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Dating the Dipsacales: comparing models, genes, and evolutionary implications[1]

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Dipsacales is an asterid angiosperm clade of ca. 1100 species, with most of its lineages occupying temperate regions of the Northern Hemisphere. A recent phylogenetic analysis based on 7593 nucleotides of chloroplast DNA recovered a well-resolved and strongly supported phylogenetic hypothesis, which we use here to estimate divergence times within the group. A molecular clock is strongly rejected, regardless of data partition. We used recently proposed methods that relax the assumption of rate constancy among lineages (local clocks, nonparametric rate smoothing, penalized likelihood, and Bayesian relaxed clock) to estimate the ages of major lineages. Age estimates for Dipsacales varied widely among markers and codon positions, and depended on the fossils used for calibration and method of analysis. Some methods yielded dates for the Dipsacales diversification that appear to be too old (prior to the presumed 125 my [million years] age of eudicots), and others suggested ages that are too young based on well-documented Dipsacales fossils. Concordant penalized likelihood and Bayesian studies imply that Dipsacales originated in the Cretaceous, as did its two major lineages, Adoxaceae and Caprifoliaceae. However, diversification of crown Adoxaceae and Caprifoliaceae mainly occurred in the Tertiary, with the origin of major lineages within these clades mainly occurring during the Eocene. Another round of diversification appears to have occurred in the Miocene. Several radiations, such as Valerianaceae in South America and Dipsacaceae around the Mediterranean, are even more recent. This study demonstrates the wide range of divergence times that can be obtained using different methods and data sets, and cautions against reliance on age estimates based on only a single gene or methodology. Despite this variance, significant conclusions can be made about the timing of Dipsacales evolution.

Key words: Adoxaceae; Bayesian relaxed clock; Caprifoliaceae; Dipsacales; local clocks; molecular clock; nonparametric rate smoothing; penalized likelihood.

The phylogeny of Dipsacales has received considerable attention over the past two decades (e.g., Donoghue, 1983; Judd et al., 1994; Donoghue et al., 1992, 2001, 2003; Backlund, 1996; Backlund and Donoghue, 1996; Pyck et al., 1999; Pyck and Smets, 2000; Bell et al., 2001; Bell and Donoghue, 2003; Zhang et al., 2003). Both morphological and molecular data, as well as combined analyses, have been used to infer relationships. Recently, Bell et al. (2001) presented a phylogeny based on 7593 nucleotides of chloroplast DNA (*rbcL, ndhF, matK, trnL-F* intergenic spacer (IGS), *trnL* intron, *atpB-rbcL* intergenic spacer, and a *mat*K intron). This analysis recovered a well-resolved phylogeny that was in strong agreement with previous analyses. To this data set Donoghue et al. (2003) added sequences from the nuclear ribosomal internal transcribed spacer (ITS) region. The support values (bootstrap percentages) were strong for all major clades in Dipsacales and in strong agreement with the recently proposed phylogenetic classification for Dipsacales (Donoghue et al., 2001; Fig. 1). This phylogenetic hypothesis provides a framework to investigate the timing of the diversification of the clade.

Several attempts have been made to estimate the age of Dipsacales. Backlund's (1996) study used a linear regression method with *rbcL* sequence data and several fossil calibration points, and estimated that Dipsacales originated around 70–60 million years ago (mya), during the late Cretaceous or Early Tertiary. This study assumed a molecular clock for the *rbcL* data and used the midpoint of major geological time periods for fossil calibrations. In an analysis of 560 species based on a three-gene phylogeny (Soltis et al., 1999, 2000), Wikstrom et al. (2001) used nonparametric rate smoothing (NPRS) to estimate divergence times across angiosperms. These authors calibrated their tree by fixing the split between Fagales and Cucubitales at 84 mya based on the fossils *Protofagacea* (Herendeen et al., 1995) and *Antiquacupula* (Sims et al., 1998), both from the Late Santonian. They estimated an age for Dipsacales of 81–78 my. Since the purpose of the Wikstrom et al. analysis was to estimate the age of angiosperms as a whole, and of major lineages within angiosperms, the sampling of Dipsacales was poor. The accuracy of the age estimates from both of these analyses may also suffer from the fact that they used the ''wrong'' topology for Dipsacales based on our current knowledge.

Several methods have recently been proposed to deal with non-rate constancy among lineages (Sanderson, 1997; Thorne et al., 1998; Huelsenbeck et al., 2000; Yoder and Yang, 2000; Kishino et al., 2001; Sanderson, 2002; Thorne and Kishino, 2002). These methods variously ''relax'' the null hypothesis of a uniform rate of molecular evolution across all lineages, allowing different parts of a tree to experience different rates. In this paper we apply a variety of these dating methods (local clocks, nonparametric rate smoothing, penalized likelihood, and Bayesian relaxed clock methods) to estimate the age of the Dipsacales and divergence times for the major lineages within this clade. We compare the results of different strategies, using several chloroplast markers (separately and in combination), and the influence of fossil calibrations and maximum and minimum constraints in such analyses. Our results bear on the tempo of diversification and morphological evo-

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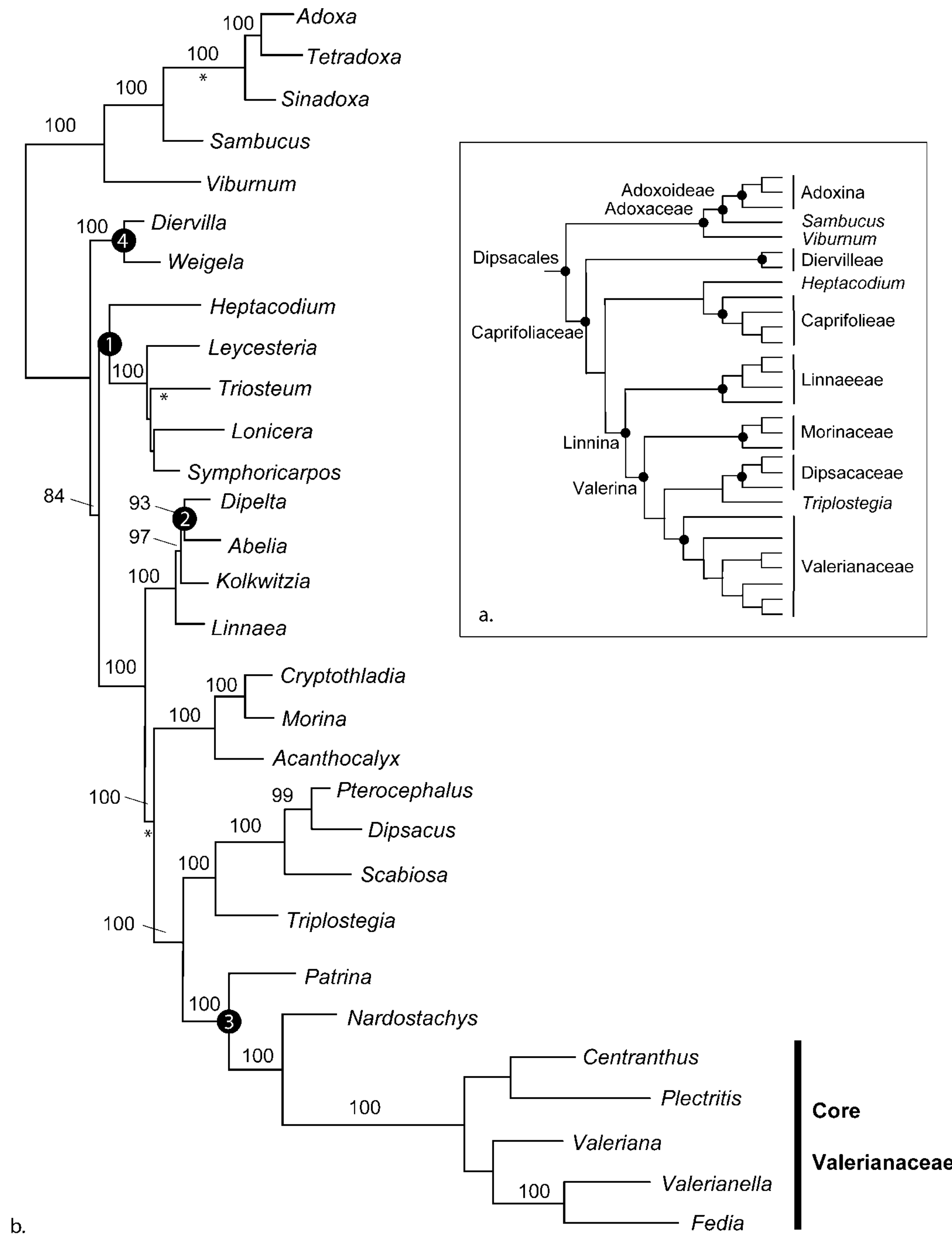

Fig. 1. (a) Application of names according to the phylogenetic taxonomy of Donoghue et al. (2001). (b) Maximum likelihood (ML) tree from the combined chloroplast DNA data set of 30 taxa of Bell et al. (2001). Numbers above branches are bootstrap values greater than 80%. Numbers at nodes correspond to calibration points used in the clock analyses (Table 4). * = inferred transitions to a herbaceous habit.

lution in Dipsacales but also provide a concrete comparison of methods that may bear on their use in other organisms.

