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Molecular phylogeny and intra- and intercontinental biogeography of Calycanthaceae

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Abstract

Based on nuclear and chloroplast sequences we resolve species relationships in Calycanthaceae and develop a biogeographic hypothesis that explains their intercontinental disjunctions and intra-continental diversification in eastern Asia. Fossil-calibrated penalized likelihood and Bayesian divergence time estimates indicate that the Northern Hemisphere *Calycanthus* and *Chimonanthus* diverged from each other in the mid-Miocene, while the Australian *Idiospermum* had already diverged by the Upper Cretaceous and likely represents a remnant of a former Gondwanan distribution of Calycanthaceae that included South America, as indicated by the occurrence of Cretaceous Calycanthaceae fossils in Brazil. Relationships within *Calycanthus* were difficult to resolve, but a shared 155-bp deletion in the *trn*L–F intergenic spacer unites the two North American species, which were also sisters in a cpDNA restriction site study. Their ancestor apparently crossed the Bering land bridge in the Miocene. The six species of *Chimonanthus*, by contrast, diverged from each other as recently as 1–2 my ago, and a DIVA analysis with four areas of endemism recognized within China suggests three vicariance and two dispersal events within *Chimonanthus*, with initial vicariance having occurred between eastern and southwestern or central China. Further divergence then appears to have involved eastern and southcentral China, and southwestern and central China.

Keywords: Bayesian divergence time estimation; Biogeography; Calycanthaceae; Internal transcribed spacer; Penalized likelihood; *trn*C–*trn*D intergenic spacer; *trn*L intron; *trn*L–*trn*F intergenic spacer

1. Introduction

Calycanthaceae are a family of 10 species in three genera, *Calycanthus* L. with three species, *Chimonanthus* Lindley with six, and *Idiospermum* Blake with a single species. In spite of its small size, the family exhibits three major range disjunctions (Fig. 1), (1) a rare intercontinental disjunction between North America (USA), eastern Asia (China), and Australia, where *Idiospermum* is endemic to Queensland; (2) a classic eastern Asian and North American disjunction among the three species of *Calycanthus*; and (3) an eastern North American and western North American disjunction between *Calycanthus floridus* and *C. occidentalis*. In terms of species richness, there is a clear imbalance, with temperate Asia having seven species, while Australia and North America have but one and two species, respectively. Calycanthaceae thus offer an exceptional opportunity to examine the history of three disjunctive patterns as well as the origin of a diversity imbalance. Possibly complicating the situation is that species of *Calycanthus* and *Chimonanthus* are commonly cultivated in Asia, North America and Europe (Bygrave, 1996; Jelitto, 1971; Lasseigne et al., 2001; Ranney, 2004; Zhang and Liu, 1998).

Calycanthaceae are characterized by opposite leaves, numerous spirally arranged tepals and stamens, and

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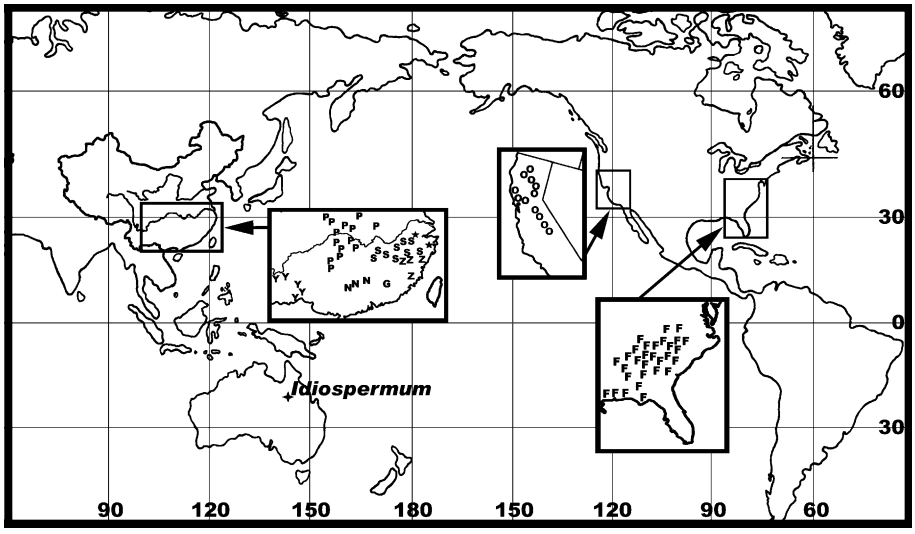

Fig. 1. Distribution of *Calycanthus*, *Chimonanthus*, and *Idiospermum* of Calycanthaceae. Star: *Calycanthus chinensis*; F, *C. floridus*; open circle: *C. occidentalis*; P, *Chimonanthus praecox*; N, *Ch. nitens*; Y, *Ch. campanulatus*; S, *Ch. salicifolius*; G, *Ch. grammatus*; Z, *Ch. zhejiangensis*; and +, *Idiospermum australiense*.

achenes that are enclosed in a concave receptacle that at maturity usually is capsule-like (Cheng and Chang, 1964; Nicely, 1965; Tsiang and Li, 1979; Zhang and Liu, 1998). Because of their deeply concave receptacles, Calycanthaceae have generally been placed in the order Laurales (Cronquist, 1981; Dahlgren, 1983; Hallier, 1905), and molecular data have shown that they are sister to the six other families of that order (Atherospermataceae, Gomortegaceae, Siparunaceae, Lauraceae, Hernandiaceae, and Monimiaceae; Renner, 1999). Four of these families have fossil records in Laurasia and Gondwana (reviewed in Renner, 2005).

One species of Calycanthaceae, *Idiospermum australiense* (Diels) S. T. Blake, has subfleshy fruits and the very rare trait (in angiosperms) of 2–6 cotyledons (Edwards et al., 2001). It is an evergreen tree endemic to the lowland tropical rain forests of Queenslands (Worboys and Jackes, 2005) . It was originally described as *Calycanthus australiensis* (Diels, 1912). After the rediscovery of living plants, Blake (1972) studied flowering and fruiting populations in the native habitat and concluded that *C. australiensis* was so distinct from other Calycanthaceae, especially in its more than two cotyledons and subfleshy fruits, that it deserved family rank (e.g., Watson and Dallwitz, 1992). Comparative studies of flower vessel supply (Wilson, 1976, 1979), leaf flavonoids (Sterner and Young, 1981), and node and wood anatomy (Carlquist, 1983) added further distinctions, while at the same time showing that *Idiospermum* is clearly calycanthaceous (Carlquist, 1983, p. 439). Molecular data support the monophyly of Calycanthaceae (Chase et al., 1993; Renner, 1999; Soltis et al., 2000).

The three species of *Calycanthus* are disjunctly distributed in eastern Asia, and eastern and western North America (above), and two of them (*C. chinensis* and *C. occidentalis*) are morphologically well delimited. *C. floridus*, which ranges from Pennsylvania to Tennessee and northern Florida, has been variously segregated into several varieties or even species (Boufford and Spongberg, 1981; Ferry and Ferry, 1987; Hardin, 1984). Most treatments, however, recognize only one species that exhibits considerable variation in leaf shape, pubescence, and floral color (Kubitzki, 1993; Nicely, 1965; Wen et al., 1996). *Calycanthus occidentalis* Hook. and Arn. from California, by contrast, is relatively uniform in morphology, and *C. chinensis* Cheng and S.Y. Chang, endemic in Zhejiang, is so distinct as to have been considered a separate genus, *Sinocalycanthus* Cheng and S.Y. Chang. This was mainly based on tepal shape (round vs. strap-like in the American species) and color (white with pale pink edges in the outer whorls, yellow in the inner whorls vs. all tepals maroon to purple or occasionally green in the American species; Cheng and Chang, 1964). Most authors, however, regard *Sinocalycanthus* as a member of *Calycanthus* (Kubitzki, 1993; Zhang and Liu, 1998), and this is supported by analyses of chloroplast DNA restriction site variation (Wen, 1999; Wen et al., 1996).

