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Phylogenetic and biogeographic complexity of Magnoliaceae in the Northern Hemisphere inferred from three nuclear data sets

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ABSTRACT

This study employs three nuclear genes (*PHYA*, *LFY*, and *GAI1*) to reconstruct the phylogenetic and biogeographic history of Magnoliaceae. A total of 104 samples representing 86 taxa from all sections and most subsections were sequenced. Twelve major groups are well supported to be monophyletic within Magnoliaceae and these groups are largely consistent with the recent taxonomic revision at the sectional and subsectional levels. However, relationships at deeper nodes of the subfamily Magnolioideae remain not well resolved. A relaxed clock relying on uncorrelated rates suggests that the complicated divergent evolution of Magnolioideae began around the early Eocene (54.57 mya), concordant with paleoclimatic and fossil evidence. Intercontinental disjunctions of Magnoliaceae in the Northern Hemisphere appear to have originated during at least two geologic periods. Some occurred after the middle Miocene, represented by two well-recognized temperate lineages disjunct between eastern Asia and eastern North America. The others may have occurred no later than the Oligocene, with ancient separations between or within tropical and temperate lineages.

1. Introduction

The intercontinental disjunctions of plants in the Northern Hemisphere are considered to be the most complex biogeographic pattern observed at the global scale (Wen, 1999, 2001; Milne and Abbott, 2002; Donoghue and Smith, 2004; Milne, 2006). Such disjunctions are generally thought to be remnants of a more continuously distributed, mixed mesophytic forest during the Tertiary, known as the "boreotropical flora" (Wolfe, 1975). It subsequently became fragmented due to geologic and climatic changes. Some of the ancient relict mesic forests that once covered much of the temperate regions of the Northern Hemisphere can be found today in the southeast region of USA as well as in eastern to central China and central to southern Japan.

Although the "boreotropical flora" hypothesis has been well accepted, questions still remain (Donoghue and Smith, 2004). These concern their overall phylogenetic relationships, morphological correlations (convergence/stasis) of temperate pairs in different areas, and morphological divergence between temperate and tropical relatives (Wen, 1999; Donoghue and Smith, 2004; Ickert-Bond et al., 2007). The disjunct taxa with relatives in the tropics are biogeographically much more complicated (Wen, 1999). To better understand the complexity requires the evaluation of phylogenetic relationships and divergence times in a broader phylogenetic framework, including closely related elements spanning from the temperate regions to the subtropical and tropical zones.

One potentially important study group showing such a disjunct pattern is the family Magnoliaceae, which is distributed from the north temperate to the tropical regions. Based on the fossil record (Tao and Zhang, 1992; Frumin and Friis, 1996, 1999), this family has been considered to be one of the earliest extant lineages of flowering plants (93.5–110 mya) and has played a crucial role toward our understanding of the origin and diversification of angiosperms. Both morphological and molecular evidence have supported this scenario (Takhtajan, 1969; Cronquist, 1981; Mathews and Donoghue, 1999; Parkinson et al., 1999; Qiu et al., 1999; Soltis et al., 1999; Graham and Olmstead, 2000).

Magnoliaceae is comprised of ca. 220–240 species characterized by an androecium of numerous spirally arranged stamens, a

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gynoecium with many simple carpels spirally arranged on an elongated axis, and separate tepals (Law, 1984, 1996, 2004; Liu et al., 1995; Figlar and Nooteboom, 2004). Roughly two-thirds of the species are currently distributed in temperate and tropical regions of eastern to southeastern Asia (Fig. 1). The other third occur in the New World from the temperate eastern North America through tropical South America as far as Brazil and Bolivia (Dandy, 1927, 1971, 1978b; Law, 1984; Nooteboom, 1993, 1998; Thorne, 1993; Frodin and Govaerts, 1996). The distribution of Magnoliaceae in the north temperate as well as tropical regions in Asia and the Americas (Fig. 1), makes it an excellent model for understanding the evolution of intercontinental temperate disjunctions and their interactions with the tropical members.

Magnoliaceae are usually divided into two subfamilies: Magnolioideae and Liriodendroideae (Law, 1984; Nooteboom, 1985). Except for the very distinct Liriodendroideae (including only *Liriodendron* with two species), taxonomists have long debated over the classification of Magnolioideae due to a paucity of phylogenetically useful characters (Dandy, 1927, 1978a,b; Law, 1984, 1996; Qiu et al., 1995a,b; Jobes, 1998; Nooteboom, 1985, 1998; Chen and Nooteboom, 1993; Azuma et al., 1999, 2001; Kim et al., 2001; Sun and Zhou, 2004; Xu and Rudall, 2006). Most recently, a new classification for Magnolioideae was proposed by Figlar and Nooteboom (2004) based on the chloroplast phylogenetic results and morphological re-examinations (also see Figlar, 2006). This classification recognized only one genus *Magnolia* in the Magnolioideae subfamily.

Molecular phylogenetic studies on Magnoliaceae have been conducted using chloroplast data (e.g., Qiu et al., 1995a,b; Azuma et al., 1999, 2001, 2004; Shi et al., 2000; Kim et al., 2001; Wang et al., 2006). One of the first important findings from molecular evidence was the polyphyly of *Magnolia* section *Rhytidospermum*, with the North American *Magnolia tripetala* closely related to the Asian counterparts, rather than to *M. macrophylla*, or *M. fraseri* from the same continent (Qiu et al., 1995a,b). The studies also indicated that *Manglietia* and *Michelia* were nested within *Magnolia*. Kim et al. (2001) used *ndhF* sequences of 99 taxa representing all of the traditional Magnoliaceae lineages and produced a most parsimonious tree containing 11 clades. However, many clades were weakly supported and the relationships among these clades remained unclear. Other molecular studies produced similar results (e.g., Azuma et al., 2000, 2001, 2004; Ueda et al., 2000).

The limited divergence of chloroplast sequences and the relative morphological homogeneity in Magnoliaceae have made it difficult to resolve the phylogenetic relationships within the family (Qiu et al., 1995a; Azuma et al., 2001; Kim et al., 2001; Li and Conran, 2003). Nuclear data have not been employed for phylogenetic studies in Magnoliaceae so far. Since nuclear markers have been generally shown to provide stronger phylogenetic signals than many plastid sequences (Wolfe et al., 1987; Small et al., 2004), we herein use three nuclear genes to resolve the phylogenetic relationships of Magnoliaceae. Phytochrome evolution in land plants has been shown to result from a series of gene duplications (e.g., *PHYA*, *PHYB*, *PHYC*, and *PHYE*) that have led to independent and functionally distinct lines (Mathews et al., 1995; Mathews and Sharrock 1996; Manabe and Nakazawa, 1997; Mathews et al., 2003). Nucleotide variation at phytochrome loci has been useful in various phylogenetic studies of basal angiosperms and of several other angiosperm families (Kolukisaoglu et al., 1995; Mathews and Donoghue, 1999). The low-copy nuclear LEAFY (*LFY*) gene has been used for phylogenetic analyses of plants with its introns showing a relatively high nucleotide substitution rate (Hoot and Taylor, 2001; Archambault and Bruneau, 2004). The *Arabidopsis* GA INSENSITIVE (*GAI*) locus and related genes of the DELLA subfamily encode growth regulators and have been implicated in quantitative variation for developmental traits (Peng et al., 1999; Silverstone et al., 1998; Thornsberry et al., 2001; Boss and Thomas, 2002). The *GAI*-like gene sequence (*GAI1*) derived from a grapevine dwarf mutant was examined in a phylogenetic context within the Vitaceae and has been shown to be a potentially important marker (Wen et al., 2007).

