

An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants

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We present molecular dating analyses for land plants that incorporate 33 fossil calibrations, permit rates of molecular evolution to be uncorrelated across the tree, and take into account uncertainties in phylogenetic relationships and the fossil record. We attached a prior probability to each fossil-based minimum age, and explored the effects of relying on the first appearance of tricolpate pollen grains as a lower bound for the age of eudicots. Many of our divergence-time estimates for major clades coincide well with both the known fossil record and with previous estimates. However, our estimates for the origin of crown-clade angiosperms, which center on the Late Triassic, are considerably older than the unequivocal fossil record of flowering plants or than the molecular dates presented in recent studies. Nevertheless, we argue that our older estimates should be taken into account in studying the causes and consequences of the angiosperm radiation in relation to other major events, including the diversification of holometabolous insects. Although the methods used here do help to correct for lineage-specific heterogeneity in rates of molecular evolution (associated, for example, with evolutionary shifts in life history), we remain concerned that some such effects (e.g., the early radiation of herbaceous clades within angiosperms) may still be biasing our inferences.

divergence times | rates of molecular evolution | angiosperms | eudicots | land plants

Our understanding of the history of life depends critically on knowledge of the ages of major clades. The timing of land plant evolution is fundamental to the interpretation of earth history and macroevolution throughout the Phanerozoic. Age estimates bear directly on our interpretation of the tempo and mode of morphological and molecular evolution of plants themselves, but also on our interpretation of the evolution of many other groups. For example, the age of origin of the angiosperms has variously been related to the evolution of other plant lineages (e.g., ferns) (1) and biomes (e.g., tropical forests) (2), as well as to the major insect clades and their feeding habits (3–7), and even to the evolution of fungi (8, 9) and dinosaurs (10).

In plants, as in other major groups (e.g., mammals) (11), the ages of clades estimated from molecular phylogenetic analyses have not always corresponded well with the accepted fossil record. In particular, the application of molecular-clock methods has tended to yield older dates (12), in some cases much older than has seemed credible based on the stratigraphic record (13–15). The approaches used in molecular dating have been problematical for several reasons, and in some cases the results have been too easy to dismiss. Early attempts used a strict molecular clock (e.g., ref. 16). Recently, so-called “relaxed clock” methods have been used, which variously allow departures from clock-like behavior (17–20), but here too there are difficulties. First, molecular dating analyses have tended to treat the tree topology as complete and fixed, as opposed to taking into account phylogenetic uncertainties (21–25). For angiosperms this is problematical, considering the limited confidence we still have in the order of branching early in their diversification (24, 26).

Second, the information provided by fossils is often treated as fixed. Uncertainties associated with fossil calibrations are inherent, given the nature of fossilization (27), the difficulty of dating

fossil localities, and the standard logic used in placing fossils into phylogenies to obtain minimum ages for lineages (28). One concern is the default practice of assigning fossils to the stem of the most inclusive crown clade to which they probably belong, thereby possibly biasing estimated ages (possibly throughout the tree) to be younger (29). In many cases, estimates have relied on lower bounds, based on what is assumed to be a tightly constrained fossil record. In angiosperms, the origin of eudicots, marked by the appearance of tricolpate pollen in the Late Barremian-Early Aptian (~125 Myr), has widely been used as a hard constraint (i.e., maximum age) to either calibrate or assess angiosperm molecular divergence times (21–25). However, using the first appearance of tricolpate pollen as a fixed calibration may underestimate the origin of eudicots and, by extension, other age estimates that have relied on this constraint. Tricolpate grains first appear in separated geographical areas and the grains themselves are not uniform in morphology (30–33), both observations implying that the tricolpate clade originated some time before its appearance in the fossil record. Although there may be value for some purposes in using the same fossil constraints in different studies, the reliance on the eudicot maximum-age constraint in multiple studies ties them all to the same underlying assumption, thereby compromising their independence.

