Rooting and Dating Maples (*Acer*) with an Uncorrelated-Rates Molecular Clock: Implications for North American/Asian Disjunctions

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Abstract.—Simulations suggest that molecular clock analyses can correctly identify the root of a tree even when the clock assumption is severely violated. Clock-based rooting of phylogenies may be particularly useful when outgroup rooting is problematic. Here, we explore relaxed-clock rooting in the *Acer*/*Dipteronia* clade of Sapindaceae, which comprises genera of highly uneven species richness and problematic mutual monophyly. Using an approach that does not presuppose rate autocorrelation between ancestral and descendant branches and hence does not require a rooted *a priori* topology, we analyzed data from up to seven chloroplast loci for some 50 ingroup species. For comparison, we used midpoint and outgroup rooting and dating methods that rely on rooted input trees, namely penalized likelihood, a Bayesian autocorrelated-rates model, and a strict clock. The chloroplast sequences used here reject a single global substitution rate, and the assumption of autocorrelated rates was also rejected. The root was placed between *Acer* and *Dipteronia* by all three rooting methods, albeit with low statistical support. Analyses of *Acer* diversification with a lineage-through-time plot and different survival models, although sensitive to missing data, suggest a gradual decrease in the average diversification rate. The nine North American species of *Acer* diverged from their nearest relatives at widely different times: eastern American *Acer* diverged in the Oligocene and Late Miocene; western American species in the Late Eocene and Mid Miocene; and the *Acer* core clade, including *A. saccharum*, dates to the Miocene. Recent diversification in North America is strikingly rare compared to diversification in eastern Asia. [Bayes factors; biogeography; Bayesian relaxed clock; Beringian disjunctions; rooting of phylogenies; substitution rates.]

Rooted phylogenetic trees are essential for inferring the sequence of evolutionary events. Trees can be rooted with outgroups (Maddison et al., 1984; Wheeler, 1990), midpoint-rooting (the tree is rooted at the midpoint of the longest path connecting any pair of taxa; Swofford and Begle, 1993), suitable gene duplications (Iwabe et al., 1989; Mathews and Donoghue, 1999), insertion/deletion-rooting (Lake et al., 2007), paleontological or other circumstantial evidence (Huelsenbeck, 1994), nonreversible models of substitution and asymmetric step matrices (Swofford et al., 1996; Yap and Speed, 2005), or molecular clocks (Huelsenbeck et al., 2002). All ultrametric trees, constructed assuming a clock, have a root as the starting point and tips, or leaves, as the end points, usually in the present. Using real and simulated datasets, Huelsenbeck et al. (2002) compared outgroup rooting, molecular clock–based rooting, and nonreversible models of DNA substitution and found that the outgroup and molecular clock criteria correctly identified the root a large proportion of the time. As expected, the outgroup criterion performed best when the length of the branch leading to the outgroup was short (Wheeler, 1990). With increasing genetic distance between the ingroup and the outgroup, the performance of outgroup-rooting decreased, while molecular clock–based rooting correctly identified the root of the tree even when the clock criterion was severely violated. Under the most extreme conditions of rate heterogeneity, the clock criterion identified the correct root roughly four times more often than did random rooting. (Rooting via a nonreversible model of DNA substitution performed poorly under most circumstances.) These results suggest that the root of a phylogenetic tree can be identified with "clock rooting" even when the substitution process is not strictly clocklike.

Unclock-like data are the rule, and many methods of estimating divergence times therefore relax the clock assumption (Rambaut and Bromham, 1998; Thorne et al., 1998; Aris-Brosou and Yang, 2002; Sanderson, 2002; Thorne and Kishino, 2002; Britton et al., 2007). These relaxed clock approaches all require a "known" rooted input tree. Recently an approach has become available that coestimates phylogeny and divergence times (Drummond et al., 2006). The new method, Bayesian evolutionary analysis sampling trees, or BEAST, by relying neither on an input topology nor on a (potentially misleading and/or too distant) outgroup, can be used to identify the root of a phylogenetic tree (Drummond et al., 2006). Here, we explore the application of relaxed-clock-rooting to resolve a difficult problem, namely the root of the genus *Acer*. This problem likely has resulted from the effects of numerous extinctions and a possible shift in net diversification rate (speciation rate minus extinction rate) in the genus, which we therefore also address.

Acer comprises 124 to 156 species (van Gelderen et al., 1994; de Jong, 2002) and is the largest tree genus of the Northern Hemisphere besides *Quercus*. Knowing the root of *Acer* is relevant for reconstructing its evolution and biogeography, and in molecular-clock dating, the correct position of the root is essential (Sanderson and Doyle, 2001). An erroneous rooting will greatly influence (and often invalidate) all estimates of divergence times. Because *Acer* is such a large and widespread clade, inferring its diversification has implications for

our understanding of the Tertiary vegetation history of the Northern Hemisphere (Wolfe and Tanai, 1987; Boulter et al., 1996; Manchester, 1999). Today, most species of *Acer* occur in Eurasia, particularly eastern Asia, whereas North America harbors only nine species: *A. circinatum*, *A. glabrum*, and *A. macrophyllum* from the western United States; *A. pensylvanicum* and *A. spicatum* from the northeastern United States; and the widespread *A. negundo*, *A. rubrum*, *A. saccharinum*, and *A. saccharum*. During the Tertiary, however, the diversity of *Acer* in North America appears to have been much greater, especially in the western United States (Wolfe and Tanai, 1987). In Europe, as well, many species of maples appear to have gone extinct during the Late Oligocene–Early Miocene (Walther, 1972; Wolfe and Tanai, 1987; Boulter et al., 1996).

The closest relative of *Acer* is *Dipteronia*, a genus of two species endemic to mainland China (Qiu et al., 2007). Whereas *Acer* has two-winged fruits (samaras) of a type that evolved several times in Sapindales (e.g., in *Thouinia*, *Thouinopsis*, and *Serjania*), *Dipteronia* has circular-winged fruits that are almost unique among angiosperms. The two species, *D. sinensis* and *D. dyeriana*, are so similar to each other that they have sometimes been ranked as subspecies of a single species (*D. sinensis* subsp. *dyeriana*). However, molecular studies of *Acer*, which have relied mainly on nuclear rDNA (the ITS region), found *D. sinensis* nested inside *Acer* (Cho et al., 1996; Suh et al., 2000; Pfosser et al., 2002, Tian et al., 2002; Grimm et al., 2006). This was especially surprising in view of Paleocene fruit fossils of both genera (Wolfe and Tanai, 1987; Crane et al., 1990; Manchester, 1999; McClain and Manchester, 2001; Kittle et al., 2005), suggesting that they existed a long time ago.

The closest relative of *Acer* and *Dipteronia* within Sapindaceae is a clade comprised of *Aesculus*, with 13 to 19 species in China, Europe, and North America; *Billia*, with two species in Central America and northern South America; and *Handeliodendron*, with a single species in China (Harrington et al., 2005). There is thus a striking asymmetry in extant species diversity between *Acer* and its relatives, perhaps pointing to differential rates of extinction and/or speciation in the geographic areas occupied by the maple lineage as well as the more inclusive Hippocastanoideae lineage.

To reconstruct the evolutionary unfolding of *Acer*, it is necessary to know both the root of the genus and the absolute divergence times of key lineages. Here we use combined data from up to seven chloroplast loci and relaxed-clock rooting, as well as outgroup and midpoint rooting, to address three issues: (i) the identity of the root of *Acer*/*Dipteronia* and the age of the *Acer* crown group; (ii) whether the diversification rate in the genus has shifted over the Tertiary; and (iii) the phylogenetic placement and temporal context of the extant North American species of *Acer*. We focus on the American species because our species-level sampling of North American maples is complete, and the timing of the American–East Asian disjunctions has been the focus of a previous investigation (Wolfe, 1981).

Materials and Methods

Taxon Sampling

Appendix 1 (http://www.systematicbiology.org) lists the 74 species and subspecies of *Acer* and *Dipteronia* that were sequenced, with their sources and NCBI GenBank accession numbers. Date matrices have been deposited in TreeBASE as study accession number S2163. Species concepts follow the monograph of van Gelderen et al. (1994). The sampled species represent all sections accepted by de Jong (1994, 2002), except for *Wardiana*, which contains only *A. wardii* W. W. Smith. Previous studies found *A. wardii* embedded in the *Palmata* clade (nuclear rDNA ITS data of Grimm et al., 2006) or in a large polytomy (chloroplast data of Li et al., 2006).

