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PHYLOGENY AND BIOGEOGRAPHY OF *JUGLANS* (JUGLANDACEAE) BASED ON *matK* AND ITS SEQUENCE DATA[1]

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We investigated phylogenetic and biogeographic relationships within *Juglans* (walnuts), a Tertiary disjunct genus, using 15 species of *Juglans* and related (Juglandaceae) outgroups. The relationships were analyzed using nucleotide sequences of the chloroplast gene *matK* and its flanking spacers and of the internal transcribed spacers (ITS) and 5.8S gene of the nuclear ribosomal DNA. The DNA sequences provided 246 informative characters for parsimony analysis. ITS data supported as monophyletic groups the four generic sections, *Cardiocaryon, Dioscaryon, Rhysocaryon,* and *Trachycaryon.* Within *Rhysocaryon,* the temperate black walnuts and the tropical black walnuts were supported as monophyletic groups. When the two data sets were combined, *J. cinerea* was nested within *Cardiocaryon.* Combined analysis with published nuclear DNA restriction site data placed *J. cinerea* in a monophyletic group with *Cardiocaryon.* These analyses consistently supported *Juglans* as a monophyletic group and as the sister group to the genus *Pterocarya.* The results of this work are consistent with the known geological history of *Juglans.* The fossil record suggests that the butternuts had evolved by the early Oligocene in North America. The presence of butternuts in Eurasia could be the result of migration from North America to Eurasia during the warming trend of the mid Oligocene.

Key words: ITS; Juglandaceae; *Juglans*; *matK*; phylogeny; Tertiary disjunct; walnut.

The study of the floristic similarity among the temperate forests of North America, Europe, and Asia dates back to the 18th century, when Halenius, a student of Linnaeus, defended a dissertation on the similarity of nine eastern North American and eastern Asian plants (Boufford and Spongberg, 1983). An array of early botanists (including Halenius, 1750; Kalm, 1770; Nuttall, 1818; Pursh, 1814; Thunberg, 1784) also recognized the floral similarity of the two regions. Asa Gray (1856, 1859, 1873), encouraged by his correspondence with Charles Darwin (Darwin, 1887, 1903; Gray, 1893), studied the disjunction from the 1850s on into the 1870s. Asa Gray felt that the contraction of a once widespread temperate flora had caused the similarity between the Asian and North American floras. Later scientists (Axelrod, 1959; Chaney, 1940, 1947) recognized that this disjunction spanned all the temperate forests of the northern hemisphere, and expanded on Gray's theory. Axelrod and Chaney believed that a temperate Arcto-Tertiary Geoflora, found throughout the high northern latitudes during the Eocene (50 million years before present), had migrated southward and been restricted in range as a result of a gradual, global cooling and drying trend. Scientists (Yurtsev, 1972; Wolfe, 1978; Tiffney, 1985a, b) now know that a complex history of tectonics, climate fluctuations, repeated emergence and submergence of land bridges, multiple migrations, and other factors have led to the current distribution of northern temperate forests, which were contiguous during the Miocene (15 million years before present), when the northern continents were themselves in contact (Davis, 1981; Tiffney, 1985a, b; Delcourt and Delcourt, 1987).

The purpose of this study was to examine the biogeography of the genus *Juglans* L. as a function of *Juglans*' phylogeny, as well as to address disagreement about the taxonomy of *Juglans. Juglans* is a Tertiary disjunct with ~21 species (Manning, 1978) occurring from temperate northern China to the arid southwestern United States to the cloud forests of tropical South America. Its range (Fig. 1) includes eastern and western Asia, southern Europe, eastern and western North America, Central America, western South America, and the West Indies (Meusel, Jager, and Weinert, 1965; Wilken, 1993). Walnuts are among the most economically important nut trees in the world, and two species, *J. nigra* L. (eastern black walnut) and *J. regia* L. (Persian walnut), are widely cultivated for this reason (Jaynes, 1969; Woodruff, 1979). *Juglans nigra* is also important in commercial wood production. Most walnut species, however, are of low economic value and are used only occasionally as timber or as a source of brown dye. Consequently, certain species of *Juglans* have been well studied, while little is known about others. This study includes three taxa not included in previous molecular studies of the genus: *J. boliviana* (C. DC.) Dode, *J. guatemalensis* Mann., and *J. cathayensis* var. *formosanum* Hayata.

