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Molecular Phylogenetic Dating of Asterid Flowering Plants Shows Early Cretaceous Diversification

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Abstract.—We present a phylogenetic dating of asterids, based on a 111-taxon tree representing all major groups and orders and 83 of the 102 families of asterids, with an underlying data set comprising six chloroplast DNA markers totaling 9914 positions. Phylogenetic dating was done with semiparametric rate smoothing by penalized likelihood. Confidence intervals were calculated by bootstrapping. Six reference fossils were used for calibration. To explore the effects of various sources of error, we repeated the analyses with alternative dating methods (nonparametric rate smoothing and the Langley-Fitch clock-based method), alternative tree topologies, reduced taxon sampling (22 of the 111 taxa deleted), partitioning the data into three genes and three noncoding regions, and calibrating with single reference fossils. The analyses with alternative topologies, reduced taxon sampling, and coding versus noncoding sequences all yielded small or in some cases no deviations. The choice of method influenced the age estimates of a few nodes considerably. Calibration with reference fossils is a critical issue, and use of single reference fossils yielded different results depending on the fossil. The bootstrap confidence intervals were generally small. Our results show that asterids and their major subgroups euasterids, campanulids, and lamiids diversified during the Early Cretaceous. Cornales, Ericales, and Aquifoliales also have crown node ages from the Early Cretaceous. Dipsacales and Solanales are from the Mid-Cretaceous, the other orders of core campanulids and core lamiids from the Late Cretaceous. The considerable diversity exhibited by asterids almost from their first appearance in the fossil record also supports an origin and first phase of diversification in the Early Cretaceous. [Angiosperms; asterids; Cretaceous; dating; fossils; molecular clocks.]

Asterids comprise more than 80,000 species or nearly 1/3 of all flowering plants. Two of the five most species-rich families of flowering plants are asterids, the sunflower family Asteraceae with 23,000 species, and the coffee family Rubiaceae with more than 10,000 species. Asterids include a large variety of plants, with familiar representatives such as dogwood (Cornaceae), heath (Ericaceae), mint (Lamiaceae), potato (Solanaceae), holly (Aquifoliaceae), and carrot (Apiaceae). Asterids are distributed all over the world, and they are well represented with species-rich subgroups in the tropics and temperate areas, in the Northern as well as Southern Hemisphere.

For a long time most asterids have been recognized as a natural group of predominantly herbaceous plants with sympetalous corollas, a group formerly known as Sympetalae (see Wagenitz, 1992). With molecular data, particularly from the *rbcL* gene of the chloroplast genome, it has been possible to circumscribe the asterids as a well-supported monophyletic group, including also several subgroups with free petals in their flowers (Chase et al., 1993; Downie and Palmer, 1992; Olmstead et al., 1992, 1993). Following the current APG classification (APG II, 2003), asterids are classified in 102 families and 10 orders, Cornales, Ericales, Aquifoliales, Apiales, Dipsacales, Asterales, Garryales, Gentianales, Solanales, and Lamiales. The last eight are known as the euasterids, and they comprise two monophyletic, roughly equally sized sister groups, campanulids and lamiids (Bremer et al., 2002; in the APG classification known as euasterids II and I, respectively).

Asterids are nested as a subgroup within the core eudicots in the flowering plant phylogenetic tree (e.g., Soltis et al., 2000). Because of their derived phylogenetic position, their predominantly herbaceous habit, and their many floral specializations, asterids may be considered a comparatively young group that diversified during the Tertiary. The majority of eudicot fossils from the Early Cretaceous belong in phylogenetically basal eudicot lineages such as Ranunculales, Proteales, and Buxaceae (Friis et al., 1988; Drinnan et al., 1991). Evidence from the fossil record indicates that the mid-Cretaceous (late Albian to early Cenomanian 105 to 95 my) flowering plant diversification was mostly restricted to the rosids (Muller, 1981). Compared to other eudicots the first appearance of asterids in the fossil record is relatively late, with the first occurrences around the late Turonian about 89 my ago (Muller, 1981; Knobloch and Mai, 1986; Magallón et al., 1999), but already by the Santonian-Campanian about 80 my ago, asterids exhibited considerable diversity and include at least one taxon, *Actinocalyx bohrii* (Friis, 1985), with sympetalous flowers.

Because asterids were circumscribed as a monophyletic group about 10 years ago, more comprehensive analyses based on several genes, in particular *ndhF*, *atpB*, and 18S rDNA, but also on morphology, have contributed to a well-resolved phylogenetic tree of asterids at the family level (Gustafsson et al., 1996; Morton et al., 1996; Plunkett et al., 1996; Backlund and Bremer, 1997; Soltis and Soltis, 1997; Oxelman et al., 1999; Backlund et al., 2000; Olmstead et al., 2000; Albach et al., 2001a, 2001b; Kårehed, 2001; Bremer et al., 2001, 2002; Anderberg et al., 2002; Xiang et al., 2002). With the presently available well-supported phylogenetic tree of

asterids, it is now time to attempt a dating of the age of asterids and their major subgroups and orders.