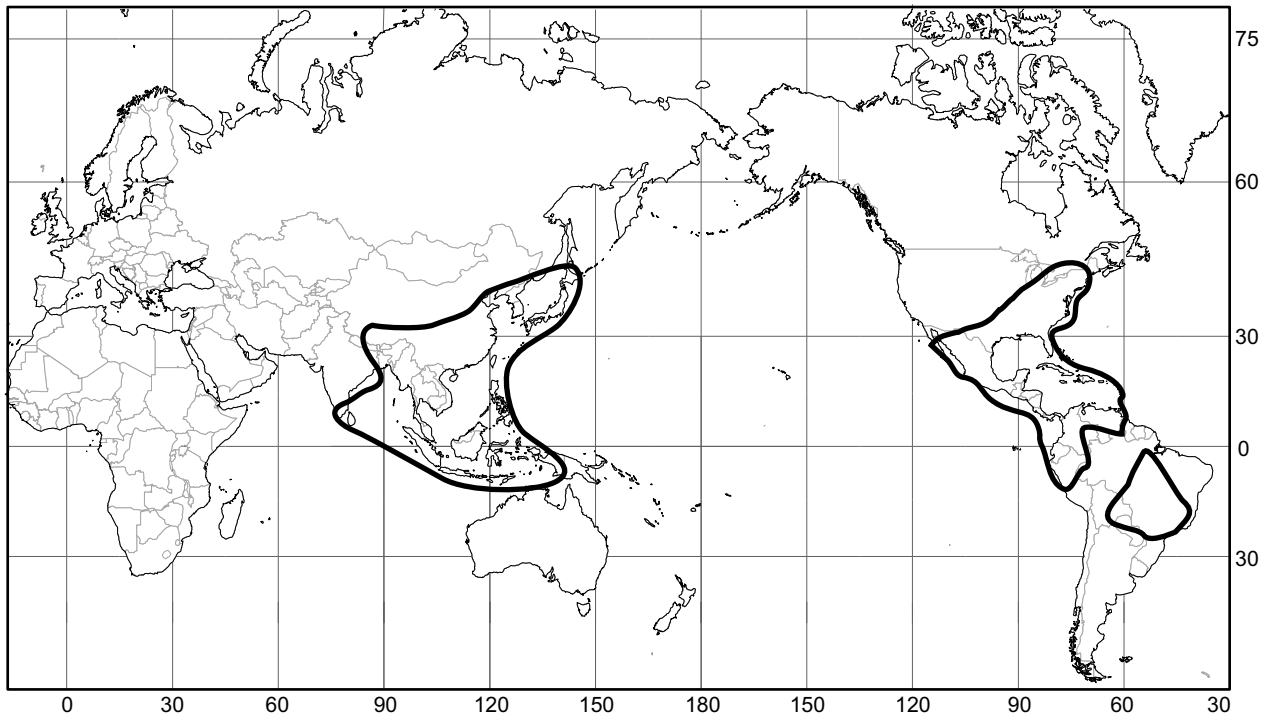

Fig. 1. Distribution map of Magnoliaceae shown with solid lines modified from Azuma et al. (2001).

Biogeographic studies have been conducted on some disjunct lineages in Magnoliaceae (Parks et al., 1983, 1994; Parks and Wendel, 1990; Qiu and Parks, 1994; Qiu et al., 1995a,b; Azuma et al., 2001). *Liriodendron* was estimated to have diverged in the mid-Miocene based on allozyme and restriction fragment length polymorphism (RFLP) analyses of cpDNA and paleobotanical evidence (Parks and Wendel, 1990). Subsequently, the divergence time of the genus was specifically estimated to be 27.9 ± 4.4 mya based on plastid *trnK*, *psbA_trnH*, and *atpB_rbcL* sequences using strict molecular clocks calibrated with fossil evidence (Azuma et al., 2001). Other lineages in the family suggested additional disjunctions. *Magnolia* sect. *Rhytidospermum*, the North American *M. tripetala* was shown to be sister to the Asian counterparts and their divergence was estimated to be during the late Miocene to early Pliocene using the allozyme and RFLP data of cpDNA (Qiu et al., 1995a,b), and 20.9 ± 3.3 to 27.9 ± 4.4 mya using the cpDNA sequences by Azuma et al. (2001). Divergence times in Magnoliaceae previously were all dated based on strict molecular clocks and discrepancies were found between different data sets. To update our biogeographic understanding of the family, we performed age estimation using "relaxed clock" analyses and multiple fossil calibrations (Renner, 2005).

This study employed sequences of three nuclear markers to infer the phylogenetic relationships in Magnoliaceae, with comparison of previous chloroplast results. Divergence times for intercontinental disjunct clades were estimated using the nuclear sequences under a relaxed molecular clock. Geologic data were placed in the phylogenetic contexts to gain insights into the biogeographic origin and interactions of temperate and tropical elements disjunct between eastern Asia and eastern North America. The phylogenetic framework and the temporal scale that we present provide a foundation to examine the complexity and relationships of biogeographic diversification of angiosperms between temperate and tropical zones in the Northern Hemisphere.

2. Materials and methods

2.1. Plant sampling and sequencing

A total of 104 samples representing 86 species and subspecies, and varieties were sequenced in this study (Appendix 1). This sampling scheme covers all sections and nearly all subsections (excepting subsections *Dugandiodendron*, *Splendentes*, and *Maingola*) of the most recent classification system of Magnoliaceae by Figlar and Nooteboom (2004). We followed Frodin and Govaerts (1996) for generic circumscription and scientific names, and Figlar and Nooteboom (2004) for the classification system. Previous classifications of the family by Dandy (1978b), Law (1984), and Nooteboom (1985) were also considered in our discussion.

Total DNAs were extracted from 15 mg of silica-gel-dried leaf material using Dneasy (QIAGEN) extraction kits or a modified CTAB extraction method (Doyle and Doyle, 1987). All primers used in the study are shown in Table 1. The primers for *PHYA* (*PHYA-F*, *PHYA-R*) were modified from those used in Mathews and Donoghue (1999) based on the sequences of *Magnolia* x *soulangeana* (AF190094) and *Degeneria vitiensis* I.W. Bailey and A.C. Sm. (AF190078) in GenBank. For some samples which were difficult to amplify with the first set of primers, a second pair of primers (*PHYA*-bF and *PHYA*-bR) was designed using sequences from the successfully amplified samples. *LFY* primers (*LFY*_F, aR, and bR) were designed based on a putative *LFY* sequence of *Liriodendron tulipifera* L. including both coding and intron regions (DQ223431, Liang et al., 2007). The primers for *GAI1* were designed in a two-step process. A few preliminary sequences were first obtained with the primers (*GAI1*_1F and 2R) as used in Wen et al. (2007). More Magnoliaceae specific primers (*GAI1*_MF and MR) then were designed.

Table 1
Primers of *PHYA*, *LFY*, and *GAI1* used for amplification and sequencing in this study

Primer name	Primer sequences (5′–3′)
PHYA-F	CCTTACGAAGTACCCATGACTG
PHYA-R	TRGCRTCCATYTCATAATCCTT
PHYA-bF	CACGGTTGCAAGCTTTGTCC
PHYA-bR	TTGAATGACGATCGCGGGTGC
GAI1-MF	ATGGCCGAGGTCGCTCACAA
GAI1-MR	ATACTCGCCTGCTTGAACGCG
LFY_F	AGGTGACTAACCAGGTGTTC
LFY_aR	GTTGTATGAATGAATGAACGG
LFY_bR	CAACCTRGTCTCTATGCACAA

All PCRs were performed in 25 μl reaction-mixture volumes using reagents and manufacturer's instructions for *Taq* polymerase (JumpStart RED Accutaq DNA Polymerase, Sigma, St. Louis, MO). The amplified products were purified via polyethylene glycol (PEG) precipitation using standard protocols. Cycle sequencing was conducted using BigDye 3.1 reagents and an ABI 3100 automated sequencer (Applied Biosystems, Foster City, California, USA). The sequences produced were then aligned with the program ClustalX version 1.83 (Thompson et al., 1997), followed by manual adjustments in Bioedit (Hall, 1999).

2.2. Phylogenetic reconstructions

Phylogenetic trees were constructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods. MP searches were performed with tree bisection-reconnection branch swapping, MulTrees on, and simple taxon addition in PAUP* version 4.0b10 (Swofford, 2003). Parsimony bootstrap (PB) support for each clade was estimated as above from 1000 heuristic search replicates, with 100 random taxon addition replicates saving all optimal trees at each step. ML was implemented in GARLI ver. 0.951 (Zwickl, 2006; distributed by D. Zwickl at http://www.zo.utexas.edu/faculty/antisense/Garli.html) starting from random trees and using 10,000,000 generations per search. ML bootstrap (MB) values were estimated from 100 bootstrap replicates in GARLI.

The optimal model of molecular evolution was determined by the Akaike Information Criterion (AIC) using the Modeltest ver. 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). In each case the optimal model was the General Time Reversible model, with rate heterogeneity modeled by assuming that some proportion of sites are invariable and that the rate of evolution at other sites may be modeled using a discrete approximation to a gamma distribution [GTR + I + Γ]. Bayesian inferences were implemented in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) with the model estimated above. We used one cold and three heated chains, with random initial trees. Trees were generated for 2,000,000 generations, with sampling every 100 generations. Following a burn-in period of the first 2000 generations, 19,800 trees were sampled from the posterior distribution to calculate the posterior probabilities (PP).

The data were analyzed separately for each nuclear gene and for the concatenated data set. Congruence among the different data sets was first tested using the incongruence length difference (ILD) test in PAUP* using 1000 data bipartitions and analyzing a maximum of 10,000 trees for each (Farris et al., 1994). Taxa missing entire markers were excluded from the matrix for the incongruence testing. The Templeton (Templeton, 1983) and the Shimodaira–Hasegawa tests (S–H) (Shimodaira and Hasegawa, 1999) also were employed to compare topological incongruence among the three nuclear data sets as well as their combined data set. The Templeton test was carried out in PAUP* under non-para-

metric pairwise conditions. The S–H test was also run in PAUP* using the best-fit model and the ML tree obtained by GARLI with RELL optimization and 1000 bootstrap replicates.

