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DNA SEQUENCES FROM MIOCENE FOSSILS: AN *NDHF* SEQUENCE OF *MAGNOLIA LATAHENSIS* (MAGNOLIACEAE) AND AN *RBCL* SEQUENCE OF *PERSEA PSEUDOCAROLINENSIS* (LAURACEAE)[1]

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We report a partial *ndhF* sequence (1528 bp) of *Magnolia latahensis* and a partial *rbcL* sequence (699 bp) of *Persea pseudocarolinensis* from the Clarkia fossil beds of Idaho, USA (Miocene; 17–20 million years [my] BP). The *ndhF* sequence from *M. latahensis* was identical to those of extant *M. grandiflora*, *M. schiediana*, *M. guatemalensis*, and *M. tamaulipana*. Parsimony analysis of the *ndhF* sequence of *M. latahensis* and previously reported *ndhF* sequences for Magnoliaceae placed *M. latahensis* within *Magnolia* as a member of the Theorhodon clade. This result is reasonable considering that: (1) the morphology of *M. latahensis* is very similar to that of extant *M. grandiflora*, and (2) a recent molecular phylogenetic study of Magnoliaceae showed that the maximum sequence divergence of *ndhF* among extant species is very low (1.05% in subfamily Magnolioideae) compared with other angiosperm families. We reanalyzed the previously reported *rbcL* sequence of *M. latahensis* with sequences for all major lineages of extant Magnoliales and Laurales. This sequence is sister to *Liriodendron*, rather than grouped with a close relative of *M. grandiflora* as predicted by morphology and the results of the *ndhF* analysis, possibly due to a few erroneous base calls in the sequences. The *rbcL* sequence of *P. pseudocarolinensis* differed from *rbcL* of extant *Persea* species by 3–6 nucleotides and from *rbcL* of extant *Sassafras albidum* by two nucleotides. Phylogenetic analyses of *rbcL* sequences for all major lineages of Magnoliales and Laurales placed the fossil *P. pseudocarolinensis* within Lauraceae and as sister to *S. albidum*. These results reinforce the suggestion that Clarkia and other similar sites hold untapped potential for molecular analysis of fossils.

Key words: Clarkia; fossil DNA; *Magnolia latahensis*; Miocene; *ndhF*; *Persea pseudocarolinensis*; *rbcL*.

DNA sequences from ancient fossils have great potential for studies of phylogeny, biogeography, and molecular evolution (Soltis et al., 1995); DNA from fossils also facilitates the rigorous testing and calibration of mutation rates among related taxa (Golenberg et al., 1990; Cano et al., 1992, 1993; DeSalle et al., 1992; Soltis et al., 1992; Poinar et al., 1993). The Clarkia fossil beds in northern Idaho, USA (17–20 million years [my] BP; Miocene), bear some of the best preserved compression fossils from the Miocene. These fossils are believed to be chemically well preserved because of the low oxygen content and cold temperatures of the water in which they were deposited. Leaves apparently fell into an ancient lake and remained unoxidized and water-saturated until the present day (Smiley, 1985; Giannasi and Nicklas, 1977). In an ultrastructural study of these fossils, 90.1% of 2300 randomly sampled cells contained chloroplasts (Niklas et al., 1985).

Golenberg et al. (1990) obtained a partial DNA sequence of the chloroplast gene *rbcL* (the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase) from a leaf specimen of *Magnolia latahensis* (Berry) Brown (Magnoliaceae) from the Clarkia fossil beds. Compared with the *rbcL* sequence of modern *M. macrophylla* (1428 base pairs [bp]), there were 17 base substitutions in the 759-bp region they sequenced, 12 of which were transitions and all but four of which were third-position substitutions. In their phylogenetic trees, *M. latahensis* was the sister to the remaining family members analyzed, *M. macrophylla* and *Liriodendron tulipifera*, when all three codon sites were included in the analysis (UPGMA using Kimura's 3 substitution type estimates). When third codon sites were eliminated, *M. latahensis* was sister to *M. macrophylla*, and *L. tulipifera* was sister to the *M. macrophylla*-*M. latahensis* clade. Successful extraction of DNA and sequencing of *rbcL* were repeated from a specimen of the gymnosperm *Taxodium* (Soltis et al., 1992) from the same site. Miocene fossil *Taxodium* differed from modern *Taxodium distichum* at 11 of the 1320 nucleotides sequenced; all substitutions were third-position changes. Phylogenetic analyses of Taxodiaceae and Cupressaceae placed the fossil sister to extant *T. distichum* (Brunsfeld et al., 1994). Despite these apparent successes, the validity of these first DNA sequences from the Clarkia fossil beds was challenged on theoretical grounds. Based on in vivo studies of DNA degradation (Lindahl and Andersson, 1972; Lindahl and Nyberg, 1972), Pääbo and Wilson (1991) suggested that DNA should be completely degraded after 4 my.

