniferous and Triassic, respectively. The 95% credibility intervals are shown for each node, and in 15 out of 17 cases the 95% intervals sit comfortably within the specified fossil constraints. However, part of the upper 95% interval exceeds the soft maximum constraint applied to the two remaining nodes (Tracheophyta and Spermatophyta). This is in agreement with the results of the cross-validation analysis and illustrates the utility of implementing soft maximum bounds (Yang & Rannala, 2006).

Impacts of changing the maximum constraints on the three most basal nodes (basalMax)

The ages of the three most basal nodes in our tree (liverwort, moss and hornwort), which all share an identical maximum constraint, are extremely uncertain. We define this shared maximum constraint as basalMax, which also marks the age of the upper constraint applied during our cross-validation analysis. To determine the impact of the age of basalMax, we explored the effect of changing this from 1042 Ma, the soft maximum we advocate, to 509 Ma, the oldest age interpretation of the oldest known fossils that exhibit any coherent embryophyte characters (Strother & Beck, 2000; Strother *et al.*, 2004; Taylor & Strother, 2008, 2009). Mean age estimates for all 17 nodes are younger when basalMax is 509 Ma (Table 2), although the effects are concentrated at the most basal divergences represented in our tree (nodes 1–5), with negligible differences seen for the more derived splits within Spermatophyta. With the use of alternative basalMax ages, estimates for Embryophyta differ by 165 Myr, Stomatophyta by 133 Myr and Hornwort + Tracheophyta by 64 Myr, whereas other age estimates are less signicantly affected: Tracheophyta by 17 Myr, Euphyllophyta by 17 Myr, Spermatophyta by 5 Myr, Acrogymnospermae by 1 Myr and Angiospermae by 7 Myr.

Impacts of nonuniform priors on the timing of clade divergence

While we used a uniform distribution to constrain node ages between the minimum and maximum bounds, most analyses that implement calibrations as minimum constraints employ nonuniform probability distributions to reflect the degree to which fossil minima approximate the true time of divergence (Ho & Phillips, 2009; Bell *et al.*, 2010; Smith *et al.*, 2010). To explore the influence of nonuniform priors on node age estimates, we used the truncated Cauchy distribution implemented in MCMCTREE (Inoue *et al.*, 2010). To reflect the different degrees to which the minimum constraints might approximate the true time of divergence, we permuted the position of the peak in prior probability (*P*) to ages 110, 125, 150 and 175% of the minimum age constraint relative to the maximum, confining the 97.5% envelope of probability within the constraints derived for each node. The results of these analyses are presented in Table 2 and Fig. 8. The results show that, as the position of the peak in the prior probability is increased, posterior estimates of divergence time also increase, but this effect is not universal. For nodes from Spermatophyta to Coniferae (inclusive), for $P = 125$ and 150%, the age estimates are younger than when $P = 110\%$.

It is clear from these results that assigning an arbitrary peak in prior probability can strongly influence posterior estimates even in a circumstance, as here, where the prior probability is allowed to vary only within the confines of minimum and maximum constraints. Inoue *et al.* (2010) have already shown that a comparable effect is produced when arbitrary nonuniform priors are fixed on minima alone. This is unfortunate as, in practice, the vast majority of studies that have implemented nonuniform priors have selected arbitrary probability distributions (Barnett *et al.*, 2005; Bell *et al.*, 2010; Smith *et al.*, 2010). Advocates of this approach have argued that exponential, normal and/or lognormal distributions may be implemented to describe, in different situations, the degree to which a fossil minimum approximates a divergence event, or to account for phylogenetic or age uncertainty of a critical fossil (Benton & Donoghue, 2007; Donoghue & Benton, 2007; Ho, 2007; Benton *et al.*, 2009; Ho & Phillips, 2009). However, given the evident undue weight placed on these arbitrary priors, it is clear that the selection of priors on node ages must be materially justified. There is no simple means of extracting this information from the fossil record; if there was, a molecular clock would not be needed. There are methods by which node age priors could be established from the fossil record based purely on stratigraphic occurrence data (Pyron, 2010), or combined with assumptions of sampling intensity, preservation rate and birth-death models (Foote *et al.*, 1999; Tavaré *et al.*, 2002), and Wilkinson *et al.* (2011) recently developed such an approach to derive priors for a clock analysis. These methods require well-catalogued stratigraphic occurrence, not merely fossil minima, and so they can only be applied readily in groups such as primates where the fossil record is sparse and extraordinarily well catalogued. Doubtless, the development of these techniques, along with a better understanding of how they influence molecular clock analyses, will lead to dramatic improvement in the accuracy and precision of divergence estimates. However, our results speak strongly against the use of arbitrary priors and, in particular, we advocate the use of priors based on conservative hard minima and soft maxima.