2.3. Molecular dating

We used the concatenated nuclear data set for dating the divergence times. A likelihood ratio test (Felsenstein, 1988) ruled out a global molecular clock ($P < 0.05$) for our data. Time estimates were done based on a relaxed molecular clock and fossil data. Penalized likelihood (PL) and Bayesian dating approaches based on a relaxed-clock model were used in the time estimates (Sanderson, 2002; Drummond et al., 2006).

An ML tree with lengths inferred from GARLI was used in the PL estimate using the program r8s 1.70 (Sanderson, 2003) with steps followed from Nie et al. (2005). The Bayesian coalescent approach to estimate the times of each clade in Magnoliaceae and their credibility intervals was implemented in BEAST version 1.4.7 (Drummond and Rambaut, 2007), which employs a Bayesian Markov chain Monte Carlo (MCMC) to co-estimate topology, substitution rates and node ages. The different nuclear genes were partitioned, and model parameters were unlinked across partitions. All analyses were performed using the GTR model of nucleotide substitution with a gamma distribution with four rate categories. Two tree prior models (constant size and exponential growth) were implemented in each analysis, with rate variation across branches assumed to be uncorrelated and lognormally distributed (Drummond et al., 2006). The final estimates were obtained using the model that yielded the highest posterior probability. Posterior distributions of parameters were approximated using two independent MCMC analyses of 20,000,000 generations with 10% burn-in. Samples from the two chains which yielded similar results were combined and convergence of the chains was checked using the program Tracer 1.3 (Rambaut and Drummond, 2004).

2.4. Fossil calibration

The fossil record for Magnoliaceae includes some extinct genera that occurred in the Cretaceous (Friis et al., 1997), but extant genera are not confirmed by reproductive structures prior to the Tertiary (Manchester, 1999). The earliest and most reliable fossil assigned to Magnoliaceae is a sample of *Liriodendroidea* seeds from the Cenomanian–Turonian (ca. 93.5 mya) sediments of the Sarbay locality in northwestern Kazakhstan, which closely resemble seeds of extant *Liriodendron*, but differ in being much smaller and winged (Frumin and Friis, 1996, 1999). We thus set the age of the crown Magnoliaceae (or stem *Liriodendron*) to be 93.5 mya.

A second calibration scheme for the crown Magnoliaceae was employed. Early fossils related to Magnoliaceae include *Archimagnolia rostrato-stylosa* from the lower Cretaceous (Aptian–Albian, ca. 110 mya) in the Dalazi Formation of northeastern China (Tao and Zhang, 1992), and *Archaeanthus* (ca. 99.6 mya) from the Early Cenomanian Dakota Formation (Dilcher and Crane, 1984). The former is an impression of a floral axis which is cylindrical or conical in shape, with 20 carpels helically arranged on an elongated receptacle. The latter is a large, multipartite floral structure with many carpels borne in a spiral arrangement along an extended receptacle (Friis et al., 2006). The upper bound age for the family overall thus was set to be 110 mya. The crown age of the family was also estimated to be 70–79 mya by Wikström et al. (2001) using of the Fagales–Cucurbitales split based on Fagales fossils at 84 mya. Therefore, we used 70 mya as the lower bound to constrain the family's crown age.

Robust fossil calibration within Magnoliaceae improved the precision of age estimates (Pérez-Losada et al., 2004; Roger and Hug, 2006). Chloroplast *ndhF* sequences (1528 bp) of *Magnolia latahensis* from the Clarkia fossil beds of Idaho, USA (Miocene; 17–20 mya) were identical to those of the extant *M. grandiflora*, *M. schiediana*, *M. guatemalensis*, and *M. tamaulipana* (Kim et al., 2004). The morphology of *M. latahensis* is also very similar to that of the extant *M. grandiflora*. Thus, a normally distributed calibration prior with the mean 18.5 mya and standard deviation 1.0 (roughly match 17–20 mya) was constrained for this group in our analyses.

3. Results

3.1. Phylogenetic analyses based on nuclear data sets

The *PHYA* matrix comprised 1070 aligned positions without indels. The putative *LFY* sequence was 887 bp in aligned length including both a coding region (1–330) with a 3-bp indel and an intron ranging from 331 to 887. The alignment of the *GAI1* sequences generated a data matrix of 1300 positions with a 9-bp indel. The strict consensus trees for each nuclear sequence significantly supported the separation of the two subfamilies (Liriodendroideae and Magnolioideae), but the phylogenetic relationships within Magnolioideae were unclear (Appendices 2–4), consisting of a large polytomy with low bootstrap values among the major subclades in the strict consensus trees of each individual data set (see Appendices 2–4).

Incongruence was detected among different nuclear sequences (ILD, $P < 0.05$). The Templeton and the S–H tests (Table 2) also suggested significant incongruence among them. Because of the stochastic manner in which lineages sort during speciation, it is common that gene trees differ in topology from each other (Maddison, 1997; Degnan and Rosenberg, 2006). Whether conflicting data sets should be analyzed separately or combined in a simultaneous analysis is a complex and controversial decision (Cunningham, 1997; Hipp et al., 2004). For the present case, our concatenated data showed significant improvement for the phylogenetic resolution of Magnolioideae (Table 2). About twelve clades were recognized with higher PB values than those from any single data set (Table 3), although the relationships among deeper nodes still remained unresolved (Fig. 2). Furthermore, few strongly supported (>90%) incongruent clades were found upon comparison of parsimony bootstrap consensus trees generated from the three data sets. We therefore chose to combine our three data sets with

Table 2
The Templeton's, and Shimodaira–Hasegawa (S–H) tests of Magnoliaceae nuclear sequences

	Templeton test		S–H tests	
	Diff length	*P* value	Diff-lnL	*P* value
PHYA	(224)	(best)	(2980.96)	(best)
LFY	95	<0.0001	394.7905	<0.0001
GAI1	71	<0.0001	353.6144	<0.0001
ALL	21	0.0004	98.80495	<0.0001
LFY	(501)	(best)	(4436.58)	(best)
PHYA	114	<0.0001	490.4632	<0.0001
GAI1	113	<0.0001	406.8553	<0.0001
ALL	40	<0.0001	142.5061	<0.0001
GAI1	(452)	(best)	(4781.048)	(best)
PHYA	62	<0.0001	451.648	<0.0001
LFY	98	<0.0001	375.3006	<0.0001
ALL	32	<0.0001	136.046	<0.0001
ALL	(1270)	(best)	(12994.74)	(best)
PHYA	92	<0.0001	542.4362	<0.0001
LFY	100	<0.0001	359.5817	<0.0001
GAI1	92	<0.0001	392.9759	<0.0001

Data were divided into *PHYA*, *LFY*, *GAI1*, or all were combined. The data matrices in the first line were used as the constraints.

Table 3
Bootstrap values of each clade inform maximum parsimony analyses of each of the three nuclear sequences and of the combined data (C: clade collapsed; –: single sample)

Clade	Taxa	*PHYA*	*LFY*	*GAI1*	Combined
A1	*Talauma*	–	94	98	100
A2	*Magnolia*	66	C	C	93
B1	*Gwillimia*	C	78	81	98
B2	*Gynopodium*	97	C	53	99
C1	*Oyama*	78	50	60	91
C2	*Rhytidosperm*	63	C	92	99
C3	*Manglietia*	C	C	98	89
C1–C3		50	C	56	98
D1	*Michelia*	59	67	88	99
D2	*Yulania*	C	C	71	87
D3	*Macrophylla*	94	95	85	100
D4	*Auriculata*	95	99	100	100

their weakly-incongruent trees, as also suggested by Wiens (1998), Sheahan and Chase (2000), and Reeves et al. (2001).

The combined data set was 3257 bp in length, with 301 positions parsimony-informative. Using the variable positions, more than one million MPTs were generated with a length of 1414 steps, a consistency index (CI) of 0.75 (CI excluding uninformative characters = 0.63), a retention index (RI) of 0.90, and a rescaled consistency index (RC) of 0.67. The phylogenetic analysis on the concatenated data set strongly supported the monophyly of each subfamily (PB = 100%, PP = 1.00, and MB = 91% for Liriodendroideae; PB = 99%, PP = 1.00, and MB = 83% for Magnolioideae, Figs. 2 and 3). In Liriodendroideae, the two species of *Liriodendron* were well separated, corresponding to their disjunct geographical distribution in eastern Asia and eastern North America. The phylogenetic relationships in Magnolioideae were not as well-defined, although several well supported clades emerged from the combined nuclear data (Figs. 2 and 3), consistent with sections and/or subsections in the recent classification system of Figlar and Nooteboom (2004).