We report two additional examples of the successful amplification and sequencing of fossil genes obtained from leaves of the Clarkia beds: (1) a partial *ndhF* (the sixth subunit of NADH dehydrogenase) sequence (1528 bp) from fossil *M. latahensis* and (2) a partial *rbcL* sequence (699 bp) from fossil *Persea pseudocarolinensis* Lesquereux (Lauraceae). Recent molecular phylogenetic studies of Magnoliaceae (Kim, 2001; Kim et al., 2001) identified major clades of extant taxa and

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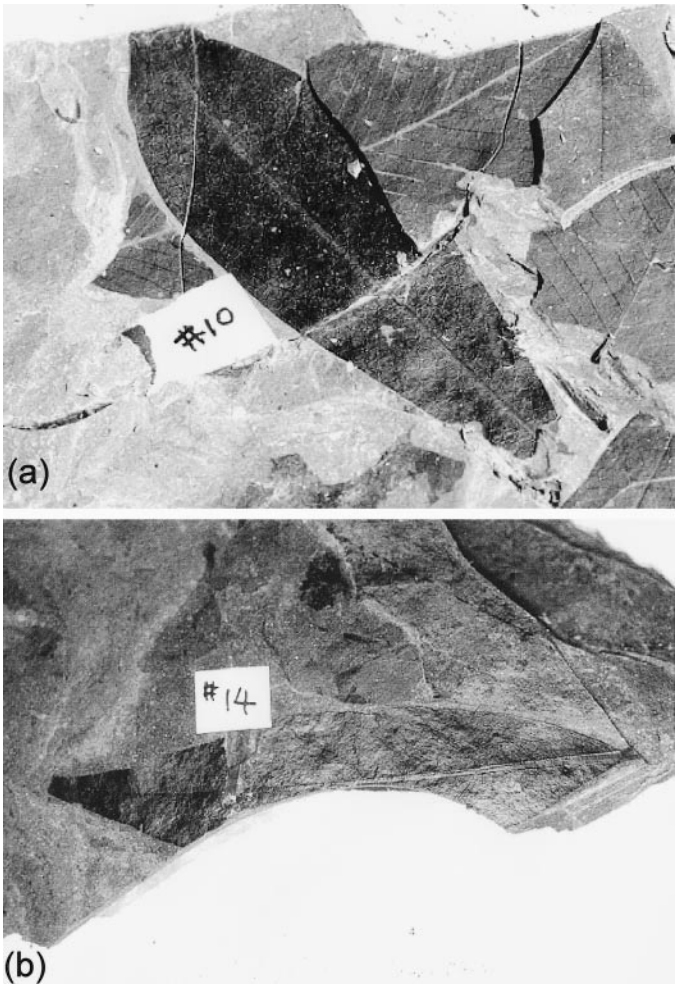


Fig. 1. Voucher photograph of fossil specimens used in this study. (a) *Magnolia latahensis* (Berry) Brown. (b) *Persea pseudocarolinensis* Lesquereux.

estimated substitution rates of various genes, including *rbcL* and *ndhF*. Complete *rbcL* sequences have also been reported for *Persea americana* and other genera of Lauraceae (Goldenberg et al., 1990; Soltis et al., 2000). We (1) reanalyzed the previously reported *rbcL* sequence of the fossil *M. latahensis* (Goldenberg et al., 1990) with data from phylogenetic studies of modern Magnoliales and Laurales (Qiu et al., 1999; Soltis et al., 2000; Kim, 2001), (2) analyzed the *ndhF* sequence of *M. latahensis* with previously published *ndhF* sequences for Magnoliaceae (Kim et al., 2001), and (3) analyzed the *rbcL* sequence of the fossil *P. pseudocarolinensis* with *rbcL* sequences for Magnoliales and Laurales.