MATERIALS AND METHODS

Calibration points—Although the fossil record is not rich for Dipsacales, a number of fossils have been well documented (Muller, 1981; Manchester and Donoghue, 1995; Backlund, 1996). In the Adoxaceae, *Sambucus* has been reported based on endocarps from the late Eocene to Pliocene of Europe (Reid and Chandler, 1926). Fruits of *Dipelta*, which are surrounded by distinctive papery bracts, have been described from the late Eocene/early Oligocene (Reid and Chandler, 1926), and those of *Heptacodium* have been identified from the late Miocene (11.2–5.3 mya) of Japan (Ozaki, 1980). The placement of this fossil (Fig. 1B) is based on the expanded calyx and ovary. *Diplodipelta* (Manchester and Donoghue, 1995), from the late Eocene Florissant flora of Colorado (36–35 mya) and several Oligocene sites in western North America, appears to be the sister group of modern *Dipelta*; both are characterized by enlarged supernumerary bracts that form a wind-dispersed unit. The distinctive winged seeds of *Weigela* have been reported from the Miocene and Pliocene of Poland (Lancucka-Srodoniowa, 1967), the Oligocene and Miocene

TABLE 1. Likelihood ratio tests (LRT) for rate constancy among lineages.

	$-\ln L_0$	$-\ln L_1$	LR = $-2(\ln L_1 - \ln L_0)$	*P*
Non-coding				
trnL	5824.17	5965.88	283.44	<0.00001
atpB	6125.59	6411.93	572.66	<0.00001
matK intron	2824.00	3017.64	387.17	<0.00001
Combined	15408.00	15967.09	1118.17	<0.00001
Coding				
rbcL	4966.99	5073.16	212.32	<0.00001
ndhF	11444.29	11740.88	593.19	<0.00001
matK	8292.46	8562.19	539.46	<0.00001
Combined	25375.76	25979.65	1207.77	<0.00001
Third codon positions				
rbcL	1226.04	1258.45	64.83	<0.00001
ndhF	5236.11	5409.91	347.59	<0.00001
matK	2602.12	2714.42	224.60	<0.00001
Combined	10509.09	10849.88	681.58	<0.00001
Combined (coding and non-coding)	41193.66	42300.15	22129520	<0.00001

of Siberia (Dorofeev, 1963), as well as from the Miocene of eastern Asia (Nikitin, 1976). In Valerianaceae, the unusual fruits (''wing'' on the ovary) of *Patrinia* have been documented from the Miocene to Pliocene of Poland and Russia (Lancucka-Srodoniowa, 1967), as well as from the late Miocene of Japan (Ozaki, 1980). Likewise, *Valeriana* is known on the basis of fossil fruits from the late Miocene and Pliocene in Europe. Fossils of other Dipsacales have been reported, but are now known to be incorrectly assigned or are considered too unreliable. These include *Abelia* (Manchester and Habley, 1997) and many specimens attributed to *Viburnum* and *Lonicera*. Until the latter are studied in greater detail we are reluctant to use them to constrain age estimates in Dipsacales.

The use of fossils in concert with molecular data can take two forms: (1) they can serve as fixed ''calibration'' points used to calculate absolute branching times, or (2) they may serve as maximum or minimum age ''constraints'' (Sanderson, 1997). For this study we selected four fossils that we believe can be confidently placed in the Dipsacales tree to use as separate calibration points (Fig. 1B), as well as minimum age constraints in concert with other fossils. In several analyses, we also explored the use of a maximum age constraint. Dipsacales is nested within eudicots, and can be no older than eudicots. The eudicots can be rather confidently dated by their distinctive and pervasive tricolpate pollen, which appears in the fossil record at about 125 mya (Doyle and Donoghue, 1993; Magallón et al., 1999; Sanderson and Doyle, 2001).

Absolute ages in this paper are based on the 1999 Geological Time Scale from the Geological Society of America (GSA, 2004). In all cases we have assigned a range of ages to fossils, as opposed to a single fixed date.

For each of the methods used, we examined the consistency of our fossil calibration points in reference to one another using the combined data set. Since all of these fossil placements are minimum age estimates, an inferred age of a clade (based on calibration with another fossil) was judged to be inconsistent only if it was inferred to be younger than a ''known'' age based on a fossil assigned to that clade.

Phylogenetic estimation and tests for rate constancy—Maximum likelihood methods were employed to infer both tree topologies and branch lengths from three coding (*rbcL, ndhF, matK*) and three non-coding (*trnL, atpB-rbcL* intergenic spacer, and a *matK* intron) chloroplast regions. These data were all previously published (Bell et al., 2001). The taxon sample represents all major lineages within Dipsacales, including representatives of all the traditional families, subfamilies, and tribes. All data sets analyzed for this study are available from TreeBASE (http://www.treebase.org) or by request from the first author. For each data set, a series of likelihood ratio tests (LRTs) was performed to select a model for maximum likelihood searches using PORN* (Bell, 2001). All searches were performed with the computer software PAUP*b10 (Swofford, 2002). Parameters for the searches were estimated on a tree topology obtained in a maximum likelihood search using a Jukes-Cantor (JC; Jukes and Cantor, 1969) model of sequence evolution. Parameters for each model were then fixed, and heuristic searches were run using 100 random taxon additions and tree-bisection-reconnection (TBR) branch swapping. A final round of searching entailed estimating all model parameters simultaneously using the tree obtained in the previous step as the starting tree and nearest-neighbor interchange (NNI) branch swapping.

Following each search, a LRT was used to test for departure from clock-like evolution in the molecular sequence data (Felsenstein, 1981). For the three coding regions, analyses were performed on two data sets: (1) including all three codon positions, and (2) including just third codon positions. In all cases, rate constancy among lineages was rejected (Table 1). For the remaining analyses, the maximum likelihood tree from the combined data, including coding and non-coding regions, was used (Fig. 1B).

Because the hypothesis of rate constancy was rejected in all cases, several recently proposed methods (Sanderson, 1997; Thorne et al., 1998; Huelsenbeck et al., 2000; Yoder and Yang, 2000; Kishino et al., 2001; Sanderson, 2002; Thorne and Kishino, 2002) were used to infer divergence times from the chloroplast sequence data. These methods variously ''relax'' the null hypothesis of a uniform rate of molecular evolution across all lineages, allowing different parts of a tree to have different rates.

Local molecular clocks—In a local molecular clock model, different rates can be assigned to different clades in a tree. All tests for local molecular clocks were performed using the software PAML, version 3.01b (Yang, 2000; see Yoder and Yang, 2000) and the tree in Fig. 1.

Because the LRT of rate hypotheses among lineages is valid only when a null hypothesis is specified (Yoder and Yang, 2000), we initially divided the tree into two rates–one rate for ''herbaceous'' and another rate for ''non-herbaceous'' taxa. This was based on previous analyses suggesting that rates might differ according to plant habit (Gaut et al., 1996; Laroche et al., 1997; Gaut, 1998). The shift to herbaceousness appears to have occurred at least five separate times in Dipsacales (Donoghue et al., 2003). However, our sampling for this analysis covers only three occurrences: (1) *Triosteum*, (2) the Adoxina clade, and (3) the Valerina clade (see Fig. 1B). The other two transitions from woody to herbaceous occur within *Sambucus* (Eriksson and Donoghue, 1997), from which we have sampled just one woody representative. Because there is no reason to think that all three lineages would necessarily have the same rate of molecular evolution, we next specified a local molecular clock with four rates—one for each of the three herbaceous lineages and another for the non-herbaceous taxa. Confidence intervals for local and global molecular clocks were calculated using BASEML in the PAML software package.

TABLE 2. Parameters for the F84+G model. L = number of nucleotides. p_T = empirical frequency of thymine. p_C = empirical frequency of cytosine. p_A = empirical frequency of adenine. p_G = empirical frequency of guanine. k = transition/transversion parameter, estimated via maximum likelihood using PAML (Yang, 2001). a = shape parameter of the gamma distribution, estimated via maximum likelihood using PAML.

Codon position	L	p_T	p_C	p_A	p_G	k	a
1	1681	0.2847	0.1656	0.2981	0.2515	0.4098	0.3542
2	1681	0.3069	0.1986	0.2737	0.1669	0.4082	0.2949
3	1681	0.4038	0.1540	0.2803	0.1618	0.9962	0.8235
Region							
rbcL	1428	0.2848	0.1994	0.2686	0.2472	0.9105	0.1839
ndhF	2199	0.3891	0.1538	0.2841	0.1731	0.7199	0.4591
matK	1416	0.3570	0.1745	0.3018	0.1668	0.5416	0.8919
Combined	5043	0.2862	0.1940	0.3496	0.1701	0.5609	0.4322

Nonparametric rate smoothing—An alternative to estimating divergence times with global or local molecular clocks, which specify an explicit parametric model of sequence evolution, is nonparametric rate smoothing (NPRS; Sanderson, 1997). NPRS estimates rates and times using a least-squares smoothing criterion that penalizes rapid rate changes from branch to branch in a phylogeny. All NPRS analyses were performed using the maximum likelihood tree topology in Fig. 1, and branch lengths were estimated for each data partition using its ''best fitting'' model as determined by a series of LRTs.

Two separate NPRS analyses were performed. We first fixed the age of the root at 1.0, smoothed all branches relative to that, and used a single fossil calibration to calculated absolute divergence dates. In a second NPRS analysis we employed minimum and maximum age constraints. In this case the root of the tree was constrained to a maximum age of 125 mya (see calibration points), and other fossils were used as minimum age constraints.