Earlier authors (Dode, 1906, 1909a, b; Miller, 1976; Manning, 1978; Fjellstrom and Parfitt, 1995; Whittemore and Stone, 1997) have been largely in agreement about

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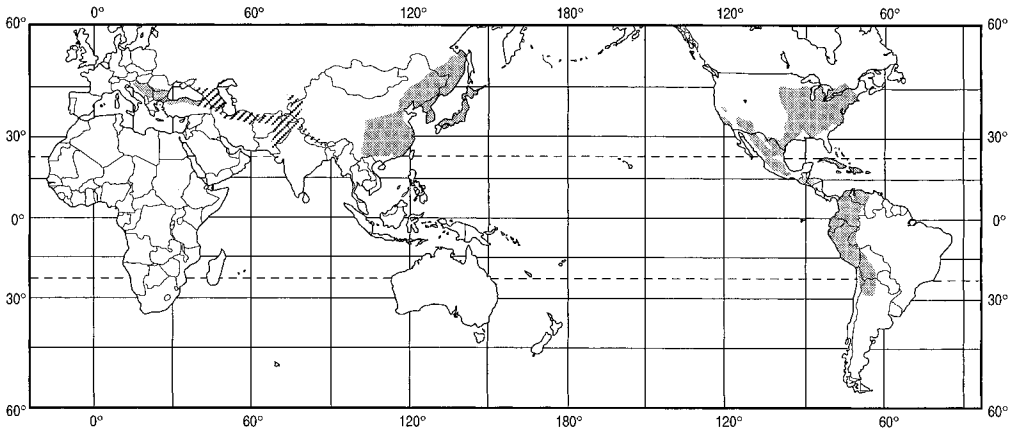

Fig. 1. The worldwide distribution of *Juglans.* Although the distribution of *J. regia* extends as far east as western China, its natural range is unclear because of a long history of cultivation. The hatched area represents the part of its distribution that may not be part of its natural range.

the classification within *Juglans.* In the only published revision of the genus, Dode (1906, 1909a, b) divided *Juglans* into four sections based on leaf and flower morphology: *Dioscaryon* Dode (traditionally *Juglans*), *Rhysocaryon* Dode (black walnuts), *Cardiocaryon* Dode (Asian butternuts), and *Trachycaryon* Dode (American butternut). The four sections were also supported by Manning's (1978) work on fruit and nut morphology. Manning did disagree with Dode about the number of species in the genus. For instance, within *Rhysocaryon,* Manning (1978) recognized *J. californica* S. Wats. and *J. major* (Torr. ex Sitsgr.) Heller as distinct species, while Dode (1909b) considered them to be synonymous. Another species of interest is *J. guatemalensis,* which Manning named as a new species (Manning, 1952), but later merged with *J. olanchana* Stadl. et L. O. Williams (Manning, 1957). All four of the aforementioned taxa (*J. californica, J. major, J. guatemalensis,* and *J. olanchana*) have been included in this study.

Dioscaryon contains just one species, *J. regia* (Persian walnut), which is native to southeastern Europe and western Asia Minor. The 16 species of black walnuts, section *Rhysocaryon,* are found only in America. Based on differences in wood anatomy between the black walnuts of the United States and northern Mexico and the tropical black walnuts of southern Mexico, Guatemala, and South America, Miller (1976) suggested dividing *Rhysocaryon* into two groups, the north-temperate black walnuts and the tropical black walnuts. The butternuts are divided into two sections, the Asian section *Cardiocaryon,* containing three species native to China, Korea, and Japan and the American section *Trachycaryon,* consisting solely of *J. cinerea* L. of the eastern United States (Dode, 1906, 1909a, b; Manning, 1978). Miller (1976) also found evidence from wood anatomy supporting the placement of all the butternuts into one group, but he did not suggest uniting the butternuts in a single section. The recent nuclear RFLP (restriction fragment length polymorphism) work of Fjellstrom and Parfitt (1995) supported *Dioscaryon* (or *Juglans*), *Rhysocaryon,* the butternuts (*Cardiocaryon* plus *Trachycaryon*), *Cardiocaryon,* and *Trachycaryon* as monophyletic groups. The molecular study (Fjellstrom and Parfitt, 1995) did not support the temperate black walnuts or the tropical black walnuts as monophyletic lineages.

According to various authors (Heimsch and Wetmore, 1939; Conde and Stone, 1970; Smith and Doyle, 1995), *Pterocarya* Kunth is the genus most closely related to *Juglans.* A cladistic analysis (Smith and Doyle, 1995) reported that *Carya* Nutt. was the sister group to the *Juglans–Pterocarya* clade, and *Platycarya* Sieb. et Zucc. the basal sister group to the *Carya–*(*Juglans–Pterocarya*) group. Three more genera of the Juglandaceae, *Alfaroa* Standl., *Engelhardia* Lesch. ex Blume, and *Oreomunnea* Oerst., were reported to be more distantly related to *Juglans* than *Pterocarya, Carya,* and *Platycarya* (Smith and Doyle, 1995). *Cyclocarya* Iljinsk., considered to be another Juglandaceous genus by some authors (Iljinskaya, 1953; Manchester, 1987), has often been considered to be congeneric with *Pterocarya* (Leroy, 1955; Conde and Stone, 1970; Manning, 1978).

To further explore the systematics of *Juglans,* our paper presents a phylogenetic analysis of nucleotide sequences from 15 walnut species and four outgroup taxa using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA and the plastid gene *matK* with its surrounding spacers. The ITS region consists of two internal transcribed spacers, ITS1 and ITS2, and the intervening 5.8S nuclear ribosomal gene. The small and rather conserved 5.8S region does not yield many characters for phylogenetic analysis, even at the division and class levels (Jorgensen and Cluster, 1988; Hillis and Dixon, 1992). However, the highly variable ITS1 and ITS2 regions are commonly used for species-level comparisons—a level at which few genes have provided sufficient characters for phylogenetic analysis (Baldwin, 1992; Baldwin, et al., 1995; Wen and Zimmer, 1995; Moller and Cronk, 1997; Stanford, Harden, and Parks, 1997; Vargas, Morton, and Jury, 1999).