We sequenced most species for the chloroplast (cp) *rbcL* gene, the *rpl16* intron, the *psbA-trnH* spacer, and the *trnL* intron and adjacent *trnL-F* spacer. The slowly evolving *rbcL* gene was not sequenced for each species of *Acer* (see Appendix 1). Sequences of the cp *trnD-trnT* and *psbM-trnD* spacers from Li et al. (2006) were downloaded from GenBank as specified in Appendix 1. Where spacer regions could not be reliably aligned between ingroup and distant outgroups, they were replaced by question marks. To maximize signal, a few analyses included just 24 species (6674 characters) selected to represent the most divergent species.

For outgroup rooting, we included species of *Aesculus* and *Handeliodendron* from subfamily Hippocastanoideae of Sapindaceae plus representatives of Dodonaeoideae (*Conchopetalum* and *Dodonaea*) and Sapindoideae (*Koelreuteria*), all based on Harrington et al. (2005). Two of the outgroup taxa were composites because some loci could not be sequenced for each species (Appendix 1). This did not affect tree topology, as tested by running analyses with and without the *Aesculus* and *Koelreuteria* composites (data not shown). To assess a possible substitution rate change in the stem of the *Acer*/*Dipteronia* clade, we added *rbcL* sequences of *Acer* and *Dipteronia* to an *rbcL* data set of all Sapindales families (Muellner et al., 2007) for a total of 98 or 128 sequences. GenBank numbers for the non-*Acer*/*Dipteronia rbcL* sequences can be found in Muellner et al. (2007). Sapindales contain several taxa with fossil records, and therefore the large *rbcL* data set also permitted estimating divergence times in *Acer* and *Dipteronia* without relying on ingroup fossils.

DNA Sequencing, Editing, and Alignment

DNA extraction, cloning, and sequencing followed the methods described in Renner et al. (2007), which also contains information on the primers used. Forward and reverse reads were obtained for most samples. Sequences were edited with Sequencher (4.6; Gene Codes, Ann Arbor, Michigan) and aligned by eye, using MacClade 4.06 (Maddison and Maddison, 2003). Species newly sequenced for this study include *A. fabri*, *A. kweilinense*, and *Handeliodendron bodinieri*.

Phylogenetic Analyses

Data matrices for the various DNA loci were analyzed separately under parsimony optimization and in the absence of statistically supported conflict were combined. Parsimony searches relied on PAUP version 4.0b10 (Swofford, 2002), using a parsimony ratchet analysis command block (Müller, 2004), with 200 replicates, 10 random taxon-addition cycles, and a single ratchet run. Gaps were treated as missing data.

Maximum likelihood (ML) searches were carried out with RAxML version 2.2.0 (Stamatakis, 2006). Selection of the best-fit models relied on ModelTest 3.7 (Posada and Crandall, 1998) and is described in Rooting and Divergence Time Estimation. Tree searches in RAxML used the GTR+Γ model, the only model implemented in RAxML version 2.2.0. Model parameters were estimated over the duration of specified runs and searches started from several random parsimony trees.

Statistical support was measured by maximum likelihood bootstrapping performed in RAxML, with 1000 replicates, and by maximum parsimony bootstrapping in PAUP, with 100,000 replicates, a single tree as the start tree, tree bisection-reconnection branch swapping, and the MulTrees option deactivated. For visualization of competing phylogenetic signal, a bipartition network based on 100 maximum likelihood bootstrap replicates (6577 bp from six chloroplast loci with two partitions and a GTR+Γ model each) was constructed using SplitsTree 4 (Huson and Bryant, 2006).

Rooting and Divergence Time Estimation

We used three methods to find the root of *Acer*: midpoint rooting, clock rooting, and outgroup rooting with more or less numerous representatives of Sapindaceae. Midpoint rooting forces the use of MINF (minimization of the *F*-value) optimization (Swofford and Begle, 1993), which assigns states at internal nodes such that the *F*-value of Farris (1972) is minimized. The effect of minimizing the *F*-value is that length is transferred from interior branches towards terminal branches whenever possible, minimizing the risk that groups will be arbitrarily resolved internally (Swofford and Begle, 1993).

Clock rooting relied on BEAST version 1.4.6 (Drummond and Rambaut, 2007). BEAST analyses were conducted on two matrices, one with the combined plastid sequences (6487 base pairs [bp]) from 53 species of *Acer* and *Dipteronia*, the other with 98 Sapindaceae *rbcL* sequences (1387 bp), including 18 from *Acer* and *Dipteronia*. Concatenated datasets were analyzed under partition-specific models, one for the *rbcL* gene, the other for all non-coding plastid regions. The best-fit substitution models under the Akaike information criterion in ModelTest were TrN+Γ+I for the coding region (*rbcL*) in the 53-taxon matrix, TVM+Γ+I for the non-coding regions in the 53-taxon matrix, and GTR+Γ+I for the coding region (*rbcL*) in the 98-taxon matrix. To reduce possible overparameterization, and given that the gamma shape parameter (Γ) and the proportion of invariable sites (I) are strongly correlated (Sullivan and Swofford, 2001), we dropped the estimation of I, instead increasing the number of rate categories for Γ to 8, and we also used the slightly simpler TrN+Γ model for both partitions in the 53-taxon matrix.

To quantify the support for alternative root positions we used Bayes factors, which measure the change in support for one model versus another given the data (Suchard et al., 2001). They are the Bayesian analogue of the likelihood ratio test but have the advantage that they can be used to compare non-nested hypotheses, such as different topologies (Suchard et al., 2001, 2005). To this end, we obtained prior probabilities for root placements at 37 different positions along the backbone of the *Acer*/*Dipteronia* topology obtained with the 53-taxon dataset (13 of these positions resulted from alternative resolutions of polytomies) by sampling from the prior only, using BEAST (computations conducted at the computer cluster of the University of Vienna Schrödinger III; http://www.univie.ac.at/ZID/schroedinger/). Despite the comparatively large number of sampling points (350,000), several root positions were estimated to have prior probabilities of zero. To be able to include these in the calculation of Bayes factors, their prior probabilities were set to 2.857×10^{-6} (= 1/350,001) as an upper bound for the prior or to 3.527×10^{-83} (the number of rooted topologies for 53 taxa) as a reasonable lower bound. We use $2\ln BF_{12}$ as the test statistic and consider $2\ln BF > 10$ as strong support for model 1. This widely used cutoff value (Kass and Raftery, 1995) has been found to give a 5% type I error rate, at least in the context of data partitioning (Brown and Lemmon, 2007).

After tuning the operators using BEAST's auto-optimization option, analyses in BEAST used UPGMA trees as the starting trees and a speciation model following a Yule process as tree prior, with two runs of 1×10^7 generations each, sampling every 250th generation, with a burn-in of 1×10^6 generations each. Results from individual runs were combined as recommended for phylogenetic MCMC analyses, instead of running single longer chains (Beiko et al., 2006), and effective sample sizes (ESS) for all estimated parameters and node ages were well above 100. Clades of interest were defined via most recent common ancestors (MRCA), and they could thus have varying taxon composition in the posterior. This effect will be particularly pronounced where relationships are uncertain because of weak signal. In our dataset, this affected our ability to calibrate the tree. In particular, the ambiguity concerning the monophyly of *Dipteronia* (see Results) affects the application of fossil calibrations for *Acer* and *Dipteronia*; if *Dipteronia* is inferred as paraphyletic or polyphyletic, a calibration point derived from *Dipteronia* fossils (not assignable to either of the two extant taxa, see below) cannot be interpreted as a minimum age of *Dipteronia* divergence.

The earliest fossils of *Dipteronia* (McClain and Manchester, 2001) are from the Paleocene Fort Union Formation in central Wyoming and are 60 to 63 Myr. *Dipteronia* fruits are also well represented at Middle Eocene localities in Washington, Oregon, and British Columbia, and

at Late Eocene localities in Colorado, western Montana, and Oregon (McClain and Manchester, 2001). The only locality of Oligocene age is from the Bridge Creek flora of Oregon, 32 Myr. There are no Asian *Dipteronia* fossils. The oldest described *Acer* fossils are Paleocene fruits from North America and Eurasia (Wolfe and Tanai, 1987; Crane et al., 1990; Manchester, 1999; Kittle et al., 2005), but still older fruits from the Maastrichtian (66.5 Ma) Hell Creek formation in South Dakota also conform morphologically to *Acer* (K. R. Johnson, personal communication, August 2008). None of these ancient isolated fruits, however, can securely be assigned to an extant clade.