Third, previous studies have relied on methods that have probably not accommodated sufficiently for heterogeneity in rates of molecular evolution (34). Rarely are datasets found to conform to a molecular clock, and broadly sampled plant phylogenies are no exception (22, 35, 36). Rates of evolution can vary among genes within a lineage, or among lineages. Incorporating data from multiple genes can help to compensate for rate heterogeneity across genes (23), but lineage-specific rate heterogeneity has not been adequately addressed and remains a potentially large source of error. Several analyses have demonstrated striking differences in molecular rates across large plant clades (36–39). These differences have been associated with traits such as growth habit, generation time, and population size, all of which are labile and might change multiple times along a branch, which will be especially difficult to detect when taxonomic sampling is sparse. When not accounted for, such rate heterogeneity can systematically bias slow-rate branches to appear younger and fast-rate branches to appear older (38, 40). Some methods for accommodating nonclock-like rates were designed to smooth differences across branches, under the assumption that rates are autocorrelated (17, 18): that is, they assume the inheritance of rate from parent node to child node. However, factors such as the evolution of life-history characteristics are expected to result in large differences between adjacent nodes, in which case autocorrelated methods are problematical (34). With the development of models of molecular evolution that are un-

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Table 1. Divergence-time estimates (in Myr) for major clades of land plants as estimated using 33 fossil calibrations (including the eudicot pollen calibration) and 32 fossil calibrations (excluding the eudicot pollen calibration)

Clade	With eudicot calibration	Without eudicot calibration
Land plants	477 (407–557)	474 (419–577)
<i>Tracheophyta</i>	432 (399–469)	434 (404–490)
<i>Spermatophyta</i>	327 (296–356)	330 (301–366)
<i>Acrogymnospermae</i>	301 (293–313)	302 (294–316)
<i>Gnetophyta</i>	155 (104–202)	158 (110–208)
<i>Angiospermae</i>	217 (182–257)	228 (193–270)
<i>Mesangiospermae</i>	174 (153–200)	184 (160–210)
<i>Magnoliidae</i>	155 (136–181)	163 (138–198)
<i>Monocotyledoneae</i>	156 (139–167)	164 (141–191)
<i>Eudicotyledoneae</i>	137 (128–147)	153 (138–172)

Dates within parentheses denote the 95% HPD.

Previous phylogenetic analyses have differed with respect to the relationships of *Amborella* and Nymphaeales to the rest of angiosperms (49) and among the magnoliids, monocots, and eudicots (24, 26). The most comprehensive analysis to date, based on 61 chloroplast genes, supported *Amborella* as sister to the rest of angiosperms but was uncertain regarding magnoliids, eudicots, and monocots (24). We conducted ML analyses on individual genes to help identify differential support for problematical relationships (Figs. S2, S3, and S4). The 18S sequences support Nymphaeales as sister to the rest of angiosperms, and weakly (51% BS) support the nonmonophyly of *Acrogymnospermae*, with cycads sister to angiosperms. *atpB* sequences support the monophyly of *Acrogymnospermae* and place magnoliids as sister to eudicots, but uncertainty remains as to the placement of *Amborella* and Nymphaeales in relation to the rest of the angiosperms. *rbcL* sequences do not clearly resolve the placement of magnoliids or monocots, and weakly support *Amborella* as sister to Nymphaeales (79% BS) and a monophyletic *Acrogymnospermae*. Our divergence time analyses take into account this uncertainty in the underlying tree topology.

Dating Analyses and Results. Our age estimates for divergences within land plants were based on 33 fossil calibrations (Table S2). The origin of land plants centered on 477 Myr (95% HPD: 407–557 Myr) (Table 1), which corresponds well with the earliest known occurrence of microfossils assigned to land plants from the middle Ordovician (~470 Myr) (50). The origin of tracheophytes was estimated at 432 Myr (95% HPD: 399–469 Myr), which corresponds to the middle Silurian. The first fossil fragments widely assigned to early *Tracheophyta* are also of Silurian age (~419) (51). We estimated the origin of *Spermatophyta* (crown seed plants) in the Middle Carboniferous (327 Myr; 95% HPD: 296–356 Myr), which broadly corresponds with the fossil record (52). A Middle Carboniferous age for crown seed plants follows the Devonian (>360 Myr) evidence of the “progymnosperm” lineages, Archaeopteridales and Aneurophytales (53, 54), and Early Carboniferous evidence of Paleozoic seed ferns (55). *Acrogymnospermae* (301 Myr, 95% HPD: 293–313) are estimated to have originated some 30 million years after crown seed plants.