MATERIALS AND METHODS

A mass of rocks (about 0.5 m^3) was removed using a pick and shovel from the Clarkia fossil beds and subsequently transported to Washington State University Pullman, Washington, USA, and stored at 4°C. Rocks were cleaved, and fossil leaves of good quality were selected for DNA extraction. We photographed each leaf to provide a voucher image and immediately scraped the leaf tissue from the rock (Fig. 1). The tissue was placed in a microcentrifuge tube (1.5 mL) with extraction buffer (buffer A in the DNeasy plant mini kit, QIAGEN, Hilden, Germany) and ground using a pellet pestle. All of these procedures, from cracking rocks to immersing the tissue in the extraction buffer, took less than 3 min. We used the DNeasy plant mini kit (QIAGEN) for DNA extraction with some modifications from the manufacturer's manual, rather than the standard cetyltrimethylammonium bromide method (CTAB; Doyle and Doyle, 1987) in order to: (1) minimize any contamination during the DNA extraction procedure; each filter included in this kit was packed separately in DNase- and RNase-free condition; and (2) obtain high quality, condensed DNA with the DNA-binding filter method. We used 3–5 volumes more of extraction buffer (buffer A) than the protocol suggested, because the amount of one scraped fossil leaf, which also included some of the surrounding rock matrix, was greater than the suggested volume for one extraction. The PCR components and temperature conditions were those of Kim et al. (2001). When we checked the PCR products in 1.0% agarose gels (loading 5 μL), faint bands were occasionally detected, but in most cases, no product was observed. After diluting the first PCR products from 1 : 10 to 1 : 50, we performed a second amplification using the same conditions. Negative controls, which were reactions without template DNA, were performed to check contamination in all PCRs.

For the amplification of *ndhF*, we tried various primer sets developed during the recent phylogenetic study of Magnoliaceae (Kim et al., 2001) and the primers of Olmstead and Sweere (1994) and Jansen (1992): 1–972R, MF561-MF1165R, 972-MF1861R, and MF1795–2110R. For the amplification of *rbcL*, we used standardly employed primer pairs: Z1-Z647R, Z1-Z895R, Z647-Z1351R, Z895-Z1351R, and Z895-Z3′. We attempted to amplify DNA segments of 500–700 bp in length rather than the entire gene or larger segments (i.e., ≥1000 bp).

Purification of PCR products followed Kuzoff et al. (1998). Sequencing reactions used ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions, except that we performed 1/4-volume reactions. Sequencing products were separated on an ABI 377 Automated Sequencer (Applied Biosystems). Proofreading and editing of each sequence were performed using Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Following the methods of Kim et al. (2001), we also determined *rbcL* sequences of eight extant members of Lauraceae thought to be closely related to fossil *P. pseudocarolinensis—P. americana*, *P. borbonia*, *P. palustris*, *Apollonias barbujana*, *Laurus nobilis*, *Litsea cubebe*, *Lindera benzoin*, and *Machilus rimosa* (GenBank accession nos. AY337727–AY337734). These new sequences were determined to facilitate comparison of the fossil with extant Lauraceae.

Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) with PAUP* 4.0b10 (Swofford, 2002). The *ndhF* sequence from fossil *M. latahensis* was analyzed with *ndhF* sequences of extant Magnoliaceae (Kim et al., 2001); trees were rooted using two *Liriodendron* species. The *rbcL* sequences of fossil *P. pseudocarolinensis* and *M. latahensis* (Golenberg et al., 1990) were analyzed together with sequences from previously reported Magnoliales and Laurales (Soltis et al., 2000) and the eight new sequences from Lauraceae. Trees were rooted between Magnoliales and Laurales, which are sister groups in the magnoliids (e.g., Zanis et al., 2002).