Several efforts have been made towards dating the age of the flowering plants using molecular data (Martin et al., 1989, 1993; Wolfe et al., 1989; Goremykin et al., 1997; Sanderson and Doyle, 2001; Wikström et al., 2001), with widely differing results. In particular, the earlier studies arrived at surprisingly old ages, apparently due to methodological deficiencies (Sanderson and Doyle, 2001). Molecular phylogenetic dating is now rapidly gaining attention, and recently several papers have appeared dealing with age estimates of groups of flowering plants (e.g., Bremer and Gustafsson, 1997; Baldwin and Sanderson, 1998; Baum et al., 1998; Bell and Patterson, 2000; Bremer, 2000, 2002; Chanderbali et al., 2001; Koch et al., 2001; Renner et al., 2000; Xiang et al., 2000; Vinnersten and Bremer, 2001; Conti et al., 2002; Davis et al., 2002; Smedmark et al., 2003). Some recent zoological dating studies in this journal include Aris-Brosou and Yang (2002), Lloyd (2003), and Yang and Yoder (2003). Here we present a phylogenetic dating of asterids, based on a 111-taxon tree representing all major groups and orders and 83 of the 102 families of asterids, with an underlying data set comprising six molecular markers (Bremer et al., 2002).

Data and Methods

Bremer et al. (2002) made a phylogenetic analysis of 129 asterids using evenly weighted parsimony and six chloroplast DNA markers. All six markers are available for 111 of the 129 taxa. Bremer et al. found 24 equally parsimonious trees, but for the subset of 111 taxa there is only a single most parsimonious tree, shown in Figure 1. Most of the clades in Figure 1 are supported by high jackknife percentages (Bremer et al., 2002), and nodes with >85% jackknife support are marked with asterisks. There is remaining uncertainty regarding interrelationships among orders of core campanulids (Apiales, Dipsacales, Asterales) and core lamiids (Gentianales, Solanales, Lamiales) and among families within Ericales and Lamiales in particular. Should interrelationships of these taxa turn out to be different than those found by Bremer et al. (2002), age estimates would be affected. To explore the effects of alternative topologies on our age estimates, we have repeated the analyses with a set of alternative topologies involving collapse of reference nodes 1, 3, and 6 (which have <85% jackknife support; Figure 1) followed by alternative resolutions of the resulting trichotomy and involving alternative interrelationships among the orders of core campanulids (Apiales, Dipsacales, Asterales) and among the orders of core lamiids (Gentianales, Solanales, Lamiales), respectively. The alternative topologies tested involve ordinal interrelationships potentially affecting the results for several subgroups whereas we omitted testing all alternative topologies within orders (e.g., Ericales, Lamiales).

The selection of 111 of the 129 taxa analyzed by Bremer et al. (2002) reduces the number of sampled asterid families from 93 to 83 (following the APG II classification, 2003) but it permits us to use all six molecular markers, totaling 9914 positions, for the dating. Regarding family classification, it should be noted that *Quintinia* is usually classified in Escalloniaceae but it is here included in Paracryphiaceae in agreement with its position as sister to *Paracryphia* and following Lundberg (2002). Icacinaceae are known to be heterogeneous, and in the following discussion Icacinaceae are taken sensu stricto excluding *Apodytes* (Kårehed, 2001).

Taxon sampling is a crucial issue not only in phylogenetic reconstruction but also in phylogenetic dating. Here we have used all asterid taxa for which the six molecular markers are available. We have, however, explored the effects of a reduced taxon sampling by repeating the analyses with 22 of the 111 taxa deleted. The taxa were more or less evenly deleted throughout the tree so as to retain at least two taxa for all families originally represented by more than one taxon. The following taxa were deleted: *Impatiens*, *Diospyros*, *Myrsine*, *Cyrilla*, *Melanophylla*, *Diervilla*, *Valeriana*, *Escallonia*, *Tribeles*, *Roussea*, *Alseuosmia*, *Scaevola*, *Icacina*, *Gentiana*, *Nicotiana*, *Grevea*, *Olea*, *Globularia*, *Scrophularia*, *Paulownia*, *Lamium*, and *Stilbe*.

The underlying data matrix from Bremer et al. (2002) comprises six molecular markers from the chloroplast genome, three coding and three noncoding. The three protein-coding genes are *rbcL*, *ndhF*, and *matK*, all well known and much used for phylogenetic analyses at higher taxonomic levels within and among families and orders of flowering plants. The three noncoding markers are first, a region including *trnL* exons and the intron and intergenic spacer between *trnT* (UGC) to *trnF* (GAA); second, a region including *trnV* exons and intron, *trnM*, and the intergenic spacer between *trnV* (UAC) and *atpE*; and third, the *rps16* intron. Before the Bremer at al. (2002) study, single copy noncoding chloroplast markers have mainly been used at lower taxonomic levels within families, tribes, or genera. Because the noncoding markers were as informative as the genes for reconstruction of phylogeny of the asterids, they should be useful also for age estimates. Our age estimates are based on the whole data set, but we have also repeated the analyses partitioning the data to the three coding sequences and the three noncoding sequences.

