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Title: Molecular Dating, Evolutionary Rates, and the Age of the Grasses

Pascal-Antoine Christin[1,2], Elizabeth Spriggs[2], Colin P. Osborne[1], Caroline A.E. Strömberg[3], Nicolas Salamin[4,5], Erika J. Edwards[2]

[1] *Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK.*

[2] *Department of Ecology and Evolutionary Biology, Brown University, 80 Waterman St., Providence, RI 02912, USA.*

[3] *Department of Biology & Burke Museum of Natural History and Culture, University of Washington, Seattle, Washington 98195, USA.*

[4] *Department of Ecology and Evolution, Biophore, Quartier Sorge, University of Lausanne, 1015 Lausanne, Switzerland.*

[5] *Swiss Institute of Bioinformatics, Quartier Sorge, 1015 Lausanne, Switzerland.*

Corresponding author: Pascal-Antoine Christin, Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK; phone +44 114 222 0034; fax +44 114 222 0002; p.christin@sheffield.ac.uk

Abstract

Many questions in evolutionary biology require an estimate of divergence times but, for groups with a sparse fossil record, such estimates rely heavily on molecular dating methods. The accuracy of these methods depends on both an adequate underlying model and the appropriate implementation of fossil evidence as calibration points. We explore the effect of these in Poaceae (grasses), a diverse plant lineage with a very limited fossil record, focusing particularly on dating the early divergences in the group. We show that molecular dating based on a dataset of plastid markers is strongly dependent on the model assumptions. In particular, an acceleration of evolutionary rates at the base of Poaceae followed by a deceleration in the descendants strongly biases methods that assume an autocorrelation of rates. This problem can be circumvented by using markers that have lower rate variation, and we show that phylogenetic markers extracted from complete nuclear genomes can be a useful complement to the more commonly used plastid markers. However, estimates of divergence times remain strongly affected by different implementations of fossil calibration points. Analyses calibrated with only macrofossils lead to estimates for the age of core Poaceae around 51-55 Ma, but the inclusion of microfossil evidence pushes this age to 74-82 Ma and leads to lower estimated evolutionary rates in grasses. These results emphasize the importance of considering markers from multiple genomes and alternative fossil placements when addressing evolutionary issues that depend on ages estimated for important groups.

Key Words: divergence time, phylogeny, molecular dating, mutation rate, Poaceae

In the absence of an exceptionally good fossil record, divergence times must be inferred from genetic markers. The accumulation of genetic mutations is not linear with respect to time, and potential variation in rates of mutation accumulation must be taken into account when inferring lineage divergence dates (Magallon 2004). Several sophisticated methods are now available that consider potential variation in evolutionary rates across the phylogeny by implementing so-called relaxed molecular clocks (Kishino et al. 2001; Drummond et al. 2006; Lepage et al. 2007; Ho 2009). Often, however, there is a low number of fossil calibration points relative to a large number of species (and thus nodes in the phylogeny). The informativeness of any fossil depends largely on the accuracy of its assignment to a taxonomic group (Magallon 2004; Parham et al. 2012). Dating methods can thus be strongly influenced by both the assumptions of the underlying models and the uncertainties around the incorporation of fossil evidence (Ho et al. 2005; Hug and Roger 2007; Battistuzzi et al. 2010; Lukoschek et al. 2012; Sauquet et al. 2012). The most commonly used methods differ mainly in how rate variation is modeled and, in particular, whether or not they assume autocorrelation of rates (Kishino et al. 2001; Drummond et al. 2006). Investigation into the appropriateness of rate autocorrelation has been inconclusive, yielding contrasting results depending on the datasets and methods used (Drummond et al. 2006; Lepage et al. 2007).

In this study, we explore the effect of variation in rates of mutation, fossil placement, and model assumptions on divergence time estimation, with the goal of inferring the age of the grasses (Poaceae; monocots). This diverse and ecologically important plant lineage of more than 11,000 species includes the world's major crops, such as rice, wheat, and maize, and natural grasslands cover large regions of the world's terrestrial land surface (e.g. Gibson 2009; Edwards et al. 2010). The vast majority of grass species belongs to two large sister groups referred to as BEP and PACMAD clades (Grass Phylogeny Working Group II 2012). Previous dating analyses of Poaceae have typically included only a limited

number of taxa outside the focal group (Vicentini et al. 2008; Bouchenak-Khelladi et al. 2009; Prasad et al. 2011). Meanwhile, molecular dating analyses of angiosperms (flowering plants) are abundant in recent literature and, despite differences in methodology, independent estimates converge on a date for the split between the two major groups of flowering plants (eudicots and monocots) between roughly 130 and 170 Ma (Bell et al. 2010; Magallon 2010; Smith et al. 2010). While studies focused on grasses estimated an origin of the BEP-PACMAD clade between 52 and 86 Ma (Vicentini et al. 2008; Bouchenak-Khelladi et al. 2009; Prasad et al. 2011), angiosperm-wide dating projects have inferred a very recent origin for this same clade, between 23 and 39 Ma (Bell et al. 2010; Magallon 2010; Arakaki et al. 2011; Magallon et al. 2013). The incongruence between large-scale phylogenetic analyses including a few representatives of Poaceae and densely sampled analyses focused on Poaceae likely results from important variation in rates of evolution between grasses and other angiosperms (Gaut et al. 1992; Graham and Olmstead 2000; Guisinger et al. 2010). New insights into this problem might be gained from analyses of markers from different genomes that consider fossil evidence within Poaceae as well as in distant lineages.

We performed divergence time analyses of different datasets of plastid and nuclear genetic markers, sampling broadly from across all angiosperms. The ages obtained for the major clades of grasses by different methods and genetic markers were compared to the known fossil record. The influence of a divergent calibration point, represented here by the most recently published phytolith fossils (Prasad et al. 2011), on the inferred ages of the major angiosperm clades and the heterogeneity of evolutionary rates was also evaluated. The conflicts between different sets of calibration points, methods and genomes highlight the importance of considering multiple sources of evidence when attempting to estimate evolutionary events that happened in distant geological time.

METHODS

Plastid Dataset

Dating analyses were first conducted on DNA regions from the plastid genome, which are the most frequently used in plant phylogenetics and are available for a large number of taxa (Soltis et al. 2011). We selected three genes that are variable enough to reconstruct relationships within lineages but are also sufficiently conserved to be compared among distantly related angiosperms (Grass Phylogeny Working Group II 2012). These three markers are coding regions of the genes for ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*), maturase K (*matK*) and NADH dehydrogenase subunit F (*ndhF*). Poaceae sequences were retrieved from a published dataset that includes 545 taxa (Grass Phylogeny Working Group II 2012). To allow additional calibration points and the comparison of evolutionary rates among all angiosperms, taxa outside the grasses were added to this initial dataset as follows: the three selected coding genes were first retrieved from complete plastid genomes available in NCBI database; then additional taxa were added that had available sequence data for all three plastid regions such that the complete dataset contained representatives for most angiosperm orders and most monocot families.

The whole dataset was aligned with MUSCLE v3.6 (Edgar 2004) and the alignment was manually refined. Variable length segments that were ambiguously aligned were manually deleted. Only 155 grasses from the original dataset were selected as follows: taxa were first discarded if the sequences were complete for less than 4900 bp (of a 4973 bp long alignment after removing the ambiguously aligned regions), a threshold that retained representatives of all subfamilies; Poaceae taxa were further randomly removed from clades that contained numerous highly similar sequences (e.g. multiple accessions for the same species or several closely related species).

The final alignment included 245 taxa sampled from across the angiosperm phylogeny (155 grasses and 90 other angiosperms) and was 99.4% complete. For comparative purposes, the same topology was used for all dating analyses (Fig. 1). In this topology, the relationships inside Poaceae were constrained to match the topology previously obtained with 545 taxa (Grass Phylogeny Working Group II 2012) and relationships among angiosperms outside Poaceae were set to those inferred with 640 taxa and 17 concatenated genes (Soltis et al. 2011), or for monocot species not included in the latter paper to those inferred for 83 angiosperms based on 81 plastid genes (Givnish et al. 2010). Members of the Nymphaeales were used as the outgroup (removed during MULTIDIVTIME dating analysis and manually removed before using other software).