The species of the third genus of Calycanthaceae, the Chinese endemic *Chimonanthus*, are difficult to circumscribe. A monograph in 1965 (Nicely, 1965) accepted only three species, *Ch. nitens* Oliv., *Ch. praecox* L., and *Ch. salicifolius* S.Y. Hu., but Chinese workers have since described six additional species, *Ch. campanulatus* R.H. Zhang and C.S. Ding (Zhang and Ding, 1980) from southeastern Yunnan, *Ch. grammatus* M.C. Liu from Jiangxi and *Ch. zhejiangensis* M.C. Liu from Zhejiang and Fujian (Liu, 1984), *Ch. baokanensis* D.M. Chen and Z.L. Dai from Hubei (Chen and Dai, 1985), *Ch. anhuiensis* T.B. Chao and Z.S. Chen from Anhui (Chen et al., 1987), and *Ch. caespitosus* T.B. Chao, Z.S. Chen and Z.Q. Li from Anhui (Chao et al., 1989). Some of these names are probably synonyms, for example, *Ch. baokanensis* has been considered conspecific with *Ch. praecox*, and *Ch. anhuiensis* and *Ch. caespitosus* as

variants of *Ch. salicifolius* (Zhang and Liu, 1998), but other entities appear to be good species. In this study, we recognize six species in *Chimonanthus* (Table 1).

Relationships within Calycanthaceae have been investigated in a morphological cladistic study (Li and Li, 2000a) that found support for the relationship *Chimonanthus* (*Calycanthus–Sinocalycanthus*) and in chloroplast (cp) DNA restriction site study (Wen et al., 1996) that found that the Chinese species of *Calycanthus* is sister to the two North American species. In order to resolve species-level relationships in Calycanthaceae we generated nuclear sequence data from the internal transcribed spacer (ITS) region of ribosomal DNA and chloroplast data from the widely used *trn*L intron and *trn*L–*trn*F intergenic spacer, located in the large single-copy region of the cp genome. In addition, we sequenced the rarely used *trn*C–*trn*D region (but see Lee and Wen, 2004). The latter is also located in the large single-copy region of the cp genome and includes the *trn*C–*pet*N intergenic spacer, the short *pet*N gene, the *pet*N–*psb*M intergenic spacer, the likewise short *pet*M gene, and the *pet*M–*trn*D spacer (Demesure et al., 1995; Wakasugi et al., 2001; Lee and Wen, 2004).

To infer the absolute ages of the main clades of Calycanthaceae, we used fossils to constrain and calibrate the obtained genetic distance, using a semi-parametric relaxed clock approach (Sanderson, 2002) and a Bayesian relaxed clock (Thorne and Kishino, 2002). Fossil fruits of *Calycanthus* (*C. lusaticus* Mai) have been reported from the Middle and Upper Miocene of the Lausitz, Germany (Mai, 1987, 2002), and a well-preserved *Idiospermum*-like flower,

Table 1
Accessions of Calyanthaceae and their localities with voucher and GenBank accession numbers

Taxon	Locality	Voucher	GenBank #[a]
Calycanthus chinensis Cheng and S.Y. Chang	Lin'an, Zhejiang, China	*S. L. Zhou 2000615* (PE)	AY786082, AY786116, AY786119, AY786130
	Tiantai, Zhejiang, China	*S.L. Zhou 2001520* (PE)	AY786083
C. floridus L.	Florida, USA	*Z.-D. Chen 990048* (PE)	AY786084
	Florida, USA	*Wen 949* (A)	AY786086
	Marion, North Carolina, USA	*Wen 867* (A)	AY786085, AY786108, AY786120, AY786131
C. occidentalis Hook. and Arn.	Berkeley, California, USA	*Wen 50-1653* (F)	AY786089, AY786109, AY786121, AY786132
	Xishuangbanna Trop. Bot. Gard., Yunnan, China	*Y.-P. Hong 99224* (PE)	AY786088
Calycanthus hybrid	Zhejiang Forestry College, Zhejiang, China	*S.L. Zhou 2002518* (PE)	AY786087
Chimonanthus campanulatus R.H. Zhang and C.S. Ding	Kunming Bot. Gard., Yunnan, China	*S.-X. Yang s. n.* (PE)	AY786091
	Longlin, Guangxi	*Y.-P. Hong H236* (PE)	AY786090, AY786110, AY786122, AY786133
	US National Arboretum, Washington, DC, USA	*Wen 375* (F)	AY786103
Ch. grammatus M.C. Liu	Zhejiang Forestry College, Zhejiang, China	*S.L. Zhou 2002519* (PE)	AY786093, AY786111, AY786124, AY786134
	Lushan Bot. Gard., Jiangxi, China	*A.-M. Lu 2056* (PE)	AY786092
Ch. nitens Oliver	Guizhou Forestry School, Guizhou, China	*Y.-P. Hong H391* (PE)	AY786094, AY786112, AY786125, AY786135
Ch. praecox (L.) Link	Beijing Bot. Gard., Beijing, China	*S.L. Zhou 0019* (PE)	AY786095
Ch. p. 'Intermedius'	Beijing Bot. Gard., Beijing, China	*S.L. Zhou 0020* (PE)	AY786096, AY786113, AY786123, AY786136
Ch. p. 'Luteus'	Beijing Bot. Gard., Beijing, China	*S.L. Zhou 0021* (PE)	AY786097
Ch. p. 'Parviflorus'	Beijing Bot. Gard., Beijing, China	*S.L. Zhou 0022* (PE)	AY786098
Ch. salicifolius S.Y. Hu	Lushan Bot. Gard., Jiangxi, China	*A.-M. Lu 2055* (PE)	AY786099, AY786114, AY786127, AY786137
	Hangzhou Bot. Gard., Zhejiang, China	*S.L. Zhou 20010608* (PE)	AY786102
	Hangzhou Bot. Gard., Zhejiang, China	*S.L. Zhou 2000621A* (PE)	AY786100
	Hangzhou Bot. Gard., Zhejiang, China	*S.L. Zhou 2000621B* (PE)	AY786101
Ch. zhejiangensis M.C. Liu	Hangzhou Bot. Gard., Zhejiang, China	*S.L. Zhou 2000621C* (PE)	AY786105
	Hangzhou Bot. Gard., Zhejiang, China	*S.L. Zhou 2000621D* (PE)	AY786106, AY786115, AY786126, AY786138
	US National Arboretum, Washington, DC, USA	*Wen s.n.* (NA 54100, F)	AY786104
Idiospermum australiense (Diels) S.T. Blake	New York Bot. Gard., New York, USA	*Wen s. n.* (F)	AY786107, AY786117, AY786128, AY786139
Illigera celebica Miq.	Ninh Binh, Vietnam	*Wen 6122* (F)	AY786118, AY786129, AY786140

[a] ITS: AY786082–AY786107; *trn*C–D: AY786108–AY786118; intron of *trn*L: AY786119–AY786129; intergenic spacer of *trn*L–*trn*F: AY786130–AY786140.

Virginianthus calycanthoides, has been described from the Puddledock flora in Virginia, which dates to the Aptian/Albian (112–105 my ago; Friis et al., 1994; throughout this paper we use the geological time scales of Berggren et al., 1995 and Gradstein et al., 1995). Friis et al. (1994) considered *Virginianthus* as exhibiting a mosaic of characters today seen in different genera of Calycanthaceae and accordingly placed it on the family's stem lineage. Formal cladistic analysis of a character matrix that included *Virginianthus*, another calycanthaceous fossil flower, and representatives of all Laurales families plus a few outgroups suggests that *Virginianthus* may lie on the stem lineage of Laurales, rather than the stem lineage of Calycanthaceae (Crepet et al., 2005). Further calycanthaceous flowers come from Early-Late Cretaceous (~97 my) deposits of the Potomac Group (Crane et al., 1994) and from Turonian (~89 my) deposits in New Jersey (Crepet et al., 2005). An important recent discovery is *Araripia florifera* from the Aptian or possibly Albian Crato formation in Brazil (ca. 115 my ago). It comprises flowers, buds, and leaves, and exhibits features that suggest that "among the Calycanthaceae, especially flowers of *Calycanthus* are most similar to the flower of *Araripia* in having similar narrow tepals" (Mohr and Eklund, 2003, p. 289;). Importantly for our study, "*Araripia* also shows similarity to flowers of the monotypic *I. australiense* [..] *Idiospermum* differs, however, by having fewer and broader tepals" (Mohr and Eklund, 2003, p. 289). Together, these geographically disjunct finds suggest that the split between northern hemisphere *Calycanthus*/*Chimonanthus* and the southern hemisphere *Idiospermum* may already have occurred by 110 my ago.