Comparison to previous studies

Although the core purpose of this study was to provide calibrations, rather than present a molecular time-scale, it is

worth considering how our divergence times compare to previous estimates.

Origin and diversification of angiosperms The origin and diversification of angiosperms have been the focus of many molecular clock studies (e.g. Ramshaw *et al.*, 1972; Martin *et al.*, 1989; Sanderson, 1997; Sanderson & Doyle, 2001; Wikström *et al.*, 2001; Sanderson *et al.*, 2004; Bell *et al.*, 2005, 2010; Magallón & Sanderson, 2005; Moore *et al.*, 2007; Soltis *et al.*, 2008; Magallón, 2010; Smith *et al.*, 2010). These studies provide a huge spread of values for the origin of crown Angiospermae, ranging from the Palaeozoic (e.g. Ramshaw *et al.*, 1972; Martin *et al.*, 1989) to the Cretaceous (Bell *et al.*, 2005; Magallón & Castillo, 2009), with the suggestion that they are converging on a range of 140–180 Ma (Bell *et al.*, 2005; Soltis *et al.*, 2008). If all of our analyses and their 95% credibility intervals are considered, the range of estimates covers 151–257 Ma, showing considerable overlap with the 140–180 Ma range. However, our mean estimates (170–229 Ma) only overlap with the higher end of this span, and continue into the Triassic, as have other recent estimates (Magallón, 2010; Smith *et al.*, 2010).

Apart from a range of methodological differences, it is our view that the manner in which we interpret palaeontological data and implement fossil constraints can account for many differences between our results and those obtained in previous studies. For example, analyses that yield Cretaceous estimates for the origin of angiosperms have invariably used a Cretaceous point calibration or maximum age for the crown (Magallón & Castillo, 2009), or the analyses have used Cretaceous fossil records of tricolpate pollen to establish a fixed calibration or maximum age on the eudicot total group or crown group (Bell *et al.*, 2010). Using these pollen records to establish minimum constraints inevitably, but justifiably, leads our estimates to be older. Although the results of our analyses are similar to those obtained in Magallón (2010) and Smith *et al.* (2010), they are consistently younger, potentially because we implement justified maximum constraints rather than affix arbitrary prior probabilities to fossil minima.

The mean age estimates for crown Angiospermae in our analyses appear particularly robust as they show little variance even in response to permuting the peak of the nonuniform probability spanning the minimum and maximum constraints (Table 2). While the posterior estimates for other nodes closely follow shifts in the peak of nonuniform prior probabilities (Table 2), the posterior estimates for crown Angiospermae do not. For example, when P = 110%, the age of the peak in the prior probability is 136 Ma, while the ensuing mean posterior estimate is 170 Ma. As the range of mean estimates for Angiospermae is 170–229 Ma, we can at least reject the hypothesis of a post-Jurassic origin of angiosperms.

Main tracheophyte divergences Apart from the origin of angiosperms, the main divergences within tracheophytes are the origin of Acrogymnospermae, Spermatophyta, Euphyllophyta and Tracheophyta (Fig. 2, nodes 4–7). The age of crown Acrogymnospermae has previously been estimated as Pennsylvanian (late Carboniferous: 301 or 302 Ma; Smith *et al.*, 2010), Mississippian (early Carboniferous: 350 Ma; Goremykin *et al.*, 1997) and Late Devonian (366 Ma; Won & Renner, 2006), or results have spanned this entire range (318–370 Ma; Magallón, 2010). Most estimates for crown Spermatophyta are Mississippian (329 and 333 Ma; Schneider *et al.*, 2004; 346 Ma; Magallón & Sanderson, 2005; 327 and 330 Ma; Smith *et al.*, 2010), although some estimates place divergence in the Middle Devonian (341–386 Ma; Magallón, 2010). The origin of crown Euphyllophyta has been dated to the Early Devonian (412 Ma; Magallón & Sanderson, 2005) or the middle Silurian–Early Devonian interval (413–428 Ma; Magallón, 2010).