In Fig. 2, clade A1 included only species from subsection *Talauma* of section *Talauma*, with the two other subsections, *Dugandiodendron* and *Splendentes*, not being sampled in our study. Subsection *Talauma* was monophyletic (PB = 100%, PP = 1.00, and MB = 100%). Clade A2 is referred to as section *Magnolia* in Figlar and Nooteboom (2004), and combined the two previously recognized sections of *Magnolia* (*M. virginiana*) and *Theorhodon* (Nooteboom, 1985). This group was robustly supported by the combined nuclear data (PB = 93%, PP = 1.00, and MB = 92%). Clade B1, representing section *Gwillimia* in Figlar and Nooteboom (2004), was strongly supported (PB = 98%, PP = 1.00, and MB = 94%). Clade B2 (subgenus *Gynopodium* in Figlar and Nooteboom, 2004) included the traditionally recognized *Parakmeria* and *Manglietiastrum* (Nooteboom, 1985), and was well supported by the nuclear data (PB = 99%, PP = 1.00, and MB = 100%). Taxa of clades B1 and B2 were from tropical-subtropical Asia, while those in clades A1 and A2 were nearly restricted to regions of the American tropics. Other groups, such as section *Kmeria* in Figlar and Nooteboom (2004), were represented by only one sample whose phylogenetic position was uncertain. A similar situation was found for the traditional *Aromadendron* group (Nooteboom, 1985), which was not well resolved in our phylogenetic analyses.

Temperate to subtropical species in both eastern Asia and North America occurred in clades C1–C3 and D1–D4 (Figs. 2 and 3). Clade C1 represented taxa of subsection *Oyama* in Figlar and Nooteboom (2004), sister to a clade including the section *Manglietia* (C3) and subsection *Rhytidospermum* (C2) with PB = 98%, PP = 1.00, and MB = 95% support. Clade D1 includes subsections of *Michelia* and *Elmerrillia*, was well supported with PB = 99%, PP = 1.00, and MB = 90%. Clade D2, sampled from subsection *Yulania* in Figlar and Nooteboom (2004), was monophyletic with support values of PB = 87%, PP = 1.00, and MB = 82%. Clades D3–D4 represented two small groups from sections *Auriculata* and *Macrophylla* in Figlar and Nooteboom (2004), which were members of the previous section *Rhytidospermum* in Nooteboom (1985). They were monophyletic with PB = 100%, PP = 1.00, and MB = 100%, respectively; however, support for their sister relationship was low (PB = 57% and MB = 65%).

3.2. Phylogenetic comparison between nuclear and chloroplast sequences

We selected representative taxa from each clade well supported by the nuclear data set in order to explore phylogenetic patterns between nuclear sequences generated in this study and the previously published chloroplast data. Thirty-seven taxa were sampled for three nuclear (*PHYA*, *LFY*, and GAI1) and three chloroplast (*ndhF*, *trnK*, and *trnL-F*) markers. The nuclear data set was 3257 bp in length, 375 of which were parsimony-informative. The strict consensus of the four MPTs produced is shown in Fig. 4 with CI = 0.77 (CI excluding uninformative characters = 0.66), RI = 0.80, and RC = 0.59. The chloroplast data set was 5651 bp in length, with 217 parsimony-informative sites. This strict consensus tree was generated from six MPTs, with CI = 0.88 (CI excluding uninformative characters = 0.79), RI = 0.87, and RC = 0.77 (Fig. 4).

Incongruence was detected between the nuclear and chloroplast data (ILD, $P < 0.05$). After comparing their strict consensus trees, we found that the conflicts involved six taxa (*Magnolia acuminata*, *M. sieboldii* subsp. *sinensis*, *M. elegans*, *Manglietia sinica*, *M. nitida* var. *lotungensis*, and *Kmeria septentrionalis*). With the exclusion of these six taxa, a non-significant $P = 0.35$ was then recovered in the ILD test. The nuclear and chloroplast data sets for the remaining 31 taxa were thus combined for a phylogenetic analysis to evaluate relationships among the deep clades of the family. The combined nuclear and chloroplast data set was 8908 bp in length, 563 of which were parsimony-informative. Two MPTs were generated with CI = 0.83 (CI excluding uninformative characters = 0.75), RI = 0.83, and RC = 0.70.

Noticeable topological differences between the nuclear and chloroplast trees were detected for six taxa (Fig. 4). *Magnolia sieboldii* subsp. *sinensis* (subsection *Oyama*) was sister to subsection *Rhytidospermum* in the chloroplast data with moderate support (PB = 78%). In the nuclear data, it was robustly supported to be close to a clade including both subsection *Rhytidospermum* and section *Manglietia* (PB = 95%). *Magnolia acuminata* (subsection *Tulipastrum*) was sister to the subsection *Yulania* with high support in the chloroplast data (PB = 92%). However, the species was nested within a clade including both subsection *Yulania* and section *Michelia* in the nuclear data set with low support (PB = 54%). *Magnolia elegans* (formerly known as *Aromadendron elegans*) was sister to the *Michelia* group with high support in the chloroplast data (PB = 100%), whereas it was quite isolated in the nuclear data (Fig. 4). The chloroplast data strongly suggested that *Kmeria* and subgenus *Gynopodium* (*Manglietia sinica* and *M. nitida* var. *lotungensis*) are nested within a clade including *Michelia* and *Yulania* taxa (PB = 93%). In the nuclear data, they were not in this group and remained unresolved (Fig. 4). The combined nuclear and chloroplast data strongly supported the close relationship of sections *Auriculata* and *Macrophylla*, *Yulania*, and *Michelia* with PB = 96% (Fig. 5).

3.3. Molecular dating

Similar results were obtained with the two different calibration schemes for the crown Magnoliaceae (93.5 vs. 70–110 mya, Table 4). Results of time estimates are presented in Table 4, and the chro-

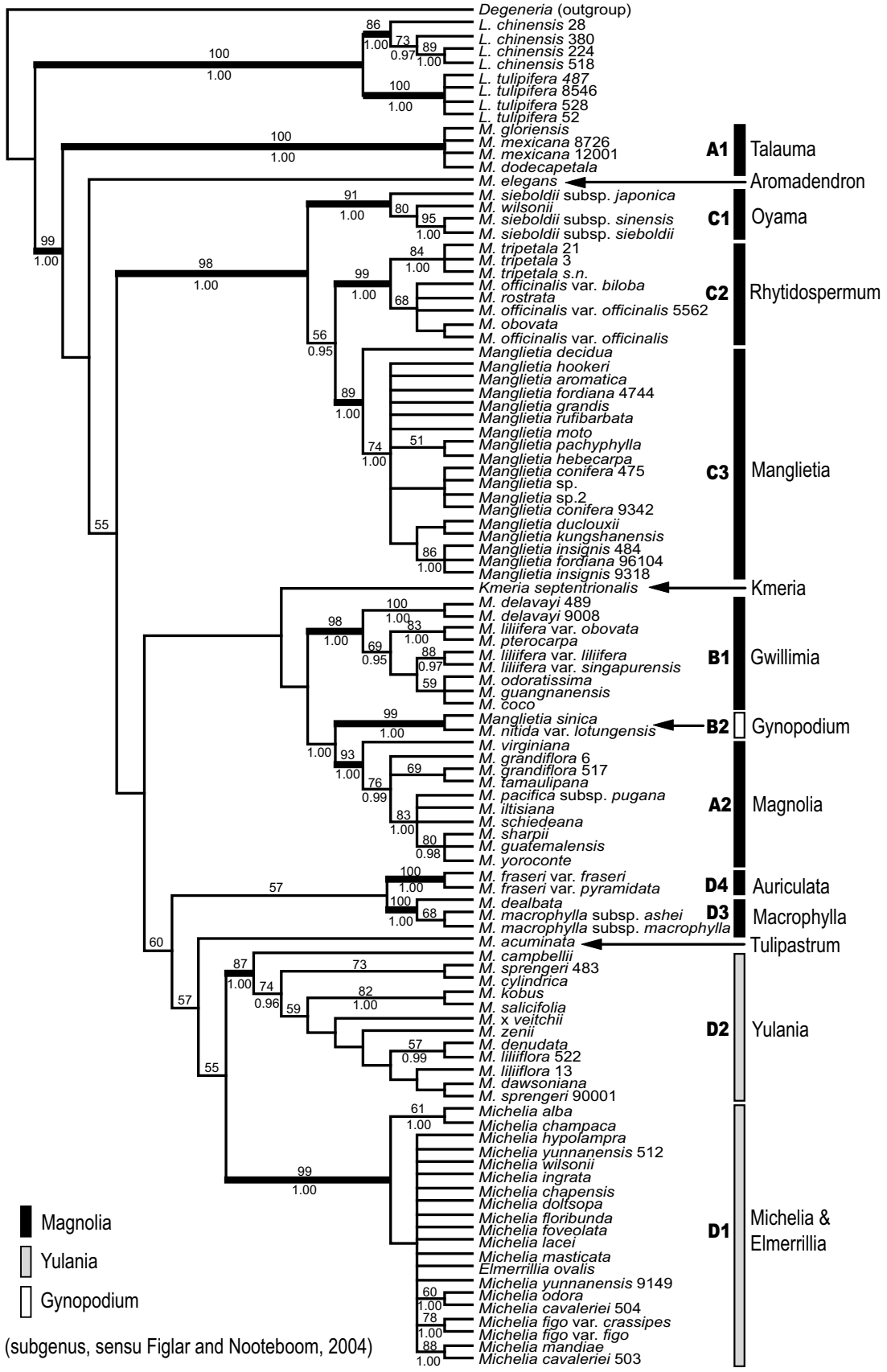

Fig. 2. Strict consensus tree of the concatenated *PHYA*, *LFY*, and *GAI1* nuclear sequences (tree length = 1414 steps, CI = 0.75, and RI = 0.90). The bootstrap values for 1000 replicates are shown above the branches and the Bayesian posterior probabilities higher than 95% are below. Arrows indicate phylogenetically isolated taxa in the family.