Based on this fossil record, we applied the following alternative constraints to the 53-taxon matrix (using BEAST). We first used an exponentially distributed prior with an offset of 60 and a mean of 1, which gave a prior mean age of 72.184 Ma and a median of 62.054 Ma for the age of the MRCA of *Acer* and *Dipteronia*. Alternatively, we employed a model that additionally constrained the *Acer* stem age. Here we used a normal prior distribution with a mean of 62 Ma and a standard deviation of 4 Ma, which resulted in a complex prior resembling an exponential distribution with an overall mean of 61.6 and a median of 61 Ma. We chose these different models for the prior distribution of fossil ages because the *Dipteronia* fossil from central Wyoming is more precisely dated than are the early *Acer* fossils. An exponential distribution of rates across branches is a one-parameter distribution (the parameter determines both the mean and the variance); it requires only the specification of the offset and the mean. By contrast, the normal prior distribution requires a mean and a standard deviation, which are not easy to choose. The normal distribution has the advantage, however, of not placing as much prior probability on a relatively narrow time frame as does an exponential distribution.

For BEAST analyses of the 98-taxon *rbcL* data set, minimal age constraints were again modeled as exponentially distributed priors, with the offsets given by fossils (below) and means of 1. The prior for the age of the root followed a uniform distribution between 0 and 137 Ma, based on the onset of angiosperm radiation (Hughes, 1994; Brenner, 1996). The minimal age of the stem lineage of *Aesculus* + *Handeliodendron* was constrained to 60 Ma (with the prior distribution following a simple exponential distribution with mean/median of 63.9/63.0 Ma), based on the oldest *Aesculus* fossils (Manchester, 2001). Of the more distant *Acer* outgroups, Biebersteiniaceae were constrained to minimally 54.8 Myr old, based on fossil pollen from the Neomugen Formation of Inner Mongolia (Late Paleocene, 57.0 to 54.8 Ma; Muellner et al., 2007); the prior distribution again followed an exponential distribution with 55.6/55.3 Ma mean/median. The *Ailanthus* stem lineage (Simaroubaceae) was constrained to minimally 52 Myr old, based on the fossil fruit taxon *A. confucii*, thought to be related to extant *A. altissima* (Corbett and Manchester, 2004), the species included in our DNA analyses. Here, the prior distributions were complex, comprising two overlapping exponential distributions with modes of ca. 52 and 55 Ma and an overall mean/median of 54.0/54.3 Ma. The stem of Cedreleae was constrained to minimally 49 Myr old, based on fruit and seed fossils of *Toona* from the London Clay (DeVore et al., 2005) that share morphological features of modern *Toona* and *Cedrela* (Muellner et al., 2007). The prior distribution for Cedreleae showed three overlapping exponential distributions with modes of ca. 49, 52, and 55 Ma and an overall mean/median of 50.9/50.2 Ma. To assess rate heterogeneity in the 98-taxon Sapindales *rbcL* dataset, we carried out local-clock analyses in PAML 3.14 (Yang, 1997) to compare the fit of models that assumed either a single rate for all Sapindales or two rates, one for *Acer* + *Dipteronia*, the other for the remaining Sapindales.

For comparison with the estimates obtained with the uncorrelated approach (in BEAST), we used three other clock approaches: the strict clock assumption of a single substitution rate (mostly carried out in PAUP); semiparametric rate smoothing (penalized likelihood) as implemented in *r8s* version 1.71 (Sanderson, 2002, 2004); and a Bayesian approach that assumes autocorrelated rates (and which therefore requires a rooted input phylogeny) as implemented in *multidivtime* (Thorne and Kishino, 2002). These analyses were necessarily carried out on data matrices that included outgroups. For strict clock analyses, we imported the highest likelihood tree found with RAxML into PAUP, outgroup-rooted it, and calculated branch lengths under a GTR+Γ+I+ clock model, with model parameters estimated in PAUP. The resulting branch length table was saved, the distance between a calibration node and the tip was then divided by the age of the calibration node to obtain a substitution rate, and this rate used to calculate the ages of other nodes of interest. For calibration of the strict clock, we sequentially explored the fossils described above.

For penalized likelihood analyses, we again imported the preferred ML topology into PAUP, outgroup-rooted it, and described its branch lengths under the GTR+Γ+I model. The resulting rooted trees with branch lengths became the input for *r8s*, outgroups were pruned from the tree, and cross-validation then used to determine the best level of clock enforcement (i.e., the smoothing parameter).

Multidivtime analyses were carried out as described in Renner et al. (2007), using an input topology obtained with RAxML and rooted on *Aesculus*. The minimal age of the *Acer*/*Dipteronia* stem lineage (i.e., the divergence of these two from *Aesculus*/*Handeliodendron*) was constrained to 63 Ma and the maximal age of the Hippocastanoideae stem to 72 Ma. This maximal age was chosen based on the age estimates for Sapindales clades of Muellner et al. (2007) and the oldest fossils of *Acer*, *Dipteronia*, and *Aesculus*, none of which predate 65 Ma; *multidivtime* does not perform well without at least one minimal and one maximal constraint (R. Thorne, *multidivtime* read-me file, 2003). Absolute ages and delimitation of fossil eras is from the geologic timescale of Gradstein et al. (2004).

Modeling the Acer *Diversification Rate*

We used a lineage-through-time (LTT) plot derived from an ultrametric tree (chronogram) for *Acer*/*Dipteronia* to graphically assess species diversification over time (Harvey et al., 1994). Taxon sampling in the chronogram included 50 of the estimated 124 or 156 species of *Acer* plus *Dipteronia* (van Gelderen et al., 1994; de Jong, 2002), and the LTT plot was therefore brought up to either 124 or to 156 living species. We also fit different survival models implemented in the APE package (Paradis, 1997; Paradis et al., 2004) to the chronogram to detect significant departures from a constant-rate null model of diversification (CR-BD). Two alternatives were compared to the CR-BD model, namely a model of a gradually changing diversification rate and one that assumes an abrupt change in rate before and after some breakpoint in the past. Because the constant-rate model is nested in both variable-rate models, it can be compared with each in a likelihood-ratio test (Paradis, 1997). Survival models can incorporate missing data (species known to belong to *Acer* but not sequenced) provided a minimum age is known for these species. In this study, we explored adding varying proportions of the 70 missing species at 5, 10, 15, 20, 25, 30, and 35 Myr. We did not use older origination times because we sampled all of the major clades within *Acer*. A worldwide analysis of maple fossils found striking decreases in occurrences of *Acer* macrofossils between 35 and 25 Myr in Europe, West Asia, and East Asia (Boulter et al. 1996: fig. 4), followed by a renewed increase after that period.

RESULTS

The Root of Acer

An outgroup-rooted ML tree obtained with the combined chloroplast data for 24 taxa (6674 characters, almost no missing data) is shown in Figure 1; *Dipteronia* and *Acer* appear mutually monophyletic, although the