Penalized likelihood—Penalized likelihood (PL; Sanderson, 2002) is a semiparametric smoothing method. Like NPRS, PL assumes that there is an autocorrelation of substitution rates and attempts to minimize rate changes between ancestral/descendant branches on a tree (i.e., at the nodes). PL attempts to combine the statistical power of parametric methods (models of molecular evolution) with the robustness of nonparametric methods. A smoothing parameter (λ) can vary from very small, in which case each branch of the phylogeny has a different substitution rate (saturated model), to very large, in which parameters are essentially clock-like. The crux of the penalized likelihood method is determining the optimal smoothing level. The program r8s (Sanderson, 2003) implements a data-driven cross-validation procedure that systematically prunes terminals from the tree, then estimates parameters from the submatrix and a given smoothing value. It then tries to predict the data for pruned taxa using the estimated parameters. Finally, it calculates a chi-squared error associated with the difference between the predicted and observed data. The optimal smoothing level is chosen as the one that minimizes the chi-squared error (Sanderson, 2002).

As in the case of NPRS, we ran two separate PL analyses: (1) we fixed the age of the root at 1.0, smoothed all branches, and calibrated with a single fossil, and (2) we employed minimum and maximum age constraints. Confidence intervals around the age estimates for all nodes were calculated using nonparametric bootstrapping (Baldwin and Sanderson, 1998). This procedure assumes that bootstrap estimates are normally distributed, which might not be the case.

Bayesian relaxed clock using Markov chain Monte Carlo (MCMC)—Bayesian methods (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002) that relax a strict molecular clock were also used to estimate divergence times using MULTIDIVTIME (available from J. Thorne, North Carolina State University). This parametric approach relaxes the assumption of a strict molecular clock with a continuous autocorrelation of substitution rates across the phylogeny, and allows the use of several calibrations/ time constraints.

Because these analyses eventually trim off the outgroup, we added three additional sequences from GenBank: *Pittosporum tobira* (*rbcL,* U50261; *ndhF,* AF130201) and *Pittosporum undulatum* (*mat*K, AJ429374). The noncoding regions were not included in the Bayesian analyses due to alignment difficulties. The three genes were analyzed separately as well as combined. A simultaneous analysis was also performed to account for the differences in the substitution process between genes using the program MULTIDIVTIME. An additional analysis was performed after partitioning the data by codon position and analyzing the three-codon positions simultaneously.

Divergence date estimation with MULTIDIVTIME involved two steps. First, ESTBRANCHES was run to estimates branch lengths from the data and a fixed tree topology using the F84 (Felsenstein, 1984; Kishino and Hasegawa, 1989) model of sequence evolution. This allows rates to vary among sites following a discrete gamma distribution with four rate categories (Yang, 1994) along with their variance-covariance matrix. Parameters for the F84 + Γ model were estimated using the BASEML program in PAML (Yang, 2000). Estimated parameters are presented in Table 2.

Next, the outgroup (in our case, the *Pittosporum* species) is pruned from the tree and MULTIDIVTIME is used to estimate the prior and posterior ages of branching events, their standard deviations, and the 95% credibility intervals via Markov chain Monte Carlo. The Markov chain was run for 1 000 000 generations and sampled every 100 generations after an initial burn-in period of 10 000 cycles. To check for convergence of the MCMC, analyses were run from at least two different starting points.

The following prior distributions were used in these analyses: 120 mya (SD = 60 mya) for the expected time between tip and root if there were no constraints; 0.0006 (SD = 0.0003) substitutions per site per million year for the rate of the root node; 0.01 (SD = 0.01) for the parameter that determines the magnitude of autocorrelation per million years; and 125 mya for the largest value of the time unit between the root and the tips.

Three separate Bayesian analyses where performed: (1) one in which all of the data were combined into a single dataset prior to analyses; (2) one in which all genes shared a common value for the autocorrelation parameter; and (3) one in which each gene had its own autocorrelation parameter.

RESULTS

Cross validation of calibration points—In all cases but one (*Diplodipelta*), the fossil calibration points yielded inconsistent results, estimating divergence times that were too young based on our knowledge of the fossil record (Table 3). For example, when *Patrinia* (C3:11.2–5.3 mya) was used to calibrate the tree, the split between *Dipelta* and *Abelia* was estimated at 10.1–4.8 mya. However, placement of the fossil *Diplodipelta* provides evidence that this split occurred at least 36–35 mya. Such inconsistencies were obtained regardless of the dating method used. Consequently, all remaining ages were calculated using only *Diplodipelta* for calibration.

Rate constancy among lineages—As noted above, all LRTs rejected ($P < 0.00001$) the hypothesis of rate constancy

TABLE 3. Cross validation of fossil calibration points in Dipsacales using nonparametric rate smoothing (NPRS) and penalized likelihood (PL) methods based on the combined data. Dates in millions of years. C1–C4 correspond to calibration points presented on Fig. 1.

	Caprifolieae + *Heptacodium* (C1)	*Diplodipelta* (C2)	Valerianceae (C3)	Diervilleae (C4)
NPRS				
Adoxaceae	4.9–10.3	53.2–54.75	7.3–15.4	22.2–25.8
Caprifoliaceae	5.8–12.2	63.2–65.0	8.7–18.3	26.4–30.7
Dipsacales	6.8–14.4	74.6–76.8	10.3–21.7	31.2–36.2
Caprifolieae + *Heptacodium* (C1)	5.3–11.2	58.1–59.8	7.9–16.9	24.3–28.2
Diplodepelta (C2)	3.2–6.7	35.0–36.0	4.8–10.1	14.6–16.9
Valerianceae (C3)	4.7–7.4	38.6–39.7	5.3–11.2	16.1–18.7
Diervilleae (C4)	4.5–9.5	49.0–50.4	6.7–14.2	20.5–23.8
PL				
Adoxaceae	4.9–10.3	69.2–71.1	7.3–15.5	27.2–31.5
Caprifoliaceae	5.9–12.4	83.43–85.8	8.8–18.66	32.7–37.9
Dipsacales	7.4–15.1	100.86–103.74	10.6–22.6	39.5–45.9
Caprifolieae + *Heptacodium* (C1)	5.3–11.2	74.8–76.9	7.9–16.8	29.3–34.0
Diplodipelta (C2)	2.4–5.2	35.0–36.0	3.7–7.8	13.7–15.9
Valerianaceae (C3)	3.5–7.5	50.1–51.5	5.3–11.2	19.6–22.7
Diervilleae (C4)	3.7–7.8	52.3–53.8	5.4–11.7	20.5–23.8

among lineages, regardless of the data partition tested. When dates where estimated on ultrametric trees using *Diplodipelta* for calibration, we obtained ages for the base of Dipsacales that, in most cases, predate the fossil record of angiosperms at greater than ca. 140 mya (Sanderson and Doyle, 2001; Table 4). Only the *rbcL* data (all sites and third codon positions) and the *matK* intron did not estimate ages for the Dipsacales older than the presumed age of eudicots (i.e., 125 mya).

Age estimates also varied dramatically when only third codon positions were examined (data not presented). For example, dates estimated for the age of Dipsacales ranged from 121.9 MYA (for *rbc*L) to 266.300 MYA (for *ndh*F). With the exception of the *rbc*L estimate, all other age estimates based on 3rd positions were older than 140 mya.

Local molecular clocks—All LRTs rejected ($P < 0.00001$) the hypothesis of the presence of local clocks among lineages, regardless of what data partition was being tested, or whether two rates or four rates were specified. As in the global clock analyses, local clock estimates for the age of Dipsacales appear to be too old, for the most part suggesting a pre-angiosperm, Triassic origin of Dipsacales.

Again, age estimates for all nodes varied considerably among genes and non-coding regions as well as if only third positions were used. Rates inferred from these analyses did support the hypothesis that rates of molecular evolution are higher in herbaceous plants than in their woody relatives, regardless of whether a two-rate model was imposed (herbaceous rate > non-herbaceous rate) or a four-rate model (*Triosteum* rate > Adoxina rate > Valerina rate > non-herbaceous rate; for all partitions of the data). Based on a likelihood ratio test, these data do not support the hypothesis that there is a uniform rate across the different herbaceous clades ($\delta = 175.4$, df $= 2$, $P < 0.001$).

Nonparametric rate smoothing (NPRS)—Age estimates from the NPRS analyses are presented in Table 4. Unlike the analyses based on global and local molecular clocks, NPRS estimates ages that generally fall within the age range of angiosperms. Ages estimated for Dipsacales using NPRS ranged from 52.94 mya (*matK* intron) to 124.12 mya (combined coding for third-codon positions). With the exception of the *matK* intron, all other partitions or combinations of partitions suggest an origin of Dipsacales at or prior to the Cretaceous-Tertiary (K/T) boundary. In the NPRS analysis of the combined data, the origin of Dipsacales is placed at 76.76 mya (± 4.4), Caprifoliaceae in the vicinity of the K/T boundary (65.00 mya ± 3.7), Adoxaceae in the Early Eocene (54.68 mya ± 4.3), and core Valerianaceae in the Miocene (12.22 mya ± 3.1).

Penalized likelihood (PL)—Smoothing levels (λ) for all analyses tended to be small (between 0.0001 and 0.01), suggesting substantial rate heterogeneity among lineages (Sanderson, 2002). Like the age estimates obtained from NPRS, estimates for the age of Dipsacales are generally more in line with the angiosperm fossil record than those obtained with global and local clocks. Age estimates for the root node ranged from 51.76 mya (for the *matK* intron) to 176.9 mya (*ndhF* all positions) (see Table 4). With the exception of the *rbcL* data, PL estimated older dates for Dipsacales than NPRS. Only the *ndhF* data estimated dates that we would reject as being too old. When a maximum age constraint of 125 mya was placed at the root of Dipsacales, age estimates for the entire clade were pushed up against this barrier (i.e., ca. 125 mya; data not presented). Age estimates for most of the clades within Dipsacales were only slightly affected by the use of the maximum age constraint.

