

Timing the Eastern Asian–Eastern North American Floristic Disjunction: Molecular Clock Corroborates Paleontological Estimates

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Sequence data of the chloroplast gene *rbcL* were used to estimate the time of the well-known eastern Asian–eastern North American floristic disjunction. Sequence divergence of *rbcL* was examined for 22 species of 11 genera (*Campsis*, *Caulophyllum*, *Cornus*, *Decumaria*, *Liriodendron*, *Menispermum*, *Mitchella*, *Pachysandra*, *Penthorum*, *Podophyllum*, and *Phryma*) representing a diverse array of flowering plants occurring disjunctly in eastern Asia and eastern North America. Divergence times of putative disjunct species pairs were estimated from synonymous substitutions, using *rbcL* molecular clocks calibrated for *Cornus*. Relative rate tests were performed to assess rate constancy of *rbcL* evolution among lineages. Corrections of estimates of divergence times for each species pair were made based on rate differences of *rbcL* between *Cornus* and other species pairs. Results of these analyses indicate that the time of divergence of species pairs examined ranges from 12.56 ± 4.30 million years to recent (<0.31 million years), with most within the last 10 million years (in the late Miocene and Pliocene). These results suggest that the isolation of most morphologically similar disjunct species in eastern Asia and eastern North America occurred during the global climatic cooling period that took place throughout the late Tertiary and Quaternary. This estimate is closely correlated with paleontological evidence and in agreement with the hypothesis that considers the eastern Asian–eastern North American floristic disjunction to be the result of the range restriction of a once more or less continuously distributed mixed mesophytic forest of the Northern Hemisphere that occurred during the late Tertiary and Quaternary. This implies that in most taxa the disjunction may have resulted from vicariance events. However, long-distance dispersal may explain the disjunct distribution of taxa with low divergence, such as *Menispermum*. © 2000 Academic Press

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INTRODUCTION

The eastern Asian–eastern North American floristic disjunction represents one of the most prominent intercontinental disjunctions of closely related species; approximately 65 genera of seed plants display the disjunction (Wen, 1999). This phylogeographic pattern has attracted the attention of botanists since the time of Linnaeus (see Boufford and Spongberg, 1983). During the 120 years since Asa Gray's first comparative study on the subject in 1878, the pattern has been discussed extensively, with emphasis on documenting its occurrence and speculating on its origin and evolution (e.g., Gray, 1878; Hu, 1935; Chaney, 1947; Li, 1952, 1972; Koyama and Kawano, 1964; Graham, 1972; Hara, 1972; Wolfe, 1972, 1975, 1981a, 1981b; 1985; Raven and Axelrod, 1974; Boufford and Spongberg, 1983; Wu, 1983; Tiffney, 1985a, 1985b; Boufford, 1992; Hong, 1993; Guo *et al.*, 1998; Guo, 1999). In general, the floristic disjunction between eastern Asia and eastern North America has been considered to be the result of the fragmentation of a once continuous mixed mesophytic forest that occurred throughout the Northern Hemisphere due to the climatic and geological changes that occurred throughout the late Tertiary and Quaternary (e.g., Li, 1952; see Graham, 1972; Wen, 1999), although long distance dispersal has also been proposed by some authors to account for the observed distributional pattern (e.g., Chaney, 1947). This "range restriction" hypothesis (corresponding to the "Boreotropical Hypothesis" of Wolfe, 1975) is based largely on paleontological evidence that is available for some disjunct taxa occurring in the two areas and has not been tested with other data. Testing the hypothesis requires the understanding of phylogenetic relationships and divergence times of taxa displaying the disjunction. However, phylogenetic relationships and divergence times of most of these taxa have not been investigated despite the long interest in this disjunc-

tion. Only recently have phylogenetic analyses been conducted for taxa displaying this disjunction, and these studies have revealed several important results (see Wen, 1999). First, *Hydrangea* L. (Hydrangeaceae), *Mitella* L. (Saxifragaceae), and section *Rytidospermum* of *Magnolia* L. (Magnoliaceae), long considered classic examples of the eastern Asian–eastern North American floristic disjunction, are each polyphyletic (Soltis *et al.*, 1991, 1995; Soltis and Kuzoff, 1995; Qiu *et al.*, 1995). Thus, some previous biogeographic inferences based on these genera may be erroneous because they involved comparisons of taxa that are not truly closest relatives.

Second, six angiosperm genera (*Cornus* L., Cornaceae; *Boykinia* Nutt., Saxifragaceae; *Tiarella* L., Saxifragaceae; *Trautvetteria* Fischer & C. Meyer, Ranunculaceae; *Calycanthus* Schumann, Calycanthaceae; *Aralia* L., Araliaceae) and one fern genus (*Adiantum* L., Adiantaceae) which have representatives in western North America, as well as in eastern North America and eastern Asia, have all shown that the eastern Asian species is sister to all of the North American species and there is a close relationship between eastern and western North American floras (Wen *et al.*, 1996b, 1998; Xiang *et al.*, 1998a). Studies of genera confined to eastern Asia and eastern North America (e.g., *Aralia* sect. *Dimorphanthus*, Araliaceae; *Diphylleia* Michaux., Berberidaceae; *Panax* L., Araliaceae; *Nyssa* L., Nyssaceae; *Symplocarpus* Salisb. ex W. Barton, Araceae; *Trillium* L., Trilliaceae; *Triosteum* L., Caprifoliaceae) revealed different patterns of species relationships (Wen and Stuessy, 1993; Kato *et al.*, 1995; Wen and Zimmer, 1996; Wen *et al.*, 1996a, 1996b; Gould and Donoghue, 1998; Kim and Jansen, 1998; Wen, 1999). For example, the genus *Diphylleia* (Berberidaceae) was found to be paraphyletic, with *Dysosma* Woodson and *Podophyllum* L. nested within *Diphylleia* (Kim and Jansen, 1998). The eastern North American species *Diphylleia cymosa* Michx is sister to a clade consisting of the Japanese species *D. grayi* F. Schmidt, *Dysosma*, and *Podophyllum*. In *Panax* (Araliaceae) the two eastern North American species, *P. quinquefolius* L. and *P. trifolius* L., do not form a monophyletic group; the former is nested among the eastern Asian species and the latter forms an isolated lineage within the genus (Wen and Zimmer, 1996). In *Symplocarpus* (Araceae) the eastern North American species, *S. foetidus* (L.) Nutt., and one of the two eastern Asian species, *S. renifolius* Schott ex Miq., form a sister pair, which is, in turn, the sister of the second eastern Asian species, *S. nipponicus* Makino (Wen *et al.*, 1996a). In *Nyssa*, morphological relationships among the eight species were found to be more complex (Wen and Stuessy, 1993). Results from these studies indicate a complex basis for the phylogeographic pattern and suggest the need for a phylogenetic analysis of each taxon displaying the disjunction.