We estimated the origin of crown angiosperms to be 217 Myr (95% HPD: 182–257 Myr), in the Late Triassic. This result was robust to the inclusion or exclusion of a 125 Myr minimum-age calibration on the node corresponding to crown eudicots (Table 1). A Late Triassic origin for crown angiosperms is typically not estimated with molecular methods. Of the several analyses carried out by Sanderson and Doyle (35), a few estimated a Late Triassic origin of crown angiosperms. However, this was sensitive to the underlying tree topology, codon position, and taxon sampling, with a majority of estimates falling instead between 140 and 190 Myr. Most molecular divergence-time analyses for crown angiosperms

have reported dates within this range of 140 to 190 Myr (reviewed by 34). For example, Bell et al. (22) and Magallón and Sanderson (23) estimated crown angiosperms to be 140 to 180 Myr (with Bayesian relaxed-clock methods) and 163 to 189 Myr (using penalized likelihood), respectively. More recently, Moore et al. (24) estimated the origin of crown angiosperms to be ~170 Myr using a data set of 61 plastid genes. Magallón and Castillo (25) reported an age of 130 Myr; however, this depended on a maximum age constraint applied to the crown corresponding to the oldest putative fossil angiosperm pollen from the Valanginian to Hauterivian (~130–140 Myr) (33).

Our age estimates for nodes within the *Mesangiospermae* suggest that crown magnoliids, monocots, and eudicots had all originated by the Late Jurassic (Table 1). Although the relationships among these three lineages remains uncertain, the short time interval between the origin of the corresponding crown groups suggests a rapid succession in the origin of the major angiosperms lineages. Although a Late Jurassic origin for magnoliids, monocots, and eudicots is much older than previous analyses have reported, it is important to note that we cannot reject an Early Cretaceous origin (the lower 95% HPD <144 Myr) (Table 1). When we removed the minimum age calibration for crown eudicots at 125 Myr, the estimates for the origin of crown magnoliids and monocots remained centered on the Late Jurassic, again with the lower 95% credibility interval encompassing the Early Cretaceous (Table 1). Taken together, our age estimates for the origin of major crown groups within mesangiosperms clearly predate the first putative angiosperm fossils (~130–140 Myr) (33).

Rate Heterogeneity. One possible explanation for the lack of correspondence between our molecular divergence estimate and the accepted fossil record is that we have failed to properly account for rate heterogeneity. Two metrics were used to evaluate the appropriateness of assuming a model of uncorrelated rates of molecular variation and to assess overall rate heterogeneity across the tree. First, the degree of autocorrelation of molecular rates from parent to child throughout the phylogeny was estimated through the covariance parameter. Across land plants, we estimated a rate covariance of $\rho = 0.074$, which was not significantly different from zero (95% HPD: –0.038 to 0.176). Although this result does not support the autocorrelation of rates, we note that the UCLN may be inadequate in detecting significant rate autocorrelation even when it exists (56). Second, we examined the coefficient of variation to assess the overall degree of rate heterogeneity across our tree. Assuming uncorrelated substitution rates, the coefficient of variation is the variance of rates scaled by the associated mean. A significant portion of the posterior density centered near zero is evidence that the data are “clock-like,” whereas a posterior density that does not encompass zero provides evidence for significant rate heterogeneity. The estimated

coefficient of variation indicated that the rate of molecular substitution varied by 69.7% (95% HPD: 63.2–76.3%) across the entire tree. This high degree of rate variation suggests an influence of rate heterogeneity.

It has been noted that nonsynonymous sites may show less rate heterogeneity than synonymous sites (37, 39, 57, 58). Different rates of evolution associated with different life histories may be more evident in synonymous sites. To examine whether the UCLN model had sufficiently accommodated for rate heterogeneity, we estimated divergence times using only the first and second codon positions for *atpB* and *rbcL*, which focused rate and date estimates on nonsynonymous sites. In this analysis, only the divergence time for land plants changed significantly, with the coding data supporting a slightly younger date of 442 Myr (95% HPD: 401–514 Myr) (Table S3). Importantly, the coefficient of variation indicated that significant rate heterogeneity remained, as the substitution rate varied 67.6% from the mean (95% HPD: 59.5–75.8%). Thus, although using only first and second sites may remove some bias caused by rate heterogeneity, it certainly does not eliminate it (39), and this is presumably true not just for the UCLN method but for methods that assume autocorrelated rates.