Bayesian estimation of divergence times—Ages estimated using Thorne's Bayesian relaxed clock method are presented in Tables 4 and 5. Prior and posterior distributions for the analyses with fossil constraints were all fairly similar, which may suggest that most of the information concerning age estimates is attributable to the prior distributions, rather than the data. However, when age constraints were not imposed, the prior distributions of divergence times showed a greater degree of variation than the posterior distributions, indicating that much of the information concerning branching times can be attributed to the sequence data (or estimated branch lengths from the sequence data). Simultaneous analyses of all three coding regions (as well as codon positions) resulted in estimates with smaller variance (see Table 5). In agreement with the PL combined results, all Bayesian analyses suggest a Late

TABLE 4. Divergence time estimations ± 95% confidence interval. All analyses constrained by *Diplodipelta*. Numbers in parentheses for Bayesian analyses represent 95% credibility intervals.

Analysis	Clade	*rbcL*	*ndhF*	*matK*	*trnL*	*atpB*	Combined
Global clock	Dipsacales	119.78 ± 11.8	241.73 ± 13.2	228.63 ± 13.9	528.04 ± 35.3	255.34 ± 13.5	216.58 ± 6.1
	Caprifoliaceae	98.52 ± 7.7	201.94 ± 9.6	171.93 ± 8.7	238.86 ± 15.9	255.34 ± 13.5	172.35 ± 4.2
	Adoxaceae	62.99 ± 7.9	128.16 ± 10.7	122.55 ± 11.9	434.07 ± 35.6	120.73 ± 13.1	118.69 ± 5.1
	Core Valerianaceae	58.56 ± 5.7	101.00 ± 5.7	72.66 ± 5.4	119.23 ± 10.2	72.60 ± 6.7	70.11 ± 2.2
Local clock[a]	Dipsacales	101.03 ± 11.6	229.88 ± 14.9	216.93 ± 15.8	481.74 ± 38.4	177.84 ± 15.3	196.72 ± 6.8
	Caprifoliaceae	72.15 ± 7.9	163.20 ± 10.8	124.32 ± 9.5	169.80 ± 16.8	142.61 ± 12.3	122.63 ± 4.5
	Adoxaceae	52.55 ± 9.4	117.80 ± 11.6	122.33 ± 13.8	355.35 ± 36.4	93.94 ± 13.2	107.29 ± 5.6
	Core Valerianaceae	34.32 ± 5.8	54.83 ± 5.9	28.01 ± 3.7	119.23 ± 10.2	24.12 ± 3.5	32.57 ± 1.7
Local clock[b]	Dipsacales	108.15 ± 12.0	231.68 ± 14.9	233.32 ± 16.3	509.24 ± 41.0	180.11 ± 15.8	206.88 ± 7.1
	Caprifoliaceae	81.62 ± 8.7	169.63 ± 10.6	130.70 ± 10.4	171.99 ± 17.7	139.60 ± 12.4	127.13 ± 4.5
	Adoxaceae	51.73 ± 8.3	107.07 ± 11.6	135.07 ± 14.2	386.64 ± 44.5	96.23 ± 14.7	112.41 ± 5.8
	Core Valerianaceae	23.45 ± 5.2	50.22 ± 6.6	25.22 ± 4.3	191.23 ± 10.4	21.31 ± 3.5	28.26 ± 1.8
NPRS	Dipsacales	70.59 ± 4.0	113.62 ± 6.5	62.90 ± 3.6	114.16 ± 6.5	69.22 ± 3.9	76.76 ± 4.4
	Caprifoliaceae	54.57 ± 3.1	98.16 ± 5.6	52.28 ± 3.0	79.90 ± 4.6	60.55 ± 3.5	65.00 ± 3.7
	Adoxaceae	48.79 ± 3.9	77.46 ± 6.1	47.11 ± 3.7	96.79 ± 7.6	44.52 ± 3.5	54.68 ± 4.3
	Core Valerianaceae	20.59 ± 3.2	27.00 ± 4.6	13.07 ± 3.2	25.10 ± 4.2	9.66 ± 1.0	12.22 ± 3.1
PL	Dipsacales	68.20 ± 3.9	176.91 ± 10.1	69.19 ± 3.9	113.93 ± 6.5	96.90 ± 5.5	103.74 ± 6.3
	Caprifoliaceae	54.79 ± 3.1	152.22 ± 8.7	57.07 ± 3.3	86.05 ± 4.9	61.90 ± 3.6	85.82 ± 5.2
	Adoxaceae	42.74 ± 3.4	115.55 ± 9.1	50.20 ± 3.9	91.85 ± 7.2	88.81 ± 6.9	71.18 ± 5.9
	Core Valerianaceae	28.71 ± 2.7	46.37 ± 7.8	38.18 ± 2.7	30.17 ± 2.7	7.95 ± 2.1	16.30 ± 4.2
Bayesian	Dipsacales	91.24 ± 17.6 (75.97–109.96)	107.93 ± 16.8 (92.15–124.98)	103.35 ± 817.6 (86.59–121.42)			102.28 ± 17.8 (85.55–120.81)
	Caprifoliaceae	82.42 ± 16.4 (67.46–99.60)	88.32 ± 13.4 (75.70–101.45)	84.31 ± 14.6 (70.44–99.23)			84.16 ± 15.2 (70.47–100.52)
	Adoxaceae	77.25 ± 23.8 (53.72–101.14)	84.47 ± 18.2 (66.85–102.59)	72.26 ± 21.2 (51.51–92.97)			70.59 ± 21.6 (49.73–92.92)
	Core Valerianaceae	31.72 ± 14.4 (19.25–46.88)	12.97 ± 5.2 (8.52–18.83)	28.08 ± 8.0 (20.27–36.49)			28.71 ± 8.8 (20.56–37.34)

[a] Two-rate local clock analyses.
[b] Four-rate local clock analyses.

TABLE 5. Comparison of penalized likelihood (PL) and Bayesian analyses for all nodes ± 95% confidence intervals (1.96 SD). See Fig. 2 for node numbers.

Node	PL	Bayesian[a]	Bayesian[b]	Bayesian[c]
1	12.46 ± 3.7	28.72 ± 16.6	29.88 ± 16.6	37.91 ± 22.0
2	16.62 ± 4.9	35.06 ± 17.2	37.71 ± 17.0	44.11 ± 23.8
3	45.49 ± 8.7	32.59 ± 18.0	33.58 ± 17.6	45.01 ± 24.26
4	71.18 ± 5.9	70.59 ± 21.6	72.34 ± 20.4	84.76 ± 39.8
5	53.81 ± 8.6	55.75 ± 18.8	56.59 ± 18.8	40.19 ± 27.8
6	13.50 ± 4.0	15.26 ± 8.6	15.08 ± 8.0	16.10 ± 10.8
7	10.71 ± 2.8	11.72 ± 7.0	11.61 ± 6.6	8.73 ± 6.4
8	16.30 ± 4.2	28.11 ± 8.6	27.61 ± 8.0	17.20 ± 11.6
9	20.34 ± 3.7	28.70 ± 8.6	28.13 ± 8.0	17.60 ± 11.8
10	42.77 ± 3.2	29.27 ± 8.8	28.62 ± 8.0	18.18 ± 12.2
11	51.58 ± 2.9	55.91 ± 5.6	55.72 ± 5.2	34.44 ± 11.6
12	12.08 ± 4.0	19.05 ± 10.2	18.93 ± 10.0	12.54 ± 9.6
13	22.26 ± 5.2	37.85 ± 13.6	37.60 ± 13.4	22.67 ± 15.2
14	47.49 ± 4.9	52.63 ± 11.4	52.55 ± 11.6	39.67 ± 24.4
15	62.48 ± 3.6	61.95 ± 9.2	62.04 ± 9.6	44.46 ± 26.8
16	17.15 ± 8.0	19.88 ± 19.2	20.20 ± 19.6	13.61 ± 14.0
17	31.09 ± 2.3	45.55 ± 15.0	45.93 ± 14.6	30.86 ± 20.8
18	69.07 ± 4.5	65.08 ± 10.2	65.27 ± 10.8	48.25 ± 28.8
19	36.00 ± 1.9	35.50 ± 5.6	55.10 ± 5.6	38.88 ± 20.4
20	38.76 ± 2.8	40.68 ± 7.4	40.42 ± 7.0	32.31 ± 21.8
21	45.30 ± 4.4	48.54 ± 10.4	48.23 ± 10.4	37.75 ± 24.0
22	71.68 ± 4.6	66.34 ± 10.6	66.39 ± 11.2	49.41 ± 29.4
23	43.37 ± 7.0	39.91 ± 17.0	40.85 ± 17.4	37.12 ± 23.0
24	44.35 ± 6.3	46.65 ± 17.6	47.13 ± 17.6	40.72 ± 25.0
25	46.48 ± 5.6	50.02 ± 17.8	50.37 ± 17.8	41.75 ± 25.4
26	76.99 ± 6.8	78.28 ± 15.0	78.85 ± 15.2	64.16 ± 35.6
27	84.00 ± 6.9	82.23 ± 15.0	82.68 ± 15.0	66.51 ± 36.4
28	85.82 ± 5.2	84.16 ± 15.4	84.53 ± 15.2	68.09 ± 36.6
29	103.74 ± 6.3	102.28 ± 17.8	103.26 ± 17.4	110.99 ± 47.8

[a] Estimates based on combined chloroplast data set.
[b] Estimates based on MULTIDIVTIME analysis of each gene (i.e., corresponding branch lengths) simultaneously with common autocorrelation parameter.
[c] Estimates based on MULTIDIVTIME analysis of each gene simultaneously with each gene having a separate autocorrelation parameter.

Cretaceous origin for Dipsacales, as well as for Adoxaceae and Caprifoliaceae.