The data were optimized on the tree using PAUP* (Swofford, 2001) and maximum likelihood applying the GTR+Γ model with the PAUP default setting of 0.5 for the gamma shape parameter. Ideally the latter is estimated from the data but it increases computation time beyond the currently possible. We did, however, try optimization with the gamma shape parameter estimated by the data and all resulting branch lengths were similar to those obtained with the default gamma shape parameter. The variation in branch lengths depending on model specification in optimization was in the central range of branch length variation obtained by the bootstrapping. Hence, the variation in estimated dates resulting from different model specifications in branch

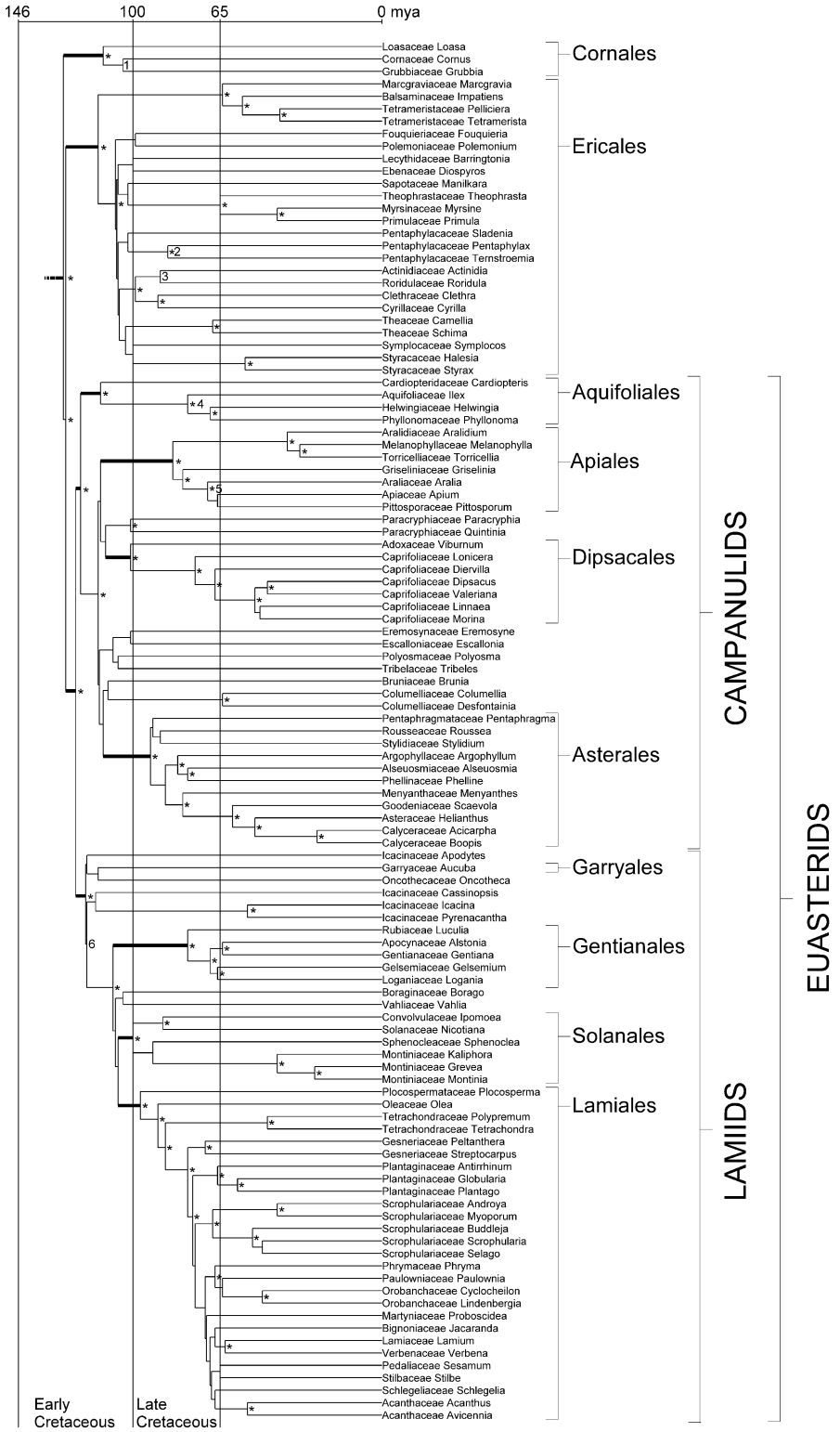

FIGURE 1. Dated phylogenetic tree of asterids obtained from semiparametric rate smoothing (penalized likelihood; Sanderson, 2002a, 2002b) of a 111-taxon asterid tree from Bremer et al. (2002), with maximum likelihood branch lengths from six molecular markers and calibration with six reference fossils, which attach above the nodes numbered 1 to 6 in the tree. Asterisks denote nodes with >85% jackknife support in the phylogenetic reconstruction by Bremer et al. (2002). Reference fossils are listed in Table 1, and further information on age estimates is found in Table 2.

length optimization is small for this data set and would hardly be visible for most groups in the two figures of this paper.

The tree with its branch lengths in estimated number of substitutions, obtained by multiplying the per site values reported by PAUP* with the number of sites (9914), was subjected to the clock test in the PATH program (Britton, 2002; Britton et al., 2002). The test reported significant clock deviations at 93 of the 110 nodes, after Bonferroni correction. The number of nodes with clock deviations may seem high but it is expected because long branch lengths from a large data set increase precision such that clock deviations are more easily detected. The results from the clock test indicate that a non–clock-based dating method should be applied.