The molecular clock concept has always been controversial and deviations from a strict molecular clock have been documented in many studies (e.g., Gaut *et al.*, 1992, 1996; Nickrent and Starr, 1994; Lutzoni and Pagel, 1997; see Gillespie, 1991; Avise, 1994; Soltis and Soltis, 1995; Hillis *et al.*, 1996; Li, 1997). Nonetheless, the molecular clock approach has been used to provide independent estimates of divergence times for testing evolutionary hypotheses in many recent studies (e.g., Savard *et al.*, 1994; Bonatto and Salzano, 1997; Feng *et al.*, 1997; Gaut and Doebley, 1997; Ayala *et al.*, 1998; Bromham *et al.*, 1998; Xiang *et al.*, 1998a,c).

Divergence times based on molecular data have been estimated for several disjunct taxa to provide insights into the timing of the eastern Asian and eastern North American floristic disjunction. These taxa include *Liriodendron* L. (using allozymes and cpDNA restriction sites (RS); Parks and Wendel, 1990); *Liquidambar* L. (using isozymes; Hoey and Parks, 1991), *Campsis* Lour. (using cpDNA RS; Wen and Jansen, 1995); *Symplocarpus* (using cpDNA RS; Wen *et al.*, 1996a), *Magnolia* sect. *Rytidospermum* (using allozyme, cpDNA RS, and *rbcL* sequences; Qiu *et al.*, 1995), *Caulophyllum* Michaux., *Menispermum* L., *Penthorum* L., and *Phryma* L. (using allozymes and ITS sequences; Lee *et al.*, 1996), *Aralia* sect. *Aralia* (using ITS sequences; Wen *et al.*, 1998); and *Boykinia*, *Cornus*, and *Calycanthus* (using cpDNA RS; Xiang *et al.*, 1998a). According to these analyses, levels of molecular divergence vary greatly among different taxa, resulting in different estimates [from 24 million years before present (Ma) between the two species of *Campsis* to ~2 Ma between species of *Magnolia* sect. *Rytidospermum* (based on cpDNA restriction sites)]. In addition, the majority of these estimates omitted a factor of 1/2 in calculating the divergence times (by definition, the divergence time between two lineages is estimated as half of their molecular divergence value divided by the rate of evolution of the molecule; Li, 1997). Furthermore, neither rate constancy tests nor rate-based corrections were performed for the estimates (except those in Xiang *et al.*, 1998a). Nonetheless, putting the rate constancy concern aside, estimation of divergence times in these taxa fall in a range from the mid-Miocene to the Quaternary after correcting for the factor of 1/2 noted above. Additional analyses of divergence times of taxa displaying the disjunction are needed to confirm the timing of the disjunction.

This study performs analyses of divergence times for 11 disjunct taxa using a carefully calibrated *rbcL* molecular clock with corrections for rate difference among lineages. The objectives of this study are dual: (1) to examine *rbcL* sequence divergence for a taxonomically diverse array of taxa occurring in eastern Asia and eastern North America and (2) to estimate the time of the disjunction by independently estimating the time

TABLE 1
Eastern Asian–Eastern North American Species Pairs Analyzed for *rbcL* Sequences