DISCUSSION

Fossil calibration—The traditional treatment of fossils for purposes of inferring divergence times using molecular phylogenetic methods has important, but not yet widely appreciated, consequences for such estimates. A particular fossil can often be assigned with some confidence to a particular branch that represents one or more extant species, but the material at hand may not allow a confident determination of whether it falls along the stem leading to the extant crown, or whether it falls instead somewhere within the crown. In such cases the fossil is typically treated as indicating the existence of the entire lineage and, therefore, of the existence by that time of the split that resulted in it and its sister lineage. This is the only treatment possible when a crown group is represented by a single species in the phylogenetic analysis. In any case, this treatment will often have the effect of underestimating divergence times at all subtending nodes. That is, if the fossil actually belonged somewhere within the crown group, the subtending divergences would be pushed deeper in time. In general, assuming that some fossils actually do fall within the crown group, we anticipate that their more accurate placement will push divergence times further back. This is an important extension of the point that fossils only provide minimum age estimates, because it highlights how a particular treatment of fossils, vis-à-vis their placement in the phylogeny, will systematically underestimate divergence times.

In the case of Dipsacales, this realization has important consequences, as we can distinguish between those fossils for which we lack evidence about whether they actually fall within or outside of the crown group (*Weigela*, *Heptacodium*, and *Patrinia*), and *Diplodipelta*, for which we have evidence that it falls outside of its crown group (*Dipelta*). Manchester and Donoghue (1995) argued that the two species of *Diplodipelta* together represent the extinct sister group of *Dipelta*, with three extant species. Thus, of the four fossils used in our analyses, we are most certain that *Diplodipelta* does not cause the underestimation problem described above. The *Weigela*, *Heptacodium*, and *Patrinia* fossils, on the other hand, might be nested within their respective crown groups, in which case our default treatment of them as falling outside of the crown will underestimate the subtending divergence times. We think this may be why overly young dates are estimated for the *Dipelta*/*Abelia* divergence when these were used as calibration points (Table 3). An alternative explanation would suppose that the fossils of *Weigella*, *Heptacodium*, and *Patrinia* really do belong outside of their respective crown groups, but that the fossil record is poor and the known fossils happen not to fall close in time to the split from the sister group, but instead fall close to the first split within the crown group.

Our greater confidence in the phylogenetic position of *Diplodipelta* provides, we believe, the best justification for relying on it for inferring divergence times in Dipsacales—better

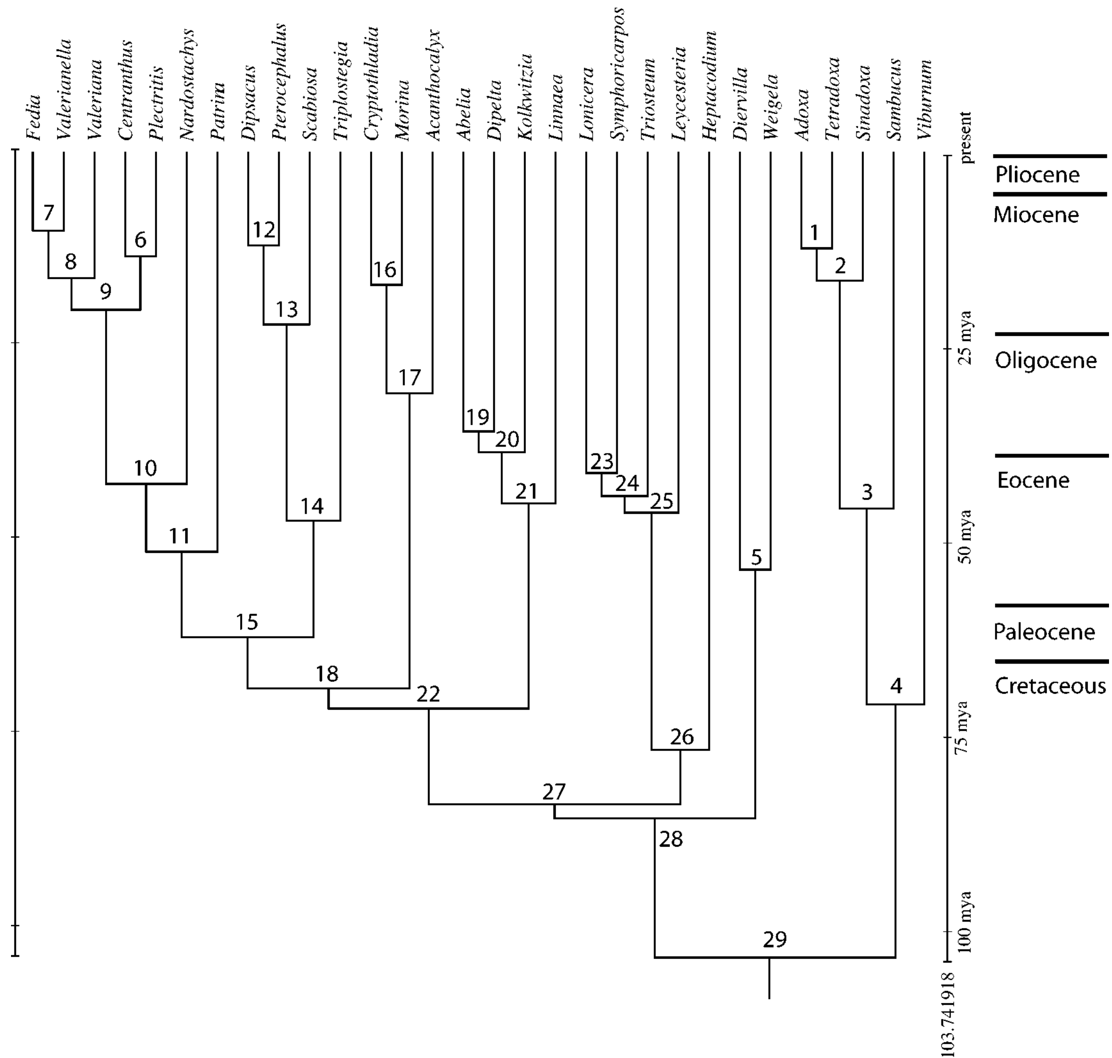

Fig. 2. Chronogram of the maximum likelihood (ML) tree from the combined chloroplast DNA data set for 30 taxa. Branch lengths transformed using the penalized likelihood (PL) method and calibrated using *Diplodipelta*. Numbers at nodes correspond to node numbering in Table 5.

than the fact that it yielded no inconsistencies with the ages of the other fossils in our cross-validation tests. *Diplodipelta*, as the oldest of the four fossils included, is not expected to yield divergence times that are too young for the clades represented by the other much younger fossils if these really are nested within their crown groups.

Divergence time variation among methods and data sets—It would be comforting to find a convergence on similar age estimates using different methods and sources of data. In Dipsacales we have not been so lucky and need to select among analyses in order to proceed. The likelihood ratio test (LRT) provides an explicit means for selecting among methods that assume a molecular clock and those that do not, though it has been suggested that the LRT may not be sufficiently sensitive to detect localized deviations from clock-like evolution (Conti et al., 2002). This is in contrast to others who feel that the global LRT represents a conservative test for rate constancy (Sanderson, 1998). In Dipsacales, rejection of the clock is also suggested by the age estimates obtained when a clock is imposed. That is, global and local molecular clock analyses both estimate dates for Dipsacales that are grossly inconsistent with the fossil record. Specifically, Dipsacales is inferred to be older than eudicots and even angiosperms. This phenomenon has been seen in analyses of other taxonomic groups (e.g., Martin et al., 1993; Heckman et al., 2001; Soltis et al., 2002).

In rejecting clock models, one faces the choice among alternative methods that relax the clock assumption. One shortcoming of the NPRS method was highlighted by Sanderson (2002, 2003). NPRS may overfit the data, leading to rapid fluctuations in rates in regions of a phylogeny where there are short branches. Data sets with little information content (i.e., few inferred substitutions across the phylogeny) may tend to have zero-length branches in areas that are fairly unresolved, which would result in the appearance of rapid rate fluctuation. Sanderson (2002) demonstrated that PL always out-performed NPRS when data departed from a constant rate and when cross-validation was used to determine the optimal smoothing level. He also pointed out that when the smoothing factor is small, the model is overfit and small changes in the data (i.e., subsamples constructed during pruning) will lead to large changes in parameter estimates. Consequently, one would ex-