There are now many methods available for phylogenetic dating (e.g., Takezaki et al., 1995; Sanderson, 1997, 2002a; Rambaut and Bromham, 1998; Thorne et al., 1998; Huelsenbeck et al., 2000; Yoder and Yang, 2000; Kishino et al., 2001; Britton et al., 2002; Thorne and Kishino, 2002; Yang and Yoder, 2003; for reviews see Benton and Ayala, 2003; Bromham and Penny, 2003). Here dating was done with the r8s program (Sanderson, 2002b) implementing semiparametric rate smoothing by penalized likelihood (Sanderson, 2002a), which combines a model-based likelihood approach with a roughness penalty that prevents too much rate variation. The size of the roughness penalty is specified by a smoothing parameter obtained by a cross-validation procedure. After cross-validation, the smoothing parameter was set to an absolute value of 3. The input file for this r8s analysis is available at www.systbot.uu.se. To explore methodological influence on age estimates, dating was done also with nonparametric rate smoothing (Sanderson, 1997) and the Langley-Fitch method (Langley and Fitch, 1974), both implemented in the r8s program. Nonparametric rate smoothing does not impose a molecular clock but allows rates to vary over the tree, however minimizing rate changes in adjacent branches. The Langley-Fitch method uses maximum likelihood for dating under the assumption of a strict molecular clock.

Confidence intervals were calculated by bootstrapping, repeating the dating procedure 100 times with 100 topologically identical trees with varying branch lengths obtained from 100 bootstrapped matrices, the latter generated by SEQBOOT in Felsenstein's (1993) PHYLIP package. The confidence interval for each node is calculated as $\pm 2 \cdot 1.96$ standard errors in the range of age estimates from the 100 dating analyses.

Six reference fossils, described below and in Table 1, were used for calibration. They attach above the nodes with numbers 1 to 6 in Figure 1 and along the branch leading to the family of the fossil. The r8s program failed to find a solution with all six fossils as simultaneous calibration points, i.e., using the fixage command of r8s for all fossils simultaneously. Instead, six preliminary dating analyses were done, each with one fossil as the calibration point using the fixage command and the other five as constraints specifying minimum ages using the constraints command. The six analyses yielded six putative crown node ages for the asterids, viz. 96, 109, 115, 138, 151, and 156 my (Table 1). The mean value, 128 my, for the crown node (the basalmost node in Figure 1) was subsequently used as the calibration point with the six numbered nodes constrained to minimum ages given by the fossils.

TABLE 1. Reference fossils used in calibration. The column to the far right (All) shows the crown node age in my for all asterids, with the fossil in this row used for calibration and the other fossils for constraining minimum ages. The first fossil was reported by Takahashi et al. (2002), the other five by Knobloch (1986).

No.	Genus	Family	Period	My	All
1	*Hironoia*	Cornaceae	Early Coniacian	88	109
2	*Eurya*	Pentaphylacaceae	Santonian	86	156
3	*Saurauia*	Actinidiaceae	Late Turonian	89	151
4	*Ilex*	Aquifoliaceae	Maastrichtian	70	115
5	*Aralia/Acanthopanax*	Araliaceae	Maastrichtian	70	138
6	*Icacinicarya*	Icacinaceae	Late Turonian	89	96

Reference Fossils

The fossil record of asterids is extensive particularly from younger strata of the Tertiary, but many older asterid fossils are continuously being added to the record as more and more fossil floras with well-preserved floral structures are being studied from the Late Cretaceous. However, despite the increasing number of highly informative Cretaceous fossils, few of these have with certainty been assigned to modern taxa at the level below the order, and assignment to an order has also in many cases been problematic. Whole plant preservation is rare, and most flowering plant fossils are typically detached organs such as leaves, fruits, seeds, flowers, or dispersed pollen. This could explain some of the problems in placing the fossils in modern taxa, but the fossils may also represent extinct lineages with characters or suites of characters that are no longer present in any living flowering plant.

The six reference fossils chosen for the present study (Table 1) are all described based on reproductive structures that usually possess more characters critical for a systematic assignment than other organs. We have been careful to use only such reference fossils that can be attached unequivocally to the phylogenetic tree. Each fossil is implicitly placed as sister group to the largest clade with which it shares one or more unique morphological synapomorphies. It means that the stem node of that clade is at least as old as the fossil. The stem nodes above which reference fossils attach are numbered in Figure 1. The fossils are all from strata that are considered relatively well dated. It should be noted, however, that exact dating is rare for terrestrial sediments, and most of the fossil floras with flowering plant remains are dated by indirect evidence and correlation to marine sequences.