By sampling all species of Calycanthaceae for nuclear and chloroplast loci, by considering all fossils available for the family, and by using two relaxed clock approaches, this study aims to provide insights into the divergence times and migration pathways of disjunct plants in the Northern Hemisphere. It also offers an opportunity to test the alternative hypotheses on northern migration routes, the Bering land bridge (Hopkins, 1967) and the North Atlantic land bridge (Tiffney, 1985).

2. Materials and methods

2.1. Taxon sampling

Twenty-seven accessions representing the 10 species accepted here (Table 1) and some variants of species of Calycanthaceae were sequenced for ITS, the *trn*L intron and adjacent *trn*L–F spacer, and the *trn*C–D region (Table 1). The genetic distances between Calycanthaceae and the remaining Laurales are large, and there is therefore no outgroup whose ITS can be unambiguously aligned with ingroup ITS (Renner, unpublished data). Chloroplast sequences of the *trn*L–F spacer could only be aligned between Calycanthaceae and *Illigera celebica* Miq. (Hernandiaceae), and this species was therefore used to root the *trn*L–F trees. To achieve unambiguous rooting, we relied on *rbc*L sequences from a set of outgroups that represent the major lineages of Laurales, with rooting coming from a member of Magnoliales, namely *Knema*, a Myristicaceae (Table 2). The *rbc*L data also formed the basis for the Bayesian divergence time estimation (below).

2.2. DNA extraction, amplification, and sequencing

DNAs of all samples were extracted from silica gel-dried leaves following a modified CTAB buffer method (Doyle and Doyle, 1987). Leaves were ground into fine powder with sand at room temperature and incubated with 2× CTAB buffer at 65 °C for 60 min. DNA was further purified with Wizard DNA Clean-Up System (Promega cat. #7280) following the manufacturer's protocol.

The *trn*L intron and *trn*L–*trn*F intergenic spacer were amplified using the primers of Taberlet et al. (1991). The entire *trn*C–*trn*D region was amplified in three segments with the following primer pairs trnC and petN1R, petN1 and psbM2R, and psbM1 and trnD (see Lee and Wen, 2004 for primer sequences). The ITS region was amplified with the primers of Wen and Zimmer (1996). Amplification reactions were performed in a 25-mL volume containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.2 mmol/L each of primer, 1 U of *Taq* polymerase, and about 25 ng DNA temperate. PCR was done on Peltier Thermal Cycler DNA engine DYAD starting at 94 °C for 2 min, followed by 35 cycles of 40 s at 94 °C, 40 s at 50 °C, 2 min at 72 °C, and ended with a final extension of 10 min at 72 °C. PCR products were gel-purified and sequencing of both strands was done on an ABI 3100 Genetic Analyzer using ABI BigDye v3.0. PCR profile of sequencing was 25 cycles of 30 s at 96 °C, 15 s at 50 °C, and 4 min at 60 °C. RAMP was set 1 °C/s.

2.3. Sequence alignment and phylogenetic analyses

DNA sequences were assembled using Sequencher ver. 3.1 (Gene Codes Corporation, 2001). Sequence alignment was initially performed using Clustal X ver. 1.81 (Thompson et al., 1997). The gap-opening penalty was 10 and the gap extension penalty 3. Sequence alignments were manually adjusted using BioEdit (Hall, 1999). Gaps were alternatively treated as missing data or as new characters.

Five data sets were analyzed to infer Calycanthaceae species relationships (the sixth data set consisted of *rbcL* sequences and was used only for divergence time estimation; below): (1) an ITS matrix of 26 OTUs (10 species), with *I. australiense* as the functional outgroup; (2) a *trn*C–D matrix with 11 OTUs (10 species), including *I. australiense* as the functional outgroup (Donoghue and Cantino, 1984); (3) a *trn*L intron and *trn*L–F spacer matrix of 27 taxa (11 species) including *I. celebica* as an outgroup; (4) the combined cp data sets with 11 taxa, including *I. celebica*, but using questions marks for its *trn*C–D sequence; and (5) the combined nuclear and cp data with 11 taxa, again with *I. celebica* as the outgroup. The ITS and *trn*C–D

Table 2
Laurales exemplars used in divergence time estimation, with GenBank accession numbers of their *rbc*L sequences used

Taxon	GenBank #	Source
Atherospermataceae/Laurales		
Atherosperma moschatum Labill.	GBAN-AF121362	Renner (1999)
Daphnandra repandula (F. Muell.) F. Muell.	GBAN-AF052195	Renner (1998)
Doryphora aromatica (F.M. Bailey) L.S. Smith	GBAN-L77211	Ablett et al. (1997)
Dryadodaphne sp. aff. *novoguineensis* (Perk.) A.C. Smith	GBAN-AF121363	Renner, 1999
Laurelia novae-zelandiae Cunn.	GBAN-AF052196	Renner (1998)
Laurelia sempervirens (R. and P.) Tul.	GBAN-AF052612	Renner (1998)
Laureliopsis philippiana (Looser) Schodde	GBAN-AF040662	Renner (1998)
Nemuaron vieillardii (Baill.) Baill.	GBAN-AF121366	Renner (1999)
Calycanthaceae/Laurales		
Calycanthus chinensis Cheng & S. Y. Chang	AY642862	This study
Calycanthus floridus L.	AY642861	This study
Calycanthus occidentalis Hook. and Arn.	AY642860	This study
Chimonanthus praecox (L.) Link	GBAN-L12639	Qiu et al. (1993)
Idiospermum australiense (Diels) S.T. Blake	GBAN-L12651	Qiu et al. (1993)
Gomortegaceae/Laurales		
Gomortega nitida R. & P.	GBAN-D89561	Ueda et al. (1997)
Magnoliaceae/Magnoliales		
Liriodendron chinense (Hemsl.) Sarg.	GBAN-L12654	Qiu et al. (1993)
Magnolia hypoleuca Siebold and Zucc.	GBAN-L12655	Qiu et al. (1993)
Myristicaceae/Magnoliales		
Knema latericia Elmer	GBAN-L12653	Qiu et al. (1993)
Siparunaceae/Laurales		
Glossocalyx longicuspis Benth.	GBAN-AF040666	Renner (1998)
Siparuna brasiliensis (Spreng.) A. DC.	GBAN-AF013246	Renner (1998)

matrices used *I. australiense* as a functional outgroup because sequences of these gene regions cannot be aligned between Calycanthaceae and other Laurales, such as *Illigera*.

Parsimony analyses relied on PAUP ver. 4.0b10 (Swofford, 2002), using heuristic searching, random taxon addition, tree bisection reconnection (TBR) branch swapping, and the Mulpars and Steepest descent options. Bootstrap analyses (Felsenstein, 1985) were performed using 500 replicates, with the random taxon addition sequence limited to 10, and branch swapping limited to 10,000,000 rearrangements per replicate. Congruency between nuclear and chloroplast datasets was assessed with the incongruence length difference (ILD) test (Farris et al., 1995). The ILD test has been criticized as a method for assessing character incongruence (e.g., Barker and Lutzoni, 2002; Yoder et al., 2001). However, Hipp et al. (2004) have recently shown that the ILD test can serve as a conservative initial test of data partition congruence.

Nucleotide substitution model parameters were determined for the ITS and cpDNA data sets using MODELTEST ver. 3.0 (Posada and Crandall, 1998). A heuristic maximum likelihood (ML) search with TBR branch swapping was then conducted. Branches were collapsed (creating polytomies) if the branch length was less than or equal to 1e−08, and the random taxon addition sequence was limited to 100.

Bayesian analyses (Mau et al., 1999; Rannala and Yang, 1996) were carried out using MrBayes ver. 3.0b3 (Huelsenbeck and Ronquist, 2001) with the same model parameters as the maximum likelihood searches. Bayesian analyses started from random trees and employed four Markov chain Monte Carlo (MCMC) runs, monitored over one million generations, re-sampling trees every 100 generations. Runs were repeated twice to confirm results. The resulting log likelihood and number of generations were plotted to determine the point after which the log likelihoods had stabilized. After discarding the trees saved prior to this point as burn-in, the remaining trees were imported into PAUP and a 50% majority-rule consensus tree was produced to obtain posterior probabilities of the clades.