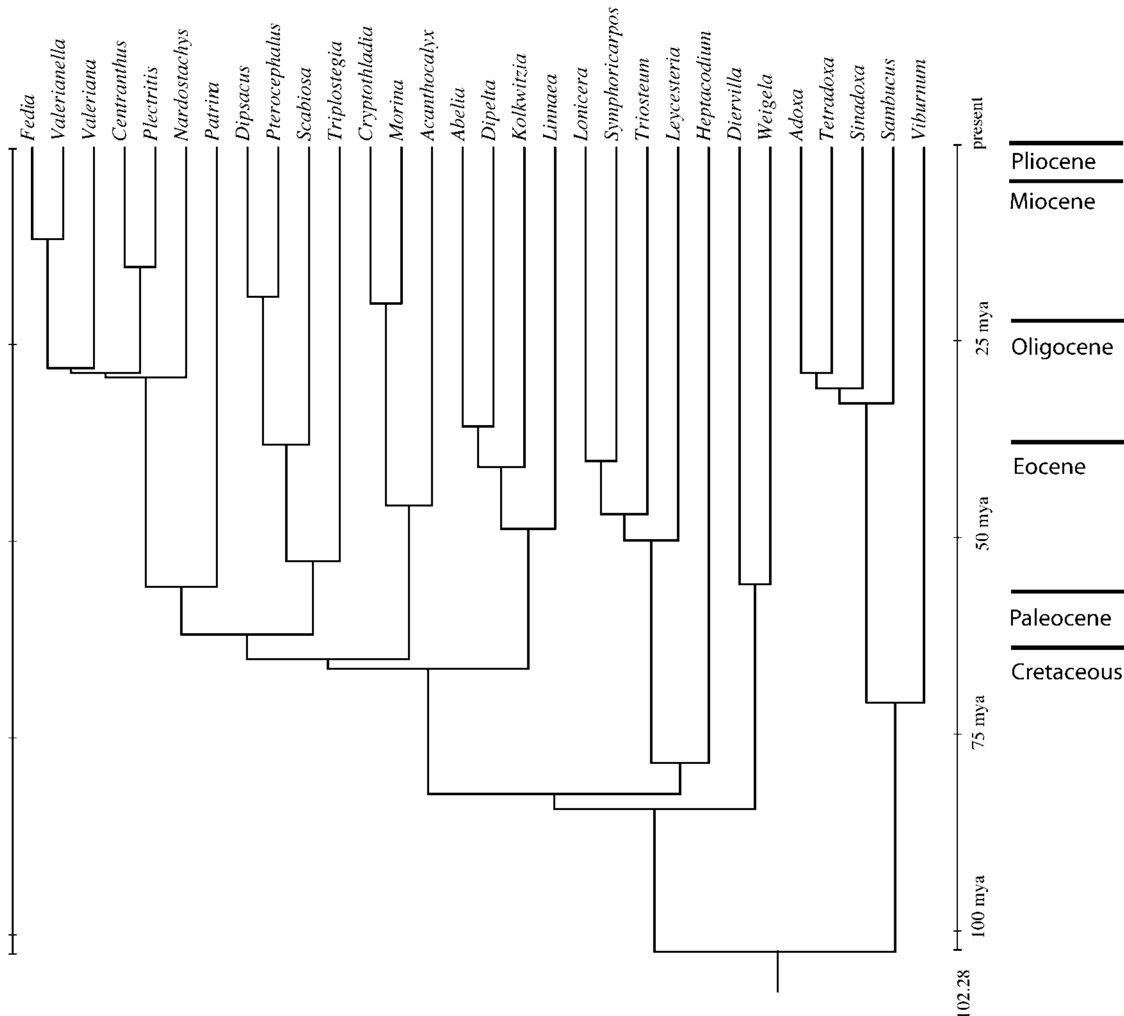

Fig. 3. Bayesian relaxed clock tree. Branch lengths estimated via Markov chain Monte Carlo (MCMC) for all three genes simultaneously.

pect some intermediate (or larger) level of smoothing to provide the best cross-validation score.

These observations may help to explain the differences we see between our NPRS and PL results for Dipsacales. In fact, in Dipsacales we find that NPRS yields consistently younger ages of major clades than PL or Bayesian. Because NPRS is sensitive to major variation in branch lengths in a tree (Sanderson, 2002), the presence of many short-internal branches could potentially lead NPRS to push the age of the root node further back in time to compensate for extreme transitions in rates from parent to offspring branches (i.e., by increasing the lengths of the short-internal branches). We suspect that we might be observing the opposite situation in Dipsacales, with several long internal branches, and shorter ones towards the tips (with only a few exceptions). Under these circumstances, NPRS may pull the root of the tree closer to the present in order to fit the data to a situation where there are decelerations in rates across nodes, potentially leading to an underestimate in divergence times.

It is noteworthy that PL and Bayesian approaches applied to Dipsacales converge on rather similar results. This may not be that surprising and may be a more general phenomenon, due to the fact that both methods weight a likelihood function by some cost—the roughness penalty in the PL method and a prior distribution in the Bayesian method. A primary concern with Bayesian approaches is how sensitive the posterior distributions of parameters are to the prior distributions. This issue has so far received very little attention (but see Kishino et al., 2001; Wiegmann et al., 2003; Yang and Yoder, 2003). The greatest differences between the PL and Bayesian estimates involved the Bayesian analyses in which each gene had its own autocorrelation parameter. Because these genes all belong to the same chloroplast genome, they may share a tendency to change rates in the same direction on certain branches due to common biological factors, such as generation time and population size (Thorne and Kishino, 2002). On this basis, we favor the Bayesian estimates that assume a common autocorrelation parameter.

With respect to different genes and different partitions within genes, the high level of variation in age estimates is noteworthy. Whether this level of variation is common or unusual is difficult to judge, as such comparisons have seldom been reported (but see Sanderson and Doyle, 2001; Soltis et al., 2002). In the face of such variation, we believe that the best results might be obtained from combined data sets. Combining data sets may dampen the impact of any one (potentially ab-

errant) gene on the overall result. On the other hand, there may be significant heterogeneity among gene regions that would render it problematical to apply a single evolutionary model. In the future, the development of methods that allow different models to be applied in the same analysis to separate partitions is especially promising (e.g., MrBayes [Huelsenbeck and Ronquist, 2001]).

Overall, our observation of great variation in age estimates based on different methods and on different gene regions suggests that estimates based on a single method applied to a single gene should be treated very cautiously. With respect to methods, those that impose global or local clocks may often be rejected by the data. PL may generally outperform NPRS (Sanderson, 2002), which may be especially sensitive to major changes in branch length in a tree. Bayesian approaches, though promising, have not been very thoroughly explored with respect to the sensitivity to different prior distributions. With respect to choosing among competing results, we favor PL and Bayesian methods as applied to the combined data. Encouragingly, for Dipsacales at least, they yield similar results.

Timing the radiation of Dipsacales—Based on the arguments above, we set aside global and local clock and NPRS age estimates for Dipsacales. The following discussion is based, therefore, on the largely congruent PL and Bayesian results from our combined data set. Because these genes all belong to the same chloroplast genome, they may share a tendency to change rates in the same direction on certain branches due to common biological factors, such as generation time and population size (Thorne and Kishino, 2002). On this basis, we favor the Bayesian estimates that assume a common autocorrelation parameter.

The first major split within crown Dipsacales appears to have taken place in the mid-Cretaceous, by 111–93 mya (Albian to Cenomanian). We note that our estimates for Dipsacales are 20–30 million years older than those of Backlund (1996) and Wikstrom et al. (2001). Crown Caprifoliaceae and Adoxaceae also probably originated prior to the Cretaceous/Tertiary (K/T) boundary, ca. 91–75 mya and 81–60 mya, respectively. Within Adoxaceae, the basal split between the *Viburnum* lineage and Adoxoideae may have taken place close to the K/T boundary. Within Caprifoliaceae, stem Diervilleae, Caprifolieae plus *Heptacodium*, and probably Linnina, were likely in existence before the Tertiary, although differentiation within their respective crown groups probably largely took place in the Tertiary.

In the Tertiary, a number of major Dipsacales clades appear to have originated during the Eocene. Within Adoxaceae the split between *Sambucus* and the herbaceous Adoxina lineage occurred in the Late Eocene according to the PL estimate, but in the Oligocene based on the combined Bayesian analyses. Within Caprifoliaceae we see the differentiation of Caprifolieae into several extant lineages, and, likewise, the beginning of the diversification of Linnaeeae and Diervilleae. Although the split between stem Valerianaceae and *Triplostegia* plus Dipsacaceae may date to the Paleocene, we infer that the initial diversification of crown Valerianaceae probably occurred in the Eocene, as did the split between the *Triplostegia* and Dipsacaceae lines.

According to the combined Bayesian analyses, the differentiation of Adoxoideae may have taken place in the Oligocene, and perhaps also the origin of core Valerianaceae. In either case, Adoxoideae and core Valerianaceae appear to have differentiated by the end of the Miocene. It would appear that the diversification of these major herbaceous lineages within Dipsacales generally coincided with the spread of colder, less equable, and in some places drier, climates around the Northern Hemisphere (Morley, 2000; Zachos et al., 2001).

This study, together with our ongoing analyses within Valerianaceae (Bell, 2004; Bell and Donoghue, In press), suggests that many extant species within Dipsacales have originated since the Miocene, probably mostly within the last 10 million years. Specifically, the two major herbaceous clades—the core Valerianaceae and the Dipsacaeae—seem to have radiated relatively recently. Together, these two clades account for nearly half of the 1100 species of Dispacales. In the case of core Valerianaceae, this diversification appears to correlate with the spread of the clade from Asia to Europe and then to the New World, mostly in alpine habitats. An especially major radiation appears to have occurred with the occupation of South America by one major clade within *Valeriana*, which now contains some 150 species. The radiation of Dipsacaceae, in contrast, has mainly taken place in relatively recently derived semi-arid areas around the Mediterranean basin. Understanding the timing of the radiation of the two major woody lineages—*Viburnum* (ca. 165 species) and *Lonicera* (ca. 180)—requires the inclusion of additional species. However, our analyses indicate that these two lineages are more ancient than the major herbaceous lineages, and preliminary molecular phylogenetic analyses within these groups (Donoghue et al., 2004; Winkworth and Donoghue, 2004; J. Li et al., unpublished data) imply that at least the basal splits within them occurred earlier in the Tertiary.