The plastid gene *matK,* previously known as *orfK,* is a maturase-encoding gene located in the intron of *trnK* (the transfer RNA gene for lysine), found in the large, single-copy region of the chloroplast genome. The *matK* gene, which is more variable than the widely sequenced *rbcL* gene, has been used in molecular systematics at the genus and family levels (Steele and Vilgays, 1994; John-

son and Soltis, 1995; Liang and Hilu, 1996; Kron, 1997; Manos and Steele, 1997).

MATERIALS AND METHODS

We were able to obtain 16 *Juglans* taxa, representing all four sections of the genus, for this analysis. Included were 15 species widely accepted in the literature plus two varieties of *J. cathayensis* Dode, *J. cathayensis* var. *cathayensis* and *J. cathayensis* var. *formosanum.* Four outgroups, *Carya, Cyclocarya, Platycarya,* and *Pterocarya,* were also sequenced for this study. These outgroups were chosen based on previous phylogenetic analyses (Heimsch and Wetmore, 1939; Conde and Stone, 1970; Smith and Doyle, 1995). The *matK* analyses were also performed using as outgroups sequences taken from GenBank for *Alfaroa* (GenBank accession number GBAN-U92849), *Betula* (GBAN-U92853), *Casuarina* (GBAN-U92858), *Comptonia* (GBAN-U92857), and *Myrica* (GBAN-U92857). (The prefix GBAN-has been added for linking the online version of *American Journal of Botany* to GenBank, but is not part of the actual GenBank accession number). All taxa were vouchered as herbarium specimens and deposited at the University of North Carolina Herbarium in Chapel Hill (NCU) (Table 1).

Total genomic DNA was extracted from fresh or silica gel-dried (Chase and Hills, 1991) leaf tissue using a modified version of the hot cetyltrimethylammonium bromide (CTAB) method outlined by Doyle and Doyle (1987). Amplifications of the ITS region of the nrDNA were obtained using the ITS5A/ITS4 primer pair (Table 2). Amplifications of the *matK* region were primarily performed using primers trnK1 and trnK2r (located within the flanking *trnK* gene), and sometimes using primers matK161f (located within the *matK* gene) and trnK2r (Table 2). Double-stranded DNA amplifications were performed in a 25-μL volume containing 12 μL of sterile distilled water, 4 μL of 200 μmol/L dNTPs in equimolar ratio, 2.5 μL of 10X *Taq* DNA Polymerase buffer (Promega, Madison, Wisconsin, USA), 1.5 μL 25 mmol/L $MgCl_2$, 2.5 μL dimethyl sulfoxide (DMSO), 0.5 μL each of 10 mmol/L primer, 0.125 μL 10% BSA, 0.5 units of *Taq* DNA Polymerase (Promega), and 1 μL of genomic DNA (1–10 ng). For DNA amplifications, the first cycle was 1 min at 94°C for denaturation, 1 min at 50°C for annealing, and 1.5 min at 72°C for primer extension. Primer extension time was increased by 3 s in each cycle; an additional 10 min of extension time followed the final cycle. Prior to manual sequencing, 40 cycles were used, and prior to automated sequencing, 30 cycles were used. Positive and negative controls were included in each set of amplifications. Except for *Cyclocarya,* all of the taxa listed in Table 1 were sequenced for both the ITS and the *matK* regions. *Cyclocarya* was sequenced for the ITS region only.

Polymerase chain reaction (PCR) product was purified using an enzymatic clean-up method: 1 μL Exonuclease I (Amersham, Arlington Heights, Illinois) and 4 μL shrimp alkaline phosphatase (Amersham) were added to 20 μL of PCR product. The mixture was incubated first for 15 min at 37°C to degrade primers and dNTPs, and then for 15 min at 80°C to denature the enzymes. Both strands were sequenced for all taxa, and, whenever possible, sequences were generated for more than one individual of each *Juglans* taxon to check for intraspecific heterogeneity (Table 1).

Initially, sequences were generated according to manual dideoxy nucleotide sequencing methods outlined by Chase et al. (1993), using Sequenase™ (USB, Cleveland, Ohio, USA) and alpha ^{32}P labeled dATP. However, most data were generated by cycle sequencing, and the sequences were produced through automated sequencing on a Perkin-Elmer (Norwalk, Connecticut, USA) Applied Biosystems, Inc. model 377 according to the manufacturer's protocols. For sequencing of the ITS region, primers ITS2, ITS3, ITS4, and ITS5A were used (Table 2). For sequencing of the *matK* region, primers trnK1, matK161f, matK720f, matK4, matK5, matK7, matK77, trnK2r, matK7r, matK820r, matK1290r, and matK1300r, were used (Table 2). Autoradiograms generated through manual sequencing were read by sight, rechecked, and recorded by hand. Sequences generated through automated methods were manually edited and assembled into a consensus sequence. Sequences were aligned manually. All sequences can be obtained through GenBank (Table 1).