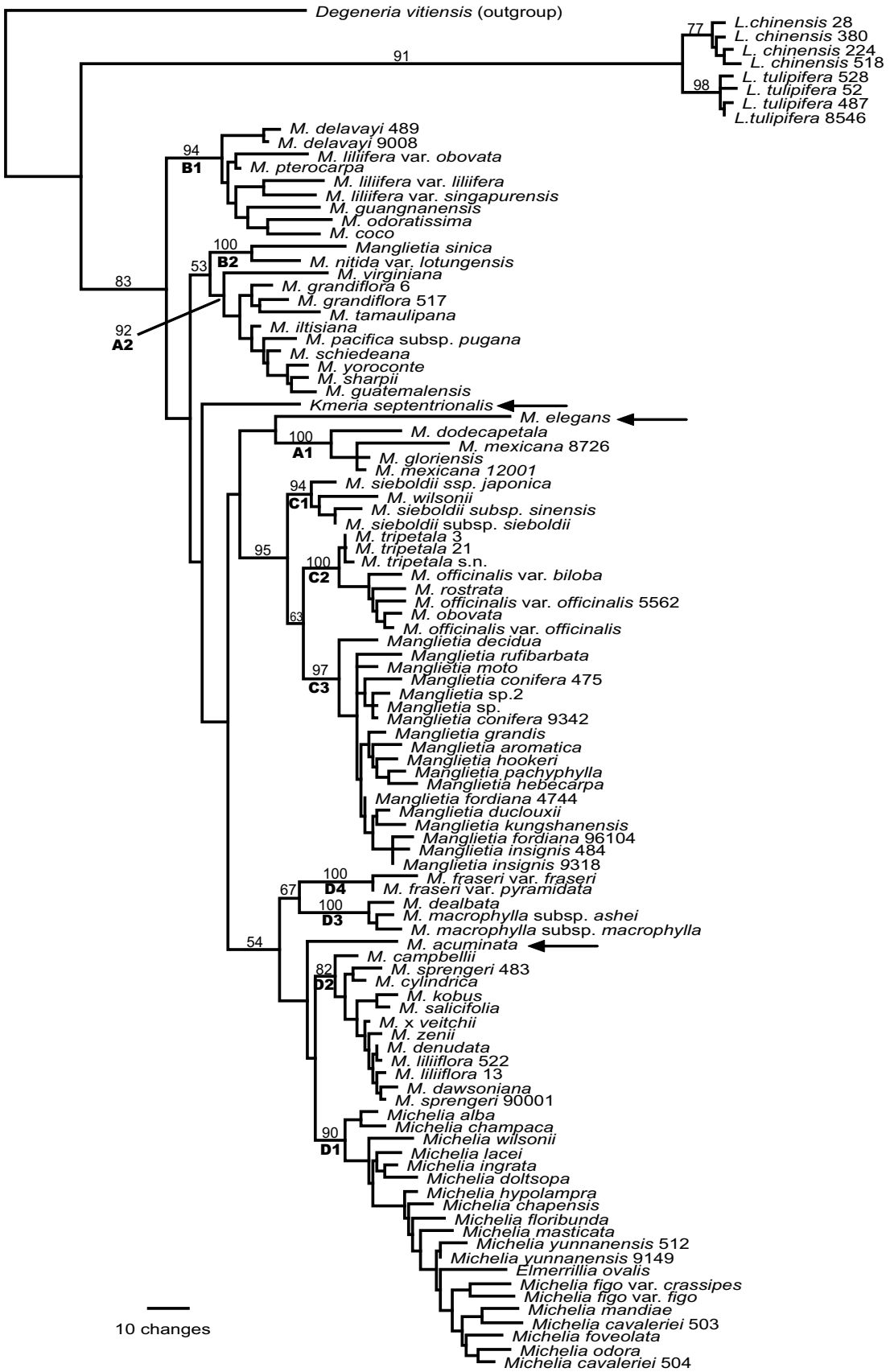

Fig. 3. Maximum Likelihood tree of the concatenated *PHYA*, *LFY*, and *GAI1* nuclear sequences inferred from GARLI, with bootstrap values for 100 replicates are shown above the branches.

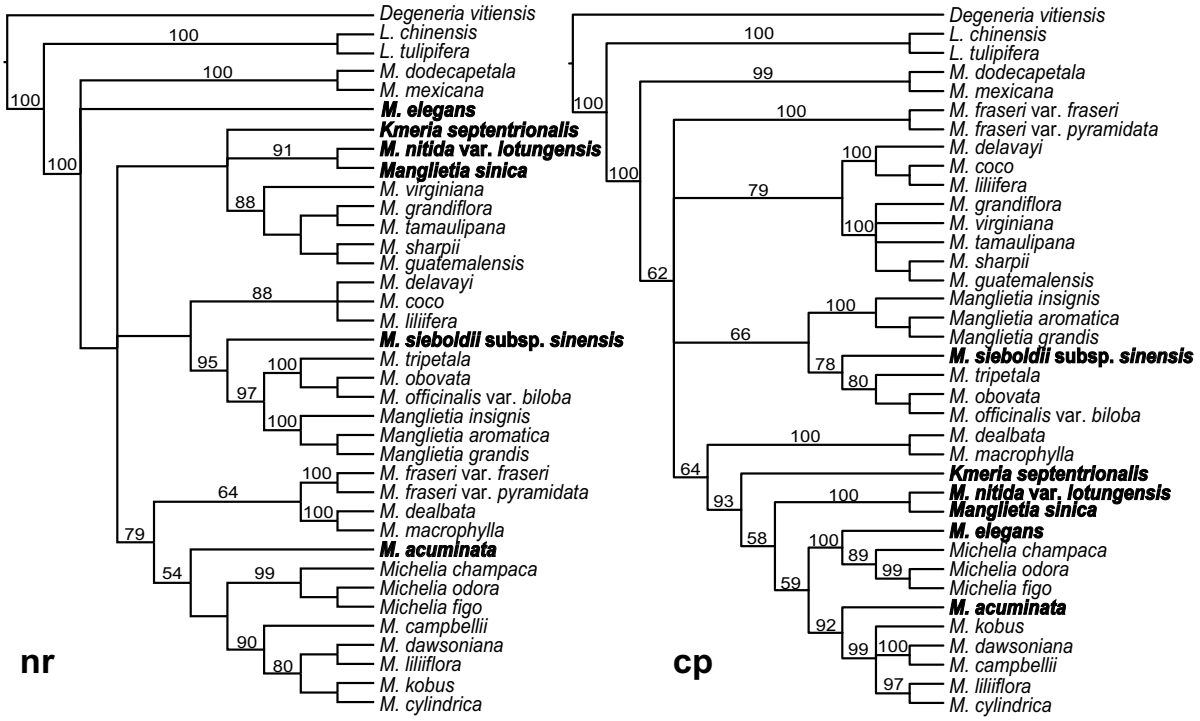

Fig. 4. Strict consensus trees based on nuclear (left: tree length = 987 steps, CI = 0.77, and RI = 0.77) and cpDNA sequences (right: tree length = 516 steps, CI = 0.88, and RI = 0.87). The bootstrap values for 1000 replicates are shown above the branches. Taxa with phylogenetic conflicts are indicated in bold.