Genus	Habit	Species analyzed (Eastern Asian/ Eastern North American)	Voucher or accession numbers
<i>Campsis</i> (2 spp., Bignoniaceae)	Temperate to subtropical deciduous climbing shrubs	<i>C. grandiflora</i> (Thunb.) Loisel <i>C. radicans</i> (L.) Seem.	J. Wen, NA, US 56522 Spangler and Omstead, unpublished AF 102642
<i>Caulophyllum</i> (2 or 3 spp., Berberidaceae)	Temperate rhizomatous perennial herbs	<i>C. robustum</i> Maxim./ <i>C. thalictroides</i> (L.) Michx.	1.989-3631, RBG, Kew 139.69-0.061, RBG, Kew
<i>Cornus</i> subgen. <i>Mesomora</i> (2 spp., Cornaceae)	Temperate deciduous trees	<i>C. controversa</i> Hemsl./ <i>C. alternifolia</i> L. f.	Arn. Arb. 20458 (WAS) Xiang <i>et al.</i> , 1993
<i>Decumaria</i> (2 spp., Hydrangeaceae)	Deciduous or half evergreen woody vines	<i>D. sinensis</i> Oliv./ <i>D. barbara</i> L.	395-22.39501, RBG, Kew 000-69.50409, RBG, Kew
<i>Liriodendron</i> (2 spp., Magnoliaceae)	Temperate deciduous trees	<i>L. chinense</i> (Hemsl.) Sargent/ <i>L. tulipifera</i> L.	Qui <i>et al.</i> , 1993 Coll. sene, 1093, VA, USA
<i>Menispermum</i> (2 spp., Menispermaceae)	Temperate deciduous vines	<i>M. dauricum</i> DC./ <i>M. canadensis</i> L.	N. S. Lee 69 (OS) N. S. Lee 92 (OS)
<i>Mitchella</i> (2 spp., Rubiaceae)	Temperate to tropical evergreen vines or herbs	<i>M. undulata</i> Sieb. & Zucc./ <i>M. repens</i> L.	Coll. sene, Toyama Pref., Japan Coll. sene, 1093, VA, USA
<i>Pachysandra</i> (3 spp., Buxaceae)	Temperate evergreen or half evergreen ascending small shrubs	<i>P. terminalis</i> Sieb. & Zucc., <i>P. axillaris</i> Franch./ <i>P. procumbens</i> Michx.	D. E. Boufford 25703 (GH) D. E. Boufford 25704 (GH) D. E. Boufford 25702 (GH)
<i>Penthorum</i> (2 spp., Penthoraceae)	Temperate perennial herbs	<i>P. chinensis</i> Pursh./ <i>P. sedoides</i> L.	H. Kato & S. Nakamura 940213, Japan Soltis <i>et al.</i> , 1990
<i>Phryma</i> (1 sp., Phrymaceae)	Temperate perennial herbs	<i>P. leptostachya</i> L. var. <i>asiatica</i> / <i>P. leptostachya</i> L. var. <i>leptostachya</i>	N. S. Lee 119 (OS) N. S. Lee 94 (OS)
<i>Podophyllum</i> (2 spp., Berberidaceae)	Temperate to subtropical rhizomatous perennial herbs	<i>P. emodi</i> Wall. ex Royle/ <i>P. peltatum</i> L.	000-69.18262, RBG, Kew Coll. sene, 1094, VA, USA

Note. Abbreviations "RBS, Kew", "Arn. Arb.", and "NA, US" represent the Royal Botanical Gardens, Kew, the Arnold Arboretum, and the US National Arboretum, respectively. Information regarding number of species, distribution, and habit is from Li (1952), How (1982), Wu (1983), and Boufford and Xiang (1992).

of divergence of the disjunct species using a molecular clock approach.

MATERIALS AND METHODS

Sampling and Sequencing

Eleven pairs of eastern Asian–eastern North American disjunct species representing 11 genera of flowering plants were sampled (Table 1). Almost all of the species pairs sampled belong to genera or subgenera known to be monophyletic that comprise only two species, one in eastern Asia and the other in eastern North America (Li, 1952; How, 1982; Wu, 1983; Wen, 1999). The only exception is *Pachysandra* Michaux. which has three species, one in eastern North America and two in Asia (Boufford and Xiang, 1992). Phylogenetic analysis of *rbcL* sequences for this genus (Xiang *et al.*, unpublished) indicated that *P. procumbens* from eastern North America and *P. axillaris* from China are sister species.

Sequences of *rbcL* were newly obtained for 20 species and those of 2 species were withdrawn from GenBank (see Table 1). DNA isolation, PCR amplification, and sequencing followed Xiang *et al.* (1993). Sequence divergence and standard errors between each species pair were estimated for synonymous, nonsynonymous, and total substitutions using the Jukes–Cantor model available on MEGA 1.0 (Kumar *et al.*, 1993).

Rate Estimation

Molecular clocks for the chloroplast gene *rbcL* have been estimated by different authors. A rate of 1.3×10^{-9} synonymous substitution per site per year was estimated for dicots by Zurawski and Clegg (1987), based on sequence comparisons between spinach and tobacco. An average rate of $1.0 \pm 0.33 \times 10^{-10}$ total substitution per site per year was estimated for woody plants by Albert *et al.* (1994), and a rate of $4.80 \pm 0.9 \times 10^{-11}$ nonsynonymous substitution per site per year was estimated for all land plants by Savard *et al.*

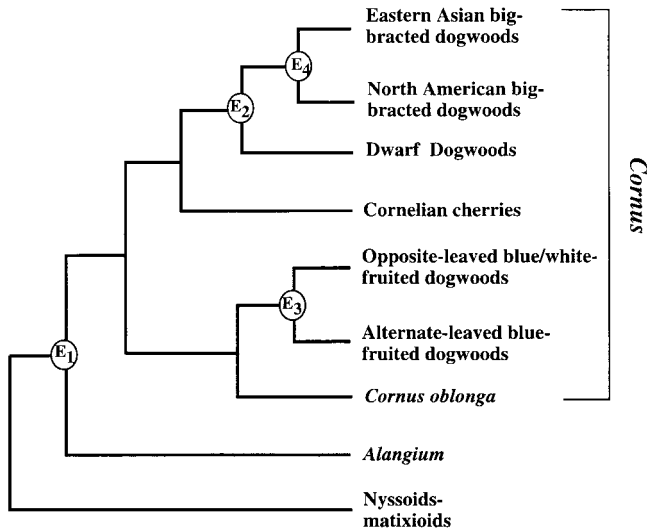


FIG. 1. Schematic phylogeny of *Cornus* derived from cpDNA restriction site data and DNA sequences of *rbcL* and *matK* (from Xiang *et al.*, 1993, 1996, 1998). Landmark events (E₁–E₄) used for constructing *rbcL* molecular clocks are indicated.