To explore whether the evolution of different life histories might help to explain shifts in the rate of molecular evolution (38, 39, 58), and specifically to test whether a shift to the herbaceous habit at the base of the angiosperms (59) could explain the long branch subtending crown angiosperms, we reconstructed growth habit over the posterior distribution of dated trees (Fig. 1B). We found an 88.6% and a 97.4% posterior probability of “woody” being the ancestral state for crown angiosperms and crown seed plants, respectively. This finding supports the results of Feild et al. (60, 61), who also argued on the basis of such reconstructions coupled with physiological data that the first angiosperms were woody plants living in “dark and disturbed” environments. Here it is relevant that fossil lineages inferred to be along the stem subtending the angiosperms, such as the glossopterids, Caytoniales, and Bennittitales (62, 63; but see ref. 64), are considered to be woody. These observations work against a simple argument that a shift to herbaceous habit along the line leading to angiosperms resulted in a faster rate of molecular evolution and, hence, to the inference that crown angiosperms are much older than they actually are. However, it is important to appreciate that the length of the branch leading to crown angiosperms spans ~100 Myr, which would allow many potentially confounding and undetected changes in life history and population size. Of course, the long branch subtending the angiosperms may not entirely reflect a faster rate of molecular evolution; it may simply indicate high extinction along that branch. We return to these concerns below.

Use of First Occurrence of Tricolpate Pollen. Tricolpate pollen grains (and derivative conditions) characterize modern eudicots, and the appearance of such grains in the fossil record is taken as evidence of the existence of the eudicot lineage, if not the crown clade. The first appearance of tricolpate pollen grains at the Barremian-Aptian boundary (~125 Myr) has commonly been used as a fossil calibration in divergence-time analyses. Whether placed along the stem or at the crown of the eudicots, this tricolpate pollen calibration has primarily been treated as a maximum-age constraint (22–25, 36, 65). This treatment could be justified based on the observation that the pollen record is substantial and that tricolpate grains, which are easy to identify, have not been recovered from any earlier sediments. It may be possible in this case to infer a likely maximum age using the statistical methods proposed by Marshall (66). In the meantime, several aspects of the appearance of tricolpate grains in the fossil record suggest that 125 Myr may not be an appropriate maximum age for eudicots. The first tricolpate grains have the same aperture configuration (tricolpate, not tricolporoidate, or triporate), but show “considerable structural variety” (33) in the sculpturing of the exine. In addition, the

Barremian-Aptian tricolpate pollen localities are geographically widespread, first at several Gondwanan sites (present-day northern and equatorial Africa), with specimens becoming more common in Laurasia during the Aptian-Albian (Fig. 1C) (30, 31). Based on these observations, it is possible that the appearance of tricolpate grains reflects the rise to dominance of the eudicot lineage as opposed to the origin of the group. Finally, it is not clear whether these pollen grains represent the emergence of the tricolpate apomorphy along the branch leading to crown eudicots or whether they represent the appearance of modern lineages of eudicots (i.e., within the crown). There are too few characters to place them with any certainty within the eudicot phylogeny (33). In view of these caveats, we favor the use of 125 Myr as a minimum age for the origin of the eudicot crown clade, with an associated probabilistic prior (67).

Reexamining Biological Patterns. Of particular interest for botanists and entomologists is the possible correlation of the early evolution of angiosperms with the rise of the major lineages of holometabolous insects (Coleoptera, Hymenoptera, Diptera, and Lepidoptera). Labandeira and Sepkoski (3) noted that a number of major insect radiations date to the late Permian (~254 Myr), with trophic diversity proliferating dramatically during this period. Based on the apparent incongruity with the angiosperm fossil record, they concluded that angiosperms had little impact on the early evolution of holometabolous insects.