2.4. Divergence time estimation

The ML topology obtained from the combined nuclear and chloroplast data was also used for estimating divergence times within Calycanthaceae. Rate constancy in the combined chloroplast and nuclear data set was tested using likelihood ratio testing (Felsenstein, 1988). Where the molecular clock was rejected, we used two relaxed-clock approaches to molecular dating, Sanderson's (2002, 2003) penalized likelihood method and the Bayesian approach of Thorne and Kishino (2002). We used PL on the combined nuclear and chloroplast data, and the Bayesian approach on *rbc*L data that did not contain any insertions/deletions (indels) or missing entries, which we felt would negatively affect MCMC runs in the Bayesian approach.

Penalized likelihood was performed with r8s ver. 1.60 (Sanderson, 2003). Because of missing data in the outgroup *Illigera*, we excluded *Illigera* from ML analyses and instead rooted trees with *Idiospermum*. Another stretch of missing data involved the start of the *trn*C–D sequence of *Idiospermum*, which lacked 870 bp. To explore the effect of these missing data on divergence time estimation, we conducted two PL analyses, one with all 3655 bp included, another that excluded the data block that contained the 870 bp missing basepairs and thus comprised only 2785 bp. We used cross-validation (implemented in r8s) to obtain the optimum smoothing parameter, and employed the recommended truncated Newton optimization, collapsing zero length branches.

Calibrations for the PL analysis were as follows: (1) crown Calycanthaceae were constrained to minimally 72 my old, as estimated from the broader Laurales analysis (below); and (2) crown *Calycanthus* was set to minimally 16 my old, based on a fossil fruit (Mai, 1987, 2002). The strata from which these fruits come date to the Middle and Upper Miocene.

To estimate the standard errors associated with divergence times estimated with the PL method, we used a parametric bootstrapping strategy similar to that in Davis et al. (2002): (1) 100 data sets were simulated on the maximum likelihood tree with the computer program Seq-Gen ver. 1.2.7 (Rambaut and Grassly, 1997); (2) the 100 simulated data sets were imported into PAUP and used to generate new maximum likelihood trees; and (3) divergence times were estimated on each of the new trees (using r8s), and the resulting node ages used to calculate the variance on the estimates obtained from the original tree.

To obtain age estimates that could rely on additional fossil calibrations (besides the Miocene *Calycanthus* fruit used above), we employed the *rbc*L gene, which has been sequenced for numerous species of Laurales (we used the ones listed in Table 2) and which is unambiguously alignable. As an alternative approach to PL, we used Bayesian divergence time estimation, which also allows relaxing the clock assumption (Thorne et al., 1998; Thorne and Kishino, 2002; analyses relied on the program package available from J. Thorne's webpage: http://statgen.ncsu.edu/thorne/multidivtime.html). We used PAML's BaseML program (ver. 3.14; Yang, 1997) and the F84 + G model with five rate categories to estimate nucleotide substitutions in the *rbcL* data matrix. The F84 + G model is the only model so far implemented in Estbranches, the component of Thorne's program that estimates branch lengths as well as the variance-covariance structure of the branch length estimates. The F84 + G model accommodates variable base frequencies, transition/transversion bias, and rate heterogeneity among sites. The topology used as input for BaseML and all subsequent steps was the same as obtained for these Laurales from six cp markers (Renner, 1999; Renner, 2005), except that intra-Calycanthaceae relationships were constrained such that *C. floridus* was sister to *C. occidentalis,* based on the ML topology obtained from the combined nuclear and chloroplast data. The output from Estbranches became the input for Multidivtime, which estimates node divergence times, given user-specified minimal or maximal constraints. Multidivtime uses an MCMC approach to approximate prior and posterior probabilities. The following data-dependent settings were used in the Multidivtime control file: length and sampling frequency of the Markov chain were set to 100,000 trees, sampled every 10th generation, with a burn-in of 1000 trees. The *a priori* expected number of time units between tip and root was set to 1.5 because the time unit was set to 100 my. The standard deviation of the prior for the time between tips and root is recommended to equal the number of time units between tips and root and therefore was set to 1.5. The a priori rate at the root node was set to 0.0003, based on Thorne's recommendation that it be calculated by dividing the median distance between the ingroup root and the ingroup tips obtained from Estbranches by the time unit. The prior for the Brownian motion parameter nu, which determines the permitted rate change between ancestral and descendant nodes, was set to 0.4, following the manual's recommendation that the time units between root and tips times nu be about 1. The standard deviation on 'nu' was also set to 0.4.

Calibrations for the Bayesian time estimation were as follows (not all were used in any one run): (1) the root of Laurales was constrained to maximally 140 my old, based on the onset of angiosperm radiation (Brenner, 1996; Hughes, 1994). (2a) The stem lineage of Calycanthaceae was constrained to minimally 112 my old, based on the fossil *Virginianthus* (Friis et al., 1994). (2b) Alternatively, crown Calycanthaceae were constrained to minimally 112 my old (even if *Virginianthus* does not represent Calycanthaceae, there are 97 and 89 my old fossil flowers similar to modern *Calycanthus* and *Chimonanthus* flowers [Crane et al., 1994; Crepet et al., 2005]). (3) The split between Atherospermataceae and Gomortegaceae was constrained to minimally 88–86 my old based on the earliest pollen of Atherospermataceae (Mohr, 1998). (4) The split between neotropical *Siparuna* and African *Glossocalyx* was constrained to minimally 90–88 my old, based on molecular-clock estimates for Laurales using a larger sample of family representatives (Renner, 2005). There are, however, no fossils of *Siparuna* or *Glossocalyx*. (5) The divergence of *Laureliopsis* was constrained to minimally 83 my old, based on the oldest *Laureliopsis* wood (Poole and Francis, 1999). (6) Crown *Calycanthus* was constrained to minimally 16 my old, based on Miocene fossil fruits (Mai, 1987, 2002).

Dispersal-vicariance analysis (DIVA ver. 1.1; Ronquist, 1996, 1997) was used to reconstruct ancestral distributions on the ML topology obtained from the combined cp and nuclear data, primarily to infer the Asian intra-continental history of *Chimonanthus* and *Calycanthus*. Seven areas of endemism were defined for the intra-continental analysis, based on species distributions: Eastern North America (A; *C. floridus*), western North America (B; *C. occidentalis*), eastern China (C; *C. chinensis*; *Ch. grammatus*; *Ch. salicifolius*, and *Ch. zhejiangensis*), southwestern China (D; *Ch.*

campanulatus), central China (E; *Ch. praecox*), southcentral China (F; *Ch. nitens*), and Queensland of Australia (G; *Idiospermum*). Areas of endemism within Asia were primarily based on the geographic boundaries defined in Li (1944) and Wu and Wu (1990).

3. Results

3.1. Nuclear and chloroplast data

Chi-square tests of homogeneity of base frequencies across taxa were run in PAUP for (1) the 26-taxon ITS data, excluding missing or ambiguous sites and using just the nonambiguous sites ($=14.2103$, $df=75$, $P=1$) or using just the 52 informative sites ($\chi^2=48.1405$, $df=75$, $P=0.9933$), and (2) the 10-taxon concatenated nuclear and cpDNA data, using just the 2785 nonambiguous sites ($\chi^2=3.0624$, $df=27$, $P=1$) or just the 54 informative sites ($\chi^2=14.6509$, $df=27$ $P=0.9740$). None of the tests revealed nucleotide bias among taxa.

The lengths of the ITS1/5.8S/ITS2 region ranged from 629 to 638 bp in *Calycanthus* and *Chimonanthus*. It was only 552 bp in *Idiospermum*. Eleven indels were required to align the sequence of *I. australiense* with those of the remaining Calycanthaceae. Eight were 1–2 bp long and three were much longer, namely an 18-bp and a 24 bp deletion in ITS 1, and a 54-bp gap in ITS 2. The ITS matrix of 26 Calycanthaceae contained 763 aligned positions, 98 (12.8%) of which were variable, with 67 (8.8%) parsimony-informative. Aligning required 27 gaps. With gaps treated as additional characters, parsimony analysis yielded 80 most parsimonious trees, with a consistency index (CI) of 0.88 (0.78 excluding uninformative characters), a retention index (RI) of 0.93, and a rescaled consistency index (RC) of 0.82. The strict consensus of the 80 trees (Fig. 2) shows *C. occidentalis* as sister to *C. chinensis*. With gaps treated as missing data, parsimony analysis produced 2527 trees (CI = 0.93, RI = 0.95, RC = 0.88), the consensus of which resembled the tree found with gaps treated as additional characters, except for less resolution within *Chimonanthus* (data not shown).