Several ecological and biogeographic aspects of the Dipsacales radiation are noteworthy. First, Dipsacales probably originated well before the spread of the Northern Hemisphere temperate-zone climate and vegetation types with which they are mainly associated today. The more limited distribution of such climates during the Cretaceous and early Tertiary (Wolfe, 1978; Upchurch and Wolfe, 1987; Zachos et al., 2001) implies that Dipsacales may have (1) occupied limited temperate territory and remained low in diversity until the expansion of the temperate zone in the mid-Tertiary; (2) diversified in more tropical areas, where they do not occur today, followed by the extinction of early lineages; or (3) initially adapted to living in more tropical habitats, but did not undergo significant diversification until they evolved adaptations that allowed their entry into the temperate zone. It may be possible in the future to choose among these alternatives, depending on the discovery of Cretaceous fossils and/or additional phylogenetic analyses within key lineages. For example, recent analyses have suggested that the root of *Viburnum* falls between *V. clemensiae*, which occupies subtropical forests in Borneo, and a clade containing the remainder of the species, almost all of which live in temperate forests (Donoghue et al., 2004; Winkworth and Donoghue, 2004). If this is substantiated and if this basal split dates at least to the Eocene, *V. clemensiae* might represent a remnant of an earlier phase of Dipsacales diversification in more tropical regions.

Second, many major lineages originated in an Eocene time frame that would have allowed spread around the Northern Hemisphere through the North Atlantic and/or the Bering land bridges (Donoghue et al., 2001; Sanmartin et al., 2001; Tiffney and Manchester, 2001). Dipsacales today provide many instances of Old World/New World disjunctions, some of these

probably dating to the Eocene (e.g., several major clades within *Viburnum*, *Lonicera*, and possibly Diervilleae, but see Donoghue et al., 2001, on the later). However, most Dipsacales clades with such intercontinental disjunctions are younger, and movements between the Old and New World in these cases presumably occurred through Beringia (e.g., disjunctions within *Sambucus*, *Adoxa*, *Triosteum*, *Symphoricarpos*, *Linnaea*, and *Valeriana*).

Third, as noted already, the major herbaceous clades within Dipsacales appear to have originated in the Oligocene or Miocene, though at least in the two largest herbaceous clades most of the species diversity may be accounted for by more recent radiations into new regions and habitats. Specifically, much of the diversification within *Valeriana* is accounted for by the recent radiation of a South American clade, and Dipsacaceae diversity has been elevated recently in connection with the occupation of semi-arid habitats.

Finally, it is interesting to consider the evolution of morphological disparity in relation to the timing of diversification within Dipsacales. The basal split between Caprifoliaceae and Adoxaceae represents the greatest shift, measured either in molecular terms or using morphological characters. In particular, these two clades differ greatly and consistently (with little homoplasy) in many flower characters (Donoghue et al., 2003): Adoxaceae have radial flowers, small calyx lobes, rotate corollas, short styles, lobed stigmas, and lack nectaries of unicellular hairs; Caprifoliaceae have bilaterally symmetrical flowers, large calyx lobes, tubular corollas, long styles, unlobed stigmas, and produce nectaries of unicellular hairs. The accumulation of so many differences between these clades favors either a concerted and relatively rapid shift in a suite of morphological characters related to pollination, or a scenario involving stepwise diversification over a longer period of time, followed by the extinction of ''intermediate'' forms before the radiation of crown Adoxaceae and Caprifoliaceae. The Adoxaceae, with ca. 200 species, is presently less species diverse than Caprifoliaceae, with over 900 species. The radiation of crown Caprifoliaceae may have commenced some 10–15 million years before that of crown Adoxaceae, but the major cause of the difference in species number appears to have been the radiation of the herbaceous Valerina clade within Caprifoliaceae, and especially the relatively recent and rapid diversification of Dipsacaceae and core Valerianaceae. The herbaceous habit evolved independently at least three times within Adoxaceae (once at the base of Adoxina and twice within *Sambucus*; Eriksson and Donoghue, 1997), but these shifts apparently were not associated with major changes in diversification rate. The key to understanding the high species diversity of the Valerina clade might lie not in herbaceousness alone, but rather in the radiation of several of its sublineages into newly available habitats.

Summary—Our analyses of Dipsacales provide a concrete example of the great extent to which age estimates can vary depending on the use of different fossil calibration points, different analytical methods, and different genes. Methods that assume either a global or local clock yield age estimates for Dipsacales that are likely to be too old based on our knowledge of the angiosperm fossil record. NPRS yields considerably younger age estimates for Dipsacales than either PL or Bayesian approaches, which may reflect sensitivities of NPRS to the distribution of long and short branches in a tree (Sanderson, 2002, 2003). The great variance observed here suggests that age estimates based on single genes and/or on single estimation methods should be treated very cautiously.

The convergence of combined PL and Bayesian analyses on a set of similar age estimates for the Dipsacales provides a basis for further work on the timing of their radiation in relation to biogeography and morphological evolution. Despite the absence of an unequivocal macrofossil record of Dipsacales from the Cretaceous, our analyses strongly imply that Dipsacales originated by the mid-Cretaceous, well before previous age estimates, and that its two major lineages diverged by the start of the Tertiary. This is consistent with reports of possible caprifolioid pollen from the Cretaceous (Muller, 1981). Several major lineages appear to have diversified during the Eocene, but the major herbaceous lineages began their diversification in the Miocene, coincident with significant climate change. Much of the diversity in these lineages is probably accounted for by even more recent diversification associated with the occupation of two new regions—alpine habitats in South America by core Valerianaceae and semi-arid Mediterranean areas by core Dipsacaceae. The greatest morphological disparity is associated with the divergence of the older lineages, whereas the generation of the greatest species diversity appears to be associated with several younger and less disparate clades.

LITERATURE CITED

BACKLUND, A. 1996. Phylogeny of the Dipsacales. Department of Systematic Botany, Uppsala University, Uppsala, Sweden.

BACKLUND, A., AND M. J. DONOGHUE. 1996. Morphology and phylogeny of the order Dipsacales. Department of Systematic Botany, Uppsala University, Uppsala, Sweden.

BALDWIN, B. G., AND M. J. SANDERSON. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). *Proceedings of the National Academy of Sciences, USA* 95: 9402–9406.

BELL, C. D. 2001. PORN*: a hierarchical likelihood ratio calculator for LINUX. http://www.phylodiversity.net/pornstar. Yale University, New Haven, Connecticut, USA.

BELL, C. D. 2004. Preliminary phylogeny of Valerianaceae (Dipsacales) inferred from nuclear and chloroplast DNA sequence data. *Molecular Phylogenetics and Evolution* 31: 340–350.

BELL, C. D., AND M. J. DONOGHUE. 2003. Phylogeny and biogeography of Morinaceae (Dipsacales) based on nuclear and chloroplast DNA sequences. *Organisms, Diversity, and Evolution* 3: 227–237.

BELL, C. D., AND M. J. DONOGHUE. In press. Phylogeny and biogeography of Valerianaceae (Dipsacales) with special reference to the South American valerians. *Organisms, Diversity, and Evolution.*

BELL, C. D., E. J. EDWARDS, S.-T. KIM, AND M. J. DONOGHUE. 2001. Dipsacales phylogeny based on chloroplast DNA sequences. *Harvard Papers in Botany* 6: 481–499.

CONTI, E., J. ERIKSSON, T. SCHONENBERGER, K. SYTSMA, AND D. BAUM. 2002. Early Tertiary out-of-India dispersal of Crypteroniaceae: evidence from phylogeny and molecular dating. *Evolution* 56: 1931–1942.

DONOGHUE, M. J. 1983. The phylogenetic relationships of *Viburnum*. *In* N.I. Platnick and V.A. Funk [eds.], Advances in Cladistics, vol. 2, 143–166. Columbia Univ. Press, New York, New York, USA.

DONOGHUE, M. J., B. G. BALDWIN, J. LI, AND R. C. WINKWORTH. 2004. *Viburnum* phylogeny based on chloroplast *trn*K intron and nuclear ribosomal ITS DNA sequences. *Systematic Botany* 29: 188–198.

DONOGHUE, M. J., C. D. BELL, AND J. LI. 2001. Phylogenetic patterns in Northern Hemisphere plant geography. *International Journal of Plant Sciences* 162: S41–S52.

DONOGHUE, M. J., C. D. BELL, AND R. C. WINKWORTH. 2003. The evolution of reproductive characters in Dipsacales. *International Journal of Plant Sciences* 164: S453–S464.

DONOGHUE, M. J., T. ERIKSSON, P. A. REEVES, AND R. G. OLMSTEAD. 2001. Phylogeny and phylogenetic taxonomy of Dipsacales, with special reference to *Sinadoxa* and *Tetradoxa* (Adoxaceae). *Harvard Papers in Botany* 6: 459–479.

DONOGHUE, M. J., R. G. OLMSTEAD, J. F. SMITH, AND J. D. PALMER. 1992. Phylogenetic relationships of Dipsacales based on *rbc*L sequences. *Annals of the Missouri Botanic Garden* 79: 333–345.

DOROFEEV, P. I. 1963. The Tertiary floras of western Siberia. Technical report, Izdatelbstvo Akedemia Nauk SSSR, Moscow, Russia.

DOYLE, J. A., AND M. J. DONOGHUE. 1993. Phylogenies and angiosperm diversification. *Paleobiology* 19: 141–167.

ERIKSSON, T., AND M. J. DONOGHUE. 1997. Phylogenetic analysis of *Sambucus* and *Adoxa* (Adoxoideae, Adoxaceae) based on nuclear ribosomal ITS sequences and preliminary morphological data. *Systematic Botany* 22: 555–573.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* 17: 368–376.

FELSENSTEIN, J. 1984. Distance methods for inferring evolutionary trees: a justification. *Evolution* 38: 16–24.

GAUT, B. S. 1998. Molecular clocks and nucleotide substitution rates in higher plants. *Evolutionary Biology* 30: 93–120.