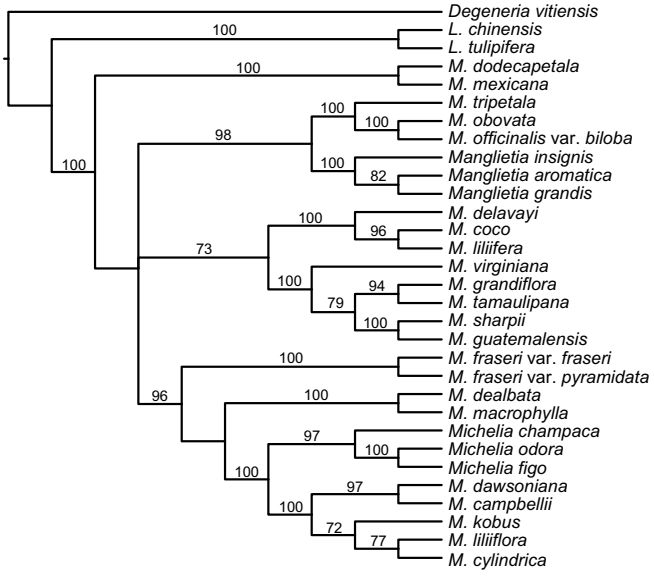

Fig. 5. Strict consensus trees of Magnoliaceae based on combined nuclear (*PHYA*, *LFY*, and *GAI1*) and chloroplast (*ndhF*, *trnK*, and *trnL-F*) sequences (tree length = 1254 steps, CI = 0.83, and RI = 0.83). Bootstrap values are indicated above the branches.

nogram obtained from the Bayesian approach is shown in Fig. 6. *Liriodendron* was estimated to diverge at 12.64 mya by r8s and 14.15 mya by BEAST. On the other hand, the divergence time of the subfamily Magnolioideae calculated with the PL analyses (31.67 mya) was significantly younger than that estimated using the Bayesian method (54.57 mya with a 95% posterior density interval [HPD] of 42.89–67.13 mya). In fact, PL produced younger estimates for all clades relative to the Bayesian method (Table 4).

Two clades with disjunct sister pairs between temperate eastern Asia and North America were recognizable in our phylogenetic analysis. One was from *Liriodendron* (node 1 in Fig. 6 and Table 4) with a divergence time of 14.15 mya. The other was from subsection *Rhytidospermum*, between *M. tripetala* and its Asian counterparts (node 2 in Fig. 6 and Table 4), which were calculated to have diverged around 10.57 mya. The disjunction in the *Yulania* group (between the sole North American *Magnolia acuminata* and all other Asian members, node 3 in Fig. 6 and Table 4) appeared to be older (28.29 mya), albeit with low support. The tropical-subtropical disjunct group of subgenus *Gynopodium* – section *Magnolia* (node 4 in Fig. 6 and Table 4) produced a divergence time of 30.22 mya. Finally, the disjunction for subsections of *Aromadendron* and *Talauma* (node 5 in Fig. 6 and Table 4) was estimated to be 47.93 mya.

4. Discussion

4.1. Phylogenetic relationships within Magnoliaceae

All nuclear data clearly supported the separation of the two subfamilies, the speciose Magnolioideae and the monogeneric Liriodendroideae, as had been shown previously based on chloroplast data (Chase et al., 1993; Qiu et al., 1993; Azuma et al., 2001; Kim et al., 2001). Each of the three nuclear genes, *PHYA*, *LFY*, and *GAI1* separately exhibited a relatively low level of sequence divergence, and thus the phylogenetic relationships within the subfamily Magnolioideae were not well resolved (their strict consensus trees are presented in Appendices 2–4). The low percentages of missing data (7.69% of *PHYA*, 3.85% of *LFY*, and 4.80% of *GAI* taxa of the total 104 samples) cannot account for this lack of resolution. Generally, *GAI1* provided higher phylogenetic resolution than *PHYA* and *LFY* in our study. Some groups were recognizable in one data set, but not in others. For example, section *Manglietia* (clade C3) was well supported in the *GAI1* tree, but collapsed in the *PHYA* and *LFY* trees.

Table 4
Divergence times (mya) with their 95% intervals of disjunct clades recognized for Magnoliaceae by calibration points

Node	Taxa	Bayesian (BEAST)		PL (r8s)
	Magnoliaceae (calibrated)	93.5	70–110	93.5
	Magnolia grandiflora–M. sharpii (calibrated)	18.5	18.5	18.5
	Magnolioideae	54.57 (42.89–67.13)	54.12 (40.47–69.83)	31.67 (23.82–50.71)
1	*Liriodendron*	14.15 (8.69–21.01)	14.34 (7.7–20.83)	12.64 (6.93–27.24)
2	*Rhytidospermum*	10.57 (5.98–15.91)	10.76 (5.9–16.18)	5.78 (2.49–17.17)
3	*Yulania*	28.29 (20.58–36.55)	29.02 (19.78–37.1)	17.45 (11.01–33.91)
4	*Gynopodium–Magnolia*	30.22 (22.2–38.43)	29.65 (21.41–39.31)	25.68 (19.10–44.55)
5	*Aromadendron–Talauma*	47.93 (31.26–61.41)	48.6 (28.1–63.75)	22.96 (16.50–40.18)

Similar situations were found in clade A1: section *Gwillimia*, and clade D2: section *Yulania*. Among the deeper nodes, some phylogenetic conflicts were observed. For example, section *Gwillimia* (A1) was included in a clade of sections *Manglietia* (C3) and *Rhytidospermum* (C2) from the *PHYA* topology, whereas in the *GAI1* data, the branches collapsed. Another case involved several groups including subgenus *Gynopodium* (A2), sections of *Macrophylla* (D3), and *Magnolia* (B1). Their close relationship was relatively well supported with the *PHYA* data, but they did not form a clade in the *LFY* and *GAI1* trees.

The analyses of the combined nuclear data set greatly improved the resolution of relationships within Magnoliaceae, with higher PB support than for any of the separate analyses (see Table 3). However, the deeper nodes of Magnolioideae were still unresolved. One interpretation for this observed pattern may be that they resulted from "soft conflicts" among the individual data sets due to the stochastic nature of the substitution process among different DNA sequences (Edwards and Beerli, 2000). Other possible explanations may include a lower phylogenetic signal or sequence homoplasy among the deeper nodes in Magnolioideae. These findings could have resulted from a rapid radiation and/or a complicated evolutionary history during the early diversification of Magnolioideae. Thus, our nuclear data are more or less consistent with the classification system of recognizing *Magnolia* broadly and defining Magnolioideae as a monogeneric subfamily proposed by Figlar and Nooteboom (2004).

4.1.1. Major clades in Magnolioideae

Figlar and Nooteboom (2004) considered Magnolioideae to contain only one genus with three subgenera and twelve sections largely based on chloroplast phylogenetic results and their comprehensive morphological re-evaluations (also see Figlar, 2006). Our results generally support their classification at the sectional and/or subsectional levels. Excluding the well-resolved genus *Liriodendron*, 11 major clades with a relatively high level of support were recognized for Magnolioideae (Figs. 2 and 3).

Section *Talauma* restricted to the tropical Americas was supported as monophyletic by the nuclear data (clade A1 in Fig. 2). In Azuma et al. (2001), section *Talauma* was shown topologically as two lineages, a basal clade consisting of only subsection *Talauma* and a separate clade consisting of subsections *Dugandiodendron* and *Splendentes*. Morphological data also suggested the polyphyly of section *Talauma* and a close relationship among *Theorhodon*, *Dugandiodendron*, and *Splendentes* (Li and Conran, 2003). Our sampling included only a few representatives of the section and additional sampling from the other two subsections will be needed to determine their phylogenetic relationships. Clade A2 includes two other New World sections of *Magnolia* (with the sole member *Magnolia virginiana*) and *Theorhodon* (Nooteboom, 1985; Vázquez-Garcia, 1994) and was strongly supported as monophyletic (Figs. 2 and 3). Recent observations of living plants have indicated that the core *Theorhodon* species have a small stiplular scar on the leaf petiole, which suggested that it may not be necessary to separate *M. virginiana* from the core *Theorhodon* group (Figlar, 2006).

The newly circumscribed subsection *Rhytidospermum* was well supported by nuclear data (clade C2, Fig. 2), identical *rbcL* sequences, a high level of genetic identity estimated from allozyme variation and chloroplast RFLP analysis, as well as similar seed and fruit morphology and high interspecific cross compatibility (Parks et al., 1983; Qiu et al., 1993; Qiu and Parks, 1994; Qiu et al., 1995a,b). Clade D1 contained the two subsections *Michelia* and *Elmerrillia* with robust support (Fig. 2). *Michelia odora* was once considered to be a member of the monotypic genus *Tsoongiodendron*, characterized by its crowded, sessile, woody, and large fruits (Chun, 1963), but molecular data suggested that this species cannot be separated from the *Michelia* group. Sections *Macrophylla* (D3) and *Auriculata* (D4) were each strongly supported as monophyletic based on our nuclear data (Fig. 2). These species had been placed in traditional section *Rhytidospermum* by Dandy (1978b) because of their whorl-like arrangement of the leaves. The leaf morphology and wood anatomy shared by the Asian and the North American *Rhytidospermum* taxa thus represent apparent convergence (Qiu et al., 1995a). Chloroplast DNA data indicated that these two groups constituted distinctive lineages, separated from the clade of the Asian-North American *Rhytidospermum* (including one North American species *M. tripetala*). The nuclear data suggested that they were closely related, albeit with weak support. However, the combined nuclear and chloroplast data set strongly supported their close relationship to *Yulania* and *Michelia* groups (Fig. 5).