Our results, taken at face value, push the origin of the angiosperm crown clade much closer in time to the diversification of the major lineages of holometabolous insects. Molecular estimates for the origin of Coleoptera (285 Myr) (5) predate crown angiosperms, but the origin of the most diverse herbivorous lineages of Coleoptera (i.e., Chrysomeloidea, Curculionoidea) are estimated to be ~230 Myr, which is in the range of our age estimate for crown angiosperms (68). Also congruent are Triassic fossils of Diptera and Hymenoptera (68, 69). Molecular age estimates for the origin of Lepidoptera, as well as for the ant and bee clades nested within Hymenoptera, correspond well with our age estimates for the major crown clades within *Mesangiospermae* (7, 70, 71), as does the fossil record of long-proboscid Mecoptera (72). However, it is important to note that even if our inferred dates were correct, the absence of clear-cut angiosperm fossils during the Triassic and Jurassic may signify that the first angiosperms were not abundant, widespread, or ecologically very significant, in which case it would be difficult to argue that the appearance of the angiosperms dramatically increased insect diversity during that time period.

Conclusions

Regarding the tempo of plant evolution, our results show generally good correspondence with the fossil record (e.g., for crown tracheophytes and seed plants). However, they also imply that crown angiosperms originated in the Triassic (or possibly in the Jurassic), well before the Cretaceous radiations that were responsible for the dramatic rise of the angiosperms. That is, they suggest that crown angiosperms were in existence for some 50 Myr (or more) before the radiation of the mesangiosperms, and some 60 Myr (or more) before the diversification of monocots and eudicots. The only living remnants of the lineages that existed in this inferred interval are *Amborella*, Nymphaeales/Hydattellales, and Austrobaileyales. Today, these lineages are species-poor, but they exhibit tremendous morphological and ecological disparity. One possibility is that these groups were once much more diverse, and that we are left today with only a few survivors. In this case, it may be that most of these plants lived in environments that were not conducive to fossilization. However, it is also possible that angiosperms were simply not diverse or ecologically dominant plants during the Late Triassic and Jurassic. For example, as Feild et al. (60, 61) have argued, the physiology and ecological prefer-

ences of the early angiosperms (living in dark, wet, and disturbed understory habitats, probably with low population sizes) may have restricted their abundance, geographical spread, and diversification. These same factors might also account for the lack of fossils during this interval.

Regarding eudicots, our results suggest that the first appearance of tricolpate grains at ca. 125 Myr underestimates the origin of the tricolpate clade by perhaps 3 to 22 Myr. This finding is problematic because the record of fossil pollen is judged to be very good through this time period (33). If our inferences are correct, the appearance of tricolpate grains may not signal the origin of the crown group, but rather the rise in abundance and geographic expansion of the tricolpate lineage.

Dismissing our angiosperm date as an artifact will be tempting. However, as the date reflects the current state of knowledge of fossils and phylogeny, as well as the current state of development of relevant analytical tools, we believe that these dates should not be set aside lightly. Yet, we hasten to acknowledge that our analysis is unlikely to be the final word on the subject and, moreover, there are several reasons to proceed cautiously. Perhaps most importantly, we remain concerned about the impacts of lineage-specific rate heterogeneity on molecular age estimates, despite having tried to accommodate this. It is increasingly clear that there may be extreme differences in molecular rate depending upon life history and other factors (39), and current methods may be unable to cope. It is possible that the effects of lineage-specific rate heterogeneity can “trickle-down” to nodes at some distance from the inferred shift in life history and molecular rate. For this reason we are concerned that, although a shift to herbaceousness may not have marked the origin on the angiosperms, multiple shifts to the herbaceous habit not far within angiosperm, followed by several rapid radiations, might result in an older age estimate for angiosperms as a whole. This possibility needs to be explored further using simulations and also suggests the need to develop methods (akin, perhaps, to so-called local-clock methods) (20) that allow shifts to different rate categories as a function of evolutionary shifts in an underlying parameter that might drive rate changes, such as life history or population size.

It is interesting to reflect, however, that as older fossils are discovered and incorporated into various lineages, this will tend to shift the angiosperm date back further in time. Furthermore, as our taxonomic sampling improves, and as knowledge of fossils increases to the point of allowing us to place them within clades with greater precision, there may be a general tendency for these to be placed further up within the clades with which they are associated. This process will also tend to push the age of crown angiosperms further back in time. It is possible that a closer match between molecular inferences and the stratigraphic record will eventually be obtained, as dating methods are improved to cope with extreme rate heterogeneity and as older fossils are discovered. However, it is also possible that a significant gap will remain and, if so, this might tell us something important about the rise of flowering plants.