The data were analyzed using PAUP 3.1 (Phylogenetic Analysis Using Parsimony; Swofford, 1993) software. Pairwise distances were calculated for the data using the PAUP 3.1 software. All informative base-pair differences were used in the analyses, and indels were coded plus(1)/minus(0) or treated as missing data. Uninformative characters were excluded from the analysis. In analyses of *matK,* all indels were treated as missing data. In analyses of ITS, data were analyzed twice: in one analysis, five indels, coded for presence or absence were included. The ITS data were then reanalyzed with indels treated as missing data. Based on previous analyses of the Juglandaceae (Heimsch and Wetmore, 1939; Conde and Stone, 1970; Smith and Doyle, 1995), outgroups were specified as paraphyletic to the ingroup. Branch-and-bound searches were executed to identify the most parsimonious trees. Several statistical measures were calculated, including bootstrap (Felsenstein, 1985) with 100 replicates, consistency indices (Kluge and Farris, 1969), decay indices (Bremer, 1994), retention indices (Farris, 1989), homoplasy indices (Kluge and Farris, 1969), and Hillis and Huelsenbeck's g1 statistic (1992), obtained by generating 1 000 000 random trees. Separate phylogenetic analyses of the *matK* spacers, the *matK* coding region, the entire *matK* region, the ITS region alone, and the combined *matK* and ITS data sets were conducted. Because transitions were 1.8 times more common than transversions in the ITS data set, an analysis of ITS data was also performed with transversions weighted 1.8 times more than transitions. A combined analysis of the molecular data (ITS and *matK*) generated in this study and the RFLP data generated by Fjellstrom and Parfitt (1995) was also conducted.

To study the biogeography of the genus, species distributions were mapped onto the phylogeny to create an area cladogram for *Juglans.*

RESULTS

The aligned *matK* data matrix consisted of 2427 nucleotide sites. Of these sites, only 173 were parsimony informative. The *matK* region yielded a minimum of 50 pairwise differences at the species level in this study. The ratio of transitions to transversions in the *matK* data set was 1 to 1.1. The aligned ITS data matrix contained 658 sites, of which 73 were parsimony-informative. The ratio of transitions to transversions in the ITS data set was 1.8 to 1. Sequences for either region proved to be identical within one species and nonidentical between species, including controversial species *J. californica, J. major, J. guatemalensis,* and *J. olanchana.*

All analyses supported *Cyclocarya* as a separate genus from *Pterocarya,* and *Juglans* as the monophyletic sister group to *Pterocarya.* Within *Juglans,* analyses of all but the *matK* data supported section *Rhysocaryon* as the sister group to the other three *Juglans* sections. All combined analyses supported a temperate clade and a tropical clade of black walnuts; the tropical clade included *J. olanchana.*

Separate analysis of the *matK* spacer regions (5′ and 3′) and coding region yielded trees that were largely unresolved due to the small number of informative characters contained in any one of the three regions.

When the entire *matK* region, including both spacers and the coding region, was analyzed, pairwise distances ranged from 0.7% within *Rhysocaryon* to 9.3% between *J. boliviana* and *J. cathayensis.* The same results were obtained both when *Alfaroa, Betula, Casuarina, Comptonia,* and *Myrica* were used as outgroups and when they

TABLE 1. Voucher information.