4.1.2. Isolated lineages and their discordance between nuclear and chloroplast data

A few taxa seem to be isolated from the other members in Magnoliaceae, such as *Magnolia acuminata*, subsection *Aromadendron*, section *Kmeria*, and subgenus *Gynopodium* (indicated by arrows in Figs. 2 and 3). Their phylogenetic placements were also inconsistent between the nuclear and chloroplast data sets (including subsection *Oyama*, Fig. 4).

Magnolia acuminata was treated as subsection *Tulipastrum*, closely related to subsection *Yulania* in Figlar and Nooteboom (2004). This relationship had been supported by chloroplast data (Azuma et al., 2001, 2004; Kim et al., 2001). However, we found it sister to a clade including subsection *Yulania* and *Michelia* with weak support (PB = 54% in Fig. 4) in the nuclear trees, rather than sister to subsection *Yulania*. The presence of yellow flowers in *M. acumi-*

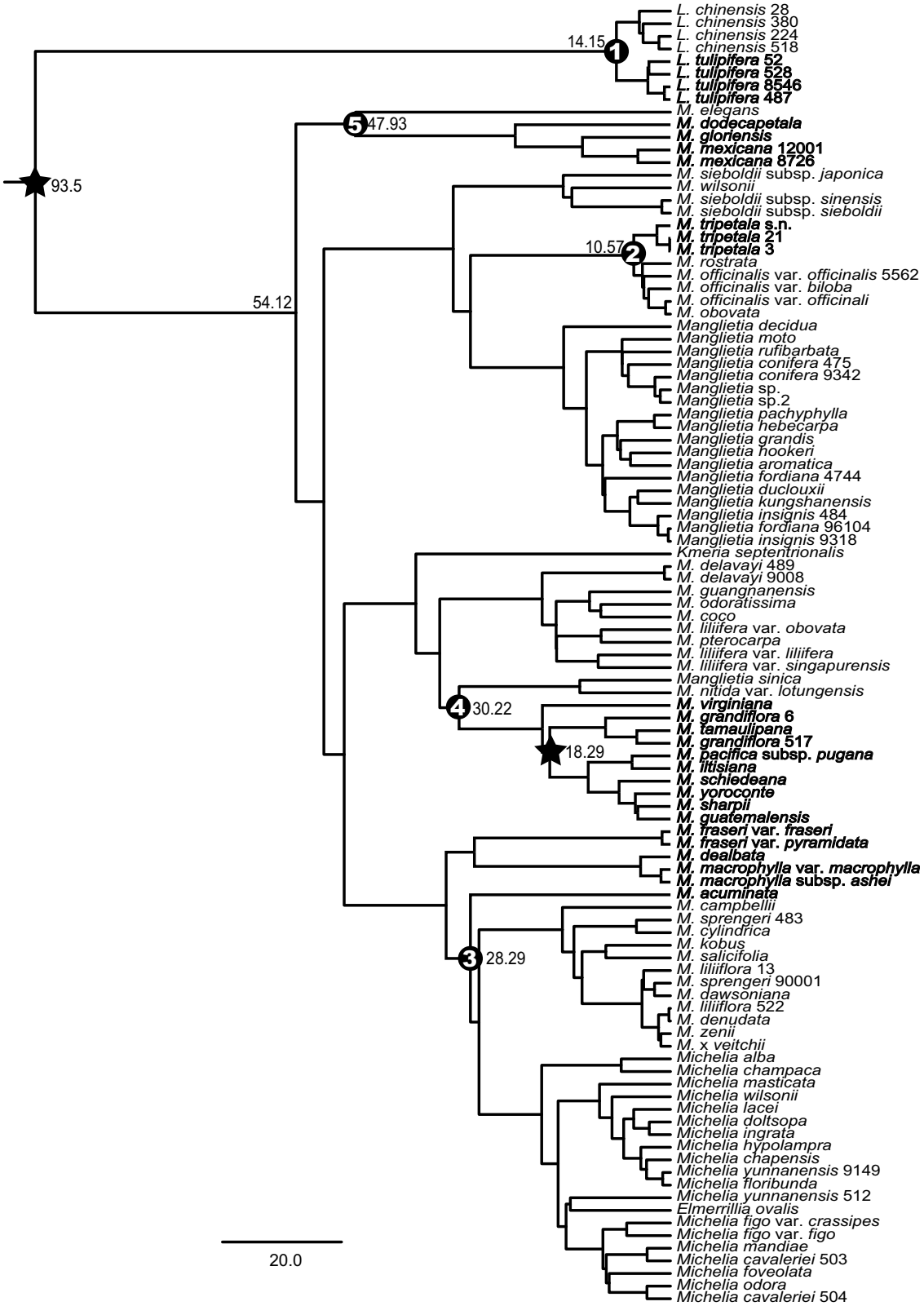

Fig. 6. Chronogram of Magnoliaceae inferred from BEAST with the combined *PHYA*, *LFY*, and *GAI1* matrix. Clade constraints are indicated with black asterisks. Samples from the New World are indicated in bold. The disjunct nodes are indentified with numbers (1–5) as in Table 4.

nata and some species of *Michelia* could have previously suggested a close relationship between them.

Subsection *Aromadendron* (represented by *Magnolia elegans* in Fig. 4) of section *Michelia* was only distantly related to *Michelia* in our nuclear gene trees, but was sister to the *Michelia* group with strong support in the chloroplast ones (Fig. 4). Section *Kmeria* has only three species, with unique unisexual flowers in Magnoliaceae (Dandy, 1927, 1978b; Law, 1984; Nooteboom, 1985; Chen and

Nooteboom, 1993). It has a sporadic distribution in tropical southeastern Asia ranging from China to Indochina. The nuclear data suggested that section *Kmeria* together with subgenus *Gynopodium* was related to sections *Gwillimia* and *Magnolia*, but with low bootstrap support (Figs. 2–4). However, cpDNA data strongly supported their nesting within a clade including members of *Yulania* and *Michelia* (Fig. 4).

Discordance between nuclear and cytoplasmic data is common in plants (e.g., Soltis and Kuzoff, 1995; Soltis et al., 1996; Setoguchi and Watanabe, 2000; Yoo et al., 2002; Ji et al., 2006; Yi et al., 2007). One of the most plausible explanations for this phenomenon has invoked introgression of the cytoplasmic genome from one species into the nuclear background of another (or vice versa) by interspecific hybridization (Soltis and Kuzoff, 1995; Wendel and Doyle, 1998). For Magnoliaceae, cross compatibility and hybridization do occur under artificial conditions (Parks et al., 1983; Qiu et al., 1995b; Gong et al., 2001; Kim et al., 2001).

The morphological evidence from *Magnolia acuminata* and *M. elegans* was congruent with their phylogenetic relationships based on the plastid data. *Magnolia acuminata* has been considered to be a close relative of *M. liliiflora* in subsection *Yulania* based on their sepaloid tepals and the flowering with leaf emergence on the internodes below the peduncle (Nooteboom, 1985; Azuma et al., 2001; R. Figlar, personal communication). *Magnola elegans* was suggested to be close to the *Michelia* group based on fruit and flowering characters, especially to taxa of *Elmerrillia* and *Maingola* (Li and Conran, 2003; Figlar, 2006). Because the plastid topology best reflects phylogenetic predictions based on the morphology, we hypothesize that the discordance between the nuclear and plastid lineages resulted from nuclear introgression without gene flow from the plastid genome, although it appeared to be rare with only a few examples reported so far (i.e., Wagner et al., 1987; Setoguchi and Watanabe, 2000; Ji et al., 2006).

For the other incongruent taxa, no clear morphological characters support their phylogenetic positions inferred from the chloroplast data set. Subsection *Oyama* was supported to be close to *Rhytidospermum* by the chloroplast tree (Fig. 4), but no morphological characters support their close relationship (Azuma et al., 2001). However, the leaf arrangement in false whorls at the end of shoots and the reticulate (wrinkled) inner seed coats are often found in taxa of *Rhytidospermum* and *Manglietia* (Qiu et al., 1995a; Azuma et al., 2001), and the close relationship of these two groups were indicated by the nuclear tree (Fig. 4). Section *Kmeria* and subgenus *Gynopodium* were included in a clade with *Yulania* and *Michelia* in the chloroplast tree and no morphological characters can be used to circumscribe this group (Figlar, 2006). Therefore, the discordance with the chloroplast-based tree that does not accurately reflect their morphological relationships may have resulted from chloroplast capture (Rieseberg and Soltis, 1991; Soltis et al., 1996). Ancient hybridizations with chloroplast introgression may have occurred among ancestors of these isolated taxa.