The *trn*L intron, including the primer sites, was 362–520 bp long, with a deletion of 203 bp uniquely shared by *C. floridus* and *C. occidentalis*. The *trn*L–F spacer was 368–375 bp long and contained no large indels. Together, the aligned intron and spacer were 1026 bp long, 190 (18.5%) of which were variable, and 25 (2.4%) parsimony-informative. Treating gaps as new characters, parsimony analysis yielded 42 trees (CI = 0.97, RI = 0.93, RC = 0.91, trees not shown). *Calycanthus* and *Chimonanthus* were each monophyletic and different from the nuclear data, *Calycanthus floridus* and *C. occidentalis* placed as sister species. Resolution within *Chimonanthus* was poor, and the *trn*L–F spacer showed no variation within either *Calycanthus* or *Chimonanthus*. A putative hybrid (Zhou 2002518) had a *trn*L–F sequence identical to that of *C. floridus*, while its ITS sequence was identical to that of *C. occidentalis*.

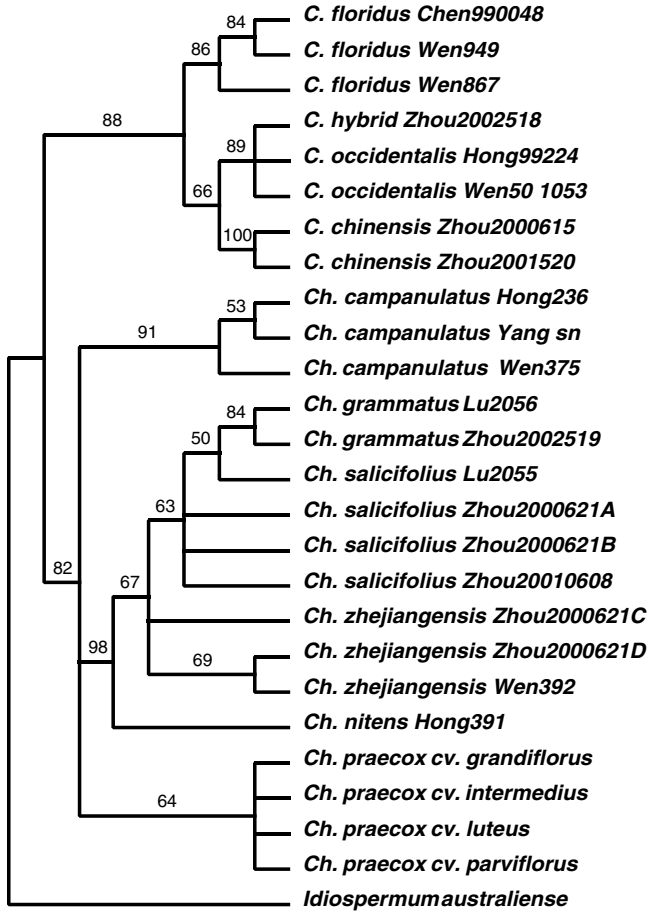

Fig. 2. Strict consensus tree of Calycanthaceae based on ITS sequences, with *Idiospermum australiense* as the functional outgroup and gaps treated as additional characters. Numbers above branches indicate bootstrap support.

The *trn*C–D intergenic region was completely sequenced for nine of the 10 species; for *Idiospermum*, we obtained only a partial *trn*C–D sequence (it lacked 870 bp; see Section 2). *I. celebica* was sequenced for *trn*C–D, but excluded from analysis because of alignment ambiguities (Section 2). Trees were instead rooted with *Idiospermum*. With gaps treated as missing data, parsimony analysis of the 1932 aligned positions of the *trn*C–D data produced 14 trees (CI = 0.96, RI = 0.89, RC = 0.85), which differed only in the relative position of *Chimonanthus grammatus*, *Ch. nitens*, and *Ch. salicifolius*. Both *Calycanthus* and *Chimonanthus* were monophyletic, and as in the *trn*L + *trn*L–F data, *C. floridus* and *C. occidentalis* formed a sister pair. Within *Chimonanthus*, clades of accessions of *Ch. campanulatus* and *Ch. praecox* were well supported. When gaps were treated as additional characters, parsimony analysis yielded two trees (CI = 0.90, RI = 0.83, RC = 0.75). The gap characters helped define two clades within *Chimonanthus*: (1) a *Ch. nitens–Ch. zhejiangensis* clade and (2) a *C. grammatus–Ch. nitens–Ch. salicifolius–Ch. zhejiangensis* clade.

When all chloroplast data were combined (and gaps treated as additional characters), parsimony analysis of the 2919 aligned positions resulted in two trees (CI = 0.95,

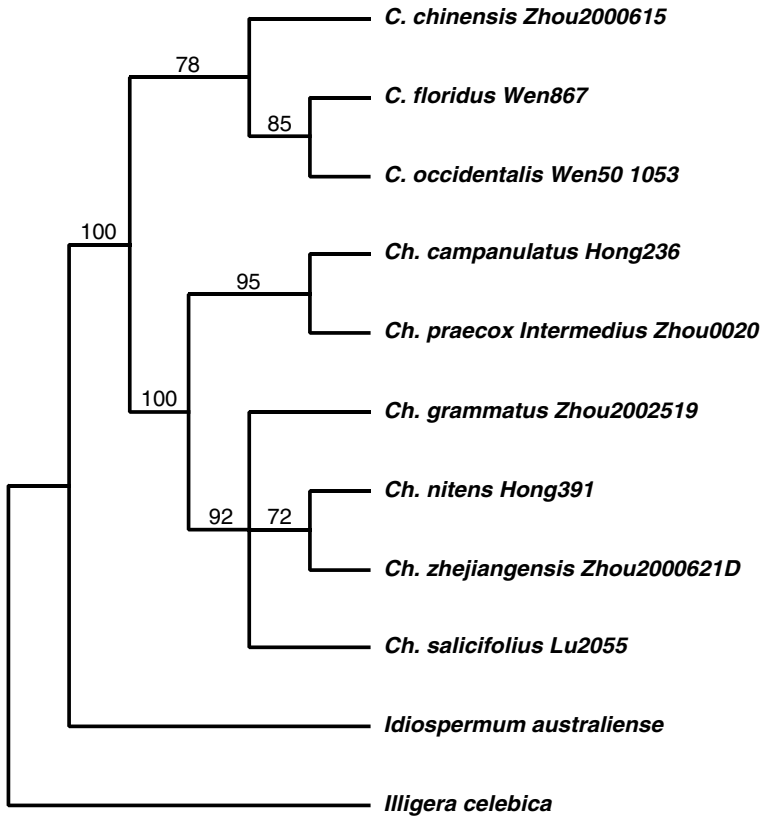

Fig. 3. Strict consensus tree of Calycanthaceae based on combined chloroplast *trn*L–F and *trn*C–D sequences with gaps treated as new characters. Numbers above branches indicate bootstrap support.

RI = 0.82, RC = 0.78); Fig. 3 shows their consensus. The two topologies differed only in the relative position of *Ch. salicifolius*, either being sister to *Ch. grammatus* or forming a trichotomy with *Ch. grammatus* and the *Ch. nitens–Ch. zhejiangensis* clade. With gaps treated as missing data, parsimony analysis produced 13 trees (CI = 0.98, RI = 0.91, RC = 0.90), which differed in the relative positions of *Chimonanthus nitens*, *Ch. salicifolius*, and *Ch. zhejiangensis*. Their strict consensus of the 13 trees was identical to the topology shown in Fig. 3.

3.2. Congruence of nuclear and chloroplast data

Assessments of conflict between the nuclear and the chloroplast data showed that they were congruent ($P = 0.09$ when gaps were treated as characters; and $P = 0.57$ when gaps were treated as missing data). Of the 3,748 aligned positions in the combined nuclear + cp data, 464 were variable, and 95 were parsimony-informative. Treating gaps as characters, parsimony analysis resulted in two trees (CI = 0.92, RI = 0.78, RC = 0.716). Topologies differ only in the position of *Ch. nitens* either as sister to *Ch. zhejiangensis* or as sister to a clade of *Ch. zhejiangensis*, *Ch. grammatus*, and *Ch. salicifolius*. Treating gaps as missing data, a single tree (Fig. 4) was obtained (CI = 0.96, RI = 0.86, RC = 0.82), which was identical to one of the two trees found when gaps were treated as additional characters.