For MP analysis, 100 replications of random taxon addition with tree bisection-reconnection (TBR) branch-swapping were used, saving all trees. One hundred replications of the bootstrap were performed using the same parameters. For the ML analyses, the program MODELTEST (Posada and Crandall, 1998) was used to determine the appropriate model of sequence evolution for this data set. The chosen model (general time reversible + I + gamma) was applied to the data matrix using PAUP* 4.0b10 (Swofford, 2002). Ten replications of random taxon addition using TBR branch-swapping were carried out, and all optimal trees were saved.

To test for saturation of base substitutions in the third codon positions of *rbcL* for this taxon sample, we computed pairwise distances between all pairs of sequences using ''uncorrected *p*'' distances. We then plotted the values for the first and second codon positions jointly vs. that for the third codon position. The *rbcL* sequence data used for this test were from Kim et al. (2001) and Soltis et al. (2000).

RESULTS AND DISCUSSION

We amplified *ndhF* from the fossil *M. latahensis* using three different primer sets and *rbcL* from the fossil *P. pseudocaro-*

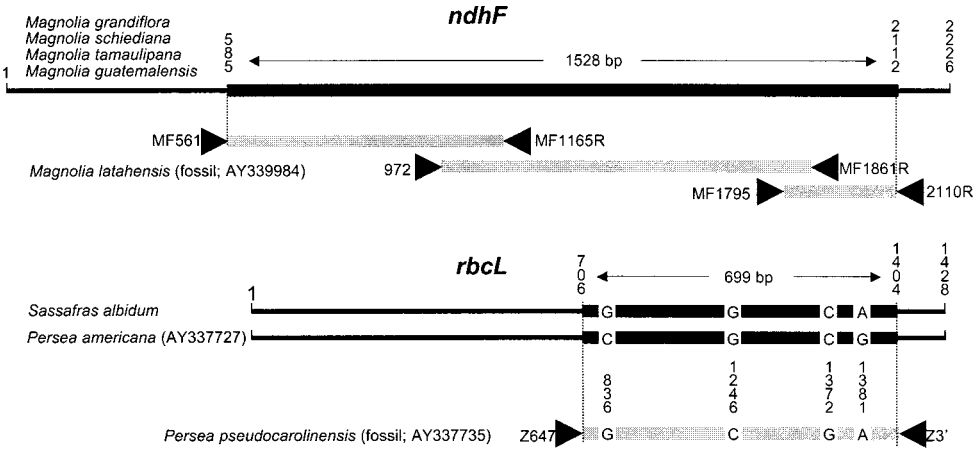


Fig. 2. Comparison among the regions of *ndhF* and *rbcL* sequences from specimens from the Clarkia fossil beds (gray bars) and related extant taxa. Arrows indicate the primers used for PCR and sequencing.

linensis using a single primer set (Fig. 2). We amplified only a part of each gene from each leaf sample because of insufficient quantities of fossil DNA. Nonetheless, we amplified approximately 70% of *ndhF* and 50% of *rbcL*. During DNA extraction, the solution became green in color, indicating the presence of chlorophyll in the extraction buffer after centrifugation and suggesting that at least some chloroplasts remained intact until extraction.

Magnolia latahensis—From *M. latahensis*, we determined 1528 bp of *ndhF*, corresponding to positions 580 to 2085 of *ndhF* of extant *M. grandiflora* (GenBank accession no. AY337727); total length of *ndhF* in subfamily Magnolioideae is 2228 bp in most cases (Kim et al., 2001). The sequence of *M. latahensis* was identical to those previously reported for *M. grandiflora*, *M. schiediana*, *M. tamaulipana*, and *M. guatemalensis*. When we included the fossil sequence in the MP analysis of *ndhF* of extant Magnoliaceae (Kim et al., 2001), this sequence was placed as part of a polytomy at the base of the Theorhodon clade, one of the major clades of Magnoliaceae (Kim et al., 2001), with *M. grandiflora*, *M. schiediana*, *M. tamaulipana*, and *M. guatemalensis* (Fig. 3). The Theorhodon clade includes Nooteboom's (1985) section *Magnolia* and a part of section *Theorhodon* (excluding the Caribbean species). The four extant species differ in morphology and are distinguished mainly by size of the mature fruit, the number of carpels per fruit, flower size, and the number of stamens (Vázquez-G., 1994). A well-preserved fossil fruit of *M. latahensis* had approximately 250 stamens and 120 carpels (Figlar, 1993), both within the range of *M. grandiflora*. Based on morphology, *M. latahensis* seems particularly similar to extant *M. grandiflora* (Figlar, 1993). The lack of sequence divergence between *M. latahensis* and the other four species is not surprising. Given the low rate of *ndhF* substitution in Magnoliaceae, maximum sequence divergence (Kimura's $K \times 100$) was only 2.45% and 1.05% in extant Magnoliaceae and subfamily Magnolioideae, respectively (Kim et al., 2001).