Nuclear Genes Extracted from whole Genomes

To construct our nuclear dataset, we focused on completely sequenced nuclear genomes of plants, which were screened for markers that can be compared across angiosperms. Although including sequenced transcriptomes would have allowed us to include a larger number of species, gene representation is generally sparse in transcriptomes, and numerous sequences are incomplete, hampering accurate phylogenetic reconstructions. Predicted gene coding sequences (cDNAs) from 26 complete nuclear genomes of angiosperms were downloaded from Phytozome (Goodstein et al. 2012; accessed on the 9th of February 2012). This included five grasses and 21 eudicots. The genome of the lycopod *Selaginella* was also downloaded and used as the outgroup. *Selaginella* is the closest relative of angiosperms that has been completely sequenced. It is a very distant outgroup, and was only used to root the ingroup in MULTIDIVTIME and was removed in downstream analyses. It was not used at all in BEAST or PHYLOBAYES analyses. In addition, the assembly 3.0 from *Phoenix dactylifera* (Arecaceae) was downloaded from Weill Cornell Medical College website (http://qatar-

weill.cornell.edu/research/datepalmGenome/download.html; accessed on the 9th of February 2012), to reach a total of 27 angiosperms plus *Selaginella*.

In order to obtain phylogenetically useful markers, we generated datasets composed of one predicted transcript per taxon that presented sufficient similarity for preliminary phylogenetic evaluation. Plant nuclear genes undergo a high number of gene duplications followed by gene losses in some lineages, which complicates the assessment of orthology, a necessary assumption in phylogenetic analyses. The BLAST algorithm (Altschul et al. 1990) can identify sets of similar sequences from different genomes, but in several instances, it returns matches that are not truly homologous, or matches that represent a different paralog. These were discarded after an assessment of orthology through phylogenetic analysis of datasets that passed a number of successive quality controls, which are described below.

Each predicted transcript (considering only one transcript model per gene) from the *Sorghum* genome, used here as the reference genome, was successively used as the query of a BLAST search against each of the other genomes with the program blastn and an e-value threshold of 0.001. Only the markers from *Sorghum* that had at least one positive match in all of the other genomes were further considered. Each of these was used again as the query of a BLAST search against the genomes of the 26 other angiosperms with an e-value threshold that was raised to 10 to increase the length of the compared region. Only the best matching region returned by the BLAST search was considered, which removed segments of the predicted cDNA that were highly divergent between distantly related taxa and would be poorly aligned. These BLAST matches were assembled in a dataset (one per *Sorghum* marker), which was then aligned using MUSCLE. TRIMAL (Capella-Gutierrez et al. 2009) was used to remove the parts of the alignment present in less than 90% of the sequences, maintaining a very low proportion of missing data. At this stage, matrices were discarded if the total alignment was smaller

than 200 bp or the smallest sequence was smaller than 100 bp. A phylogenetic tree was inferred for each of the remaining single-gene matrices using PhyML (Guindon and Gascuel 2003) under the substitution model deemed adequately parameter-rich for each dataset using likelihood ratio tests done with PhyML while fixing the topology to that inferred under a HKY model. Orthology was assessed by comparing the inferred topology with the expected species tree (based on Soltis et al. 2011 concatenated analysis) using the S-H topology tests (Shimodaira and Hasegawa 1999) as implemented in Baseml. All the datasets that rejected the species tree (p-value < 0.05) were discarded, with the assumption that they might contain different paralogs, non-homologous genes, or other problematic sequences. An accurate estimation of the p-value by the S-H test theoretically requires that a large pool of plausible trees be sampled (Goldman 2000), which is not the case here. The selected datasets might consequently include some false negatives, especially in the case of closely related paralogs. The test however represents a rapid way to compare topologies for a large number of datasets and to identify most cases of paralogy problems. Differences between nuclear and plastid phylogenies can also be caused by incomplete lineage sorting or hybridization, but with 27 species spread so broadly across angiosperms, the resulting topological differences would be small if existent at all (Maddison and Knowles 2006), and topology tests would likely not be significant. On the other hand, significant topological differences due to lateral gene transfer between distantly related species cannot be differentiated from paralogy problems without a careful evaluation of the gene diversity present in diverse genomes (see Christin et al. 2012). Our approach removes such sequences and is consequently conservative. The remaining alignments were assumed to be composed of only co-orthologs (*sensu* Sonnhammer and Koonin 2002) and were used for dating analyses. The topology corresponding to the expected species tree based on Soltis et al. (2011) was used for all dating analyses (Fig. 1).

Of the 27,608 coding sequences predicted from the *Sorghum bicolor* genome, 3,180 had a homolog in all of the 27 other plant genomes. After removing all the alignments that were too short (2,165 datasets) or that produced phylogenies incompatible with the species tree (826 datasets), a total of 189 datasets were retained. Of these, five were further removed because they represented duplicates that arose in the ancestor of *Sorghum* after the diversification of Poaceae (they matched the same loci as other *Sorghum* markers in at least some other grasses). The final dataset included 184 loci for a total of 83,851 aligned bp.

Molecular Dating

Each dataset was analyzed with two sets of calibration points (see below) and with four different methods. These methods all use a Bayesian procedure and allow for rate variation among branches of the phylogenetic tree, but they differ in their assumptions. In the method implemented in MULTIDIVTIME (Thorne et al. 1998; Kishino et al. 2001), rates are autocorrelated along the phylogenetic tree while in the procedure implemented in BEAST, rates are uncorrelated (Drummond et al. 2006; Drummond and Rambaut 2007). In addition to differences in the implemented molecular clock models, BEAST and MULTIDIVTIME differ in the models used for priors and the available nucleotide substitution models. To ensure that these differences were not responsible for variation in the results, we also used PHYLOBAYES, a program that can compare uncorrelated and autocorrelated models while keeping everything else constant (Lartillot et al. 2009).

For analyses using BEAST, two independent MCMC tree searches were run for 20,000,000 generations, with a sampling frequency of 3,000 generations after a burn-in period of 5,000,000. The GTR substitution model with a gamma shape parameter and a proportion of invariants (GTR+G+I) was used, being the adequately parameter-rich model for all datasets, identified through hierarchical

likelihood ratio tests. The adequacy of the length of the analysis and burn-in period was confirmed using Tracer (Rambaut and Drummond 2007) through a visual inspection of the traces for the tree likelihood and the substitution model parameters and checking that their ESS was larger than 100. The prior on the distribution of node ages was approximated by a Yule speciation process and evolutionary rates among branches by followed a log-normal distribution. For computation purposes, the time-calibrated tree obtained with MULTIDIVTIME (see below) was set as the starting tree. The topology was kept constant throughout the analyses, which was necessary to directly compare results across multiple software programs, models, and priors. The different markers were concatenated into a single plastid and a single nuclear dataset, which were first used without data partitioning. Additional BEAST analysis of the plastid and genome datasets allowed different substitution model parameters for 1st, 2nd and 3rd positions of codons, which did not significantly alter the results (Supplementary Fig. 1; available on Dryad). For all analyses, ages and rates were computed as the median across the set of sampled trees. In addition, standard deviations were calculated to obtain estimates comparable across software packages.

For PHYLOBAYES, two parallel analyses were run for ten days (minimum of 6,600 cycles with the nuclear dataset and an uncorrelated gamma model) on the Vital-IT computer cluster (based on Intel Xeon architecture with up to 16 cores, 2.5 to 3.4 GHz and 2 to 4 BG RAM per core), under a GTR+G model with uniform prior of divergence times. Both the uncorrelated gamma (similar to BEAST) and correlated log-normal (similar to MULTIDIVTIME) models were used. The analyses were also done with the correlated CIR model (Lepage et al. 2007), but the results were highly similar to the correlated log-normal model and are not discussed separately. Ages were retrieved from the sampled trees, with a burn-in period of 1,000 cycles and a sampling frequency of 10 cycles. In addition, the thermodynamic integration implemented in PHYLOBAYES was used to compare the fit of the

224 different models available in this software (Lartillot and Philippe 2006). The 'long' option was used.
225 Data partitioning is not implemented for relaxed clock models in PHYLOBAYES and so analyses were
226 performed on concatenated datasets only.