GAUT, B. S., B. R. MORTON, B. C. MC CAIG, AND M. T. CLEGG. 1996. Substitution rate comparisons between grasses and palms: synonymous rate differences at the nuclear gene *adh* parallel rate differences at the plastid gene *rbc*L. *Proceedings of the National Academy of Sciences, USA* 93: 10274–10279.

GEOLOGICAL SOCIETY OF AMERICA. 2004. GSA geologic time scale. http://www.geosociety.org/science/timescale/timescl.htm.

HECKMAN, D. S., D. M. GEISER, B. R. EIDELL, R. L. STAUFFER, N. L. KARDOS, AND S. B. HEDGES. 2001. Molecular evidence for the early colonization of land by fungi and plants. *Science* 293: 1129–1133.

HERENDEEN, P. S., P. R. CRANE, AND A. N. DRINNAN. 1995. Fagaceous flowers, fruits, and capsules from the Campanian (Late Cretaceous) of central Georgia, USA. *International Journal of Plant Sciences* 156: 93–116.

HUELSENBECK, J. P., B. LARGET, AND D. L. SWOFFORD. 2000. A compound Poisson process for relaxing the molecular clock. *Genetics* 154: 1879–1892.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17: 754–755.

JUDD, W. S., R. W. SANDERS, AND M. J. DONOGHUE. 1994. Angiosperm family pairs: preliminary phylogenetic analyses. *Harvard Papers in Botany* 5: 1–51.

JUKES, T. H., AND C. R. CANTOR. 1969. Evolution of protein molecules. *In* H. N. Munro [ed.], Mammalian protein metabolism III. 21–132. Academic Press, New York, New York, USA.

KISHINO, H., AND M. HASEGAWA. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution* 29: 170–179.

KISHINO, H., J. L. THORNE, AND W. J. BRUNO. 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. *Molecular Biology and Evolution* 18: 352–361.

LANCUCKA-SRODONIOWA, M. 1967. Two new genera: *Hemiptelea* Planch. and *Weigela* Thurnb. in the younger Tertiary of Poland. *Acta Palaeobotany* 8: 1–17.

LAROCHE, J., P. LI, L. MAGGIA, AND J. BOUSQUET. 1997. Molecular evolution of angiosperm mitochondrial introns and exons. *Proceedings of the National Academy of Sciences, USA* 94: 5722–5727.

MAGALLÓN, S., P. R. CRANE, AND P. S. HERENDEEN. 1999. Phylogenetic pattern, diversity, and diversification of eudicots. *Annals of the Missouri Botanical Garden* 86: 297–372.

MANCHESTER, S. R., AND M. J. DONOGHUE. 1995. Winged fruits of Linnaeeae (Caprifoliaceae) in the Tertiary of western North America: *Diplodipelta* gen. nov. *International Journal of Plant Sciences* 156: 709–722.

MANCHESTER, S. R., AND L. HABLY. 1997. Revision of ''Abelia'' fruits from the Paleogene of Hungary, Czech Republic, and England. *Review of Palaeobotany and Palynology* 96: 321–240.

MARTIN, W., D. LYDIATE, H. BRINKMANN, G. FORKMANN, H. SAEDLER, AND R. CERFF. 1993. Molecular phylogenies in angiosperm evolution. *Molecular Biology and Evolution* 10: 140–162.

MORLEY, R. J. 2000. Origin and evolution of tropical forests. Wiley, Chichester, UK.

MULLER, J. 1981. Fossil pollen records of extant angiosperms. *Botanical Review* 47: 1–142.

NIKITIN, V. P. 1976. Flora Mamontovoj Gory. Nauka, Moscow, Russia.

OZAKI, K. 1980. Late Miocene Tatsumitoge flora of Tottori Prefecture, southwest Honshu, Japan. *Science Reports of the Yokohama National University* 2: 40–42.

PYCK, N., P. ROELS, AND E. SMETS. 1999. Tribal relationships in Caprifoliaceae: evidence from a cladistic analysis using *ndh*F sequences. *Systematics and Geography of Plants* 69: 145–159.

PYCK, N., AND E. SMETS. 2000. A search for the position of the seven-son flower (*Heptacodium*, Dipsacales): combining molecular and morphological evidence. *Plant Systematics and Evolution* 225: 185–199.

REID, E. M., AND M. E. J. CHANDLER. 1926. Catalogue of Cenozoic plants in the Department of Geology. *In* The Bembridge flora, vol. 1. British Museum (Natural History), London, UK.

SANDERSON, M. J. 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. *Molecular Biology and Evolution* 14: 1218–1231.

SANDERSON, M. J. 1998. Estimating rates and time in molecular phylogenies: beyond the molecular clock? *In* D. E. Soltis, P. S. Soltis, and J. J. Doyle [eds.], Molecular systematics of plants II, 242–264. Kluwer, Boston, Massachusetts, USA.

SANDERSON, M. J. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution* 19: 101–109.

SANDERSON, M. J. 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. *Bioinformatics* 19: 301–302.

SANDERSON, M. J., AND J. A. DOYLE. 2001. Sources of error and confidence intervals in estimating the age of angiosperms from *rbc*L and 18S rDNA data. *American Journal of Botany* 88: 1499–1516.

SANMARTIN, I., H. ENGHOF, AND F. RONQUIST. 2001. Patterns of animal dispersal, vicariance and diversification in the Holarctic. *Biological Journal of the Linnaean Society* 73: 345–390.

SIMS, H. J., P. S. HERENDEEN, AND P. R. CRANE. 1998. New genus of fossil Fagaceae from the Santonian (Late Cretaceous) of central Georgia, USA. *International Journal of Plant Sciences* 159: 391–404.

SOLTIS, P. S., D. E. SOLTIS, AND M. W. CHASE. 1999. Angiosperm phylogeny inferred from multiple genes: a research tool for comparative biology. *Nature* 402: 402–404.

SOLTIS, D. E., P. A. SOLTIS, M. W. CHASE, M. E. MORT, D. C. ALBACH, M. ZANIS, V. SAVOLAINEN, W. H. HAHN, S. B. HOOT, M. F. FAY, M. AXTELL, S. M. SWENSEN, L. M. PRICE, W. J. KRESS, K. C. NIXON, AND J. S. FARRIS. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL,* and *atpB* sequences. *Botanical Journal of the Linnaean Society* 133: 381–461.

SOLTIS, P. S., D. E. SOLTIS, V. SAVOLAINEN, P. R. CRANE, AND T. G. BARRACLOUGH. 2002. Rate heterogeneity among lineages of tracheophytes: integration of molecular and fossil data and evidence for molecular living fossils. *Proceedings of the National Academy of Science, USA* 99: 4430–4435.

SWOFFORD, D. L. 2002. PAUP* 4.0: phylogenetic analysis using parsimony (* and other methods). Sinauer, Sunderland, Massachusetts, USA.

THORNE, J. L., AND H. KISHINO. 2002. Divergence time and evolutionary rate estimation with multilocus data. *Systematic Biology* 51: 689–702.

THORNE, J. L., H. KISHINO, AND I. S. PAINTER. 1998. Estimating the rate of evolution of the rate of molecular evolution. *Molecular Biology and Evolution* 15: 1647–1657.

TIFFANY, B. H., AND S. R. MANCHESTER. 2001. The use of geological and paleontological evidence in evaluating plant phylogeographic hypotheses in the Northern Hemisphere Tertiary. *International Journal of Plant Sciences* 162: S3–S17.

UPCHURCH, G. R., AND J. A. WOLFE. 1987. Mid-Cretaceous to early Tertiary vegetation and climate: evidence from fossil leaves and woods. *In* E. M. Friis, W. G. Chaloner, and P. R. Crane [eds.], The origin of angiosperms and their biological consequences, 75–105. Cambridge University Press, Cambridge, UK.

WIEGMANN, B. M., D. K. YEATES, J. L. THORNE, AND H. KISHINO. 2003. Time flies, a new molecular time-scale for Brachyceran fly evolution without a clock. *Systematic Biology* 52: 745–756.

WIKSTROM, N., V. SAVOLAINEN, AND M. W. CHASE. 2001. Evolution of the angiosperms: calibrating the family tree. *Proceedings of the Royal Society of London, B* 268: 2211–2220.

WINKWORTH, R. C., AND M. J. DONOGHUE. 2004. Viburnum phylogeny:

evidence from the duplicated nuclear gene GBSSI. *Molecular Phylogenetics and Evolution* 33: 109–126.

WOLFE, J. A. 1978. A paleobotanical interpretation of Tertiary climates in the northern hemisphere. *American Scientist* 66: 694–703.

YANG, Z. 1994. Maximum likelihood phylogenetic estimations from DNA sequences with variable rates over sites: approximate method. *Journal of Molecular Evolution* 39: 306–314.

YANG, Z. 2000. Phylogenetic analysis by maximum likelihood (PAML), 3.0 ed. University College London, London, UK.

YANG, Z., AND A. D. YODER. 2003. Comparison of likelihood and Bayesian methods for estimating divergence times using multiple gene loci and calibration points, with application to a radiation of cute-looking mouse lemur species. *Systematic Biology* 52: 705–716.

YODER, A. D., AND Z. YANG. 2000. Estimation of primate speciation dates using local molecular clocks. *Molecular Biology and Evolution* 17: 1081–1190.

ZACHOS, J., M. PAGANI, L. SLOAN, E. THOMAS, AND K. BILLUPS. 2001. Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* 292: 686.

ZHANG, W.-H., Z.-D. CHEN, J.-H. LI, Y.-C. CHEN, AND H-B. TANG. 2003. Phylogeny of the Dipsacales s.l. based on chloroplast *trn*L and *ndh*F sequences. *Molecular Phylogenetics and Evolution* 26: 176–189.