The placement of *M. latahensis* in the *ndhF* tree is reasonable considering: (1) the morphology of *M. latahensis* is very similar to that of *M. grandiflora*, although other species placed with *M. latahensis* in the Theorhodon clade possess very different morphologies and (2) the maximum pairwise sequence divergence of *ndhF* in extant Magnoliaceae is very low (1.05% in subfamily Magnolioideae) compared with other angiosperm families (Kim et al., 2001). The possibility of contamination is extremely low because no PCR products were detected in any negative controls, and the laboratory at Washington State University in which DNA of *M. latahensis* was extracted, amplified, and sequenced never possessed samples of the four extant species of *Magnolia* that share an *ndhF* sequence with *M. latahensis*.

Golenberg et al. (1990) obtained a sister relationship between *M. latahensis* and *M. macrophylla*, with *L. tulipifera* as their sister, when third codon positions were excluded in *rbcL*. However, our hierarchical test of saturation of third codon positions in *rbcL* indicates that third positions are not saturated in subfamily Magnolioideae, in the Magnoliaceae, or even in the Magnoliales. In all of these groups, the relationship between substitutions in first + second positions and third positions is linear (Fig. 4). Therefore, there is no reason to exclude third positions. Furthermore, several recent studies have demonstrated the value of third positions in *rbcL* in phylogenetic analyses (Lewis et al., 1997; Källersjö et al., 1998, 1999). We therefore included all codon positions in our reanalysis of the *rbcL* sequence from *M. latahensis* with additional sequences of extant Magnoliales and Laurales not available at the time of the Golenberg et al. (1990) analysis.

The MP analysis of an *rbcL* data set of 37 sequences that included the Golenberg et al. (1990) sequence of *M. latahensis* generated 373 equally parsimonious trees, and *M. latahensis* formed a polytomy with the subfamilies Magnolioideae and Liriodendroideae of Magnoliaceae, Annonaceae, Himantandraceae, Degeneriaceae, Myristicaceae, and Eupomatiaceae in the Magnoliales in the strict consensus tree (not shown). In the ML tree, *M. latahensis* was grouped with *Liriodendron* (Fig. 5). Considering the morphology of *M. latahensis* and the base substitution rate of *rbcL* in extant Magnoliaceae (1.03% in subfamily Magnolioideae and 2.46% in Magnoliaceae; Kim, 2001), the placement of the previously reported *rbcL* sequence of *M. latahensis* in trees from MP and ML analyses suggest that there may have been errors in the *rbcL* sequence of *M. latahensis* (Golenberg et al., 1990).

Persea pseudocarolinensis—We determined a second sequence from the Clarkia fossil beds, a 699-bp portion of *rbcL* from *Persea pseudocarolinensis* (GenBank accession no.