The GTR+I+G model was the best fit for the combined data, using the Akaike Information Criterion. Base frequencies were A = 0.30, C = 0.21, G = 0.22, T = 0.27, the Ti/Tv ratio was 1.62, the proportion of invariable sites was 0.77, and the gamma shape parameter was 0.64. ML analysis using these parameters yielded a single tree with the topology of one of the two most parsimonious trees found when gaps were treated as characters, namely the one that showed *Ch. nitens* and *Ch. zhejiangensis* as sisters. The ML tree (Fig. 5) differed from the parsimony tree (Fig. 4) in that *C. floridus* and *C. occidentalis* formed a monophyletic group, sister to *C. chinensis*.

Bayesian analyses with the same model and parameters showed that a burn-in period of 5000 generations was sufficient for stabilization of the likelihood scores. The majority rule consensus of the trees kept after discarding the burn-in, resulted in a topology congruent with the strict consensus of the two most parsimonious trees found when gaps were treated as characters (Fig. 4 shows the respective posterior probabilities).

3.3. Estimates of divergence times

Cross validation under PL found a smoothing parameter of 100 to be optimal for the matrix of 2785 bp of the combined ITS and cpDNA data excluding the block of 870 characters where *I. australiense* lacked the start of its *trn*C–

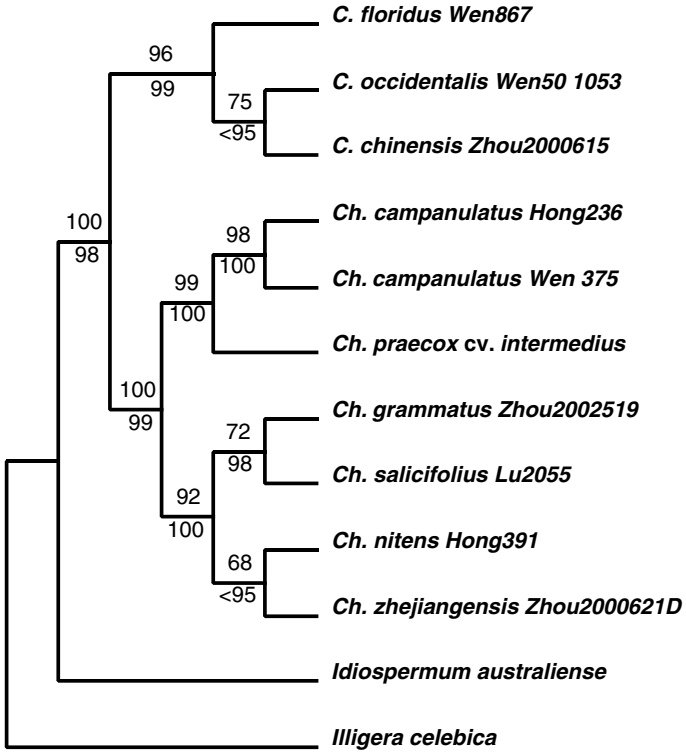

Fig. 4. Strict consensus tree of Calycanthaceae from the combined ITS, *trn*L–F, and *trn*C–D sequences (gaps are treated as missing data). Numbers above branches indicate bootstrap support, numbers below branches Bayesian posterior probabilities.

D sequence. Use of the full ITS and cpDNA matrix of 3655 bp, yielded the same optimal smoothing parameter. With crown Calycanthaceae constrained to minimally 72 my old, based on results of the Laurales *rbc*L analysis, and crown *Calycanthus* set to minimally 16 my old, penalized likelihood yielded an age of 17.5 my for the *Calycanthus*/*Chimonanthus* divergence, 12.5 my for the *C. floridus*/*C. occidentalis* divergence, and 5.5 my for the age of *Chimonanthus*. Table 3 shows the corresponding results when the reduced matrix was used.

Bayesian divergence time estimation from the *rbc*L matrix, which included Atherospermataceae, Gomortegaceae, Myristicaceae, and Siparunaceae, with multiple simultaneous constraints from several outgroups and the ingroup (Calycanthaceae) (Section 2, *Divergence Time Estimation*), yielded an age of 72 my (with a 95% credibility interval of 31–121 my) for the age of crown Calycanthaceae (Fig. 6), an age of 37 my (credibility interval: 10–79 my) my for the *Calycanthus*/*Chimonanthus* divergence, and 13 my for the *C. floridus*/*C. occidentalis* divergence (credibility interval: 8–40 my). The age of *Chimonanthus* could not be estimated because only one species of *Chimonanthus* was sequenced for *rbcL*.

3.4. DIVA analyses

When left unconstrained, DIVA often yields fairly uninterpretable results, with multiple ancestral areas for several nodes (Davis et al., 2002; Donoghue et al., 2001). This was also the case here, with widespread ancestral areas being inferred at nearly all nodes. Thus, with the maximum number of areas at nodes constrained to seven, the following nodes had alternative solutions: ancestor of terminals *Ch. grammatus–Ch. zhejiangensis* (C or CG), ancestor of terminals *Ch. campanulatus–Ch. zhejiangensis* (CDE, DEG, or CDEG), ancestor of terminals *C. floridus–Ch. zhejiangensis* (ABCDE or ABCDEG), and the ancestor of terminals *C. floridus–Idiospermum* (ABCDEF or ABCDEFG) (area codes, see Section 2). Results from an analysis in which the number of ancestral areas was constrained to two are shown in Fig. 7.

4. Discussion

4.1. Idiospermum, a Gondwanan relict

Several molecular analyses have shown that *I. australiense* is sister to *Calycanthus* plus *Chimonanthus* (Chase et al., 1993; Renner, 1999; Soltis et al., 2000). Judging from genetic distances, the divergence between *Idiospermum* and the other two genera is ancient. For example, ITS uncorrected pairwise sequence divergence between *Idiospermum* and the other two genera is 19.1–20.3%, while that between *Calycanthus* and *Chimonanthus* is only 3.1–4.2% (this study). We know of no other flowering plant lineage that exhibits a range disjunction between tropical Australia and temperate/subtropical Asia plus North America (Camp, 1947; Good, 1974; Thorne, 1972). There are a few aquatic

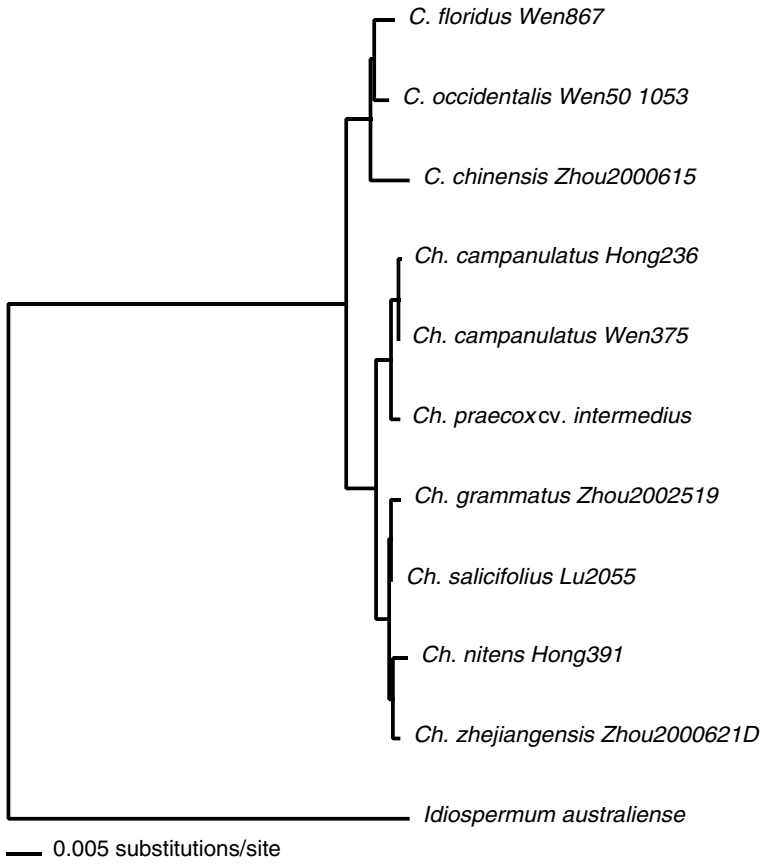

Fig. 5. Maximum likelihood tree of Calycanthaceae from the combined ITS, *trn*L–F, and *trn*C–D sequences.