227 For MULTIDIVTIME, model parameters were first estimated with Baseml (Yang 2007), and
228 branch lengths and the variance-covariance matrix were then optimized by Estbranches (Thorne et al.
229 1998) under a F84+G model, which is the most complex model implemented in this software. These
230 parameters were then used by MULTIDIVTIME to approximate the posterior distribution of rates and
231 divergence times on the concatenated dataset. The MCMC procedure was run for 1,000,000
232 generations, with a sampling frequency of 1,000 generations after a burn-in period of 100,000. Each
233 MULTIDIVTIME analysis was run with priors following the recommendations of Rutschmann (2005).
234 The effect of the prior was evaluated by rerunning the analysis under external calibration only (see
235 below) with different values for four priors. With the scale in twenties of million years ago, the mean
236 and standard deviation of the rate at the root were set successively to 0.01/0.1, 0.1/1 and 1/2. For each
237 of these combinations, the mean and standard deviation of the Brownian motion constant were
238 independently changed to the following values; 0.01, 0.1, 0.5, 1, 2, and 5. For these additional analyses,
239 the burn-in period was decreased to 10,000 generations and the sampling frequency and number of
240 samples to 100, to allow additional comparisons. Plastid and nuclear concatenated datasets were used
241 without data partitioning, but analyses were repeated with markers within each dataset (three and 184
242 for the plastid and nuclear datasets, respectively) treated as different loci. The sampling size for these
243 partitioned analyses was reduced to 1,000 trees sampled every 100 cycles after a burn-in period of
244 100,000 cycles. A comparison with the concatenated analyses indicated that the effect of the
245 partitioning was small (Supplementary Fig. 1).

To evaluate the effect of sampling density, the plastid dataset was reanalyzed with a species sampling similar to that of the nuclear genomes. Plastid sequences for 28 species that were identical or closely related to those in the nuclear dataset (Fig. 1) were used for molecular dating with BEAST and MULTIDIVTIME as described below. In addition, to evaluate the effect of sequence length, dating analyses were repeated with a number of nucleotides corresponding to the plastid dataset (4973) sampled without replacement from the nuclear dataset. One hundred pseudoreplicates were reanalyzed with BEAST and MULTIDIVTIME as described below, except that the number of generations was decreased to 10,000,000 with a sampling frequency of 1,000 after a burn-in period of 5,000,000 in BEAST and 100,000 generations sampling every 100 generations after a burn-in period of 1,000 with MULTIDIVTIME.

Primary Calibration Points

Dating analyses were run without taking into account Poaceae fossils, which were compared *a posteriori* to the ages inferred for various nodes within grasses (Table 1). The exclusion of Poaceae fossils as calibration points in the initial analysis allowed their later use to validate or invalidate the results of alternative dating hypotheses. Fossils with reliable dates and taxonomic placement for eudicots and non-grass monocots were used to set minimal ages on stem nodes of clades to which they have been previously assigned. To mirror the minimal and maximal bounds used by MULTIDIVTIME and PHYLOBAYES, calibration points in BEAST were implemented as a uniform distribution between the minimal age of the constraint and the maximal age of the root. These calibration densities are not equal to the marginal prior distributions, which are also influenced by the topological constraints and tree prior (Heled and Drummond 2012). BEAST analyses were first run without molecular data, which showed that the marginal prior distributions take non-uniform distributions when the topology is fixed

(Supplementary Fig. 2-5). Based on the review by Magallon and Sanderson (2001), minimal bounds were set at 77.4 Ma for the crown of Typhales, 83.5 Ma for the stem of Zingiberales, 77.4 Ma for Arecales, 45.15 for Liliales, 88.2 for Myrtales, 91.2 for Malpighiales, and 102.2 for Buxales. In addition, a minimal age of 125 Ma was set on the stem node of core eudicots, based on the appearance of tricolpate pollen in the fossil record (Friis et al. 2006). The appearance of tricolpate pollen was also used to set a maximal age for the crown of core eudicots at 135 Ma. The rationale behind this constraint is that, given the rich fossil record of pollen and the distinctive morphology of tricolpate pollen, it is unlikely that tricolpate pollen grains would be undetected for a long period of time after their evolution (Anderson et al. 2005). The use of maximal age constraints is controversial, but its absence can lead to unacceptably ancient divergence-time estimates (Hug and Roger 2007; Ho and Phillips 2009).

These nine constraints are congruent with each other (Christin et al. 2011) and were set simultaneously to run a first dating analysis (external calibration only) on the different markers. The maximal age of the root was set to 200 Ma, a time that exceeds the monocot/eudicot divergence in all recent dating analyses (Bell et al. 2010; Magallon 2010; Smith et al. 2010; Magallon et al. 2013). Not all of the calibration points listed above could be placed in the phylogeny based on markers from whole genomes or the reduced phylogeny based on plastid markers. Because of the reduced species sampling, the corresponding node was not present in these smaller phylogenies. Consequently, constraints on Buxales, Typhales, Liliales and Zingiberales were not used for these analyses.

A second calibration (external calibration plus phytoliths) was run on the plastid and nuclear datasets with the fossil evidence described above and the addition of phytoliths and attached cuticle (hereafter referred to simply as “phytoliths”) found in fossilized dinosaur dung from the Late Cretaceous (ca. 67-66 Ma; Prasad et al. 2005) of India and assigned to the Oryzeae tribe of the BEP clade of grasses based on morphological characters (Prasad et al. 2011). Phytoliths are microscopic

292 silica bodies precipitated in and around plant cells in many land plants that remain in the soil when
293 plants die and decay (Piperno 2006). The morphology of grass phytoliths varies among extant taxa,
294 suggesting that fossil phytoliths might be assigned to specific taxonomic groups and be informative
295 regarding the timing of speciation events (Prasad et al. 2005; Strömberg 2005; Piperno 2006; Prasad et
296 al. 2011). Fossilized phytoliths, and especially the associated cuticles, are relatively rare in ancient soils
297 and the described fossils are unlikely to represent the earliest appearance of the group. The 67 Ma
298 phytoliths fossils were consequently included as a minimal age on the stem of Oryzeae (last common
299 ancestor of *Oryza sativa* and *Microlaena stipoides*). In the nuclear genomes dataset, *O. sativa*
300 (Oryzeae) is the only representative of Ehrhartoideae and the minimal age of 67 Ma was consequently
301 set to the stem of Ehrhartoideae (last common ancestor of *O. sativa* and *Brachypodium distachyon*),
302 which likely underestimates the effect of this fossil evidence.
303

304 RESULTS

305 *Inferences from Plastid Markers*

306 Strong variation in branch lengths were present in the plastid phylogeny (Fig. 1). In particular, the
307 average length from the root to the tips of the BEP-PACMAD clade greatly exceeded that of branches
308 leading to most other monocots, including the other graminid lineages (*sensu* Givnish et al. 2010) that
309 split before the appearance of the BEP-PACMAD clade (Fig. 1). Based on the thermodynamic
310 integration method implemented in PHYLOBAYES, the uncorrelated gamma model seems to be a
311 better fit for the data although the 95% credibility intervals of natural logarithm of the Bayes factors for
312 the uncorrelated and correlated models overlap (Table 2).

In the absence of constraints inside Poaceae (external calibration only), BEAST estimated an age of 54.9 Ma (± 7.0) for the crown of the BEP-PACMAD clade (Table 3). The ages estimated by BEAST are compatible with the known macrofossils, but not with phytoliths attributed to Oryzeae, even if these are attributed to more ancient ancestors of Oryzeae (Table 1). BEAST estimated relatively low evolutionary rates for branches inside the BEP-PACMAD clade; however, it assigned very high rates to branches leading to the BEP-PACMAD crown and other graminids (Fig. 2; Supplementary Fig. 6). The highest value ($\mu = 4.1 \pm 1.7$ expected mutations per site per billion years) was assigned to the branch leading to the common ancestor of *Joinvillea* and Poaceae, and the second and third highest rates also occurred on graminid branches leading to the BEP-PACMAD clade (Fig. 1).