4.2. Disjunctions within Magnoliaceae

Divergence times estimated from PL were more recent than those from the Bayesian method (Table 4). It is difficult to compare the fit of the model used in the Bayesian analysis with PL (Roger and Hug, 2006). Penalized likelihood assumes that substitution rates change gradually over a fixed tree and introduces a penalty for abrupt rate changes between them. The Bayesian method in BEAST, on the other hand, assumes the rates as uncorrelated, with the rate in each branch being independently drawn from a lognormal distribution (Drummond et al., 2006). Simulation studies have suggested that PL tend to underestimate rates when the clock assumption was relaxed, especially when sequences are short and substitution rates are low, whereas the Bayesian method with a log-normal mode did not show such obvious bias (Ho et al., 2005). The limited degree of divergences observed with our nuclear data sets may have led to an underestimate with the PL method. We thus used the Bayesian rather than the PL results to discuss the biogeographic history of Magnoliaceae as below.

Two independent temperate disjunctions in Magnoliaceae were confirmed by the nuclear data with robust support (Fig. 6). The first disjunction was between *Liriodendron tulipifera* and *L. chinense*, which exhibit the classical disjunct pattern with one species each in eastern North America and eastern Asia, respectively (node 1 in Fig. 6). Our analysis suggested that the divergence time between these two species was about 14.15 mya (95% HPD: 8.69–21.01 mya) in the middle Miocene. This time estimate is consistent with that from Parks and Wendel (1990), who had estimated the divergence time for the two species as 10–16 mya (middle to late Miocene) based on allozyme and cpDNA RFLP data as well as on paleobotanical evidence. The second disjunction we dated was within subsection *Rytidospermum* (node 2 in Fig. 6), with new characters including a ridged seed coat surface and fruit shape identifying a narrower group (Qiu et al., 1995a,b). The divergence time between *M. tripetala* and its Asian counterparts was estimated to be 10.57 mya, a value from nuclear genes quite different from previous estimates. Qiu et al. (1995b) reported the divergence time to be 4.1–5.5 or 1.9 mya using allozyme data and 1.7 ± 0.8 mya using cpDNA RFLP data. The age of 27.9 ± 4.4 or 20.9 ± 3.3 mya reported by Azuma et al. (2001) was older than our estimate. Thus, time estimates with molecular clocks, especially from strict models, should be treated with the greatest care.

The disjunction of temperate taxa after the middle Miocene is consistent with the palaeoclimate and fossil evidence. The Miocene was a period with globally warmer climates than those in the preceding Oligocene, or the subsequent Pliocene (Zachos et al., 2001). The middle Miocene warming period from 13 to 18 mya (Wolfe, 1985; White et al., 1997) may have stimulated the diversification of temperate elements in the Northern Hemisphere. Later in the Miocene a distinct climatic cooling period may have resulted in the range reduction of both tropical and coniferous forests, with grasslands and savannas predominating in their stead. Numerous fossils resembling modern *Magnolia* species have been identified as being from the late Miocene (Nooteboom, 1993; Mai, 1995). Extensive fossil evidence from the early to middle Miocene reveals the diverse, temperate, deciduous, and mesophytic vegetation that was widely distributed in the Northern Hemisphere (Wolfe, 1969, 1977; Tanai, 1972; Muller, 1981; McCartan et al., 1990). These Miocene floras had many elements in common with the modern mesophytic floras of eastern Asia and eastern North America (Parks and Wendel, 1990), supporting the proposal that the divergence of the modern north temperate elements occurred during that period.

Other disjunctions primarily concern relict temperate to tropical taxa, and were not convincingly supported by our nuclear data (nodes 3–5 in Fig. 6). *Magnolia acuminata* from temperate North America was sister to an Asian clade including both the *Yulania* and *Michelia* clades with low bootstrap support (node 3), whereas the chloroplast data suggested its close relationship to the Asian *Yulania* (Azuma et al., 2001; Kim et al., 2001). Our estimate suggested an age of 28.29 mya in the middle Oligocene for this disjunction. Seeds of the *Yulania* group are usually flat, symmetrical, and cordiform to bean-like in shape (Tiffney, 1977; Azuma et al., 2001). Fossil seeds agreed with the divergence of the *Yulania* group in the middle Oligocene to the early Miocene (Dorofeev et al., 1974; Figlar, 1993; Tiffney, 1977; see reviews in Azuma et al., 2001). The other disjunct relationships were observed for subgenus *Gynopodium*–section *Magnolia* (node 4 in Fig. 6) with an estimated divergence time of 30.22 mya, and for subsection *Aromadendron*–section *Talauma* (node 5 in Fig. 6) with a divergence time of

47.93 mya. Due to the low support values for these groups, further investigation with additional taxon sampling and sequence data will be needed to resolve their biogeographic relationships.

Although the disjunct lineages involving some subtropical-tropical taxa were weakly supported, we can propose that the disjunctions in Magnoliaceae exhibit two patterns. Some disjunctions occurred relatively recently (at or after the middle Miocene) and their phylogenetic relationships are well supported by the molecular data (i.e., *Liriodendron* and subsection *Rhytidospermum*). The others were relatively ancient (no later than the Oligocene), involving the tropical-subtropical and the relict temperate disjunctions between the Old and the New Worlds (e.g., between tropical sections *Gwillima* from the Old World and *Magnolia* from the New World, and between *Magnolia acuminata* and its Asian counterparts). These phylogenetic relationships are not well-resolved with either nuclear or previous chloroplast data (Azuma et al., 2001; Kim et al., 2001). Overall, the heterogeneous pattern of molecular divergence between Asian and North American species suggests that the current distribution of Magnoliaceae was accomplished by multiple migrations via both the Bering and the North Atlantic land bridges (Qiu et al., 1995b). Subsequently, cooling events might have caused movement of the elements of the boreotropical flora to the lower latitudes, leading to disjunction of ancient lineages of modern tropical plants between North America and Eurasia (Parks and Wendel, 1990).

4.3. Biogeographic implications of Magnoliaceae evolution in the Tertiary

The crown group of subfamily Magnolioideae was estimated to be 54.57 mya old, inferring an early diversification of the extant magnolias in the boundary between the Paleocene and the Eocene in the early Tertiary. This correlates with the "Paleocene-Eocene thermal maximum" event, which marked the start of the Eocene, the most rapid and extreme global warming event recorded in geologic history (Lourens et al., 2005). The divergence of Magnolioideae was assumed to be about 42 mya (the middle Eocene) by Azuma et al. (2001), but used a molecular clock for *matK* gene sequences calibrated with fossil evidence of 25 mya for the *Yulania* group (*Magnolia acuminata* v.s. Asian relatives). When the age of the stem Magnolioideae was determined as 93.5 mya based on fossil evidence (Frumin and Friis, 1996, 1999), the crown age was estimated to be roughly half as old as its stem age (Table 4). Fossils suggest that Magnolioideae originated before the Tertiary and that the divergence of the extant magnolias (excluding *Liriodendron*) occurred no earlier than the Eocene.

The global gradual cooling climate after 50 mya in the Eocene (Zachos et al., 2001) may well explain the early diversification of Magnolioideae into its major extant clades. Plant life at that time was a boreotropical flora, comprised primarily of broad-leaved evergreen taxa, reaching the regions of high paleolatitudes in the Northern Hemisphere during the early Tertiary (Wolfe, 1972, 1975). Our results are largely consistent with the fossil evidence. The fossil record provides unequivocal evidence for the presence of diverse *Magnolia* assemblages in the Eocene, and indicates that many major lineages of extant Magnolioideae were already differentiated by the Eocene. Only a few plausible Magnolioid fossils appeared before the Late Cretaceous and cannot be assigned with certainty to modern genera before the Cretaceous (Zhang, 2001). In the Tertiary, many fossils of magnolia-like leaves (e.g., Tralau, 1963; Dorofeev et al., 1974; Rember, 1991; Mai, 1995; Liu et al., 1996; Walther, 1999) and seeds (Peigler, 1989; Collinson et al., 1993; Mai, 1995) have been reported and the group became widely distributed in the Northern Hemisphere (Tiffney, 1977; Cevallos-Ferriz and Stockey, 1990; Graham, 1999). During the Eocene, Magnoliaceae had its widest distribution (Zhang, 2001). Our results support that there were expansions of distinctive clades well separated in Magnolioideae over the Eocene. The major Eocene diversification of the family is also supported by the marked increase in fossils of the magnolias during the same period.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.06.004.

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