(1994). First, because *rbcL* is a conservative and functionally highly constrained gene (Albert *et al.*, 1994; Davis *et al.*, 1998), the nonsynonymous substitutions are likely to be nonclocklike in closely related lineages; thus, nonsynonymous substitutions and perhaps even total substitutions of *rbcL* may not be suitable for estimating divergence times for recently diverged lineages. Second, *rbcL* in different lineages may evolve at different rates. Third, these *rbcL* clocks may not be accurate due to the difficulties in determining the divergence times for the taxa used in the clock calibration.

To minimize these problems, we made a new molecular clock calibrated on landmark events from one of the genera with species pairs sampled in this study, the dogwood genus *Cornus*, and assessed rate heterogeneity among lineages (see below). The estimates of divergence time between disjunct species were further corrected based on the rate differences between *Cornus*

and other disjunct species pairs. *Cornus* was chosen for constructing a local *rbcL* clock for two reasons. First, there is a robust phylogenetic hypothesis derived from molecular data for the genus (Fig. 1; Xiang *et al.*, 1996, 1998b). Second, there is an adequate fossil record for subgroups that can be reliably identified (see Eyde, 1988). According to Crane *et al.* (1990), the oldest unquestionable fossil leaves and fruits of *Cornus* are from the late Paleocene (~58 Ma); the oldest fossils of the big-bracted dogwoods are from the mid-Eocene (~44 Ma; Manchester, 1994) while those of the alternate-leaved blue-fruited dogwoods are from the late Miocene (~8 Ma), and those of the Asian big-bracted dogwoods are from the latest Miocene (Eyde, 1988). The oldest fossil of *Alangium*, the sister group of *Cornus*, is from the early Eocene (~55–56 Ma) (Taylor, 1990; S. R. Manchester, personal observation).

Based on the molecular phylogeny of *Cornus* and Cornaceae inferred from cpDNA restriction site variation and DNA sequences of *rbcL* and *matK* (Xiang *et al.*, 1996, 1998b) (see Fig. 1), as well as the fossil evidence, the divergence time between *Cornus* and its sister genus *Alangium* (E₁) can be conservatively estimated at approximately 58 Ma (in the late Paleocene), based on the earliest records of both genera confirmed by their fossil fruits (Eyde, 1988; Crane *et al.*, 1990). The divergence time between the big-bracted dogwoods and their sisters, the dwarf dogwoods (E₂), can be estimated at 44 Ma (in the mid-Eocene), based on the earliest record of fruit stones from the Clarno Formation of Oregon (Manchester, 1994). That between the alternate-leaved blue-fruited dogwoods and the opposite-leaved blue- or white-fruited dogwoods (E₃) can be estimated at 6–10 Ma (in the late Miocene), and between the Asian and the North American big-bracted dogwoods (E₄) also at 6–10 Ma, based on the earliest record of fruit stones of the alternate-leaved blue-fruited dogwoods and the Asian big-bracted dogwoods (Eyde, 1988). These four landmarks were used to construct molecular clocks for *rbcL* at the synonymous sites, at nonsynonymous sites, and at all sites. The clocks or rates (*R*) were estimated as half of the *rbcL*

TABLE 2

Estimation of Molecular Clocks for *rbcL* in *Cornus*

Landmark event	Divergence time (Ma)	No. of nucleotide substitutions per site			Substitution rate per site per year		
		<i>Ks</i>	<i>Ka</i>	<i>Kt</i>	<i>Rs</i>	<i>Ra</i>	<i>Rt</i>
E ₁	58	0.1185 ± .0199	0.0176 ± .0041	0.0465 ± .0056	1.02 × 10 ⁻⁹	1.52 × 10 ⁻¹⁰	4.00 × 10 ⁻¹⁰
E ₂	44	0.1267 ± .0208	0.0086 ± .0029	0.0364 ± .0052	1.42 × 10 ⁻⁹	0.98 × 10 ⁻¹⁰	4.14 × 10 ⁻¹⁰
E ₃	8	0.0219 ± .0082	0.0123 ± .0040	0.0184 ± .0036	1.37 × 10 ⁻⁹	7.68 × 10 ⁻¹⁰	11.5 × 10 ⁻¹⁰
E ₄	8	0.0185 ± .0076	0.0127 ± .0035	0.0146 ± .0033	1.12 × 10 ⁻⁹	7.93 × 10 ⁻¹⁰	9.13 × 10 ⁻¹⁰
		Average: <i>Rs</i> = 1.23 ± 0.128 × 10 ⁻⁹					

Note. E₁, *Cornus*–*Alangium* split; E₂, big-bracted–dwarf dogwoods split; E₃, alternate-leaved–opposite-leaved blue-fruited dogwoods split; E₄, eastern Asian big-bracted–eastern North American big-bracted dogwoods split.

sequence divergence value (K) between two sister lineages divided by the divergence time (T) between the two lineages (Table 2). The sequence divergence value between two sister lineages was estimated as the average of all pairwise sequence divergence values between species from the two different clades sampled in the *rbcL* phylogeny.

The rates of synonymous substitutions estimated using the four landmark events are very close (Table 2) and their average ($R_s = 1.23 \pm 0.128 \times 10^{-9}$ substitution per site per year) is very close to Zurawski and Clegg's (1987) estimate. However, the estimated rates of total base substitutions (synonymous and nonsynonymous) (K_t) derived from E_1 and E_2 differ by a factor of 2–3 times from those derived from E_3 and E_4 , and those of nonsynonymous base substitutions derived from the four separate landmarks differ dramatically (see Table 2). These results further confirm that nonsynonymous substitutions may not evolve in a clock-like fashion. Therefore, we decided to use only synonymous substitution rate to estimate time of divergence.