Compared to BEAST, PHYLOBAYES produced similar results when using the uncorrelated gamma model (Fig. 3 and Supplementary Fig. 6). By contrast, the correlated log-normal model implemented in the same software led to younger estimates for nodes within graminids, as well as older estimates for multiple nodes outsides graminids (Fig. 3; Table 3). These estimates were obtained by inferring evolutionary rates for graminids outside the BEP-PACMAD clade that are comparable to other clades and comparatively higher rates for nodes within the BEP-PACMAD clade (Supplementary Fig. 6). The results obtained under the similarly correlated model implemented in MULTIDIVTIME are comparable, but the difference is more extreme, with very young ages estimated for graminids and very high rates for nodes within the BEP-PACMAD clade (Fig. 3 and Supplementary Fig. 6; Table 3). If the prior for the standard deviation of the Brownian motion constant is very small (0.01), MULTIDIVTIME results are heavily dependent on the prior for the mean of the Brownian motion constant (Supplementary Fig. 7). We interpreted age estimates to be incompatible with the fossil record if the maximum credible age for a given node was younger than a known fossil belonging to that clade. Results obtained by PHYLOBAYES under the uncorrelated model are generally compatible with fossil

evidence, with the exception of the 67 Ma phytoliths, unless these are assigned to the stem of the BEP clade (Table 1). By contrast, several estimates obtained under the correlated model are incompatible with fossil evidence and all estimates produced by MULTIDIVTIME are younger than known fossils (Table 1).

Using phytolith fossils as a calibration point (external calibration plus phytoliths) strongly affected estimated ages with all methods (Table 4). As illustrated with BEAST results, this extra calibration point leads to older estimates for all nodes within graminids, but has little effect on nodes within eudicots (Fig. 4). These different results were obtained by inferring elevated rates for some nodes of the graminids and slightly decreased rates within the BEP-PACMAD clade (Fig. 2).

Analysis of Markers Extracted from Complete Nuclear Genomes

Differences in root-to-tip length between BEP-PACMAD and other taxa was smaller in the trees inferred with nuclear genomes than in those from plastid markers, with the exception of the Brassicaceae which had longer root-to-tip distances than other taxa (Fig. 1). The best-fit model selected by thermodynamic integration implemented in PHYLOBAYES was the uncorrelated gamma (Table 2).

In the absence of constraints within grasses, the ages estimated from the 184 transcripts were very similar among the different methods, with an age for the crown of BEP-PACMAD at 51.2 (±12.3) and 62.6 (±7.6) Ma, with BEAST and MULTIDIVTIME respectively (Table 3). With the exception of one node within eudicots (at the base of Brassicaceae), these ages were, moreover, very similar to those inferred from plastid markers with BEAST (Fig. 5). However, they were not compatible with putative Oryzeae phytoliths at 67 Ma, as the crown of the BEP clade (the group containing Oryzeae) was estimated at 39.9 (±12.2) and 52.4 (±8.0) in the two analyses respectively (Table 3). Differences between plastid and nuclear markers were not due to different species numbers or sequence length, as

359 the dataset sampled to the same size produced similar results (Supplementary Fig. 8). The evolutionary
360 rates of grasses inferred from the 184 transcripts were similar to those inferred for other groups
361 (Supplementary Fig. 6).

362 The inclusion of the phytoliths assigned to Oryzeae produced an older age for the BEP-
363 PACMAD clade, at 82.4 (±14.8) and 79.1 (±3.0) Ma with BEAST and MULTIDIVTIME respectively
364 (Table 4). This constraint led to the inference of lower evolutionary rates within grasses, which fell
365 below those for the root and most branches in eudicots and monocots (Fig. 2).

366

367 DISCUSSION

368 *Rate Heterogeneity in Plastid Markers Creates Incongruence between Dating Methods*

369 The investigated plastid genes show strong variation in branch lengths (Fig. 1), with long distances
370 from the root to the tips of Poaceae, a pattern previously reported with markers spread across the
371 chloroplast genome (Graham and Olmstead 2000, Saarela and Graham 2009; Magallon et al. 2013).
372 Since the time elapsed from the root to the tips is the same for all extant species, this branch-length
373 variation must be interpreted as strong differences in evolutionary rates (Gaut et al. 1992, Saarela and
374 Graham 2009). A cluster of long branches within one clade (the BEP-PACMAD clade in this case)
375 could be explained by two alternative scenarios. First, higher evolutionary rates could have been
376 sustained throughout the whole history of the clade, which would mean that the clade is of relatively
377 recent origin. Second, evolutionary rates could have been high during the early evolution of the clade
378 and then later decreased, in which case the clade would be older, a scenario favored in several recent
379 studies (Leebens-Mack et al. 2005; Jansen et al. 2007; Zhong et al. 2009; Guisinger et al. 2010).

In the absence of calibration points inside Poaceae, methods that assume a correlation of rates among adjacent branches, as implemented in MULTIDIVTIME and PHYLOBAYES, inferred a gradual increase of evolutionary rates in branches leading to Poaceae and, depending on the priors, very high rates for many branches inside the BEP-PACMAD clade (Supplementary Fig. 6). The ages produced under these hypotheses are, however, incompatible with macrofossil evidence, as the estimated ages for most nodes are more recent than the corresponding fossils (Table 1). The methods that assume uncorrelated rates, as implemented in BEAST and PHYLOBAYES, solve the branch-length variation observed in the plastid phylogeny by assigning extremely high rates to branches that lead to the BEP-PACMAD clade and low rates inside the BEP-PACMAD clade (Fig. 2, Supplementary Fig. 6). The ages estimated with these methods are compatible with macrofossil evidence as well as geochemical proxy data (i.e. for C_4 lineages; Table 1). It has been demonstrated that both types of methods are strongly misled when their underlying model is violated (Ho et al. 2005; Battistuzzi et al. 2010), and the incompatibility of correlated methods with fossil evidence suggests that plastid rates are not autocorrelated among angiosperms.

Uncorrelated methods inferred high evolutionary rates in graminid branches leading to the BEP-PACMAD clade, with the two sets of calibrations (Fig. 2 and Supplementary Fig. 6). This increase of mutation accumulation is followed by a return to rates that are typical of angiosperms in descendant taxa, as inferred by previous authors (Zhong et al. 2009; Guisinger et al. 2010). Several phenomena have been presented as potential explanations for this pattern of rate variation (e.g. faulty DNA repair and/or adaptive evolution; Zhong et al. 2009, Guisinger et al. 2010), although none of them are yet supported by experimental data. In all cases, the strong rate variation observed in chloroplasts of Poaceae and other graminids is a great challenge for dating analyses, and explains the incongruence

between previous angiosperm-wide analyses and our current understanding of Poaceae evolutionary history based on fossil evidence.

Whole Nuclear Genomes as a Promising Alternative to Plastid Markers

Due to the rate heterogeneity among lineages in the plastid genome, dating methods that differ in their assumptions produce incongruent results. Markers from other genomes can provide support in favor of one method or the other, but most phylogenetic studies in plants rely solely on markers that are easy to amplify, such as plastid markers and the nuclear internal transcribed spacers (ITS), which are extremely difficult to align among distant taxa (Smith and Donoghue 2008; Soltis et al. 2010; Zimmer and Wen 2010). Genome projects are generating nuclear genetic markers for an increasing number of angiosperms, which can provide new insights into plant evolution (Cibrian-Jaramillo et al. 2010; Lee et al. 2011). Extracting phylogenetically informative markers from these genomes is not straightforward because repeated gene duplications and losses in nuclear genomes makes the assessment of orthology difficult (Chiu et al. 2006; Gabaldon 2008). Nevertheless, we have shown here that a large number of reliable markers can be obtained from these genomes, which help disentangle contrasting evolutionary scenarios. The nuclear datasets we investigated are not free of branch-length variation, but the variation is less pronounced than with plastid markers, especially in grasses (Fig. 1). Differences in model assumptions were therefore less important than with plastid markers and the different methods yielded similar results (Fig. 2; Table 3). Moreover, unlike analyses based on plastid markers, the estimated dates are compatible with Poaceae macrofossils (Table 1), increasing our confidence in molecular dating analyses conducted with nuclear markers for the grasses. The low number of nuclear markers presently available however limits the evolutionary insights that can be gained because many questions require large species sampling. The problem is likely to decrease with the rapid accumulation of