Species	Collection no.[a]	Collector	Collection site	GenBank accession nos.[b]	
				matK	ITS
Juglans ailantifolia Carr.	#1, 92-JH-8e	C. Parks	China	GBAN-AF118024	GBAN-AF179567
	LP95-029	C. Parks	China		
Juglans australis Griseb.	DJUG0429.1	G. White	National Germplasm Repository, Davis, CA	GBAN-AF118025	GBAN-AF179568
Juglans boliviana (C. DC.) Dode	SB1-191	S. Beck	La Paz, Bolivia	GBAN-AF118026	GBAN-AF179569
	SB2-192	S. Beck	La Paz, Bolivia		
Juglans californica S. Wats.	DJUG14.1	G. White	National Germplasm Repository, Davis, CA	GBAN-AF118027	GBAN-AF179570
	LA8-1218	B. Prigge	Los Angeles, CA		
Juglans cathayensis var. *formosanum* Hayata	LP95-028	C. Parks	China	GBAN-AF118028	GBAN-AF179571
Juglans cathayensis var. *cathayensis* Dode	LP95-045	R. Harden	Arnold Arboretum, MA	GBAN-AF118028	GBAN-AF179571
	LP95-046	R. Harden	Arnold Arboretum, MA		
Juglans cinerea L.	LP95-039	R. Harden	Arnold Arboretum, MA	GBAN-AF118029	GBAN-AF179572
	LP95-041	R. Harden	Arnold Arboretum, MA		
Juglans guatemalensis Mann.	JFH1-1068	J. Hernandez	Guatemala City, Guatemala	GBAN-AF118030	GBAN-AF179573
Juglans hindsii (Jeps.) Rehder	1200-1	C. Parks	Jackson Co., OR	GBAN-AF118031	GBAN-AF179674
	1200-2	C. Parks	Jackson Co., OR		
Juglans major (Torr. ex Sitsgr.) Heller	LP95-040	R. Harden	Arnold Arboretum, MA	GBAN-AF118032	GBAN-AF179575
Juglans mandshurica Maxim.	DJUG0431.2	G. White	National Germplasm Repository, Davis, CA	GBAN-AF118033	GBAN-AF179576
	DJUG0436.0	G. White	National Germplasm Repository, Davis, CA		
Juglans microcarpa Berl	LP95-042	R. Harden	Arnold Arboretum, MA	GBAN-AF118034	GBAN-AF179577
	LP95-043	R. Harden	Arnold Arboretum, MA		
	LP95-044	R. Harden	Arnold Arboretum, MA		
Juglans neotropica Diels	DJUG0330.3	G. White	National Germplasm Repository, Davis, CA	GBAN-AF118035	GBAN-AF179578
	DJUG0285.1	G. White	National Germplasm Repository, Davis, CA		
Juglans nigra L.	LP95-026	C. Parks	Chapel Hill, NC	GBAN-AF118036	GBAN-AF179579
	LP95-036	R. Parks	San Francisco, CA		
Juglans olanchana Stadl. et L. O. Williams	DJUG212.1	G. White	National Germplasm Repository, Davis, CA	GBAN-AF118037	GBAN-AF179580
	DJUG213.1	G. White	National Germplasm Repository, Davis, CA		
Juglans regia L.	LP95-027	C. Parks	Coker Arboretum, Chapel Hill, NC	GBAN-AF118038	GBAN-AF179581
	LP95-037	R. Parks	San Francisco, CA		
Carya glabra (Mill.) Sweet	LP94-050	L. Prince	Coker Arboretum, Chapel Hill, NC	GBAN-AF118039	GBAN-AF179582
Cyclocarya palouris (Batal.) Iljinsk.	E43 AA MA '91	K. Wurdack	J. C. Ralston Arboretum, Raleigh, NC		GBAN-AF179583
Platycarya strobilea Sieb. et Zucc.	W01	K. Wurdack	Orangeburg, SC	GBAN-AF118040	GBAN-AF179584
Pterocarya caucasica C. A. Mey.	W17	K. Wurdack	J. C. Ralston Arboretum, Raleigh, NC	GBAN-AF118041	GBAN-AF179585
Pterocarya stenoptera C. DC.	LP95-030	R. Harden	Coker Arboretum, Chapel Hill, NC	GBAN-AF118042	GBAN-AF179587
Pterocarya tonkinensis (Franch.) Dode	W03 920561/1	K. Wurdack	J. C. Ralston Arboretum, Raleigh, NC	GBAN-AF118043	GBAN-AF179586

[a] These vouchers are stored at University of North Carolina Herbarium (NCU).
[b] The prefix GBAN- has been added for linking the online version of *American Journal of Botany* to GenBank, but is not part of the actual GenBank accession number.

TABLE 2. Primers used in this study.

Primer name	5′ to 3′ Primer sequence	5′ primer position	Primer source
ITS2	GCTGCGTTCTTCATCGATGC	306	White et al., 1990
ITS3	GCATCGATGAAGAACGCAGC	310	White et al., 1990
ITS4	TCCTCCGCTTATTGATATGC	>655 (downstream)	White et al., 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	<1 (upstream)	White et al., 1990
ITS5A	CCTTATCATTTAGAGGAAGGAG	<1 (upstream)	Kenneth Wurdack
maK 4	CTTCGCTACCGGGTGAAAGATG	1241	Manos and Steele, 1997
matK 5	GGATCCTTTCATGCATT	1571	Steele and Vilgalys, 1994
matK 7	GTATTAGGGCATCCCATT	1908	Steele and Vilgalys, 1994
matK 7r	ACTAATTGGATGCCCTACTGC	1928	Manos and Steele, 1997
matK 77	AACGCAGTAGGGCATCCAATT	1905	Manos and Steele, 1997
matK 161f	GAATGGAAAAAGTAGCATGTC	161	Alice Stanford
matK 720f	GGTTGAACAAATAAAGGGATCTC	720	Alice Stanford
matK 820r	CCCGGGAACAGATAAGAATTATTC	820	Alice Stanford
matK 1290r	ACGAAGGGTTGAACCATT	190	Alice Stanford
matK 1300r	GTAGCGAAGGGTTTGAATCAAGA	1300	Alice Stanford
trnK 1	CTCAACGGTAGAGTACTCG	1	Steele and Vilgalys, 1994
trnK 2r	AACTAGTCGGATGGAGTAG	2573	Steele and Vilgalys, 1994

were not. (These genera have therefore been excluded from our cladograms.) The search yielded four most parsimonious trees (Fig. 2), which differed only in the placement of *J. major*. The consensus tree contained a *Cardiocaryon* clade with *J. ailantifolia* Carr. and *J. cathayensis* as the sister group to *J. mandshurica* Maxim. *Juglans regia* was the sister group to *Cardiocaryon*, and the *Cardiocaryon–Dioscaryon* clade was the sister group to a *Rhysocaryon–Trachycaryon* clade. Bootstrap values were <50% for most branches, and decay values were between one and two steps.