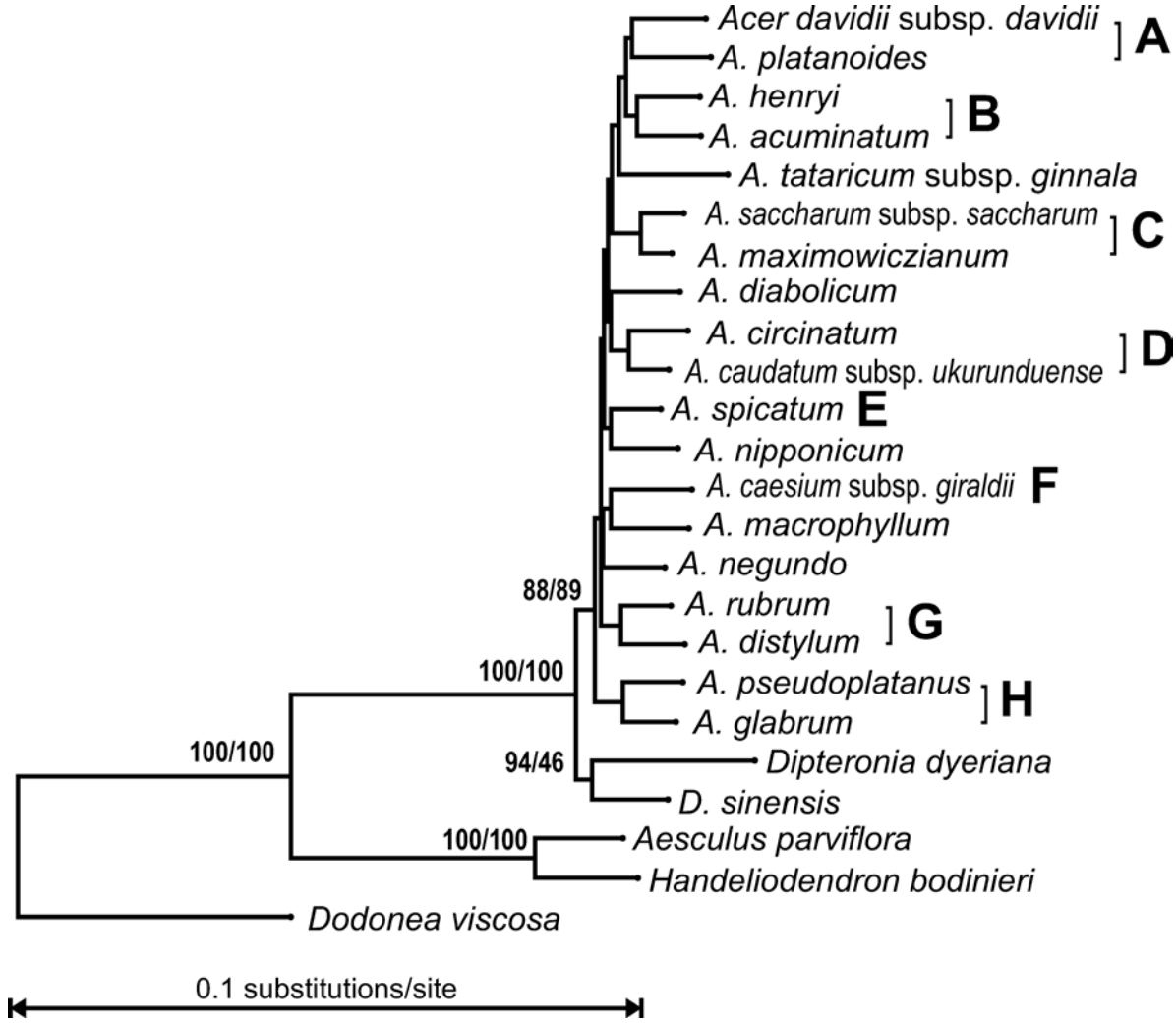

FIGURE 1. Outgroup-rooted maximum likelihood tree for 24 species of *Acer*, *Dipteronia*, and outgroups based on 6674 aligned nucleotides from six chloroplast loci analyzed under the GTR+*r* model. Values at nodes indicate bootstrap support under maximum likelihood and parsimony. Labels A to H refer to major well-supported clades and are also used in Figures 2 and 3.

monophyly of *Dipteronia* is only weakly supported. With the dataset enlarged from 24 to 74 taxa by the inclusion of species that had not necessarily all been sequenced for *rbcL*, there was continued weak support for the mutual monophyly of *Acer* and *Dipteronia*. Midpoint rooting of the ML trees obtained for the 24 and 74 taxon datasets placed the root between *Dipteronia* and *Acer*. The backbones of the *Acer* trees were essentially unresolved, while there was considerable support for tip clades (labeled A to H in Figs. 1 to 3).

A relaxed clock model with uncorrelated rate change (in BEAST) applied to the 53-taxon *Acer*/*Dipteronia* dataset usually placed the root between *Acer* (posterior probability [PP] 0.95) and *Dipteronia* (PP 0.51), rendering each monophyletic (Fig. 3). The minority of topologies showed the root between the two *Dipteronia* species (with *D. dyeriana* usually sister to all other species), rendering *Dipteronia* paraphyletic. A strict clock model applied to the same data also placed the root between *D. dyeriana* and all other species.

To assess the decisiveness of the data, we compared 37 alternative rootings using Bayes factors. The results (Table 1) show that most root positions with prior probabilities of zero either were not significantly worse than the preferred root position (when using the upper bound prior probability values; see Materials and Methods) or received strong support in their favor (when using the lower bound prior probability values). Because Bayes factors measure the *change* in support for one hypothesis versus the other coming from the data, they can be very high when prior probabilities are essentially zero. Thus, a root position at branch 5, between a clade including all *Acer* species except *A. glabrum* and *A. pseudoplatanus* and the remaining taxa, changes from a prior probability of essentially zero to a posterior propability of 0.011, resulting in a large BF in its favor (Table 1). In the following, we only consider root positions that received posterior probabilities at or above an arbitrary threshold of 0.01 (branches 2 to 5 and 21 in Table 1 and Fig. 3). Among those, root positions at branches 3 (to *D. sinensis*) and 21 (to *A. tataricum* subsp. *ginnala*) are rejected in favor of the preferred root position by BFs > 10, whereas roots at branch 2 (to *D. dyeriana*) or 4 (to the clade of *A. glabrum* and *A. pseudoplatanus*) are not (Table 1, Fig. 3). When the root position at a given branch is compared with that at any other branch (Table 1, column 5), BFs again "overrate" positions 1, 2, 4, and 5, which receive significant support in their favor (BF > 10) because their prior probabilities are essentially zero.

Substitution rate heterogeneity along the tree is substantial as judged by the high coefficient of variation (mean 0.234; 95% highest posterior density [HPD] 0.111 to 0.362). Also, a model of autocorrelated rate change, as assumed by penalized likelihood and *multidivtime* relaxed clock models, is rejected (covariance mean 0.007; 95% HPD −0.186 to 0.202). However, testing for autocorrelation in BEAST may be problematic because the current implementation does not allow the two branches chosen at random to have their rates switched to have identical rates, which biases against finding positive autocorrelation (Rannala and Yang, 2007). However, because the 95% HPD interval found here safely includes zero, the inferred lack of autocorrelation appears robust.

Divergence Times and Shifts in Diversification Rates

Table 2 lists the estimates for when the North American species of *Acer* split from their closest Asian relatives. In spite of poor model fit, divergence time estimates obtained with all three relaxed clock approaches agree with each other (Table 2). Figure 3 shows a BEAST chronogram with the time estimates and their errors. Increasingly wide error bars towards the older parts of the BEAST chronogram indicate the lack of phylogenetic signal in deeper parts of the tree. This is supported by the bipartition network (Fig. 4), which depicts the distribution of conflicting signal in the data. Competing splits are confined almost entirely to the central part of the network (the grey-shaded area in Fig. 4), where they cause very low bootstrap support values (generally below 25%); in contrast, the mid-level and near-tip clades A through H are relatively well circumscribed and contain few contradictory splits.

Acer circinatum, from the northwestern United States, apparently diverged from its Asian relatives (in the *Palmata* clade) in the Early to Mid Miocene, *A. macrophyllum* diverged from its Asian sisters *A. caesium* and *A. pilosum* already in the Late Eocene, and the third western North American species, *A. glabrum*, separated from *A. pseudoplatanus* in the Late Eocene or Oligocene (Table 2). The northeastern American *A. spicatum* appears to have split from the Japanese *A. carpinifolium* in the Oligocene, whereas *A. pensylvanicum* split from *A. crataegifolium* and *A. tegmentosum* in the Late Miocene. The estimates for the crown of the *Acer* core clade, which includes the North American *A. saccharum*, range from Eocene and Oligocene to the Miocene (Table 2), whereas *A. rubrum* and *A. saccharinum* (both widespread throughout the eastern United States) appear to have split from their Asian relatives in Late Oligocene to Early Miocene and from each other in the Pliocene. The ninth North American species, *A. negundo*, is an isolated, apparently early-divergent species (Mid to Late Eocene), as are the Japanese *A. distylum* (sister to the remainder of clade G in Figs. 2 and 3), *A. nipponicum*, the Eurasian *A. tataricum*, and the two species of *Dipteronia* from China. The most tropical species of *Acer*, *A. laurinum*, which is widespread in Hainan, Vietnam, Sumatra, Borneo, Celebes, Java, and Indonesia's Lesser Sunda Islands, belongs to a clade that is at least 30 Myr old (Table 2).