The oldest fossil that can be related to Cornaceae is *Hironoia fusiformis*, an extinct genus and species collected from Futaba Group sediments at Kamitikaba, northeastern Japan (Takahashi et al., 2002). The fossil is based on small three-dimensionally preserved fruits (drupes) with

remnants of a perianth. Synapomorphies of the endocarp wall and dehiscence valves observed for the fossil occur in Cornaceae only in the two genera *Nyssa* and *Mastixia*. The fossil taxon cannot be placed with certainty in one of the two genera, and *Hironoia* is either an early member of the *Nyssa*/*Mastixia* branch or it may belong to the stem group leading to this clade. The age of the Futaba group sediments is established as early Coniacian to early Santonian, and fossil plants are thought to belong to the lower part of the sequence, which would give a minimum age for the *Nyssa*/*Mastixia* branch of early Coniacian about 88 my ago (Takahashi et al., 2002). Other fossils that are considered as closely related to *Mastixia* (*Beckettia*, *Eomastixia*, *Mastixiocarpum*, *Mastixiopsis*) are common in the Maastrichtian of Central Europe (Knobloch and Mai, 1986).

Fossils with synapomorphies indicating affinity to members of Pentaphylacaceae and Ternstroemiaceae (the two families are synonymized in APG II, 2003, but may be optionally treated as distinct) are common in the Late Cretaceous and represented by a variety of floral structures as well as fruits and seeds. The oldest fossils of this group are seeds with campylotropous organization and characteristic deep and strongly pitted cells of the outer seed wall. By these synapomorphies they are clearly assignable to the modern genus *Eurya* and included in an extinct species, *E. crassitesta* (Knobloch and Mai, 1986). *Eurya* is known from several core samples and localities in Central Europe ranging in age from the Santonian about 86 my ago and onwards (Knobloch and Mai, 1986). Several other Late Cretaceous fossils are related to this clade. *Paradinandra suecica* is a fossil flower from the Santonian/Campanian of Scania (about 80 my) similar to *Adinandra* (Schönberger and Friis, 2001).

The oldest fossils assignable to Actinidiaceae are from the late Turonian (about 89 my) of Central Europe and include characteristic anatropous and reticulate seeds synapomorphic with those of the modern genus *Saurauia* (Knobloch and Mai, 1986). Two extinct species were established, *Saurauia alenae* and *S. antiqua* both recorded from several assemblages in Central Europe and ranging in age from the late Turonian to the Maastrichtian (Knobloch and Mai, 1986). Further evidence of the presence of the family in the Late Cretaceous are fossil flowers described as *Parasaurauia allonensis* from the Campanian (about 80 my) of Georgia (Keller et al., 1996). It is placed in Actinidiaceae as sister to extant *Saurauia* and *Actinidia*.

The fossil record of Aquifoliaceae is sparse, particularly from the Cretaceous. The oldest representatives of the family are endocarps from the Maastrichtian (about 70 my) flora of Eisleben, Germany, assigned to the modern genus *Ilex* and included in the extinct species *I. antiqua* (Knobloch and Mai, 1986). Endocarps of *Ilex* represent distinctive synapomorphies with longitudinal and sometimes anastomosing ridges common in the fruit and seed floras of the Tertiary. According to Muller (1981) characteristic pollen related to *Ilex* is reported from older strata (Turonian and Coniacian) of Africa, but none of these have been confirmed.

The earliest Araliaceae fossils are from the Maastrichtian (about 70 my) floras of Eisleben and Walbeck, Germany, and include five extinct species assigned to the modern genera *Acanthopanax* (*A. friedrichii*, *A. gigantocarpus*, *A. mansfeldensis*, *A. obliquocostatus*) and *Aralia* (*A. antiqua*), based on synapomorphies in the endocarps. The fossil species have endocarps that are distinctly ribbed in *Acanthopanax* and rugulate in *Aralia* (Knobloch and Mai, 1986).

The oldest report of Icacinaceae is from the Turonian (about 89 my) of Central Europe. The record is based on endocarps with a characteristic, deeply foveolate-reticulate surface pattern, a synpomorphy shared by other Icacinaceae. The fossil is assigned to the extinct genus and species *Icacinicarya budvarensis* (Knobloch and Mai, 1986). The species occurs in late Turonian (89 my) to Santonian sediments of the Klikov sequence of the Czech Republic. Several other taxa related to Icacinaceae were recorded from younger strata of the Late Cretaceous with the earliest record of the modern genus *Iodes* in the Maastrichtian (Knobloch and Mai, 1986).

RESULTS

Results are shown in Figures 1 and 2 and in Table 2. The exact deviations in my reported in Figure 2 are available at www.systbot.uu.se. The crown node age of Garryales could not be estimated because one member only is sampled. We provide no stem node age for asterids; it would need inclusion of a large sample of taxa outside the asterids. Also we do not report variations in the crown node age for asterids in Figure 2, because it was set to 128 my in all analyses except the preliminary ones when calibration was done with single reference fossils. The varying crown node ages from the preliminary analyses, 96 to 156 my, are given in Table 1.

The bootstrap confidence intervals are generally small (b in Figure 2), indicating that error due to variation in the data set is small. This is expected due to the large amount of underlying data. Considerable uncertainty exists for the crown node ages of Styracaceae, Theaceae, Paracryphiaceae, and Tetrachondraceae, however.