Our date estimates for the deepest nodes (Fig. 7) are comparable to previous estimates, indicating a Pennsylvanian origin for crown Acrogymnospermae (316 Ma), a Mississippian origin for crown Spermatophyta (351 Ma) and an early Silurian origin for crown Euphyllophyta (434 Ma). For Acrogymnospermae and Spermatophyta, we use a potentially conservative minimum (based on the oldest fully reconstructed Cordaitales) and potentially tight maximum constraint (represented by Late Devonian seeds),which may explain why our estimates are more in line with recent studies suggesting younger (Smith *et al.*, 2010) rather than older estimates (Magallón, 2010). However, our estimate for crown Euphyllophyta is slightly older than that obtained in Magallón (2010), probably because here a fixed calibration is imposed on the node below (Tracheophyta) at 421 Ma. The minimum constraint we applied to crown Euphyllophyta is also 3.3 Myr older than the constraint used in Magallón (2010), although we both refer to the same fossil monilophyte, *Ibyka*.

The final node to consider is crown Tracheophyta itself. There are surprisingly few studies that have estimated the age of this node, instead fixing it as a point calibration at 419, 421 or 430 Ma (early to late Silurian; Magallón & Sanderson, 2005; Heinrichs *et al.*, 2007; Magallón, 2010). As we have argued above, the maximum age of this node is among the least well constrained as the appearance of a macrofossil record for crown tracheophytes appears to coincide with the first widespread occurrence of terrestrial sedimentary sequences in the Phanerozoic rock record (Inoue *et al.*, 2010). Smith *et al.* (2010) dated the origin of crown Tracheophyta at 432 and 434 Ma, considerably younger than our 446 Ma estimate. This disparity may be accounted for, in part, by differences in the age of the minimum constraint applied to this node (416 Ma here vs 377.4 Ma in Smith *et al.*, 2010).

Bryophyte divergences and the origin of land plants The fundamental split within embryophytes, the liverwort–stomatophyte divergence, has been dated at Late Silurian to Late Cambrian (425, 435, 483 and 490 Ma; Sanderson, 2003), Late Ordovician (454 Ma; Heinrichs *et al.*, 2007), and Early Ordovician (477 and 474 Ma; Smith *et al.*, 2010), while the divergence of crown stomatophytes, equivalent to the moss–tracheophyte split, has been dated to the early Cryogenian (703 Ma; Heckman *et al.*, 2001; 707 Ma; Hedges *et al.*, 2004). We estimate the liverwort–stomatophyte divergence at 670 Ma (568–815 Ma), the moss–tracheophyte divergence at 632 Ma (548–750 Ma) and the hornwort–tracheophyte divergence at 524 Ma (475–596 Ma). Methodological and topological differences notwithstanding, the formulation and implementation of different calibrations provide the best explanation for the disparity observed between previous estimates and our own.

Our estimate for the origin of crown land plants is older than those obtained in the majority of previous clock studies, whose estimates fall within the Ordovician and Silurian (Sanderson, 2003; Heinrichs *et al.*, 2007; Smith *et al.*, 2010), with the exception of the studies by Heckman *et al.* (2001) and Hedges *et al.* (2004), which imply an origin for land plants at least as old as their Cryogenian moss–tracheophyte divergence estimate.

However, none of these previous studies applied a minimum constraint to Embryophyta, and, because of changes in clock methodology, only Smith *et al.* (2010) included any minimum constraints, with the closest calibration to Embryophyta being at the tracheophyte crown node, with a minimum of 377.4 Ma. A point calibration of 330 Ma for crown seed plants in Sanderson (2003) is the next closest calibration. Therefore, it is no surprise that, when we use a minimum constraint of 449 Ma based on the first trilete spores (see 'Results', node 1), we obtain older estimates (Fig. 7, Table 2). Interestingly, regardless of the use of a less conservative maximum at 509 Ma for Embryophyta, or the use of alternative prior probability peaks, none of our analyses resolve an Ordovician origin for crown land plants (Table 2). Thus, based on these analyses we can reject a post-Cambrian origin for Embryophyta.