The LTT plot (Fig. 5) derived from an optimal chronogram suggests that diversification (speciation minus extinction) in *Acer* began leveling off between 30 and 20 Myr. The results of censored survival analyses under three models of diversification varied with the way the missing 70 species were added to the LTT plot (Materials and Methods). Models of constant diversification were favored when about half the missing species were allocated to the period between 35 and 20 Myr, whereas models of a gradual decrease in diversification were

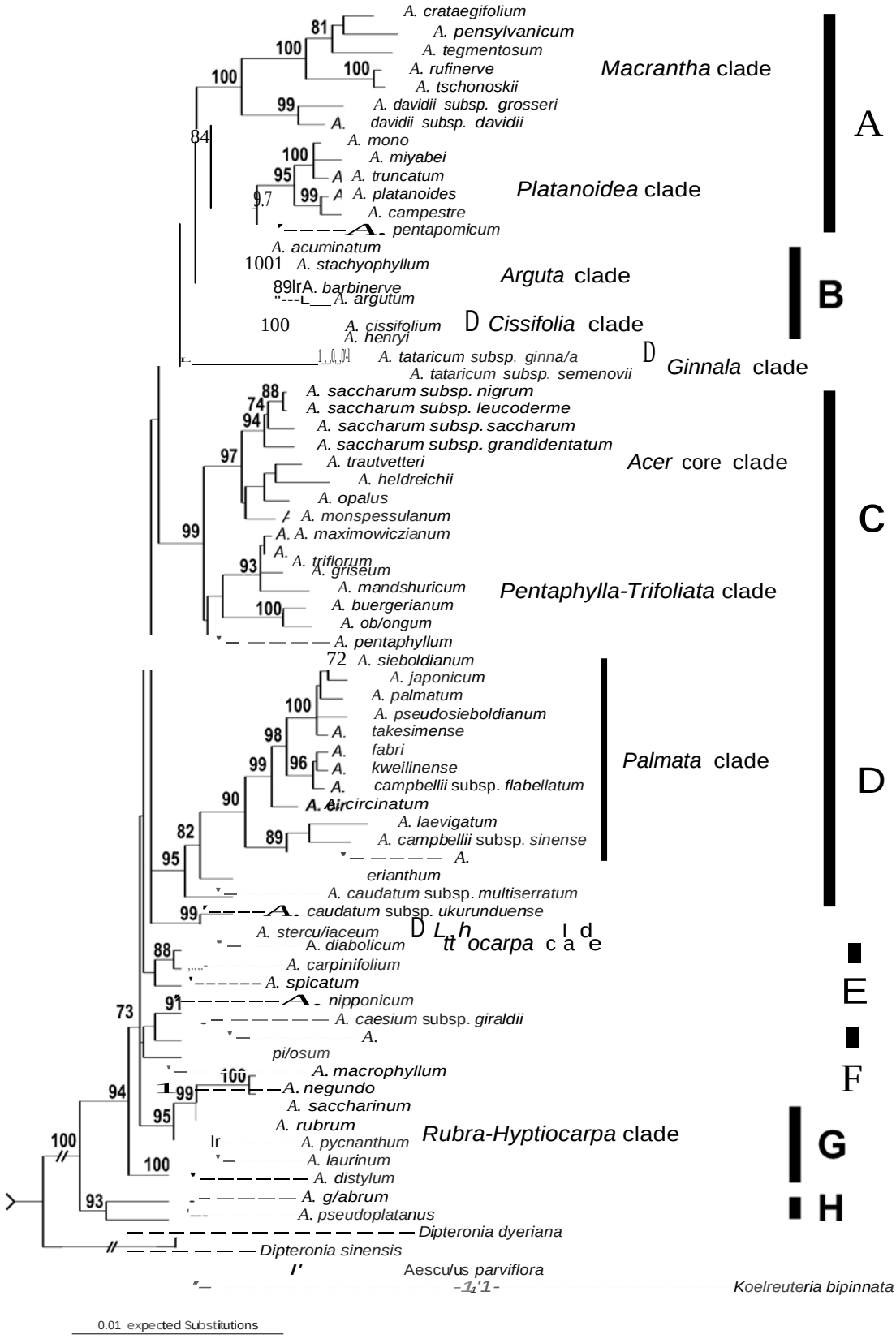

FIGURE 2. Ou tgroup-moted rnaxirnurn likelihood tree for species and subspecies of *Acer*, *Di pteronia*, and outgroups based on the sarne six chloroplast loci as a nalyzed in Figure 1 but including 14 species that lacked sequences for the *rbcL* gene. Values at nodes indicate bootstrap support under rnaxirnurn likelihood optirnization. The section narnes to right are frorn the classification of van Gelderen et al. (1994). Narnes of North Arnerican species are printed in bold. Clades are labeled as in Figures 1 and 3.

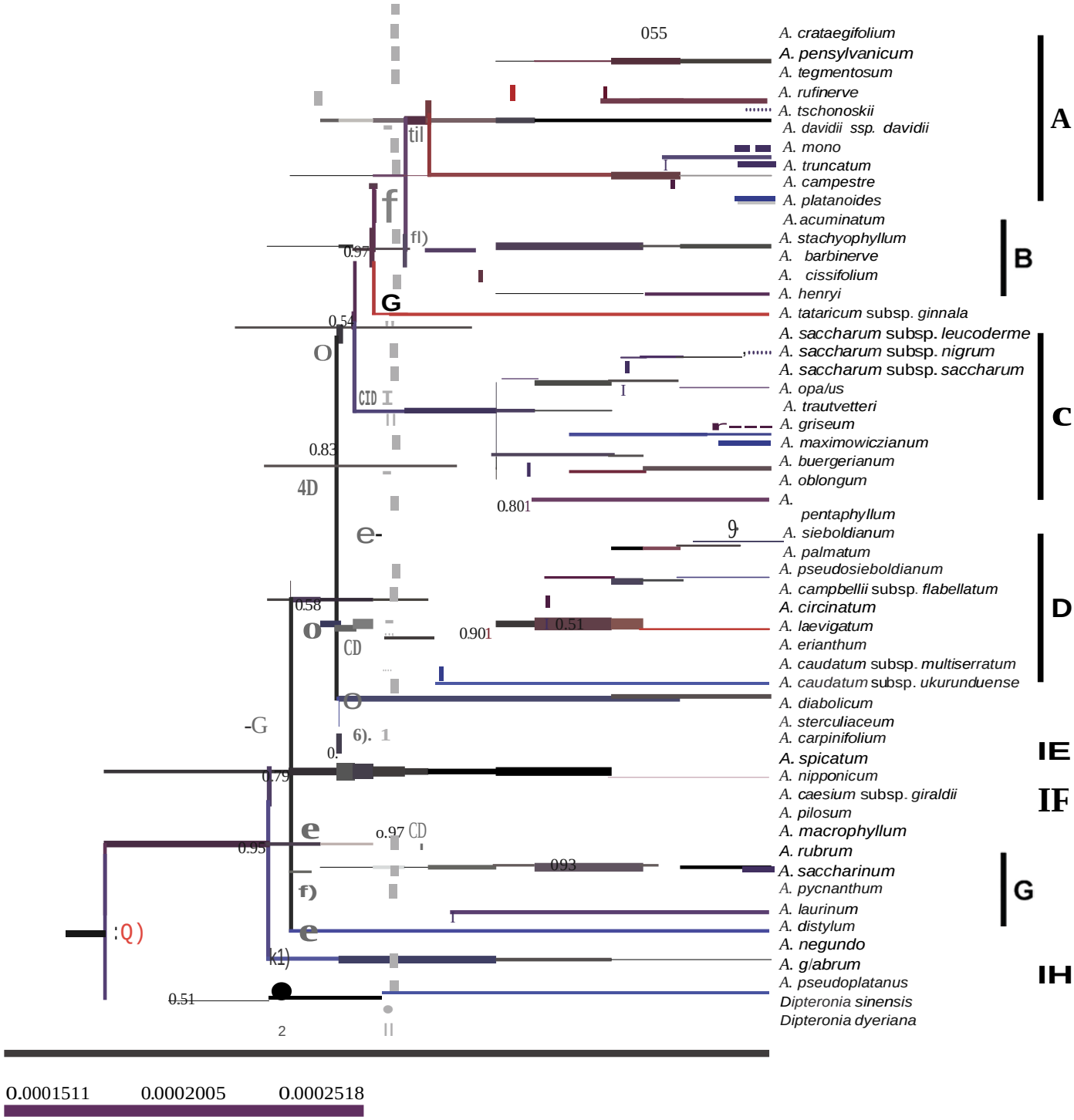

FIGURE 3. Chronogram based on a majority rule consensus tree of 72,000 trees, for 53 species and subspecies of *Acer* and *Dipteronia* from six combined chloroplast loci obtained under a model of uncorrelated rate change using two calibration points (see text for details). Node heights are median ages, with bars indicating the 95% highest posterior density intervals. Branches are color-coded from cold to increasingly warm colors, the latter indicating increasing Substitution rates (substitution per site and million years). The vertical hatched grey line indicates a turnover seen in the fossil record at about 35 Myr between an "amorphous" *Acer* pool and modern *Acer* lineages (Boulter et al., 1996). Names of North American species are printed in bold. The numbered branches along the backbone of the tree refer to the experimental positions of the root that were compared using Bayes factors (BF; see Table 1). Positions with posterior probabilities <0.01 are marked in grey and are not considered further. Using a cutoff of 2lnBF > 10, the root position at branch 1 (marked in red) is not significantly better than root positions 2, 4, and 5 (marked in white), but significantly better than root positions 3 and 21 (marked in black).