Relative Rate Tests

Before constructing the molecular clocks for *Cornus*, lineage relative rate tests were performed to assess rate constancy between the lineages providing landmark events, using the method of Li and Bousquet (1992) employing Nei and Gojobori (1986) distance and a computer program provided by B. S. Gaut (University of California, Irvine, CA). For each test, one to three species from the sister group of the lineage pair under comparison were chosen as the reference group based on the molecular phylogeny of *Cornus* (Fig. 1). Lineages with a significantly different rate may be removed from use in calibrating the clock. Results of these relative rate tests indicated no significant heterogeneity in synonymous substitution rates of *rbcL* evolution between any two lineages of all four pairs of taxa used to provide time marks for molecular clock construction (also see Xiang *et al.*, 1998b).

Approximate rate constancy among the disjunct species pairs was also assessed using relative rate tests. The *rbcL* sequence of *Nymphaea* L., one of the first-branching angiosperms suggested by 18S rDNA sequence data (Soltis *et al.*, 1997) and by a combined analyses of *rbcL*, 18S rDNA, and *atpB* sequences (Soltis *et al.*, unpublished), was used as the reference sequence for these relative rate tests. For synonymous and nonsynonymous substitutions, relative rate tests were performed using Li and Bousquet's (1992) method, and for total substitutions, the tests were conducted following a simple method of Li (1997). Li's (1997) method compares the sequence divergence values between the two lineages under test to their standard errors. If the difference between the two sequence divergence values is equal to or greater than twice the

standard error, the rate difference in the two lineages is considered to be significant at the 0.05 level.

Estimation of Time of Divergence

For all species pairs, the divergence time between the two species was estimated using the estimated synonymous substitution rate of $R_s = 1.23 \pm 0.128 \times 10^{-9}$ substitution per site per year. The divergence time between the two species of each pair was estimated as half of their sequence divergence value divided by the rate. The standard errors of the divergence time were estimated as half of the standard error of the sequence divergence value divided by the rate. These estimates of time of divergence were then further corrected for each species pair according to its rate difference in *rbcL* compared to that of *Cornus*.

RESULTS

Sequence Data

Among the 22 species examined, the length of *rbcL* ranges from 1428 to 1464 bp (Table 3). No base substitutions were observed between the two species of *Menispermum*. Sequence divergence values between the remaining species pairs range from 0.0031 to 0.0254 for synonymous substitutions, from 0.0009 to 0.0061 for nonsynonymous substitutions, and from 0.0021 to 0.0081 for total substitutions (see Table 3; values for nonsynonymous substitutions are not presented).

Relative Rates

Rates of *rbcL* evolution estimated using *Nymphaea* as the reference vary with lineages; however, relative rate tests indicated that the differences among species pairs are mostly not significant (Table 4), with exceptions in *Liriodendron* (Magnoliaceae) and *Mitchella* L. (Rubiaceae). For example, the synonymous substitution rate is significantly lower in *Liriodendron*, but significantly higher in *Mitchella*; for total substitutions, *Liriodendron* has a significantly slower rate than other species pairs, while *Mitchella* has a significantly faster rate than *Menispermum*, *Decumaria* L., *Caulophyllum*, and *Pachysandra* (Table 4).

Comparing rates in *Cornus* and other species pairs, there are no significant differences, except in a couple of cases. The synonymous substitution rate is slower in *Liriodendron* than in *Cornus*, while it is higher in *Mitchella* than in *Cornus* (Table 4). *Liriodendron* has a significantly slower rate in total substitutions than *Cornus* (Table 4).

Time of Divergence

Divergence times estimated based on synonymous substitutions for the disjunct species pairs vary among lineages, with a range from recent (<0.28 Ma) (in *Menispermum*, in which no sequence divergence was

TABLE 3

**Estimates of Time of Divergence for Disjunct Species Pairs from Eastern Asia and Eastern North America,
Using an *rbcL* Clock Calibrated from *Cornus***

Species pairs	<i>rbcL</i> length (bp)	Base substitutions per site		Time of divergence (Ma) based on <i>Ks</i>
		<i>Ks</i>	<i>Kt</i>	
<i>Campsis</i> (<i>gran./radi.</i>)	1440	0.0090 ± 0.0052	0.0072 ± 0.0023	3.62 ± 2.10 (3.78 ± 2.07)
<i>Caulophyllum</i> (<i>thal./roba.</i>)	1434	0.0059 ± 0.0042	0.0028 ± 0.0014	2.38 ± 1.69 (2.47 ± 1.76)
<i>Cornus</i> (<i>cont./alte.</i>)	1428	0.0121 ± 0.0061	0.0043 ± 0.0018	4.88 ± 2.46
<i>Decumaria</i> (<i>sine./barb.</i>)	1428	0.0059 ± 0.0042	0.0021 ± 0.0012	2.38 ± 1.69 (2.57 ± 1.83)
<i>Liriodendron</i> (<i>chin./tuli.</i>)	1431	0.0104 ± 0.0056	0.0072 ± 0.0023	4.19 ± 2.26 (6.15 ± 3.31)
<i>Menispermum</i> (<i>daur./cand.</i>)	1428	0.0000 ± 0.0000	0.0000 ± 0.0000	<0.28 (<0.31)
<i>Mitchella</i> (<i>undu./repe.</i>)	1452/1464	0.0146 ± 0.0059	0.0070 ± 0.0020	5.89 ± 2.38 (4.54 ± 1.84)
<i>Pachysandra</i> (<i>axil./proc.</i>)	1455	0.0254 ± 0.0087	0.0063 ± 0.0022	10.24 ± 3.51 (12.49 ± 4.28)
<i>Penthorum</i> (<i>chin./sedo.</i>)	1428	0.0121 ± 0.0061	0.0073 ± 0.0023	4.88 ± 2.46 (5.13 ± 2.59)
<i>Podophyllum</i> (<i>emod./pelt.</i>)	1428	0.0172 ± 0.0079	0.0081 ± 0.0025	6.94 ± 3.19 (6.71 ± 3.08)
<i>Phryma</i> (<i>asia./lept.</i>)	1458	0.0145 ± 0.0066	0.0056 ± 0.0020	5.85 ± 2.66 (5.45 ± 2.47)
Average		0.0141 ± 0.0055	0.0060 ± 0.0018	4.98 ± 2.39 (5.41 ± 2.62)