The analyses with alternative topologies, reduced taxon sampling, and coding versus noncoding sequences (t, s, and d in Figure 2) all yielded small and in some cases no deviations in age estimates. Apparently, the results are robust to changes in topology affecting nodes without high jackknife support, the taxon sampling is sufficient at least for age estimates of the major groups and the larger orders, and the dating is insensitive to the nature of the data, coding or noncoding. Also, the amount (length) of sequence data seems sufficient, because reductions by 50% did not cause any considerable change in results (Figure 2).

Deviation in age estimates due to the use of alternative dating methods (m in Figure 2), viz. nonparametric rate smoothing and the Langley-Fitch clock-based method, indicates that choice of method may influence the age estimates of particular nodes considerably (e.g., Theaceae, Paracryphiaceae). In general, however,

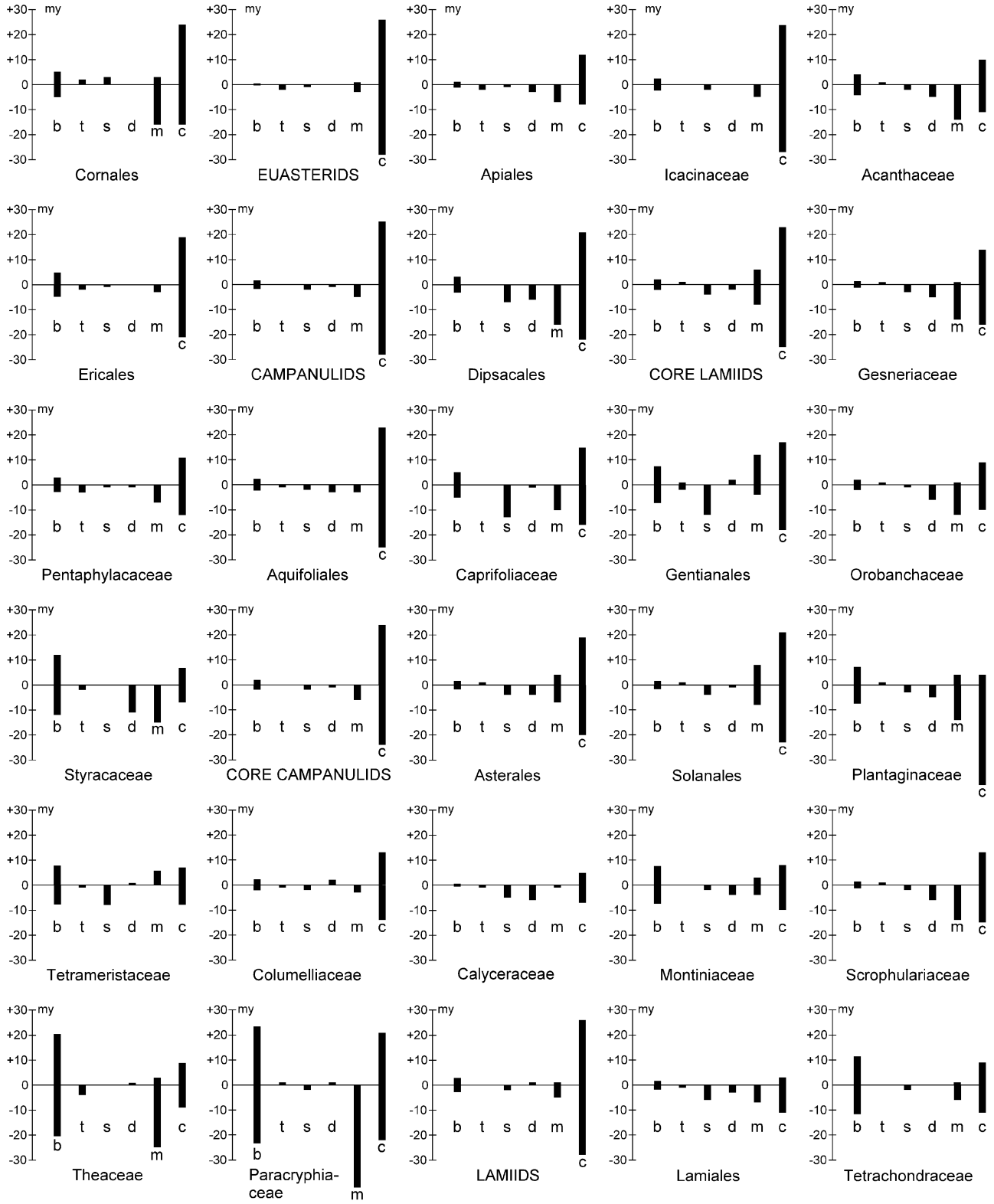

FIGURE 2. Variation in crown node age estimates compared to results in Table 2 and Figure 1; exact deviations in my are available at www.systbot.uu.se. b = confidence intervals obtained by bootstrap analysis of 100 replicates of the data matrix; t = deviation obtained with varying topologies (given under Data and Methods); s = deviation obtained after reduced sampling by deletion of 22 of the 111 taxa; d = deviation obtained with data from the three coding or the three noncoding sequences only; m = deviation obtained with alternative methods, nonparametric rate smoothing or the Langley-Fitch clock-based method; c = deviation obtained with calibrations by one only of the six reference fossils.