The ITS data set, with pairwise differences from 0.3% between *J. major* and *J. nigra* to 7.4% between *J. californica* and *J. mandshurica*, contained 73 potentially informative characters. Analysis resulted in two most parsimonious trees (Fig. 3), which differed only in the placement of *J. nigra*. Analyses that treated indels as missing data yielded the same trees as analyses in which indels were treated as a fifth character state. The monophyly of all four *Juglans* sections was also supported. *Trachycaryon* was the sister group to *Cardiocaryon*, and *Dioscaryon* was the sister group to the butternut clade.

In analysis of the ITS data set, weighting transitions/transversions resulted in five most parsimonious trees, differing only in the placement of *J. major* and *J. microcarpa* Berl. The consensus tree was consistent with the consensus tree resulting from unweighted analysis except within *Cardiocaryon*. The trees resulting from the weighted analysis featured *J. ailantifolia* and *J. cathayensis* as the sister group to *J. mandshurica*, while unweighted analysis featured *J. ailantifolia* and *J. mandshurica* as the sister group to *J. cathayensis.*

When the combined ITS and *matK* data sets were analyzed, three most parsimonious trees, differing only in the arrangement within the *J. major–J. microcarpa–J. nigra* clade, resulted (Fig. 4). The consensus tree was sim-

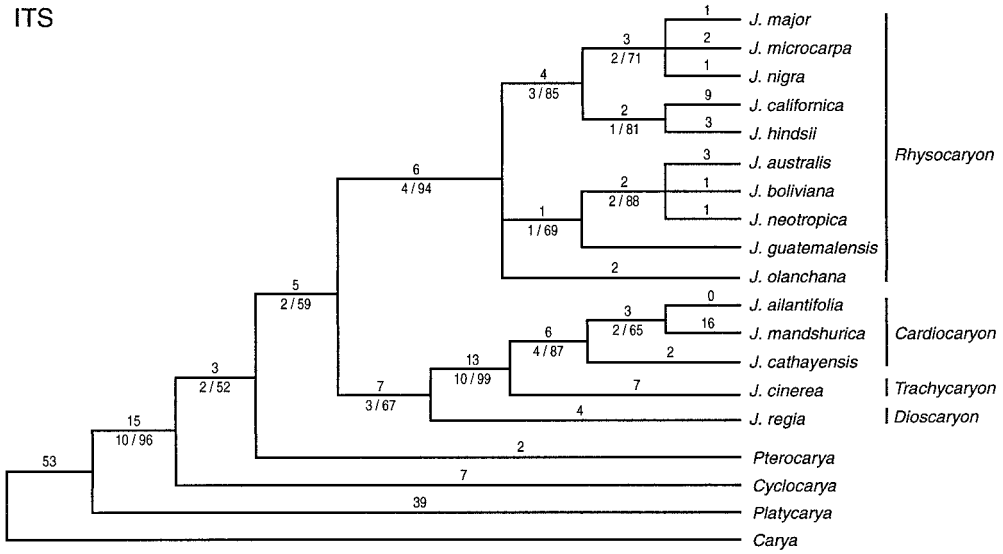

Fig. 2. One of the four most parsimonious trees resulting from analysis of the entire *matK* region. Branch lengths are above the branches, and decay indices (left or alone) and bootstrap values (right or absent) are below (tree length = 1269 steps, consistency index = 0.656, retention index = 0.705, and g1 statistic = −0.839).