Our mean estimate for the origin of crown stomatophytes is near the Edicaran–Cryogenian boundary (632 Ma; Table 2), *c.* 70 Myr younger than previous estimates that are fully within the Cryogenian (Heckman *et al.*, 2001; Hedges *et al.*, 2004). Issues regarding the calibrations used in Heckman *et al.* (2001) and Hedges *et al.* (2004), such as the large distance between the nodes used for calibration and the target nodes being estimated, have been highlighted previously (Sanderson, 2003; Graur & Martin, 2004). Using calibrations in this way allows any rate heterogeneity to strongly influence estimates and could account for significant overestimation. Thus, any similarity between our estimates and previous estimates seems entirely coincidental, although the 70 Myr difference is still substantial.

The hornwort–tracheophyte divergence presents our final major divergence involving a bryophyte lineage, an event we estimate to have occurred in the Cambrian (524 Ma), but for which no other estimates are available in the literature for comparison.

A discussion of previous estimates (Magallón & Hilu, 2009) also highlights two additional novelties of our analyses. Magallón & Hilu (2009) suggested that previous estimates for the establishment of the major plant lineages before seed plants (nodes 1–5) were falling in two clusters, a Precambrain cluster and an Ordovician–Devonian cluster. Thus, one novel insight from our main analysis (Fig. 7) is that it bridges the gap between the two clusters. The second insight is that, whereas both previous clusters suggested that the time difference between the origins of crown land plants and crown Euphyllophyta is *c.* 70 Myr, our analysis suggests a diversification that is far more gradual, spanning 236 Myr.

We have no doubt that a number of our palaeobotanical colleagues will consider untenable a Precambrian origin of Embryophytes and Stomatophytes, an origin of total group Tracheophytes that predates the Middle Ordovician, and a Triassic-Early Jurassic origin of crown Angiosperms. However, it is important to note that, because our analyses were constrained by fossil minima and maxima, our molecular clock estimates are fully compatible with the available palaeobotanical data. We hope that the justified procedure presented here will stimulate debate regarding the minima and maxima most suitable for calibrating land plant evolution. New fossil discoveries and further anatomical and systematic work on known fossils, together with a better understanding of anatomical character evolution in living plants, will surely lead to revision of the 17 calibrations presented here. This work will serve to test our findings and ultimately lead to further progress in creating a realistic time-line for plant evolution, so that the benefits from an accurate evolutionary time-scale can be realized.

Conclusions

Bayesian molecular clock methods can now accommodate calibrations of rate as constraints, an approach that better reflects the nature of fossil data. Establishing hard minimum and soft maximum constraints requires justifying the phylogenetic position of fossils critical to calibration, as well as their age assignment. This requires the reinterpretation of palaeobotanical data with much great transparency so that the impacts of new phylogenetic hypotheses, of new fossil discoveries, and of new interpretations of the ages of fossil-bearing strata can be determined and the calibration constraints on molecular clock analyses revised.

We have devised a set of calibration constraints for 17 nodes in plant phylogeny that represent the divergences between species whose genomes have been sequenced and, thus, for which the majority of molecular sequence data are available and on which the majority of molecular clock analyses are conducted. These are presented as exemplars of the quality and clarity with which molecular clock calibrations should established. Because not just the quantity but the quality of these constraints needs to be considered, methods to assess quality such as cross-validation remain a valuable area for future work, and appear to have provided some illumination here in relation to the Tracheophyta and Euphyllophyta calibrations.

We have shown that the specification of prior probabilities on the timing of divergence relative to fossil minimum and maximum constraints has a pervasive impact upon molecular clock estimates. Arbitrary nonuniform prior probabilities should not be used. Approaches for deriving nonuniform prior probabilities from fossil occurrence data are in their infancy but offer the best hope of obtaining both accurate and precise molecular clock estimates. In the interim, nonuniform priors bounded by conservative hard minimum and soft maximum constraints offer an approach to calibration that makes best use of fossil data in molecular clock analyses.

Our molecular clock analyses provide a time-scale of land plant evolution that is constrained to be compatible with the available palaeobotanical data and yet is very different from the literal reading of the fossil record used in explanations of, for instance, biogeochemical evolution of the planet. We reject a post-Cambrian origin of land plants and a post-Jurassic origin of angiosperms.

These hypotheses on the timing and tempo of land plant evolution can and will be tested through new fossil discoveries, refinements in our interpretation of the anatomy of known fossils, revised phylogenies and reinterpretations of the ages of fossils critical to constraining the calibration of future molecular clock analyses.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Taxa used and GenBank accession numbers for markers collected

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