TABLE 2. Stem node ages (sna) and crown node ages (cna) in my for major groups, orders, and some families of asterids obtained from semiparametric rate smoothing (penalized likelihoods; Sanderson, 2002a, 2002b) of a 111-taxon asterid tree from Bremer et al. (2002), with maximum likelihood branch lengths from six molecular markers and calibration with six reference fossils.

Group	sna	cna
Asterids	—	128
Cornales	128	112
Ericales	127	114
Pentaphylacaceae	107	102
Styracaceae	100	55
Tetrameristaceae	56	41
Theaceae	103	68
Euasterids	127	123
Campanulids	123	121
Aquifoliales	121	113
Core campanulids	121	114
Columelliaceae	110	64
Paracryphiaceae	111	101
Apiales	113	84
Dipsacales	111	101
Caprifoliaceae	101	75
Asterales	112	93
Calyceraceae	51	26
Lamiids	123	119
Icacinaceae	119	115
Garryales	114	—
Core Lamiids	119	108
Gentianales	108	78
Solanales	106	100
Montiniaceae	92	42
Lamiales	106	97
Acanthaceae	67	54
Gesneriaceae	78	71
Orobanchaceae	64	48
Plantaginaceae	76	66
Scrophulariaceae	75	68
Tetrachondraceae	87	46

the differences due to method are not great in our results. Except for results within Lamiales in which families are comparatively young, the differences between semiparametric and nonparametric rate smoothing are small. This is expected because the smoothing parameter found by the cross-validation procedure is small (3) compared to what is found in many other data sets (M. J. Sanderson, personal communication), allowing for considerable rate changes across the tree as in nonparametric rate smoothing. The differences between semiparametric rate smoothing and the Langley-Fitch clock-based method are larger. This is also expected, due to the small value of the smoothing parameter but also because the clock test revealed significant deviation from clocklike change at a majority of the nodes in the tree. Most of the deviation reported in Figure 2 is from comparison with the results of the Langley-Fitch clock-based method. Results from this method are included simply for demonstrating the magnitude of error that may result from choosing an inappropriate dating method, and they should not be taken as indicating a range of uncertainty in our age estimates.

Deviation in age estimates due to the use of different calibrations (c in Figure 2), setting the crown node age of asterids to 96 or 156 my (cf. Table 1), indicates that calibration is a critical issue in molecular phylogenetic dating. The use of several reference fossils is certainly to be recommended. We have included the deviation with two extreme results found by using one only of the six reference fossils to demonstrate the magnitude of error that may result from different choices of reference fossils. We do not, however, consider the range of variation in age estimates setting the asterid crown node age to 96 or 156 my as a range of uncertainty in our results. Because we have accepted all six reference fossils of Table 1 as valid, there is no reason to do anything else but include the information from all six in the analysis, and this is what is done in setting the asterid crown node age to the mean value 128 my.

DISCUSSION

A major concern in phylogenetic dating is the influence of various error sources (Sanderson and Doyle, 2001; Soltis et al., 2002). We have explored several of these by our analyses with alternative topologies, reduced taxon sampling, coding versus noncoding sequences, different dating methods, and varying reference fossils. Sanderson and Doyle (2001) investigated the effects of the same error sources, except that of the reference fossils, on estimating the age of flowering plants in a phylogenetic tree of land plants. They found considerable variation in results, much larger than in our analysis of asterids. The origin of flowering plants is of course further back in time than that of asterids, so greater uncertainty in estimating the former is to be expected. However, it seems that in our analysis of asterids the variation due to error is less than expected. One reason may be our use of six reference fossils. The minimum age constraints imposed by these fossils in different parts of the tree are likely to restrict variation caused by a variety of other factors.

There is considerable variation in the crown node age for asterids estimated from the six different reference fossils, and it may seem that some of them must be wrong. As explained above, we have been careful to use only those reference fossils that could be attached unambiguously to the tree. Nevertheless, the reference nodes include errors of different kinds, related to age of the fossil and to the branch lengths of the tree from the reference node towards the root and towards the terminals. Furthermore, these different errors may be small or large. There is, however, no way to tell which of the six calibrations are "wrong" and which are "right." All six give approximations of the true crown node age for asterids. Hence, we considered it best to estimate the crown node age by taking the mean value of the six estimations.

It may seem that the oldest estimates are unreasonable given the alleged age of eudicots indicated by the appearance of triaperturate pollen 125 my ago (Crane et al., 1995), but some authors (e.g., Wikström et al., 2001) argue that eudicots must be much older than 125 my because this is indicated by other reference fossils (e.g., the Fagales cupule used by Wikström et al., 2001). Others would argue that the youngest estimates seem unreasonable given the age of other fossils, and by

this type of reasoning, you may exclude some of the oldest and some of the youngest estimates. But how many? There is no way to tell and the reasonable approach is to accept all six as more or less good approximations, some too old and some too young, and include them all in a mean value.

We have based our age estimates on both the coding genes (all three codon positions) as well as the noncoding markers. We found few differences between the two types of data (Figure 2), and both data types show nonclocklike substitution rates. This is in contradiction to what Renner et al. (2000) found in their study of Atherospermataceae (Laurales). They found *rbcL* to have a clocklike substitution rate, but the noncoding markers were not, and thus the latter were excluded from Renner et al.'s age estimates. Sanderson and Doyle (2001), on the other hand, showed that *rbcL* for their analyses of the flowering plants behaved in a nonclocklike manner. They also found that the use of all three codon positions best agreed with the fossil record. A possible reason for Renner et al.'s (2000) observation of *rbcL* clocklike behaviour is that they studied a single family representing only a restricted number of closely related taxa.