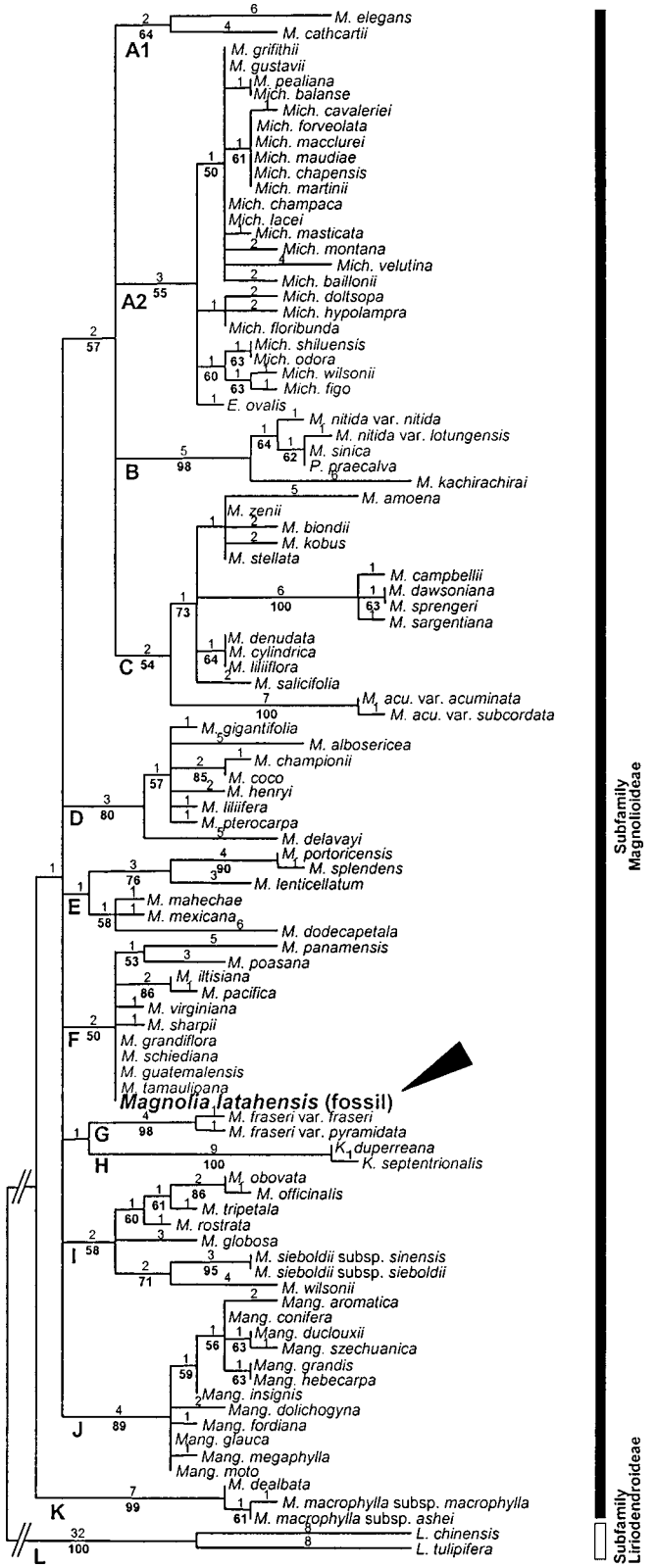


Fig. 3. One of three equally parsimonious trees based on *ndhF* sequences of selected taxa of Magnoliaceae (256 steps, consistency index = 0.79, and retention index = 0.93). The topology of this tree is identical to that of the strict consensus tree. Numbers above and below the line indicate the number of nucleotide changes and bootstrap percentages, respectively. Bootstrap values greater than 50% are indicated. Capital letters indicate 12 major clades of Magnoliaceae recognized by analysis of 10 regions of cpDNA data (Kim, 2001). Arrow indicates the placement of *M. latahensis*. Abbreviations: *M.*, *Magnolia*; *Mang.*, *Manglietia*; *Mich.*, *Michelia*; *L.*, *Liriodendron*; *K.*, *Kmeria*; *P.*, *Pachylarnax*; *E.*, *Elmerrillia*; *acu.*, *acuminata*.