425 nuclear datasets based on genome-scale projects. In the meantime, phylogenetic datasets composed of a
426 large number of nuclear markers and multiple species can be generated through high-throughput
427 sequencing following target enrichment (e.g. Faircloth et al. 2012; Lemmon et al. 2012)
428
429 *Consequences of Incorporating the Phytolith Fossils for Ecological Scenarios*
430 In the absence of fossil constraints within Poaceae, all the genetic markers investigated produced dates
431 that were incompatible with the hypothesized presence of members of the Oryzeae tribe in the Late
432 Cretaceous (ca. 67-66 Ma; Prasad et al. 2005, 2011), regardless of the method used (Table 1;
433 Supplementary Fig. 8). Nevertheless, it is possible to integrate the phytolith fossils as a calibration
434 point and obtain dates that are compatible with our current knowledge of the ages of other major
435 angiosperm lineages; the putative Oryzeae phytoliths merely imply lower rates of molecular evolution
436 in BEP-PACMAD grasses and higher rates in other graminids (Fig. 2). Fossil remains provide an
437 independent proxy for divergence times, but a reliable assignment to a specific group requires
438 synapomorphies that are unlikely to be shared with other groups (Parham et al. 2012). The 67 Ma
439 phytolith fossils have multiple traits that are found in Oryzeae or Ehrhartoideae (subfamily containing
440 the Oryzeae tribe), but these also occur in some Bambusoideae and PACMAD species. The only
441 characters exclusively shared by some phytolith fossils and extant Oryzeae are the distribution of
442 vertical bilobates in costal rows and their scooped shape (Prasad et al. 2011). Whether these traits
443 evolved only once is unknown. A reevaluation of Poaceae diversification and therefore evolutionary
444 rates should wait until the potential homoplasy of these phytolith characters has been adequately
445 assessed through comparative studies based on a wide sample of extant monocots. In the meantime, our
446 analyses can predict the consequences of the phytolith-based hypothesis for evolutionary and
447 ecological scenarios.

448 The timing of the basal splits within the BEP and PACMAD clades influences the most likely
449 scenario for early grass biogeography. If these splits occurred at or after 55 Ma (Table 3), then grass
450 lineages must have spread from their Gondwanan center(s) of origin (Bremer 2002; Bremer and
451 Janssen 2006; Bouchenak-Khelladi et al. 2010) long after the breakup of this southern supercontinent
452 (e.g., McLoughlin 2001), pointing to long-distance dispersal as an important mechanism by which
453 grass lineages achieved their world-wide distribution. In contrast, under the phytolith-based age
454 hypothesis, these divergences would have occurred during a time when there were still land
455 connections between the southern continents; hence, vicariance may have played a larger role in early
456 grass diversification (Prasad et al. 2011).
457 The difference in age estimates is also crucial to understanding the causal factors driving the
458 evolution of C_4 photosynthesis in PACMAD lineages (Christin and Osborne 2013; Edwards and
459 Donoghue In press). The earliest C_4 acquisition occurred in Chloridoideae, by at least 32.0 (±3.8;
460 BEAST, external calibration only) or 41.2 (±4.1; BEAST external calibration plus phytoliths) Ma. The
461 younger of these two dates places the oldest origin of C_4 Chloridoideae potentially after the drop in
462 pCO_2 in the early Oligocene (Pagani et al. 2005; Beerling and Royer 2011), consistent with the
463 commonly cited hypothesis that the evolution of this new photosynthetic pathway became
464 advantageous in a low-CO_2 atmosphere (Christin et al. 2008; Vicentini et al. 2008; Bouchenak-Khelladi
465 et al. 2009). In contrast, the phytolith-based ages for Poaceae result in a scenario by which C_4 grasses
466 appeared in the Eocene, when atmospheric CO_2 was elevated (Beerling and Royer 2011; Zachos et al.
467 2008). While this would necessitate a reevaluation of potential environmental drivers (Urban et al.
468 2010; Prasad et al. 2011), this early C_4 origin would concern only Chloridoideae as all other C_4 origins
469 could have occurred during or after the Oligocene, even when phytoliths are incorporated as calibration
470 points. Finally, based on analyses that did not include the fossil phytoliths from India, it has been

suggested that core Pooideae evolved cold tolerance in response to climatic cooling following the Eocene-Oligocene boundary (33.9 Ma; Sandve and Fjellheim 2010), which is compatible with our analyses without phytolith fossils. If the phytolith-based ages are used, core Pooideae are significantly older than 33.9 Ma, and would have evolved in the warm, middle Eocene (Zachos et al. 2001).

Microfossils offer the potential to add a great deal of data to an otherwise scant grass fossil record, but until the phylogenetic informativeness of their characters is better known, their placement should be considered as hypothetical. With the current state of knowledge, we suggest that the dates obtained with phytolith evidence should be considered as an alternative to those obtained with macrofossils only.

CONCLUSION

Molecular dating methods are widely used in ecology and evolution to address diverse questions, but sufficient attention is not always given to the influence of the underlying model assumptions and placement of fossils. Unfortunately, the estimates of evolutionary rate variation (linked to the model assumptions) and divergence times of key nodes (linked to the placement of fossils) are tightly connected and one can be confidently estimated only with an accurate knowledge of the other (Magallon 2004). The comparison of different molecular markers, different calibration points and different models of evolution must be advocated to evaluate the uncertainties linked to the inferred dates and evolutionary rates. Using the grasses as a case study, we show that strong rate variation of plastid markers among branches of the phylogeny mislead analyses when using a method that assumes an autocorrelation of evolutionary rates. This problem is diminished by assuming that evolutionary rates are not correlated, as indicated by the congruence between uncorrelated analyses of plastid

markers and nuclear markers. Unfortunately, the best model for the evolutionary rates is difficult to predict *a priori*. Models can be compared based on their score, but the computationally less demanding approaches involving Bayes factors have been proven unreliable (Xie et al. 2011; Baele et al. 2012). Other methods exist, such as the thermodynamic integration (Lartillot and Philippe 2006), but the approach was not able to categorically differentiate the models compared here. The biological relevance of different assumptions must consequently be evaluated independently for each case, through a comparison between different markers that can be extracted from different genomes (Lukoschek et al. 2012). Completely sequenced genomes are becoming available for an increasing number of taxa, and they constitute a prolific source of phylogenetic information for evolutionary studies interested in divergence time estimates, adequately complementing the haploid markers that are available for a greater number of species.

SUPPLEMENTARY MATERIAL

Supplementary material, including data files and/or online-only appendices, can be found in the Dryad data repository.

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Table 1: Compatibility of dating analyses with fossil evidence[1]

Clade	Age	Type[10]	BEAST	PB_ug[11]	PB_ln[12]	MD[13]
Cenchrinae[2]	7	M	17.5 (2.2)	21.6 (4.0)	15.8 (1.9)	5.8 (1.1)*
Stipeae[2]	17[8]	M	40.4 (3.9)	47.0 (4.5)	27.8 (2.3)	12.7 (1.9)*
Puelioideae+	55[9]	M	64.4 (4.3)	71.6 (4.7)	49.1 (2.7)*	31.1 (3.3)*
BEP-PACMAD[3]						
First grass pollen[4]	70	Po	69.0 (4.7)	84.4 (4.7)	64.5 (3.3)*	34.1 (3.6)*
First C_4[5]	23	I	38.5 (3.9)	45.2 (4.6)	28.6 (2.2)	12.9 (1.8)*
Oryzeae[6]	67	Ph	38.5 (6.3)*	44.1 (8.2)*	30.9 (2.5)*	15.3 (2.1)*
Ehrhartoideae[7]	67	Ph (H1)	53.0 (3.6)*	60.3 (4.5)*	36.9 (2.3)*	19.6 (2.3)*
BEP[7]	67	Ph (H2)	54.9 (3.6)*	62.3 (4.6)	37.6 (2.3)*	20.2 (2.3)*
BEP-PACMAD[7]	67	Ph (H3)	57.9 (3.8)*	64.8 (4.6)	39.2 (2.4)*	21.6 (2.5)*

[1] Ages of the stem node of each group are given for the analyses based on plastid markers without calibrating point in Poaceae (in million years ago; standard deviations in parentheses). Ages not compatible with fossil evidence are indicated by an asterisk; [2] Elias 1942; [3] Crepet and Feldman 1991; [4] Herendeen and Crane 1995, compared with age of the crown Poaceae; [5] Fox and Koch 2003, compared with stem of core Chloridoideae; [6,7] Prasad et al. 2011 for the fossils and Prasad et al. 2005 for the date; [6] preferred placement according to Prasad et al. 2011; [7] alternative placement on successively ancestral nodes to Oryzeae; [8] Age of the formation based on Janis et al. 2000; [9] age estimate based on Bremer 2002 and Vicentini et al. 2008; [10] M=macrofossil, Ph=phytolith, Po=fossilized pollen, I=isotope ratio; [11] uncorrelated gamma method implemented in PHYLOBAYES; [12] log-normal autocorrelated method implemented in PHYLOBAYES; [13] MULTIDIVTIME.