Table 3
Comparison of age estimates with or without a large 870-bp missing data block in the ML analysis

Clade	Age estimates with missing data excluded (my)	Age estimates with missing data included (my)
Idiospermum	72	72
Calycanthus/*Chimonanthus*	16.9 ± 0.48	17.4 ± 0.77
Calycanthus	16.0	16.0
Chimonanthus	5.5 ± 1.15	6.0 ± 1.33
C. floridus/*C. occidentalis*	12.5 ± 2.29	6.8 ± 1.88
Ch. campanulatus/*Ch. praecox*	2.0 ± 0.77	1.8 ± 0.53
Ch. grammatus–Ch. salicifolius–Ch. nitens–Ch. zhejiangensis	2.3 ± 0.45	2.1 ± 0.47
Ch. gramatus–Ch. salicifolius	1.0 ± 0.35	1.1 ± 0.42
Ch. nitens–Ch. zhejiangensis	1.7 ± 0.48	1.5 ± 0.39

plants with Australian/eastern Asian disjunction, such as the pair *Torrenticola* and *Cladopus* in the Podostemaceae, estimated as 28 my old, and *Myriophyllum ussuriense* and *M. variifolium*, estimated as 2.1 my old (Les et al., 2003). However, these pairs do not involve additional Beringian links as found in Calycanthaceae. Different from the young ages estimated for the disjunct ranges of these aquatics, we obtained a Campanian (Upper Cretaceous) divergence time for the split between the tropical Australian *I. australiense* and the temperate/subtropical Asian/North American *Calycanthus–Chimonanthus* (using a relaxed clock approach and multiple simultaneous fossil calibrations). This great age fits with the discovery of *Calycanthus*- and *Idiospermum*-like flowers and leaves from the Aptian/Albian of southeastern Brazil (Mohr and Eklund, 2003) and the presence of *Calycanthus*/*Chimonanthus*-like flowers in the Turonian of North America (Crane et al., 1994; Crepet et al., 2005). Based on these fossils and their extant range, Calycanthaceae in the Cretaceous appear to have been part of the southern Gondwana province (southern South America, South Africa, Australia, Antarctica, New Caledonia, and New Zealand). The southeastern part of this province, comprising the biota of South America, Antarctica, and Australia, did not fragment until the Early Eocene, and stepping-stone dispersal was possible well into the Eocene (52 my), when a shallow marine seaway formed between Australia and Antarctica (Lawver et al., 1992; Veevers et al., 1991). Increasingly cooler climates by the Middle Eocene (46 my) then began to limit overland dispersal even for temperate-adapted plants. Because the South American Calycanthaceae are extinct, we are today left with the Australian branch of Calycanthaceae and the American/Chinese branch. Based on our molecular clock estimates, these two branches appear to have diverged at least since Campanian times.

We thus favor a scenario in which *Idiospermum* is a Cretaceous leftover from a once continuous southern

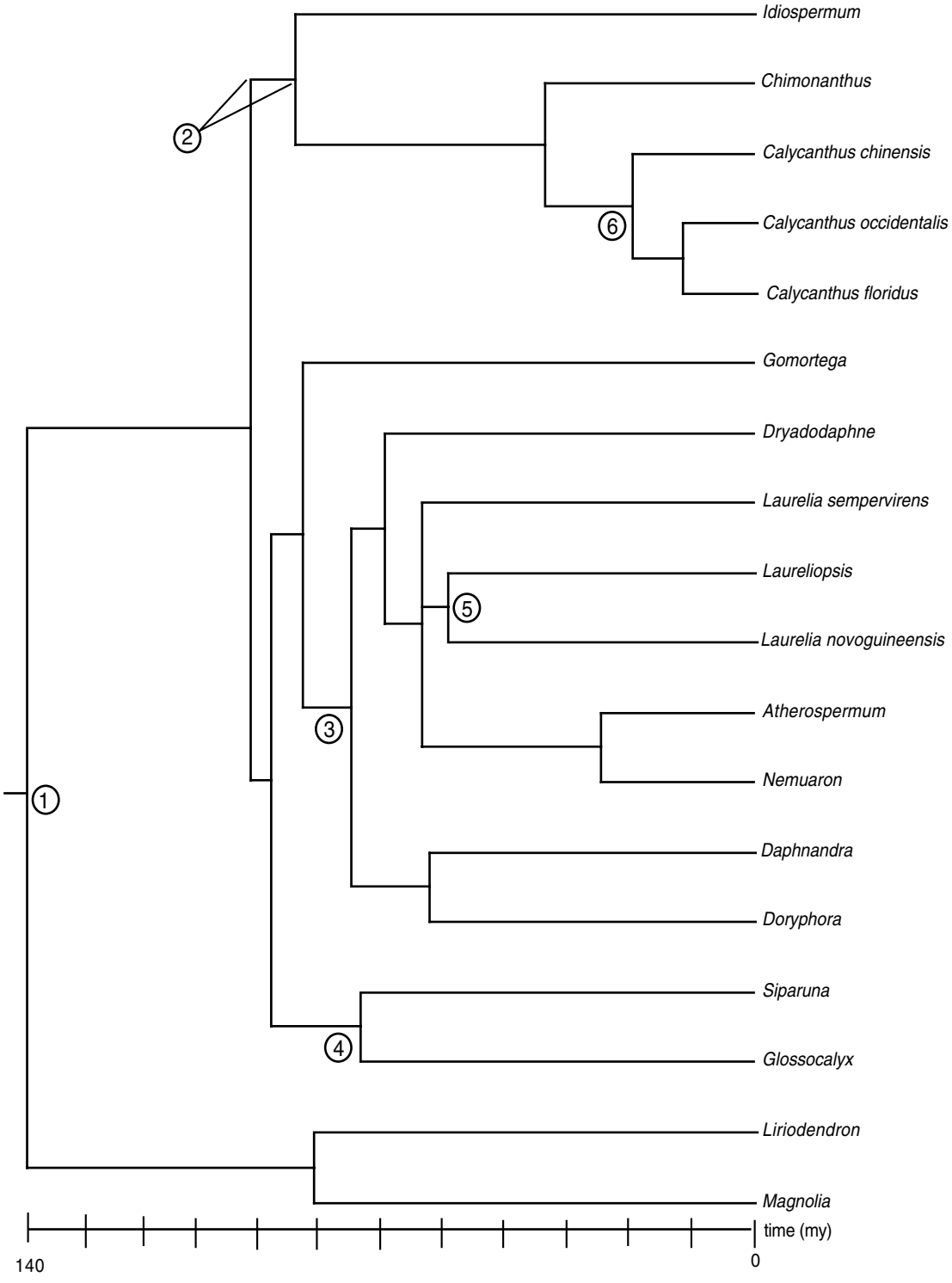

Fig. 6. Bayesian chronogram for Calycanthaceae from an *rbc*L matrix that included Magnoliaceae, Siparunaceae, Atherospermataceae, and Gomortegaceae, with the numbered nodes constrained as stated in Section 2 Not all constraints shown were used in all analyses.

hemisphere/Gondwana range of the stem group of Calycanthaceae. Whether the North American *Virginianthus* and other calycanthaceous Cretaceous flowers (Crane et al., 1994; Crepet et al., 2005; Friis et al., 1994) represent the ancestors of today's North American *Calycanthus* species or whether the family died out in North America and the ancestor of *C. floridus* and *C. occidentalis* came from China cannot be decided from our data. Should today's North American Calycanthaceae represent the endpoints of a lineage that survived in situ, then *Chimonanthus* and *Calycanthus chinensis* would have to derive from two West to East crossings of Beringia at quite different times.