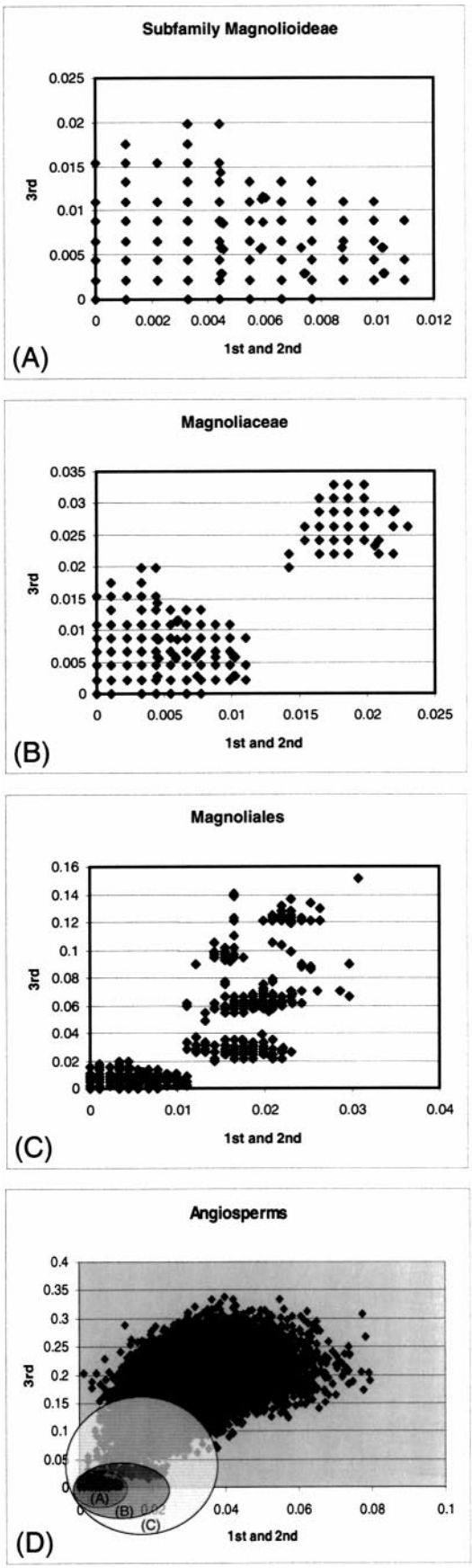
AY337735). The *rbcL* fragment (total length of *rbcL* in Lauraceae is 1428 bp; Soltis et al., 2000) from the fossil specimen corresponds to positions 706 to 1404 of the *rbcL* sequence from extant *P. americana* (Fig. 2). The fewest substitutions in pairwise comparisons of *P. pseudocarolinensis* and extant species occurred with *Sassafras albidum* rather than *Persea* species (Table 1). Two base pairs differed between *P. pseudocarolinensis* and *Sassafras albidum* (0.29%), and the number of base substitutions between *P. pseudocarolinensis* and extant species of *Persea* ranged from three to six (0.43–0.86%).

To check for possible errors in the original sequence of *S. albidum* and to eliminate the possibility of contamination of the *P. pseudocarolinensis* sample from *S. albidum*, we newly determined the *rbcL* sequence of *Sassafras albidum* and compared it with the sequence from GenBank (AF206819). The newly determined sequence was exactly the same as the sequence from GenBank. Thus, because the fossil sequence differs from all other sequences of Lauraceae reported to date, contamination seems highly unlikely.

In both MP and ML analyses, *P. pseudocarolinensis* is placed in the Lauraceae clade (Fig. 5) and grouped with *Sassafras albidum* rather than with extant *Persea* species. However, *Persea* does not form a clade in our analyses (Fig. 5), and its monophyly is not confirmed in recent multigene analyses of the family (Chanderbali et al., 2001). Thus, the failure of the fossil sequence to group with other *Persea* sequences does not invalidate the fossil sequence but instead reinforces evidence from extant species that the circumscription of *Persea* should be reconsidered.

It is also possible that the identification of the Clarkia fossil material as a species of *Persea* is incorrect. The leaf from which the DNA was extracted (Fig. 1b) is typical of most genera of Lauraceae, and the identification of genera and species of Lauraceae is extremely difficult without flowering and fruiting material (Judd et al., 2002). However, the only Lauraceae species reported in the Clarkia fossil bed is *P. pseudocarolinensis* (Rember, 1991). Because *P. pseudocarolinensis* and *Sassafras* were sister in our analysis and because *Sassafras* has both lobed and unlobed leaves (Judd et al., 2002), we cannot exclude the possibility that the Clarkia sample is not really a member of *Persea* but is instead an unreported *Sassafras* fossil with unlobed leaves.