Note. Bracketed numbers represent time of divergence estimated with corrections of relative rates between *Cornus* and each other species pair. *Ks* is number of synonymous substitutions per site and *Kt* is number of total substitutions per site. Names of species are abbreviated in parentheses following the genus names. For complete names of species, see Table 1.

observed; at least 0.28 Ma is required to have one nucleotide substitution occur between the two species according to the molecular clock) to 10.24 ± 3.51 Ma (the mid-Miocene; in *Pachysandra*). The overall average for all comparisons is 4.98 ± 2.39 Ma (the late Miocene through Pliocene) (Table 3). Corrections for rate differences between *Cornus* and other species pairs change the estimates of divergence times slightly (see Table 3), ranging from <0.31 to 12.49 ± 4.28 Ma (average 5.40 ± 2.62 Ma).

DISCUSSION

Evolution of *rbcL*

Rates of *rbcL* evolution seem to be fairly constant among the lineages of dicots examined based on the relative rate tests, a result similar to that found for Cornales (Xiang *et al.*, 1998b). Significantly different rates were observed in *Mitchella* and *Liriodendron*. Relative rate tests suggest that evolution of *rbcL* has accelerated in *Mitchella* and slowed in *Liriodendron* compared to that in other dicots. *Mitchella* is an evergreen trailing herb and *Liriodendron* is a large, long-lived deciduous tree. Therefore, the rate changes in these taxa may reflect generation time, as has been

suggested for monocots and other lineages (Gaut *et al.*, 1992, 1996; Soltis and Soltis, 1995; Hillis *et al.*, 1996; Li, 1997).

Divergence Times and Geographic Isolations

The time of divergence of each species pair provides a rough estimate of the time of geographic isolation of the two species. Based on *rbcL* sequence data, estimated divergence times of the species pairs differ among lineages, with a range from less than 0.31 to 12.49 ± 4.28 Ma, spanning the mid-Miocene to the Quaternary, with most estimates <10 Ma, in the late Miocene and Pliocene (Fig. 2; Table 3). This suggests that the isolation of these species pairs occurred in different geological times in different lineages, but did not start until the mid-Miocene. For all species except those of *Pachysandra*, which appear to have been isolated in the mid-Miocene, the isolation events occurred in the late Miocene or later.

It is noteworthy that divergence times of disjunct species pairs estimated earlier using other molecular data also fall in this range if these estimates are corrected for the factor of 1/2 (see Introduction). These include *Liriodendron* (Parks and Wendel, 1990), *Campsis* (Wen and Jansen, 1995), *Symplocarpus* (Wen

TABLE 4
Results of Relative Rate Tests for *rbcL*

OTUs	1	2	3	4	5	6	7	8	9	10	11
1		-0.086	-0.101	-0.054	-0.126	-0.194	-0.077	-0.089	-0.030	-0.094	-0.112
2	-0.0245		-2.735	-1.628	-3.097	-4.055	-2.141	-2.401	-0.898	-2.375	-3.640
3	±0.0087			0.032	-0.040	-0.108	0.009	-0.004	0.055	-0.009	-0.026
4	-0.0235	0.0010	-0.454		0.863	-1.139	-2.419	0.261	-0.098	1.560	-0.253
5	±0.0087	±0.0094		0.048	-0.024	-0.092	0.025	0.012	0.057	0.007	-0.011
6	-0.0151	0.0095	0.0085		1.324	-0.664	-2.173	0.889	0.320	1.545	0.208
7	±0.0084	±0.0091	±0.0091	-0.072	-1.902	-2.972	-0.641	-1.139	0.649	-1.052	-1.643
8	-0.0269	-0.0024	-0.0033	-0.0032		-0.068	0.049	0.037	0.095	0.032	0.014
9	±0.0088	±0.0095	±0.0095	±0.0092		-1.665	1.446	0.954	2.546	1.653	0.337
10	-0.0420	-0.0175	-0.0185	-0.0270	-0.0152		0.117	0.105	0.163	0.100	0.082
11	±0.0092	±0.0099	±0.0099	±0.0096	±0.010		2.929	2.184	3.519	2.578	1.968
1	-0.0124	0.0122	0.0112	0.0027	0.0145	0.0290		-0.013	0.045	-0.017	-0.035
2	±0.0083	±0.0091	±0.0090	±0.0088	±0.0092	±0.0095		-0.352	1.465	-0.541	-0.937
3	-0.0200	0.0045	0.0036	-0.0050	0.0069	0.0220	-0.0077		0.059	-0.005	-0.023
4	±0.0086	±0.0093	±0.0093	±0.0090	±0.0094	±0.0098	±0.0089		1.658	-0.122	-0.560
5	-0.0093	0.0153	0.0143	0.0009	0.0176	0.0328	0.0031	0.0108		-0.064	-0.068
6	±0.0083	±0.0090	±0.0090	±0.0086	±0.0091	±0.0095	±0.0087	±0.0089		-1.774	-1.948
7	-0.0232	0.0013	0.0035	-0.0131	0.0037	0.0188	-0.0109	-0.0032	-0.0140		-0.021
8	±0.0087	±0.0094	±0.0094	±0.0090	±0.0095	±0.0099	±0.0090	±0.0093	±0.0090		-0.508
9	-0.0259	-0.0014	-0.0024	-0.0158	0.0010	0.0161	-0.136	-0.0059	-0.0167	0.0027	
10	±0.0089	±0.0096	±0.0096	±0.0093	±0.0097	±0.0101	±0.009	±0.0095	±0.0092	±0.0096	