Materials and Methods

Phylogenetic Analyses. Sequences for each gene region were aligned separately with MUSCLE (73) within three partitions: angiosperms, acrogymnosperms, and the remaining green plants. These separate alignments were then combined using profile alignment techniques (73; see also ref. 74), and the aligned gene regions were concatenated using Phyutility (75).

Maximum-likelihood analyses were conducted with RAxML (Ver. 7.0.1) (76). Runs were partitioned into gene regions with parameters unlinked. We used

the GTRMIX substitution and rate heterogeneity model. ML analyses were conducted by first running 100 rapid bootstrap analyses; every tenth bootstrap tree was used as a start tree for a full ML search. The best tree from those searches was considered to be the ML tree. Bootstraps were summarized with Phyutility (75). We conducted these analyses on the concatenated dataset as well as on the individual gene regions (Figs S1, S2, S3, and S4).

Bayesian phylogenetic analyses were conducted with MrBayes (Ver. 3.1.2) (77, 78) using the Metropolis coupled Markov Chain Monte Carlo (MCMC) algorithm. Two analyses, each consisting of four incrementally heated chains, were run for 10 million generations, sampling every 1,000th tree. The posterior distribution of trees was summarized after removing 1 million generations as burn-in. A GTR+ Γ model was applied to each gene region and the associated parameters were unlinked. Posterior distributions for parameter estimates and likelihood scores were visualized in Tracer (Ver. 1.4) to approximate convergence.

Divergence-Time Estimation. Simultaneous divergence-time and phylogenetic analyses were conducted using MCMC methods implemented in BEAST (Ver. 1.4.7; 42). BEAST employs an uncorrelated relaxed-clock (UCLN) model to estimate divergence times and allows topologies to be considered “fixed” or estimated to accommodate for phylogenetic uncertainty (41, 42). Here, we allowed BEAST to estimate the topology. For each branch, the UCLN independently draws substitution rates from a lognormal distribution, allowing substitution rates to be uncorrelated across the phylogeny. The absolute estimates of divergence times are then calculated from fossil calibrations, each with an associated probabilistic prior. We attached a lognormal prior probability (67) to the minimum-age estimates obtained from 33 fossil calibrations (using the International Commission on Stratigraphy 2007), 27 of these for crown clades within angiosperms (Table S2). The mean and standard distributions of these calibrations were chosen to acknowledge that, although the fossil age represents the minimum age of the lineage, there remains a probability that the true age extends (in most cases ~10–15 Myr) further back in time.

Our divergence-time analyses were carried out using two partitioning strategies. The first partitioned the data by gene region (*atpB*, *rbcl*, and 18S), with the rate parameters unlinked and assuming a GTR+ Γ substitution model. The second partitioned the first and second codon positions of *atpB* and *rbcl* only. Again, the parameters were unlinked and we assumed a GTR+ Γ substitution model. For each partitioning strategy, we initiated five separate MCMC chains, each consisting of 10 to 50 million generations with convergence monitored by Tracer (Ver. 1.4). We determined the number of runs to conduct based on the effective sample sizes of each estimated parameter, where we required the posterior, prior, and likelihood to be at least 200. We heuristically removed a percentage of each run as burn-in and the resulting trees for each replicate were combined. Trees were summarized with TreeAnnotator and represent the maximum clade credibility tree. Ninety-five percent HPD were estimated using the R package (79) Bayesm (Ver. 2.2–1).

Ancestral Life-History Reconstructions. We used the Bayesian implementation of the program MultiState in BayesTraits (80) to reconstruct the probable life-history of crown seed plants and crown angiosperms across the posterior distribution of dated trees. Multistate implements a reversible-jump MCMC procedure for single multistate characters. Predominantly herbaceous clades were scored as 0, and predominantly woody clades as 1; any clade for which ancestral life form was judged to be equivocal was scored as missing data. We used an exponential hyperprior on the rate coefficients and sampled every 1,000th point from 10 million total generations. We discarded the first 2.5 million iterations as burn-in. The probability distributions obtained from the reversible-jump MCMC were examined using Tracer (Ver. 1.4).

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