TABLE 1. Prior and posterior probabilities of the 37 different root positions experimentally enforced in the *Acer/Dipteronia* tree shown in Figure 3, which also shows each of the numbered root positions. Column 4 ($H_{1/n}$) compares the root position at branch 1 (marked in red in Fig. 3) with a position at the branch in the respective row, whereas column 5 ($H_{n/not-n}$) compares a root position at the respective branch with that at any other branch. Root positions receiving posterior probabilities >0.01 are marked in bold. BF stands for Bayes factor. Using a cutoff of 2lnBF > 10, a root position at branch 1 is not significantly better than root positions 2, 4, and 5 but significantly better than positions 3 and 21.

Branch	Prior probability[a]	Posterior probability	$2 \times \ln BF_{12}$	
			$H_{1/n}$	$H_{n/not-n}$
1	0.00002571	**0.43656944**		**20.63**
2	0.00078571	**0.41670833**	**6.93**	**13.62**
3	0.00076000	**0.05256944**	**11.01**	**8.58**
4	0.00004286	**0.01270833**	**8.10**	**11.41**
5	3.53×10^{-83} (0.00000285)	**0.01106944**	**−351.22 (2.96)**	**370.72**
6	0.00071143	0.00709722	14.88	4.61
7	3.53×10^{-83} (0.00000285)	0.00452778	−349.43 (4.74)	368.92
8	3.53×10^{-83} (0.00000285)	0.00083333	−346.05 (8.13)	365.53
9	3.53×10^{-83} (0.00000285)	0.00181944	−347.61 (6.57)	367.09
10	0.00097143	0		
11	0.00002000	0		
12	0.00000286	0.00072222	8.41	11.07
13	3.53×10^{-83} (0.00000285)	0.00240278	−348.17 (6.01)	367.65
14	0.00076286	0.00076389	19.48	0.00
15	0.00004286	0.00002778	20.35	−0.87
16	0.00001429	0.00208333	9.51	9.97
17	3.53×10^{-83} (0.00000285)	0.00362500	−348.99 (5.19)	368.48
18	3.53×10^{-83} (0.00000285)	0.00108333	−346.57 (7.60)	366.05
19	3.53×10^{-83} (0.00000285)	0.00172222	−347.50 (6.68)	366.98
20	3.53×10^{-83} (0.00000285)	0.00693056	−350.29 (3.89)	369.78
21	0.00064857	**0.01593056**	**13.08**	**6.43**
22	3.53×10^{-83} (0.00000285)	0.00500000	−349.63 (4.54)	369.12
23	3.53×10^{-83} (0.00000285)	0.00077778	−345.91 (8.27)	365.39
24	3.53×10^{-83} (0.00000285)	0.00111111	−346.62 (7.55)	366.11
5a	3.53×10^{-83} (0.00000285)	0.00284722	−348.51 (5.67)	367.99
5b	3.53×10^{-83} (0.00000285)	0.00063889	−345.52 (8.66)	365.00
5c	3.53×10^{-83} (0.00000285)	0		
5d	3.53×10^{-83} (0.00000285)	0.00008333	−341.44 (12.73)	360.92
5e	3.53×10^{-83} (0.00000285)	0.00025000	−343.64 (10.54)	363.12
5f	3.53×10^{-83} (0.00000285)	0.00186111	−347.66 (6.52)	367.14
5g	3.53×10^{-83} (0.00000285)	0.00001389	−337.86 (16.32)	357.34
5h	3.53×10^{-83} (0.00000285)	0.00016667	−342.83 (11.35)	362.31
5i	3.53×10^{-83} (0.00000285)	0.00079167	−345.95 (8.23)	365.43
5j	3.53×10^{-83} (0.00000285)	0.00066667	−345.60 (8.57)	365.08
13a	3.53×10^{-83} (0.00000285)	0.00029167	−343.95 (10.23)	363.43
13b	3.53×10^{-83} (0.00000285)	0.00063889	−345.52 (8.66)	365.00
13c	3.53×10^{-83} (0.00000285)	0.00011111	−342.02 (12.16)	361.50

[a] See Material and Methods for details; prior probabilities of zero were set to two extreme values, one the probability of a single rooted topology with 53 taxa and the other (in parentheses) the probability 1/350,001.

favored when the bulk of missing species was allocated to younger periods. In other words, when the censored species were moved towards the present, the decrease in diversification through time is less steep (as expected). These results suggest that the inferences are robust to this amount of missing data.

DISCUSSION

Tree Rooting with a Relaxed Clock

With some 6500 base pairs of chloroplast sequence data, outgroup rooting, and a species sample that spanned the root of each genus, *Dipteronia* and *Acer* are mutually monophyletic, although this has weak statistical support. Midpoint rooting also places the root between *Dipteronia* and *Acer*. Accepting the sister group relationship between *Dipteronia* and *Acer* as true, a relaxed clock model with uncorrelated rate change between ancestors and decedents correctly specified this root without an outgroup, albeit also without significant support (Table 1, Fig. 3). To our knowledge, this might be the first exploration of the use of clock analyses in BEAST for rooting purposes. Explicit tests of alternative root positions via Bayes factors revealed that a rooting between *Dipteronia* and *Acer* is better supported than most alternative rootings, although it is not significantly better than root positions 2, 4, and 5 (Table 1, Fig. 3). Bayes factors crucially depend on good estimates of both prior and posterior probabilities. More intensive sampling of tree space to obtain stronger prior probabilities might have improved the reliability of the BFs calculated here, but the possible resulting changes are unlikely to affect our main conclusions because we only considered root placements with posterior probabilities ≥0.01.

Regardless of the specifics of the *Acer/Dipteronia* system, the possibility of using uncorrelated rates relaxed

TABLE 2. Age estimates (in Myr) for Northern Hemisphere disjunctions in the genus *Acer* based on three relaxed clock approaches: penalized likelihood, a Bayesian molecular clock with an autocorrelated rates model, and a Bayesian clock with an uncorrelated rates model (Materials and Methods). Values in brackets refer to the 95% highest posterior density interval. Names of North American species are printed in bold. Geologic eras based on Gradstein et al. (2004). MRCA = most recent common ancestor; nt = nucleotides.

Nodes involving North American species of *Acer*	Penalized likelihood 74 taxa, 6674 nt; constraints: *Dipteronia*/*Acer* min. 63 Ma; root min 65 Ma, max 100	Autocorrelated rates 59 taxa, 6619 nt; constraints: *Aesculus* vs. *Acer-Dipteronia* min 63 Ma and max. 72 Ma	Uncorrelated rates lognormal 53 taxa, 6487 nt; exponential prior with a median of 62 Ma on the MRCA of *Dipteronia*/*Acer negundo*
Acer/*Dipteronia* split	N/A	60 (46–70) Mid Paleocene	61 (60–63) Mid Paleocene
A. negundo (in unresolved backbone)	N/A	37 (22–52) Late Eocene	44 (32–56) Mid Eocene
A. glabrum/*A. pseudoplatanus*	25	35 (22–49) Late Eocene	26 (16–40) Late Oligocene
A. macrophyllum/*A. caesium* + *A. pilosum*	37	34 (19–49) Late Eocene	34 (23–45) Late Eocene
A. spicatum/*A. carpinifolium*	27.8	32 (21–46) Early Oligocene	25 (15–36) Late Oligocene
Rubra-Hyptiocarpa clade/ *A. distylum*	21.9	31 (17–45) Early Oligocene	29 (20–41) Early Oligocene
A. rubrum* + *A. saccharinum/ *A. pycnanthum* + *A. laurinum*	21.9	23 (12–36) Oligocene Miocene border	17 (11–26) Early Miocene [N Am/E As] 20 (13–29) *Rubra*-clade/*A. laurinum*
A. saccharum/Asian *Acer* core clade	14.6	18 (9–29) Early Miocene	14 (8–20) Mid Miocene
A. circinatum/Asian *Palmata* clade	13.6	12 (6–21) Mid Miocene	12 (7–17) Mid Miocene
A. pensylvanicum/*A. crataegifolium* (+ *A. tegmentosum*)	9.9	8 (3.4–14) Late Miocene	9 (5–13) Late Miocene 11 (7–16) MRCA of all 3 taxa
A. rubrum/*A. saccharinum*	3.0	4 (0.5–11) Late Pliocene	2.6 (0.6–5.6) Late Pliocene

clock models to root datasets for which outgroup rooting is problematic (or not possible) presents an exciting application of clock analyses. This was presaged by the simulation study of Huelsenbeck et al. (2002), who compared outgroup rooting, molecular clock–based rooting, and nonreversible models of DNA substitution and found that the molecular clock criterion correctly identified the root a large proportion of the time. Clock-based rooting, however, was not possible in practice before the development of an approach that can handle large (real-life) datasets. With the availability of uncorrelated rates models of molecular evolution (Drummond et al., 2006), one can now use clock-based rooting to explore whether outgroup rooting might be biased by long-branch attraction or simply as an independent test of correct rooting (as done here).