730 **Table 2: Comparison of the fit of different molecular clock models**[1]

Model	Plastid dataset	Nuclear dataset
Strict clock	[-801.449:-646.171]	[-3246.09:-3243.05]
Log-normal autocorrelated	[-18.7212:111.48]	[10.146:17.7047]
CIR process[2]	[-16.4669:139.191]	[8.8931:11.4258]
Uncorrelated gamma	[98.0219:110.115]	[19.498:20.7114]

731 [1] The 95% credibility intervals for natural logarithms of Bayes factors against the unconstrained model

732 were estimated through thermodynamic integration with PHYLOBAYES (See Lepage et al. 2007); [2]

733 Lepage et al. 2006.

Table 3: Ages estimated under external calibration only[1]

	plastid				nuclear			
node	**BEAST**	**PB_ug[2]**	**PB_ln[3]**	**MD[4]**	**BEAST**	**PB_ug[2]**	**PB_ln[3]**	**MD[4]**
eudicot/monocot split	163.5 (9.0)	143.4 (3.6)	151.1 (3.5)	157.4 (5.5)	143.1 (10.4)	134.6 (5.2)	138.9 (5.9)	149.0 (4.4)
Arecales stem	117.7 (7.1)	117.4 (4.3)	120.2 (3.6)	116.5 (5.1)	115.7 (17.9)	104.5 (9.6)	117.8 (9.4)	133.6 (4.6)
BEP/PACMAD split	54.9 (3.6)	62.3 (4.6)	37.6 (2.3)	20.2 (2.3)	51.2 (6.2)	50.9 (7.4)	55.0 (7.0)	62.6 (7.6)
BEP crown	53.0 (3.6)	60.3 (4.5)	36.9 (2.3)	19.6 (2.3)	39.9 (6.3)	39.3 (6.8)	46.3 (7.1)	52.4 (8.0)

[1] Ages are given in million years ago, with standard deviations in parentheses; [2] uncorrelated gamma method implemented in PHYLOBAYES; [3] log-normal autocorrelated method implemented in PHYLOBAYES; [4] MULTIDIVTIME.

Table 4: Ages estimated from plastid markers under external calibration plus phytoliths[1]

	plastid				nuclear			
node	**BEAST**	**PB_ug[2]**	**PB_ln[3]**	**MD[4]**	**BEAST**	**PB_ug[2]**	**PB_ln[3]**	**MD[4]**
eudicot/monocot split	176.0 (8.3)	147.0 (4.2)	197.4 (1.7)*	183.8 (5.4)*	158.7 (11.2)	150.5 (8.6)*	157.3 (7.9)	150.6 (4.8)
Arecales stem	131.8 (6.8)*	124.1 (3.9)	165.1 (2.6)*	144.4 (5.6)*	143.6 (13.5)	137.1 (10.7)*	150.2 (10.8)*	136.4 (4.6)
BEP/PACMAD split	74.5 (2.6)*	75.6 (2.5)*	73.1 (1.0)*	71.8 (2.2)*	82.4 (8.4)*	83.8 (6.7)*	81.7 (4.4)*	79.1 (3.0)*
BEP crown	72.6 (2.3)*	74.0 (2.3)*	72.5 (1.0)*	70.8 (2.1)*	70.7 (5.6)*	72.6 (5.4)*	71.9 (4.3)*	70.5 (3.2)*

[1] Ages are given in million years ago, with standard deviations in parentheses. Asterisks indicate ages that are not compatible with those obtained with external calibration only (Table 3) [2] uncorrelated gamma method implemented in PHYLOBAYES; [3] log-normal autocorrelated method implemented in PHYLOBAYES; [4] MULTIDIVTIME.

Figure 1: Phylograms for plastid and nuclear markers

Branch lengths are shown for the different markers. Branches belonging to graminids but not the BEP-PACMAD clade are in bold. **a)** Branch lengths inferred from plastid markers by PhyML under a GTR+G+I substitution model with a fixed topology, **b)** Branch lengths inferred from the concatenated transcripts from whole genomes under a GTR+G+I substitution model with a fixed topology, **c)** Branch lengths inferred from plastid markers by PhyML under a GTR+G+I substitution model with a fixed topology with a species sampling comparable to panel **b**. The clades discussed in the text are delimited on the right; Ory = Oryzeae (represented by only one tip in panels **b** and **c**).

Figure 2: Effect of different calibrations on inferred evolutionary rates.

The distribution of rates (in expected mutations per site per billion years) inferred by BEAST for different taxonomic groups is indicated by boxplots for external calibration only (black) and external calibration plus phytoliths (grey), for **a)** plastid markers and **b)** nuclear markers.

Figure 3: Comparison of age estimates produced by different methods on plastid markers.

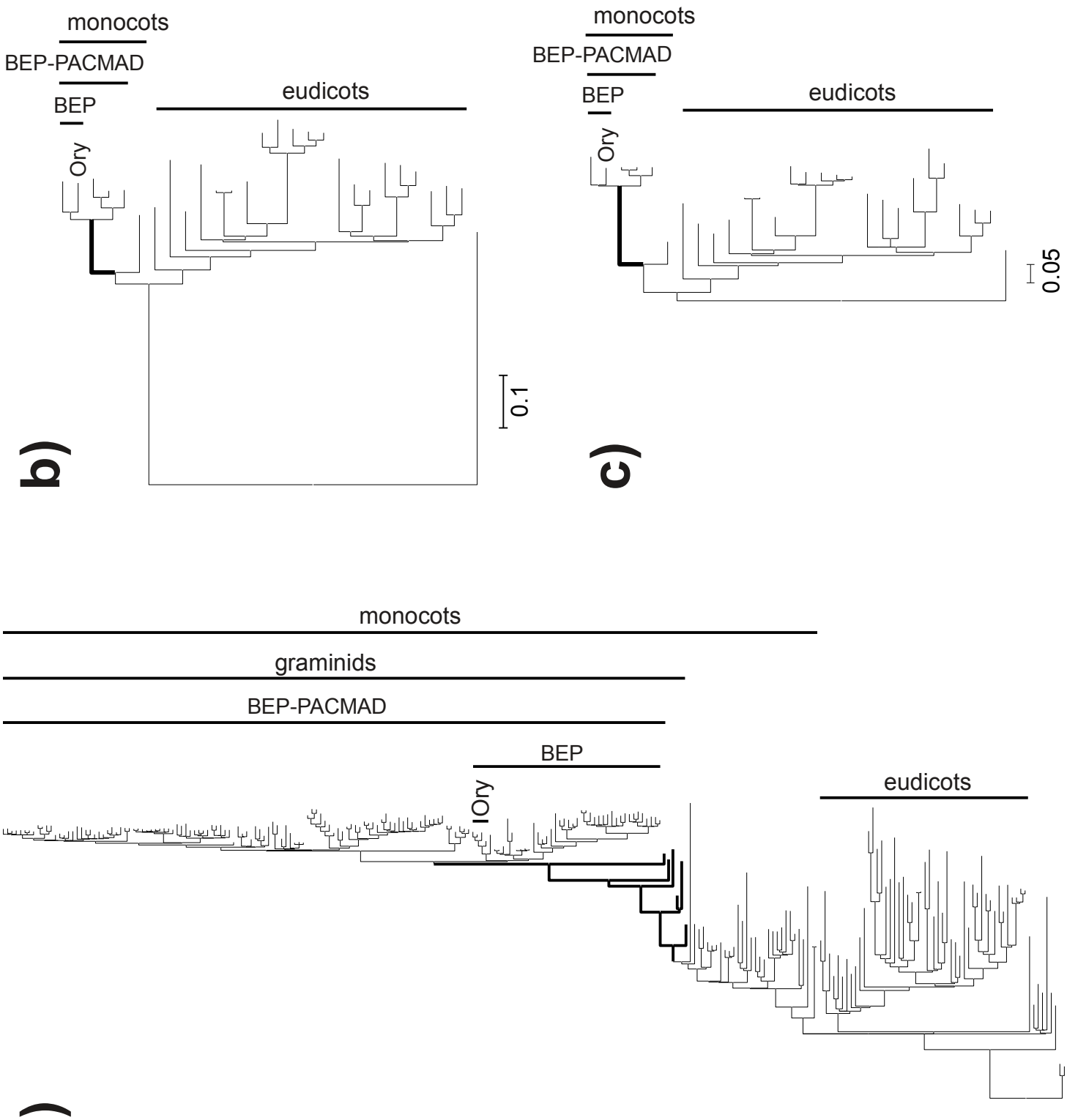
For external calibration only, ages estimated by BEAST (in million years ago) are compared to those produced by other methods. Nodes inside the BEP-PACMAD clade are in black dots, those in graminids but outside the BEP-PACMAD in grey triangles and those outside the graminids in light grey dots. Black lines indicate 1:1 relationships. PM_ug = uncorrelated gamma model implemented in PHYLOBAYES; PM_ln = correlated log-normal model implemented in PHYLOBAYES; MD = MULTIDIVTIME.