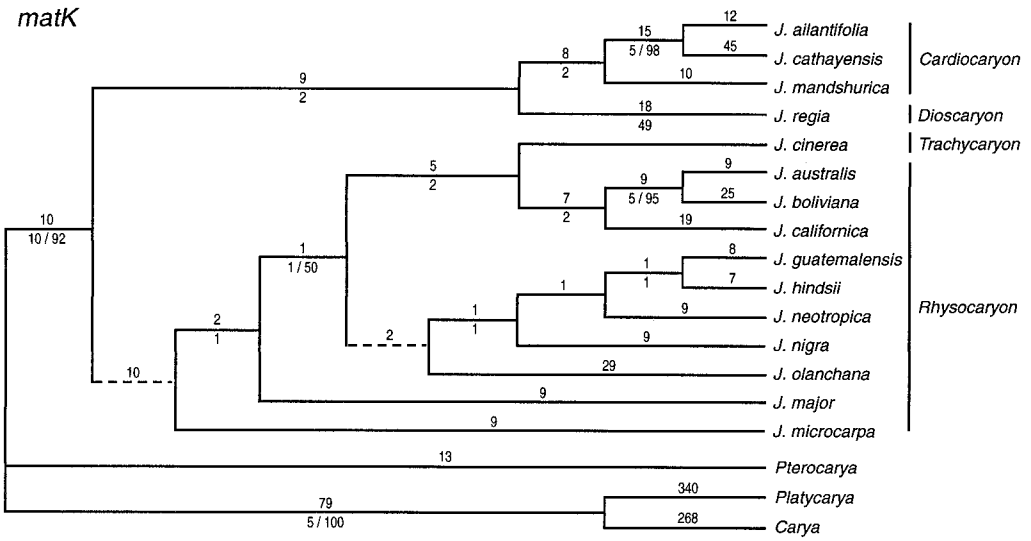

Fig. 3. One of two most parsimonious trees resulting from analysis of the ITS region. Branch lengths are above the branches, and decay indices (left or alone) and bootstrap values (right or absent) are below (tree length = 238 steps, consistency index = 0.644, retention index = 0.808, and g1 statistic = −0.646).

ilar to the tree obtained from analysis of the ITS data set alone. The topology within *Rhysocaryon* was both more resolved and better supported than within the ITS-based tree. Within the tropical black walnut clade, *J. olanchana* was the sister group to the four species found farther south, *J. guatemalensis* was the sister group to the species found south of it, and *J. neotropica* Diels was the sister group to *J. australis* Griseb. and *J. boliviana.* The temperate black walnut clade contained two groups, a ''western'' clade containing *J. californica* and *J. hindsii* (Jeps.) Rehder and an ''eastern'' clade containing *J. major, J. microcarpa,* and *J. nigra.*

Finally, a combined analysis of the molecular data (ITS and *matK*) generated for this study with the RFLP data generated by Fjellstrom and Parfitt (1995) was conducted. Analysis of 289 potentially informative characters yielded just two most parsimonious trees (Fig. 5) that differed only in the placement of *J. cinerea* and *J. mandshurica.* In these trees, *Rhysocaryon* was better resolved than in the ITS/*matK* trees. The trees had essentially the same topology as the trees resulting from the combined analysis of the ITS and *matK* data. The Mexican species *J. mollis* Engelm. ex Hemsl. (which was not included in the ITS and *matK* studies) fell within the temperate black walnut clade. The United States' black walnut species formed a monophyletic sister group to *J. mollis.*

DISCUSSION

Phylogeny—Section *Rhysocaryon* was well supported as a monophyletic group in these analyses. Analyses of

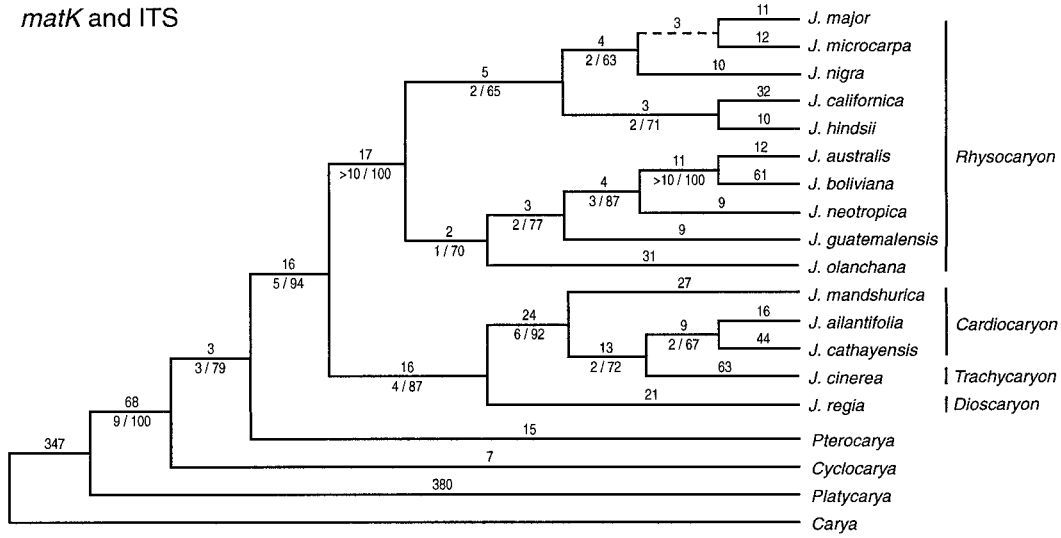

Fig. 4. One of three most parsimonious trees resulting from analysis of the combined *matK* and ITS regions; the trees differed only in the placement of the ''dotted'' branch. Branch lengths are above the branches, and decay indices (left or alone) and bootstrap values (right or absent) are below (tree length = 1520 steps, consistency index = 0.809, retention index = 0.912, and g1 statistic = −0.771).