Arrival of the ancestor of *Idiospermum* from Laurasia to Cretaceous Australia seems unlikely in view of the absence

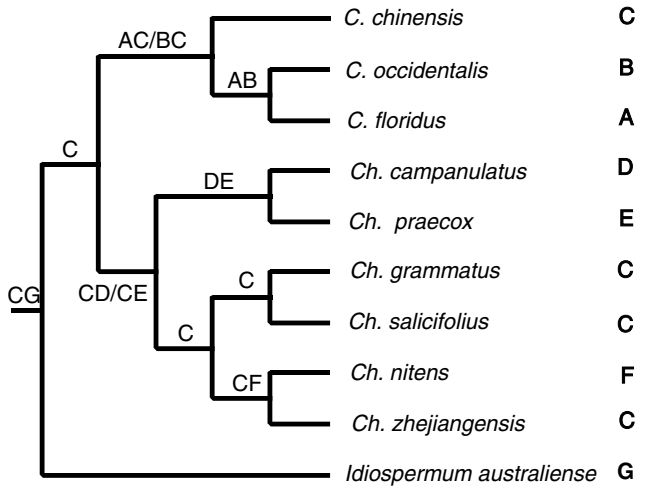

Fig. 7. Results of dispersal-vicariance analyses of Calycanthaceae. Letters refer to areas as follows: A, eastern North America; B, western North America; C, eastern China; D, southwestern China; E, central China; F, south-central China; and G, Queensland, Australia.

of facilitating sea currents as well as the dispersal biology of *Idiospermum*. Although the large diaspores of *Idiospermum* do not provide a strong argument against long-distance dispersal because they could have evolved recently as an adaptation for penetrating the leaf litter in forest understories (e.g., Grubb, 1996; Grubb, 1998), they certainly do not make it especially likely. A single seed of *I. australiense* measures 4.5–5 cm in length and 5.5–6.5 cm in diameter, and weighs 70–235 g (mean about 130 g; Edwards et al., 2001). At maturity, the single-seeded fruits fall to the ground below the trees, where their wall and testa decay, and the embryo germinates (Edwards et al., 2001; Endress, 1983). There may be some secondary dispersal by scatter-hoarding large rodents and musky rat kangaroos, but the fruits are too large to be consumed by even the largest of Australia's frugivorous pigeons, and there is no evidence of Cassowaries or other large ground-dwelling birds consuming them (S. Worboys, Natural Resource Assessment, Cairns, Queensland, Australia, personal communication, 2004). Dispersal of the juicy receptacles of *Calycanthus* and *Chimonanthus* (including the fruitlets/achenes inside) is by frugivorous birds (van der Pijl, 1982), but it is unclear whether the fruitlets germinate after gut passage.

4.2. Calycanthus, a Beringian disjunct lineage

Relationships among the three species of *Calycanthus* were difficult to resolve. Chloroplast *trn*C–D and *trn*L and *trn*L–F data indicate that *C. chinensis* is sister to the *C. floridus–C. occidentalis* clade, while nuclear ITS data indicate a sister group relationship between *C. chinensis* and western North American *C. occidentalis*, as does *rbc*L and the combined ITS and cpDNA data (Figs. 4 and 5). However, the two North American species share a deletion of 155 bp in the *trn*L intron, which constitutes a potential synapomorphy. The incongruence between chloroplast and nuclear ribosomal data may be due to the persistence of ancient ITS paralogues (Buckler et al., 1997) or introgression of such paralogues via hybridization (Baldwin et al., 1995). Additional nuclear sequence data, preferably single-copy genes, may resolve the relationships among the three species of *Calycanthus*. Morphologically the North American *C. floridus* and *C. occidentalis* share maroon, straplike tepals (Section 1) as well as food bodies (protein-rich small tissue outgrowths for grazing beetle pollinators) at the tips of staminodes and tepals (Rickson, 1979; Zhang and Liu, 1998). We know of no uniquely shared morphological characters between *C. chinensis* and *C. occidentalis*, but *C. chinensis* and *C. floridus* share the unusual condition of having lateral buds that are partially embedded below the petiole base (J. Wen, personal observation).

The fossil record of *Calycanthus* dates back to the Miocene (Mai, 1987, 2002), which fits with our molecular clock estimate of the divergence between *C. floridus* and *C. occidentalis* as minimally 6.72 ± 1.15 my old. An earlier study (Xiang et al., 1998) obtained an age of only 3.1 my for the Chinese/North American divergence in *Calycanthus* (and a correspondingly younger age for the divergence between *C. floridus* and *C occidentalis*), using divergence in cpDNA restriction sites (Wen et al., 1996) and an assumed divergence rate of 0.1% per million years (Parks and Wendel, 1990). Even with the large error ranges typical of molecular clock-based estimates, it is clear, however, that the *C. chinensis* lineage is at least of Tertiary age as also argued on the basis of morphology (Cheng and Chang, 1964; Li, 1986, 1988, 1989, 1990; Li and Li, 1999, 2000a,b).

The North Atlantic land bridge (NALB) was an important migration route in the early Eocene for plants and animals (Tiffney and Manchester, 2001). By the Middle to Upper Miocene, the NALB was broken, and our age estimate of 6–7 my for the initial divergence between the North American species of *Calycanthus* therefore argues for migration across Beringia rather than the NALB. The precise affinity of the fossil *Calycanthus lusaticus* from East Germany to any extant species is not clear (Mai, 1987), and it is probably best explained as reflecting migration of the genus *Calycanthus* from eastern Asia into Europe after the disappearance of the Turgai Strait in the Oligocene (Tiffney, 1985; Wen, 1999).

4.3. Chimonanthus, recent diversification within China

Overall, the genus *Chimonanthus* ranges fairly widely (Table 1, Fig. 1), but except for *Ch. praecox*, its species are restricted to unglaciated areas in eastern to southwestern China, and most species have narrow, allopatric ranges. The combined nuclear and chloroplast data (Figs. 4 and 5) show two major clades in the genus, one comprising *Ch. praecox* and *Ch. campanulatus*, the other the remaining species, *Ch. grammatus*, *Ch. nitens*, *Ch. salicifolius*, and *Ch. zhejiangensis*. Relationships among the latter four species are poorly resolved (see also Figs. 2 and 3). The molecular

topology contradicts the close relationship among *Ch. campanulatus*, *Ch. nitens*, *Ch. salicifolius* inferred by Zhang and Ding (1980) based on these species' evergreen or semi-evergreen habit. Instead, the deciduous, *Ch. praecox* from mountainous areas in central to southwestern China and the semi-evergreen *Ch. campanulatus* from southwestern China (Yunnan and Guizhou) are sister groups. The poorly resolved four-species clade comprises the semi-evergreen *Ch. salicifolius* and three evergreen species, namely *Ch. grammatus*, *Ch. salicifolius* and *Ch. zhejiangensis* in the Anhui, Fujian, Jiangsu, Jiangxi, and Zhejiang provinces of eastern China.

The molecular clock estimates suggest that species within *Chimonanthus* diverged in the Pliocene, as recently as 1–2 my ago (Table 3), although the genus itself appears to date back to the Miocene or Oligocene with the estimates of the *Calycanthus*/*Chimonanthus* divergence as 37 my with the Bayesian method, and 16.9–17.4 my with the PL method. The young age of *Chimonanthus* species may explain some of the difficult species delimitation, especially among the evergreen species, with small differences in leaf color, shape, size, pubescence, and texture, as well as petal shape and size, and ornamentation on the fruits all serving to distinguish entities at different hierarchical levels (Zhang and Liu, 1998). A good example is *Ch. zhejiangensis*, described as a close relative of *Ch. salicifolius* (Liu, 1984), but differing in the color of its lower leaf surface (green in *Ch. zhejiangensis*, glaucous in *Ch. salicifolius*), and the evergreen vs. semi-evergreen habit. The ITS sequence profiles of these two entities show intra-specific sequence variation, with three of the four samples (except "Lu2056") of *Ch. salicifolius* exhibiting heterozygosity at one or more nucleotide sites. The sample "Zhou2000621C" of typical *Ch. zhejiangensis* showed polymorphisms at the same nucleotide sites.

With four areas of endemism recognized within China, the DIVA analysis suggested three vicariance and two dispersal events within *Chimonanthus* (Fig. 7). Initial vicariance appears to have occurred between eastern China (C) and southwestern China (D) or central China (E). Two additional separation events (Fig. 7) then could have occurred between eastern China (C) and southcentral China (F) (split of *Ch. nitens* and *Ch. zhejiangensis*), and between southwestern China (D) and central China (E) (split of *Ch. campanulatus* and *Ch. praecox*). Eastern China is the most frequent ancestral area for clades within *Chimonanthus*. These results support the role of eastern China (area C) in the evolution and diversification of *Chimonanthus*, fitting with the high level of species diversity and apparent genetic polymorphism, although additional genetic data are required for a fuller assessment. Nevertheless, it is clear from the topology, branch lengths, and molecular clock-based estimates that Calycanthaceae among their 10 living species comprise a striking mix of Cretaceous and Tertiary relicts as well as very young products of population divergence.

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