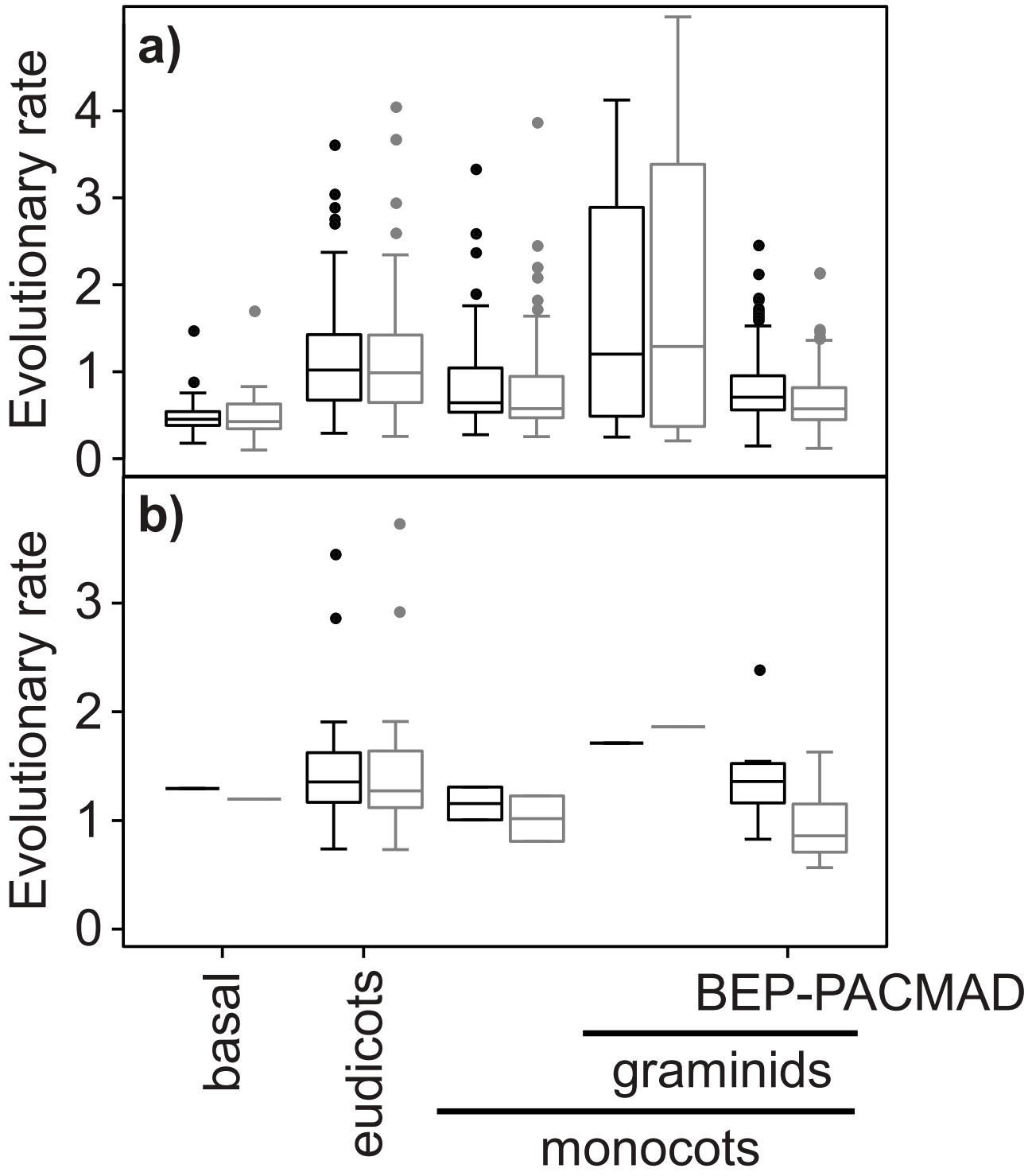
Figure 4: Comparison of age estimates produced by BEAST on plastid markers under different calibrations.

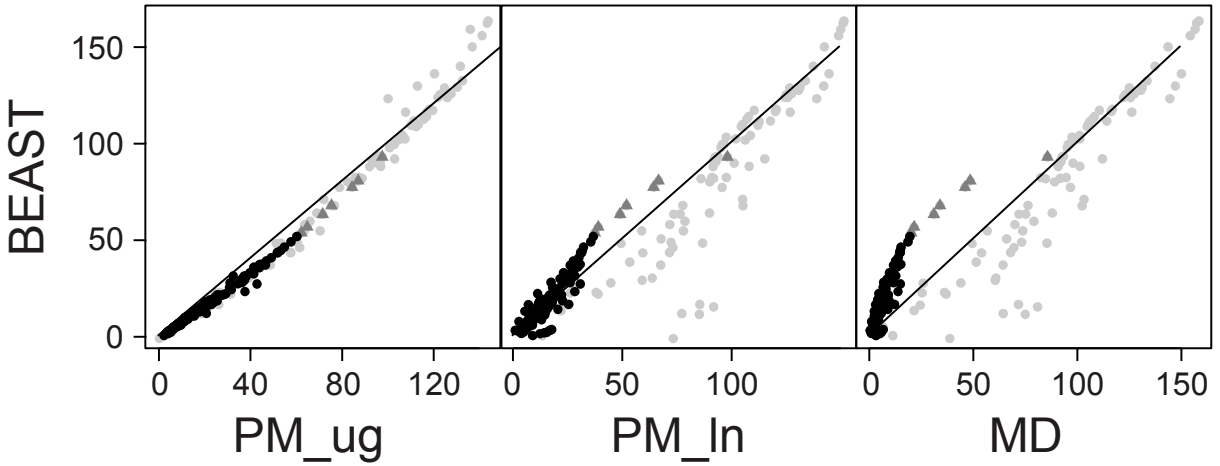
Ages estimated by BEAST (in million years ago) under external calibration plus phytoliths are plotted against those obtained under external calibration only. Nodes inside the BEP-PACMAD clade are in black dots, those in graminids but outside the BEP-PACMAD in reversed grey triangles, those in monocots but outside the graminids in grey triangles, those in eudicots in light grey circles, and those in basal groups in light grey squares. The black line indicates 1:1 relationship.

Figure 5: Comparison of age estimated produced by BEAST and MULTIDIVTIME on different datasets.