Note. 1, *Liriodendron*; 2, *Penthorum*; 3, *Cornus*; 4, *Menispermum*; 5, *Phryma*; 6, *Mitchella*; 7, *Decumaria*; 8, *Caulophyllum*; 9, *Pachysandra*; 10, *Campsis*; 11, *Podophyllum*. Top numbers in the upper-right portion of the matrix are pairwise differences of number of synonymous substitutions per site between OTUs in the column and those in the row and bottom numbers are test statistics. An absolute value of 1.96 of the test statistic indicates that the difference in synonymous substitution rates between the two lineages is significant at 0.05. Top numbers in the lower-left portion of the matrix are pairwise differences of number of total substitutions per site between OTUs in the row and those in the column and bottom numbers are standard errors. Boldface type indicates comparisons that are statistically significant at the 0.05 level.

et al., 1996a), *Magnolia* sect. *Rytidospermum* (Qiu *et al.*, 1995), *Caulophyllum*, *Menispermum*, *Penthorum*, and *Phryma* (Lee *et al.*, 1996), *Aralia* sect. *Aralia* (Wen *et al.*, 1998); and *Calycanthus* (Xiang *et al.*, 1998a). Thus, the time of disjunction of the eastern Asian and eastern North American floras estimated using *rbcL* sequence data are congruent with previous estimates using other molecular data in suggesting that the floristic exchanges between eastern Asia and eastern North America continued into the mid-Miocene; gene flow in most disjunct taxa did not cease until the late Miocene or later due to geographic isolation.

These molecular clock data are remarkably well correlated with the paleontological evidence and in agreement with the "range restriction" hypothesis regarding the timing of the eastern Asian-eastern North American floristic disjunction. According to paleontological evidence, the widespread mixed mesophytic forest developed from the late Oligocene to the early Miocene and expanded in the mid-Miocene (Tiffney, 1985a, 1985b). Exchange of plant elements between North America and eastern Asia may have occurred into the Miocene via the North Atlantic Bridge and into the late Miocene and even into the latest Tertiary for cool-adapted elements via the Bering Strait (see Raven and

Axelrod, 1974; Tiffney, 1985a, 1985b; Wolfe, 1985; Cunningham and Collins, 1994). Exchanges within the North American continent were possible via forest corridors until the late Miocene or even later for some herbaceous taxa (Graham, 1993). Paleontological evidence indicates a dramatic decrease of temperature in the Northern Hemisphere since the mid-Miocene. The cordillera in western North America became an effective barrier to biotic interchange between eastern and western North America in the mid-Miocene, but complete isolation of the two floras was a gradual process throughout the late Miocene and Quaternary (Graham, 1993). In the late Miocene, the reduced rainfall and lowered minimum winter temperatures caused by the midcontinental rain shadow to the east of the Rocky Mountains further restricted woody vegetation to the valley habitats and favored the development of herbaceous vegetation. Development of extensive grasslands began at the Miocene-Pliocene transition (7–5 Ma), the driest part of the Tertiary, which restricted forests and woodlands. The true prairie did not develop extensively until the Quaternary. The appearance of this widespread prairie vegetation in midcontinental North America plus the sclerophyllous and coniferous vegetation in western and northern North

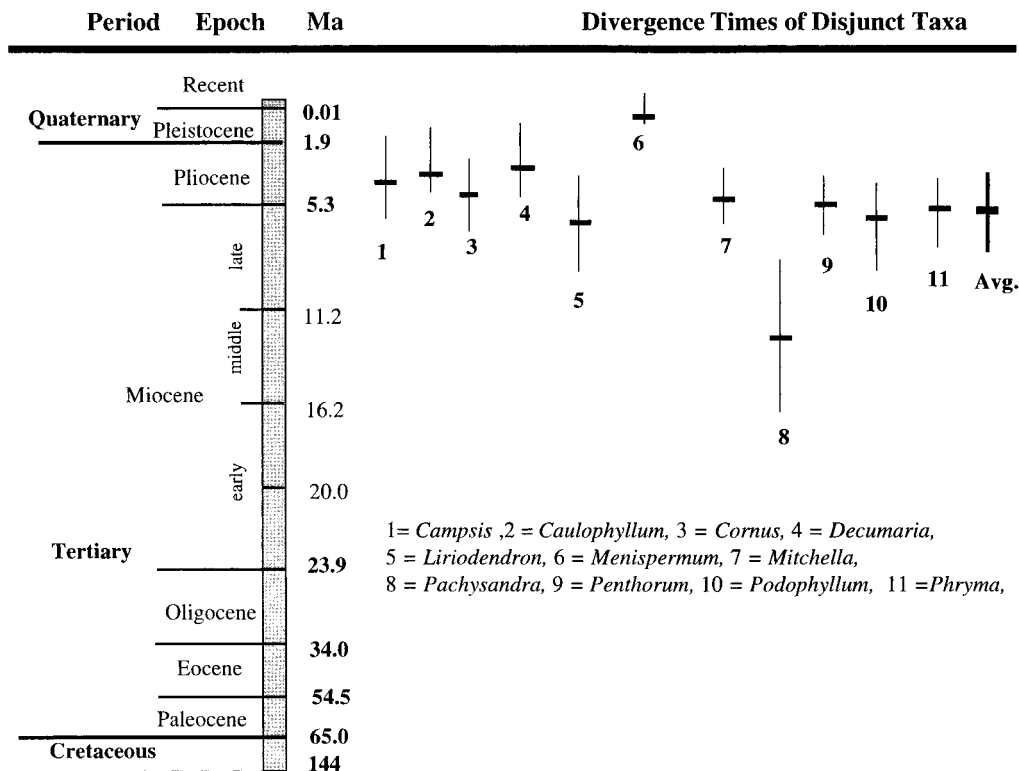


FIG. 2. Geological time scale (followed John Alroy, <http://www.nceas.ucsb.edu/~lroy/TimeScale.html> and Berggren *et al.*, 1995) and distribution of divergence times of disjunct species pairs estimated based on *Ks* presented in Table 3.