Wikström et al. (2001) estimated ages for asterids and many of their subgroups as well as for many other flowering plants, but their analysis was preliminary in the sense that a single reference fossil was used as a calibration point for the whole tree. This fossil is in Fagales of the rosids, hence topologically rather far away from subgroups of asterids in the tree, resulting in uncertainty in the dating. In their dating of flowering plants, Wikström et al. (2001) obtained consistently younger age estimates for asterids, mostly 10 to 20 my younger for major groups and orders compared to our estimates (Table 2). For example, our crown node age for asterids is 128 my whereas Wikström et al. estimated it to 117 to 107 my. Asterids are in a phylogenetically derived position in the flowering plant tree and comparison with fossils—not used as constraints in the analysis—shows that the age of such derived groups is underestimated in their results (Wikström et al., 2001).

The history of the genus *Ilex* in Aquifoliaceae has been studied by Cuénoud et al. (2000) and Manen et al. (2002). These authors suggested that extant species of *Ilex* diversified during the Tertiary, although the family must be much older. We know this since there are *Ilex* fossils from the Late Cretaceous c. 70 my ago (Table 1). Until recently Aquifoliales consisted of the genus *Ilex*, and a few small, apparently closely related genera in Aquifoliaceae and two other families (Helwingiaceae and Phyllonomaceae). The order has now been expanded with Stemonuraceae (not sampled in our analysis) and Cardiopteridaceae (Kårehed, 2001), and with this expansion it is dated to the Early Cretaceous, with a crown node age of 113 my (Table 2).

Backlund (1996) carried out a provisional dating of Dipsacales based on *rbcL* sequences and calibration with the fossil flower *Silvianthemum*, which he placed among core campanulids close to *Quintinia*. He estimated the crown node age of the "Dipsacales-Apiales lineage" to 115 to 130 my. This "lineage" corresponds roughly to our core campanulids, the crown node of which we have estimated to 114 my. This good correspondence is not found; however, in Backlund's and our estimates of the crown node age of Dipsacales, 60 to 70 and 101 my, respectively. The position of *Silvianthemum* among core campanulids close to *Quintinia* is also uncertain.

The crown node age of Asterales was estimated to 96 my by Bremer and Gustafsson (1997) using *rbcL* sequences and calibration with fossil Asteraceae pollen. Here we have no reference fossil in Asterales, yet the age estimate is close, 93 my (Table 2). In addition to the well-known sunflowers (Asteraceae) and bellflowers (Campanulaceae), this order comprises several small, mainly southern hemisphere families that originated (split from their sister groups) during break-up of Gondwana in the Late Cretaceous and Early Tertiary (Bremer and Gustafsson, 1997).

We have included stem and crown node ages for those families represented by more than one member in the analysis, but the estimates should be taken with some caution due to the restricted sampling. Notably, Pentaphylacaceae and Paracryphiaceae date back to Mid-Cretaceous, albeit with uncertainty for Paracryphiaceae as indicated by the bootstrap confidence interval, and Icacinaceae are even older. We estimate the crown node age of Tetrachondraceae, i.e., the split between *Polypremum* and *Tetrachondra* to 46 my, roughly in accordance with the Early Tertiary split suggested by Wagstaff et al. (2000). They also observed that *rbcL* sequences of the two species of *Tetrachondra*, one in South America and one in New Zealand, are virtually identical, indicating that the two species are comparatively young and that one of their ancestors must have dispersed across the Pacific.

Our results show that asterids and their major subgroups euasterids, campanulids, and lamiids diversified during the Early Cretaceous. Cornales, Ericales, and Aquifoliales also have crown node ages from the Early Cretaceous, meaning that they diversified during that period. Dipsacales and Solanales are from the Mid-Cretaceous, the other orders of core campanulids and core lamiids from the Late Cretaceous. All orders split from their sister groups during the Early Cretaceous; that is, their stem node ages are from this period. The results conflict with the fossil record in the sense that no fossil asterid has so far been discovered from the Early Cretaceous. Furthermore, the earliest unequivocal record of eudicots is from the Barremian-Aptian (Hughes, 1994) about 3 my after our estimated time of asterid origin.

However, the first entrance of a taxon in the fossil record is most unlikely to represent the time of origin for this taxon. An origin for eudicots prior to 125 my as indicated by their first occurrence is therefore not controversial. There is a clear increase in complexity in organisation and structure in flowering plant organs (leaves, pollen, flowers) through the Early Cretaceous (Doyle and Hickey, 1977; Friis and Crepet, 1987; Crane et al., 1995), indicating that the major flowering plant diversification must have taken place within a relatively short interval during the Early Cretaceous. Although no asterid fossils

have been found prior to the Turonian about 89 my ago, the considerable diversity exhibited by the group almost from their first appearance in the fossil record supports an origin and first phase of diversification in the Early Cretaceous.

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