For external calibration only, the age estimates (in million years ago) are represented for nodes that were shared between phylogenetic trees of plastid and nuclear markers. Ages estimated on nuclear genomes are represented by black squares (BEAST) and black triangles (MULTIDIVTIME) and those based on plastid markers are represented by grey circles (BEAST) and grey triangles (MULTIDIVTIME). Taxonomic groups are indicated on the bottom. The last point corresponds to the crown of BEP, and the horizontal bar indicates the minimal age for the clade that would be congruent with the 67 Ma phytolith fossil (Prasad et al. 2011). Numbers can be used to identify the corresponding nodes in Supplementary Figure 9.


monocots
BEP-PACMAD
BEP
eudicots
Ory
0.1
b)
monocots
BEP-PACMAD
BEP
eudicots
Ory
0.05
c)
monocots
graminids
BEP-PACMAD
BEP
eudicots
Ory
0.05
a)


a)
b)
Evolutionary rate
Evolutionary rate
0
1
2
3
4
0
1
2
3
basal
eudicots
BEP-PACMAD
graminids
monocots


BEAST
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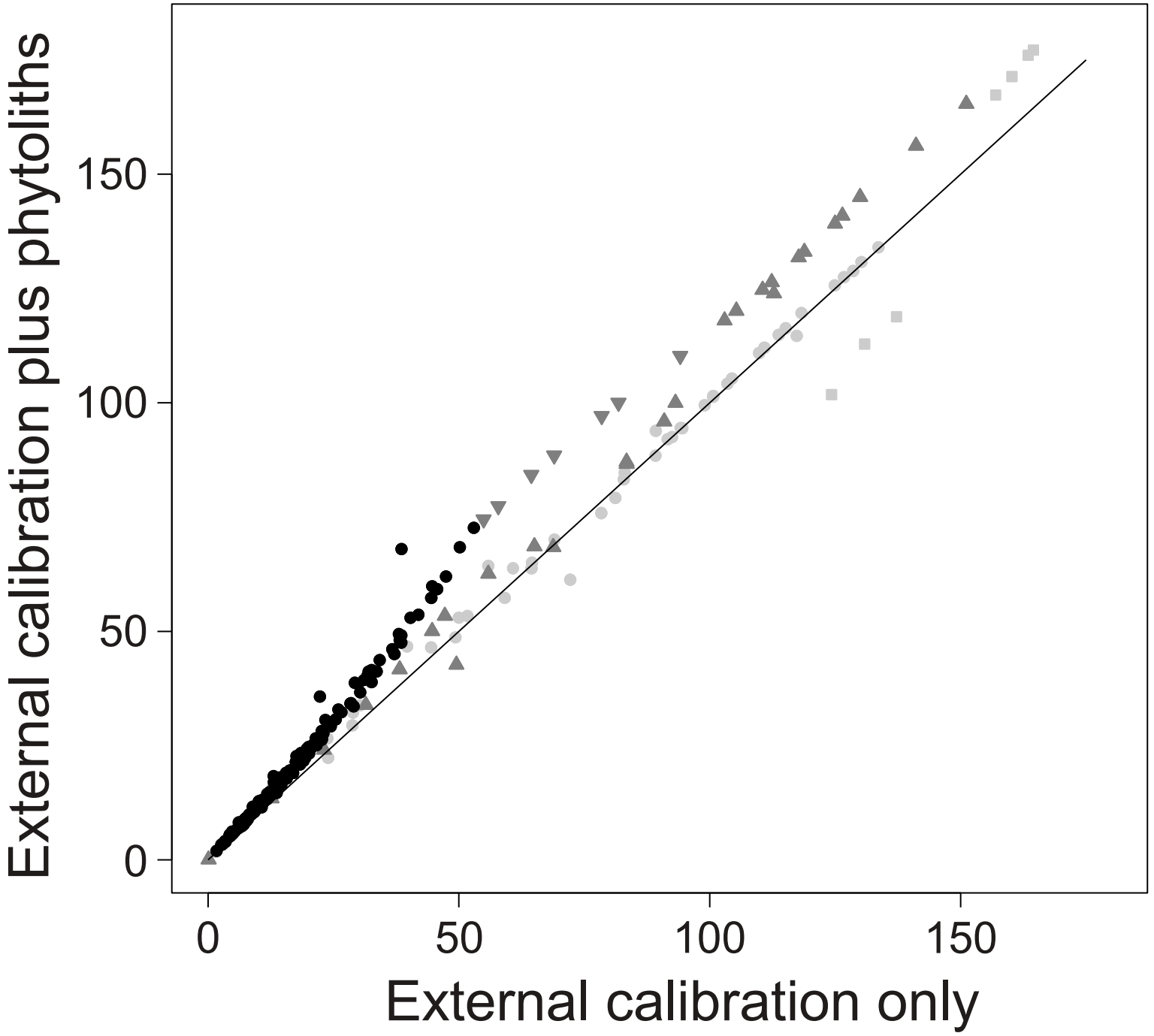
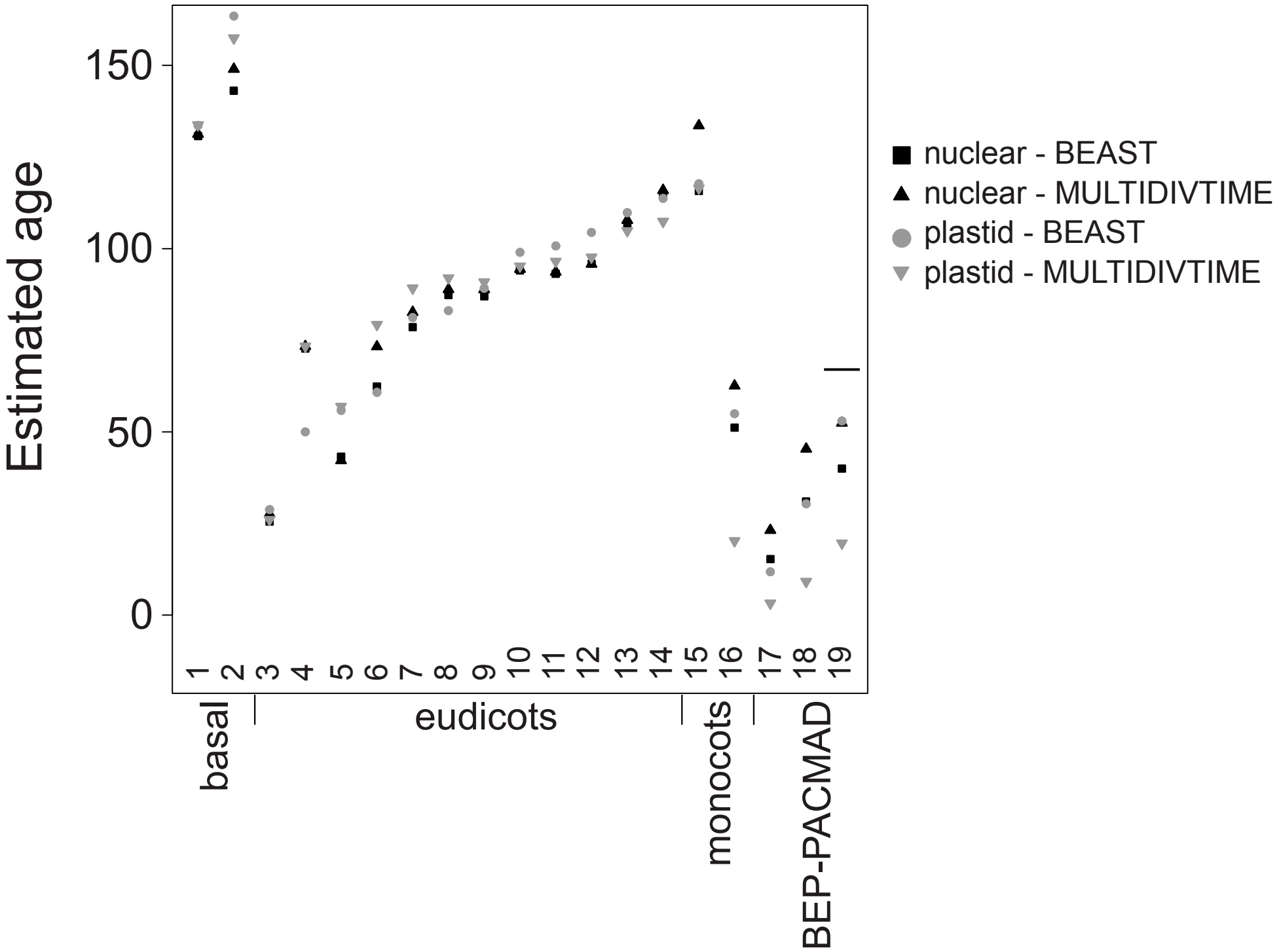
50
0
0
40
80
120
PM_ug
0
50
100
PM_ln
0
50
100
150
MD


External calibration plus phytoliths
0
50
100
150
0
50
100
150
External calibration only


Estimated age
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
basal
eudicots
monocots
BEP-PACMAD
nuclear - BEAST
nuclear - MULTIDIVTIME
plastid - BEAST
plastid - MULTIDIVTIME