Conclusions—This paper confirms that DNA sequences can be obtained from Miocene-age plant remains, in contrast to the dire predictions of the models of some authors (Lindahl and Andersson, 1972; Lindahl and Nyberg, 1972; Pääbo and Wilson, 1991). Furthermore, the success rate is increased over previous studies (Soltis et al., 1995) through the use of improved methods of DNA extraction and the amplification of small DNA segments of the fossil DNA. Continued experimentation with DNA extraction methods and the sequencing of additional fossils from Clarkia should have tremendous potential for studies of phylogeny, biogeography, and molecular evolution.



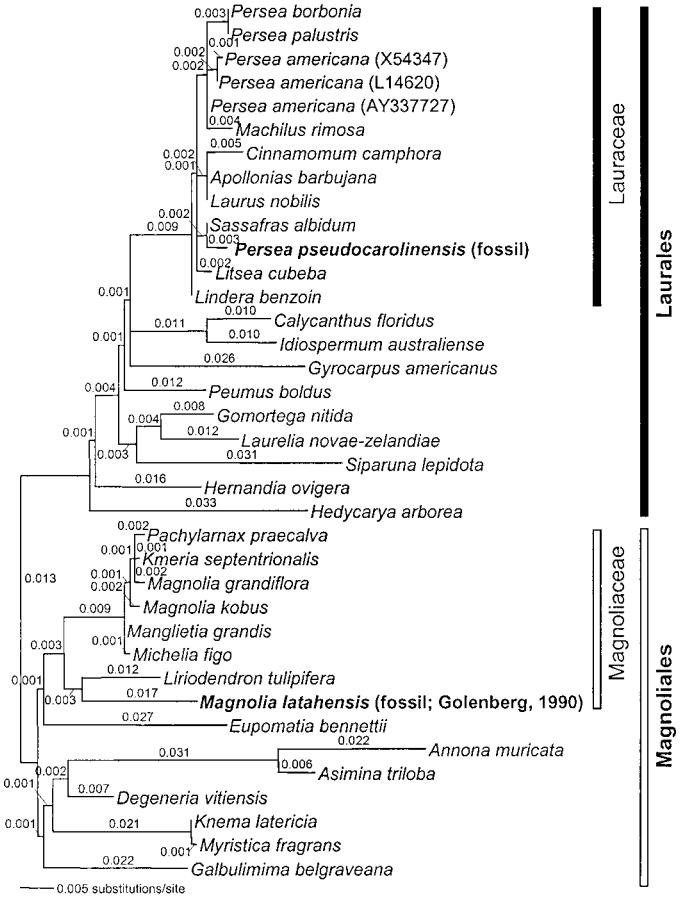


Fig. 5. Maximum likelihood tree based on *rbcL* sequences from fossils and selected taxa of Magnoliales and Laurales (−ln = 4834.73228). Numbers above the branches are substitutions per site.

TABLE 1. Number of base substitutions in 699 bp of *rbcL* sequence between the fossil *Persea pseudocarolinensis* and the corresponding region of extant species of Lauraceae.

Extant taxa (GenBank accessions)	No. of base substitutions
Persea americana (L14620)	4
Persea americana (X14620)	3
Persea americana (AY337727)[a]	4
Persea borbonia (AY337728)[a]	6
Persea palustris (AY337730)[a]	6
Apollonias barbujana (AY337729)[a]	5
Laurus nobilis (AY337731)[a]	5
Litsea cubeba (AY337734)[a]	5
Lindera benzoin (AY337732)[a]	4
Machilus rimosa (AY337733)[a]	5
Sassafras albidum (AF206819)	2

[a] Newly determined sequences in this study.

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Fig. 4. The ''uncorrected *p*'' distance values of *rbcL* were plotted by first–second codon position vs. third codon position for clades of increasing size. Ranges of subfamily Magnolioideae (A), family Magnoliaceae (B), and Magnoliales (C) were marked as circles in the plot of angiosperms (D).

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