In the 53-taxon-6847-bp *Acer*/*Dipteronia* data, the correct root (assuming that it lies between *Dipteronia* and *Acer*) was found only under a relaxed clock, while under a strict clock (in BEAST), the root was placed between *D. dyeriana* and all other species. The separation of *D. dyeriana* from the remaining species might be explained by its higher substitution rate compared to *D. sinensis* and the majority of *Acer* species (Fig. 3). Although a strict clock model performed well in most of the experimental datasets analyzed by Huelsenbeck et al. (2002), misleading results could be obtained if a crucial taxon close to the root had a substitution rate substantially different from taxa nearby.

Shifts in Diversification Rates and Timing of the Beringian Disjunctions

Inspection of the lineage-through-time plot for *Acer* and *Dipteronia* suggests a gradual decrease in the diversification rate sometime between 30 and 20 Myr (Fig. 5), but the ratio of some 50 sequenced species to 70 (or more) not-sequenced species cautions against attaching too much weight to the LTT plot. A worldwide analysis of maple fossils revealed a striking decrease in occurrences of *Acer* megafossils between 35 and 25 Myr in Europe, West Asia, and East Asia, but not in North America (Boulter et al., 1996). These apparent waves of extinctions were followed by new radiations in Europe, East Asia, and Western Siberia (Boulter et al. 1996; Kvaček, 1996). Doubtless, both *Acer* and *Dipteronia* have suffered substantial extinction since the Early Oligocene (33 to 32 Ma), when *Dipteronia* was still present in Oregon (McClain and Manchester, 2001). The number of combinations of extinction and speciation rates that can result in the same net diversification is infinite, and LTT plots are therefore poor tools for distinguishing these two processes. Nevertheless, changes in diversification can be inferred as long as early lineages are reasonably well sampled (Harvey et al., 1994; Pybus and Harvey, 2000; Nee, 2006; Ricklefs, 2007), which we believe is the case for *Acer.* Overall, we sampled 74 of the 124 to 156 species (Fig. 2), of which the chronogram includes 53 selected to span the root.

Divergence time estimation under a model of uncorrelated rate change, by not relying on a resolved input topology, may be relatively robust against unresolved relationships in the deeper part of a phylogeny (as is the case here), and even clock approaches that assume autocorrelation are surprisingly little affected by polytomies or zero-length branches. Thus, Won and Renner (2006) analyzed a gymnosperm dataset, using either different resolutions of a polytomy or maintaining the polytomy in the input tree, and found that time estimates were essentially unaffected. Apparently, polytomies impact posterior branch lengths only where branch length uncertainty occurs throughout the tree, which clearly is not the case

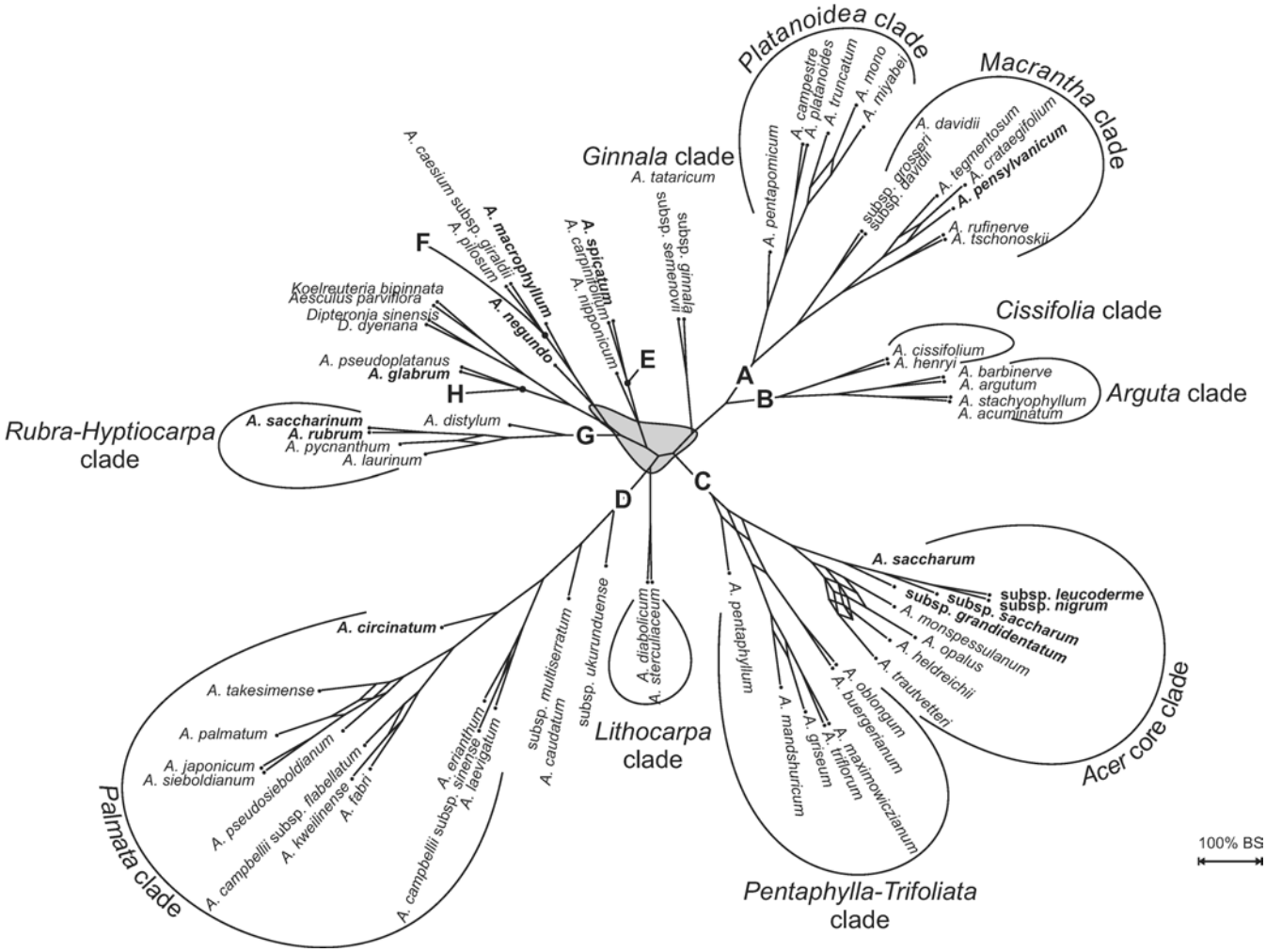

FIGURE 4. Bipartition network for 70 species and subspecies of *Acer*, *Dipteronia*, and outgroups based on 100 ML bootstrap replicates (6577 aligned nucleotides from six chloroplast loci; Fig. 2). Only bipartitions occurring in more than 20% of replicates are shown. The grey-shaded area is characterized by many competing splits, with bootstrap support values below 25%. Clades are labeled as in Figures 1 and 2; names of North American species printed in bold.

in the *Acer* data. In the present study, Bayesian relaxed clocks yielded similar time estimates, whether they relied on an input topology and autocorrelated rates or not (Table 2). In general, however, autocorrelated models outperform uncorrelated models when age estimation is the goal (Lepage et al., 2007).