America expanding in the late Tertiary caused the major disruption of the mixed mesophytic forest (Graham, 1993).

According to fossil evidence, *Liriodendron*, *Pachysandra*, *Cornus*, and *Menispermum* had a wider and more or less continuous distribution in the past (Bůžek *et al.*, 1976; Wolfe and Wehr, 1987; Baghai, 1988; see Parks and Wendel, 1990; Taylor, 1990; Budantsev, 1997; Meyer and Manchester, 1997; also see review in Manchester, 1999; Plant Fossil Record at <http://ibs.uel.ac.uk/palaeo-bin/pfrocc.pl>). Unequivocal leaf and fruit fossils representing the extant *Liriodendron* are found in North America in the Miocene of Idaho (Baghai, 1988). Leaf fossils of *Liriodendron* with cuticle like that of the extant North American species *L. tulipifera* L. are found in the Oligocene of Markvartice (Bůžek *et al.*, 1976). The latest European record of the genus was upper Pliocene (Wilde *et al.*, 1992). The genus was also present in Japan as *L. honshuensis* Endo and *L. fukushimaensis* Suzuki in the middle and late Miocene (Uemura, 1988). Buxaceae pollen of the *Pachysandra* type was found in British Columbia, Canada in the Oligocene (see Taylor, 1990). Fossils of the alternate-leaved blue-fruited dogwoods (*Cornus controversa* Hemsl. and *C. alternifolia* L. f.) were found in Europe in the late Miocene and those of the extant Asian species *C. controversa* were found in Japan in

the Pliocene and Quaternary (Eyde, 1988). Endocarps morphologically identical to those of extant *Menispermum* are known from the Oligocene of Oregon (Meyer and Manchester, 1997). Although relationships of these fossil species to the extant species are not clear, all of this evidence suggests that these genera may have evolved and obtained a more or less continuous distribution in the Northern Hemisphere before the isolation of species in eastern Asia and eastern North America occurred; the restriction of their distributions (or the divergence of the eastern Asian–North American taxa) may have occurred in the late Miocene for *Liriodendron*, in the Pliocene for *Cornus*, and not earlier than the Miocene for *Pachysandra* and *Menispermum*. This scenario is well correlated with the inference from molecular data, except for *Menispermum* (Table 3), and suggests a vicariance origin for the disjunct species in these genera.

Molecular data from *rbcL* sequences suggested that the two species of *Menispermum* diverged very recently (<0.31 Ma). Comparative ITS sequencing (Lee *et al.*, 1996) also revealed a low level of sequence divergence between the two species. This evidence suggests that *Menispermum* may have experienced remarkably slow rates of molecular evolution. Alternatively, the genus may have obtained its disjunct distribution recently

via long-distance dispersal from North America to eastern Asia.

The fossil records of Araceae (containing *Symplocarpus*), Bignoniaceae (containing *Campsis*), Berberidaceae (containing *Podophyllum* and *Caulophyllum*), *Hydrangea* (Hydrangeaceae containing *Decumaria*), and Rubiaceae (containing *Mitchella*) date back to the Eocene (see Tiffney, 1985a; Manchester, 1987, 1999). However, reliable fossil records for the particular genera displaying the disjunction are lacking. According to Tiffney (1985a), *Symplocarpus* (representing monocots) may have obtained a wide distribution in the Northern Hemisphere in the pre-Tertiary time; *Mitchella*, *Podophyllum*, and *Caulophyllum* (representing plants of the forest floor) may have obtained their wide distribution in the early Eocene and *Campsis*, *Decumaria*, and *Penthorum* (representing deciduous woody vines and perennial herbs) in the Miocene, although fossils of these taxa are not preserved.

CONCLUSIONS

Molecular analyses of these diverse groups of angiosperms indicate that the divergence times for species with the eastern Asian–eastern North American disjunct pattern range from the mid-Miocene to recent. Most of them appear to have diverged in the late Miocene and Pliocene. This time frame is highly correlated with paleontological evidence and in agreement with previous hypotheses that the floristic disjunction in eastern Asia and eastern North America was mainly a result of fragmentation, starting in the late Tertiary, of the mixed mesophytic forest once widespread in the Northern Hemisphere. The Bering Strait may have played an important role in supporting the floristic exchange between eastern Asia and North America in the Miocene and later geological times.

The estimate of isolation time for each species pair provided in the study should be viewed as approximate for two reasons. First, the time for the landmark events used for calibrating the clocks could have been slightly underestimated because the actual divergence time between two lineages could be earlier than the age of the earliest fossils. The underestimate of time for the landmark events would result in a faster molecular clock and lower estimate of time of divergence of the species pair. Second, extinction of closely related species of extant taxa could have occurred in both continents in any of the genera examined, resulting in an overestimate of time of disjunction in the genus. Nonetheless, the present study adds another example demonstrating the usefulness of molecular data and a molecular clock approach in testing biogeographic hypotheses, when used with caution.

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