The following inferences about the Beringian disjunctions in *Acer* concern only nodes towards the tips of the tree that have statistical support; for absolute times we rely on the estimates obtained with the two Bayesian relaxed clock approaches, calibrated with a *Dipteronia* fossil (Table 2). The pre-Oligocene burst of *Acer* radiation (Figs. 3 and 5) inferred in this study fits with the abundant Eocene and Oligocene record of the genus (Wolfe and Tanai, 1987; Boulter et al., 1996; Manchester, 1999; McClain and Manchester, 2001; Kittle et al., 2005). Much of this diversity, especially in Europe and North America, appears to have gone extinct during the Late Oligocene–Early Miocene (Walther, 1972; Wolfe and Tanai, 1987; Boulter et al., 1996). Nevertheless, living *A. macrophyllum* may descend from a lineage with fruits and foliage resembling the extinct *A. osmontii*, which occurred in Oregon during the Early Oligocene at ca. 33 Ma. Similarly, the living European species *A. heldreichii* (incl. *A. trautvetteri*) and *A. pseudoplatanus* have leaf traits that closely resemble those of *A. haselbachense*, which lived in Central Europe some 35 Myr (Walther, 1972; Grimm et al., 2007). A difficulty with assigning fossils to living clades of *Acer*, however, is that similar leaf and fruit characters have evolved repeatedly. For example, leaves of North American *A. spicatum* closely resemble those of *A. caudatum* subsp. *ukurunduense*, and leaves of North American *A. negundo* resemble those of Japanese *A. cissifolium* and Chinese *A. henryi* (van Gelderen et al., 1994: 64), yet these species are not closely related (Fig. 2; further examples are illustrated in Grimm et al., 2007: fig. 7). This uncertainty is exemplified by the recent reassignment of most "*Acer arcticum*" leaves from the North American

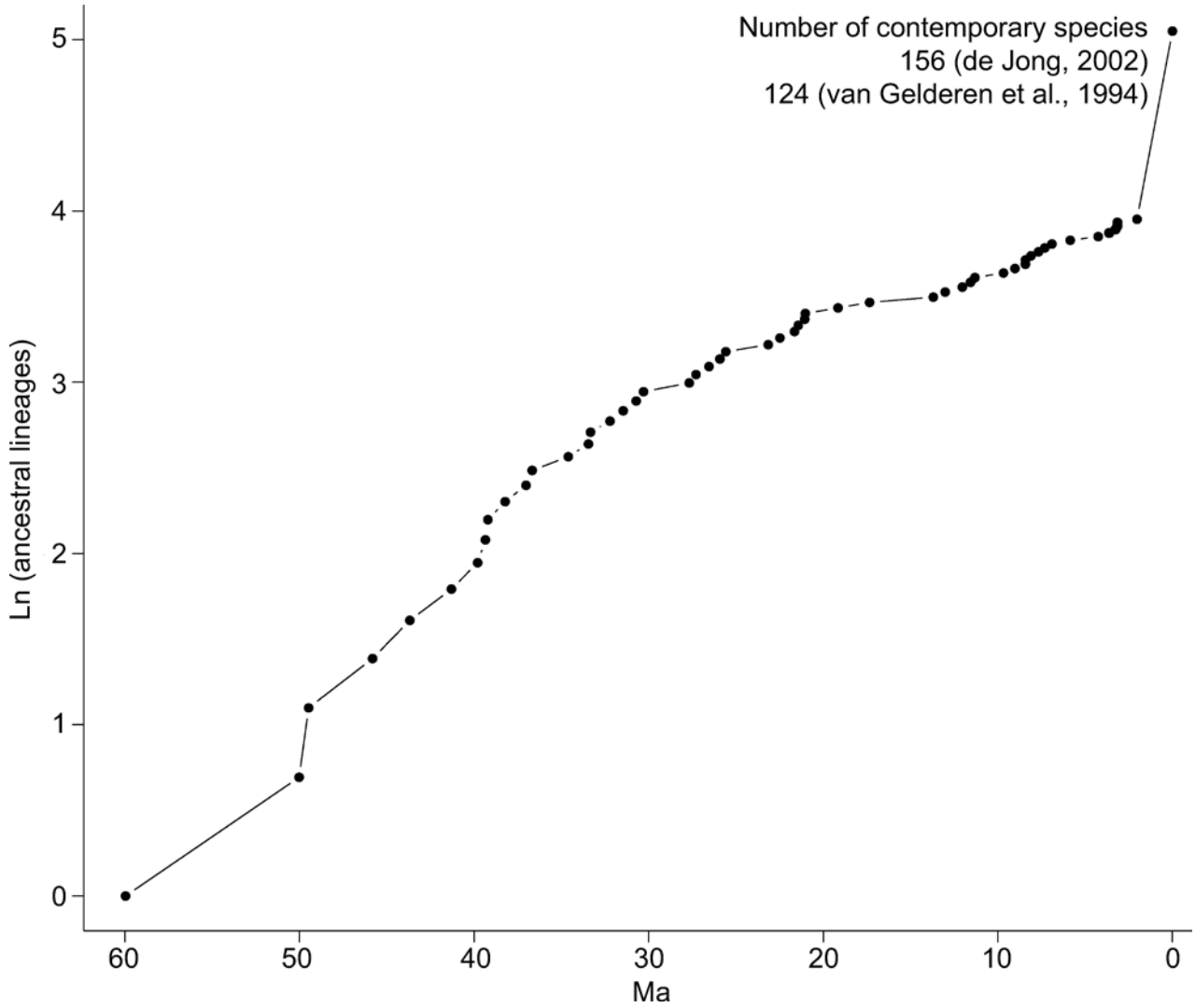

FIGURE 5. Lineage-through-time plot for *Acer* and *Dipteronia*, based on an optimal ultrametric tree rooted between *Acer* and *Dipteronia*. Visual inspection and survival analyses (see text) suggest a decrease in diversification between 30 and 20 Myr.

Paleocene and Eocene (and also Eocene floras of northeastern China) to *Deviacer wolfei*, whereas "*Acer arcticum*" fruits have been reassigned to Polygalaceae (Manchester, 1994; Pigg et al., 2004).

In spite of large errors around the molecular divergence times it is clear that the extant North American species of *Acer* diverged from their respective Asian sister clades at widely different times (Table 2). Wolfe (1981), based on an extensive knowledge of *Acer* macrofossils, arrived at the same conclusion. He thought that *Acer* originated in (western) North America (his fig. 10.1) and that some lineages spread to eastern Asia in the Early Eocene, others from Asia to North America in the Early Miocene (the *A. pensylvanicum*–containing section *Macrantha*) or Late Pliocene (*A. circinatum*, section *Palmata*). Wolfe also thought that the *Negundo* and *Rubra* lines (which in his view did not include *A. laurinum*) originated in North America during the Middle Eocene and that the *A. macrophyllum* line originated in western North America in the Late Eocene. The rough coincidence with several of our divergence time estimates (Table 2) is striking since we did not use any internal constraints on node times. One of Wolfe's main conclusions was that "the distribution patterns of fossil *Acer* do not support the hypothesis of an Arcto-Tertiary Geoflora," because many of the vicariance events occur later than the abrupt climate cooling at the end of the Eocene. Nor do they support the concept of fragmentation of one biota. "Rather, the history of *Acer* is replete with repeated dispersals and vicariant events through a period of about 60 million years" (Wolfe, 1981:422).

Based on the average of the estimated divergence times, North American taxa split from their Asian sister taxa at a rate of about once per 5 Ma beginning about 40 Myr. What is remarkable about diversification in *Acer* is that except for the recent divergence of *A. rubrum* and

A. saccharinum, the North American lineages have not formed new species, at least among those that are still extant. This is consistent with the discrepancy in diversity favoring eastern Asia over eastern North America in the number of species in disjunct genera (Qian and Ricklefs, 2000, 2004) and analyses that found diversification of sister lineages to have been more rapid in Asia than in North America (Xiang et al., 2004).

Our PAML and BEAST analyses of the 98-taxon Sapindales *rbcL* dataset rejected a molecular clock but yielded no evidence of a strong rate change in the common ancestor of *Acer* and *Dipteronia*. A PAML-estimated non-clock model had a likelihood score of −12,476.6, a single rate clock model a score of −12,858.4, and a two-rate local clock model with one rate for *Acer*/*Dipteronia*, the other for the remaining Sapindales had a score of −12,858.0, not significantly different from the single-rate model. When the 98-taxon set was analyzed in BEAST with minimal age constraints only on the *Aesculus* and *Dipteronia* crown groups, the *Acer* crown was dated to 30 (18–44) Ma and the divergence of *Acer* and *Dipteronia* to 34 (20–48) Ma. Even younger ages were obtained when neither *Aesculus* nor *Dipteronia* were constrained (in runs relying on other Sapindales fossils); the crown-group age of *Acer*/*Dipteronia* was then less than 20 Myr old (data not shown). These age estimates are in the range of those obtained by Wikström et al. (2001), who in an angiosperm-wide chronogram dated the split between *Acer* and *Aesculus* to 20 to 26 Ma (using a single calibration point far from Sapindaceae, namely the divergence between Fagales and Cucurbitales). Such young ages for the *Acer*/*Dipteronia* split are strongly contradicted by the Paleocene fossil records of both genera. One explanation for the discrepancy might lie in the saturation of conserved genes, such as the Rubisco enzyme (*rbcL*), which is under strong selection (Kapralov and Filatov, 2007), suggesting that molecular dating might fare better with a mix of coding and non-coding gene regions. Indeed, some of the worst artifacts in angiosperm-wide time estimation occurred when non-synonymous substitutions were used, and the bias was often in opposite directions (Sanderson and Doyle, 2001: fig. 12). Alternatively, strongly delayed sorting of ancestral chloroplast lineages in *Acer* might explain some of the relatively young molecular estimates. Testing this possibility would require a comparison with time estimates from nuclear data.

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