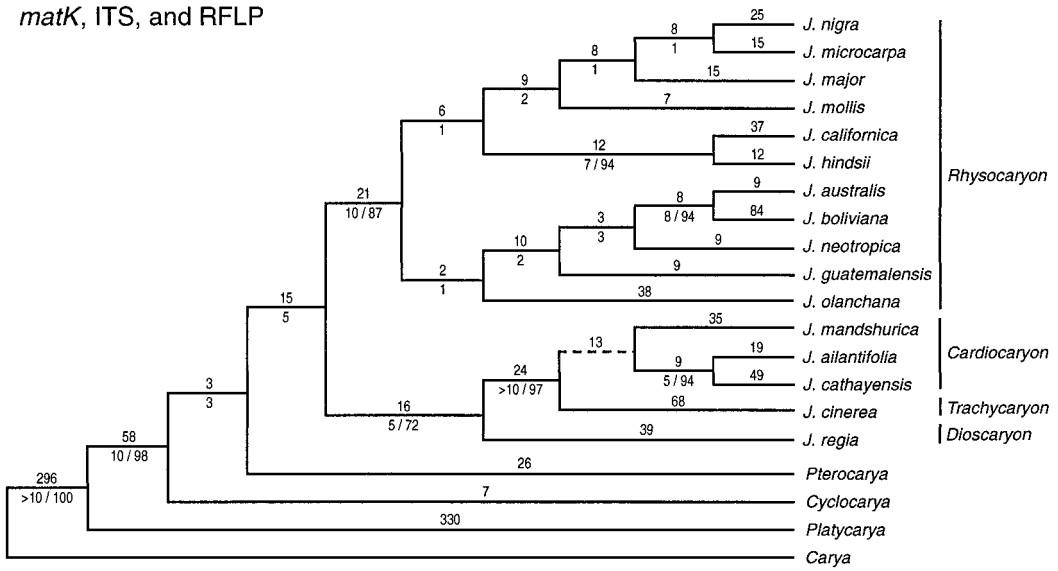

Fig. 5. One of two most parsimonious trees resulting form analysis of combined *matK*, ITS, and nuclear RFLP (Fjellstrom and Parfitt, 1995) data; the trees differed only in the placement of the ''dotted'' branch. Branch lengths are above the branches, and decay indices (left or alone) and bootstrap values (right or absent) are below (tree length = 1528 steps, consistency index = 0.824, retention index = 0.633, and g1 statistic = −0.809).

ITS, total sequence data, and combined sequence and RFLP data yielded a monophyletic *Rhysocaryon,* with a decay index greater than ten and a bootstrap value of 100% in analysis of total sequence data (Fig. 4). Although *matK* data did not present any well-supported hypothesis for relationships within *Rhysocaryon,* ITS data and total data analyses suggested that *Rhysocaryon* contains two monophyletic groups. The analysis supported dividing *Rhysocaryon* into two subclades, a temperate black walnut clade and a tropical black walnut clade. This same division was suggested by Miller (1976). Within the temperate black walnut clade, two monophyletic groups were supported: a clade of *J. californica* and *J. hindsii* (the two California species) and a clade of *J. microcarpa, J. major,* and *J. nigra.* Within the tropical black walnuts, the northernmost species, the Mexican *J. olanchana,* is the basal sister group to all the more southerly taxa, *J. guatemalensis, J. neotropica, J. australis,* and *J. boliviana.* Of those four species, the northernmost, *J. guatemalensis,* is the basal sister group to the remaining three. It seems likely that southward migration has been closely followed by speciation in the tropical species of the genus. All analyses supported *J. californica, J. major, J. guatemalensis,* and *J. olanchana* as distinct species.

Cardiocaryon was supported as a monophyletic group in these analyses, but it was supported less strongly than *Rhysocaryon.* Analyses of the *matK* data and of weighted ITS data indicated that *J. ailantifolia* was more closely related to *J. cathayensis* than to *J. mandshurica,* while analysis of unweighted ITS data indicated that *J. ailantifolia* was more closely related to *J. mandshurica* than to *J. cathayensis.* The closer relationship of *J. ailantifolia* and *J. cathayensis* resulting from analysis of *matK* data had much stronger support (with a bootstrap value of 98% and a decay index of 5) than the closer relationship of *J. ailantifolia* and *J. mandshurica* (with a bootstrap value of 67% and a decay index of 2) resulting from analysis of unweighted ITS data. However, Fjellstrom and Parfitt's (1995) analyses consistently supported a closer relationship of *J. ailantifolia* and *J. mandshurica.*

The butternuts as a whole were strongly supported as a monophyletic group in analyses of ITS data and of total data, but analysis of *matK* data placed *J. cinerea* in a clade with *Rhysocaryon.* With a decay index of 10 and a bootstrap support of 99%, the monophyly of the butternuts suggested by ITS data was better supported than the relationship (with decay indices of 2 or less and bootstrap values of 50 or less) suggested by *matK* data.

Both *matK* and ITS data, when analyzed separately or together, supported section *Dioscaryon* as the sister group to the butternuts. Analysis of total data indicated that 16 characters supported the monophyly of the butternuts and the Persian walnut (*J. regia*). The butternut–*Dioscaryon* clade was consistently supported as the monophyletic sister group to the *Rhysocaryon* clade.

In general, the phylogenetic trees generated by these analyses became more resolved and better supported as additional data sets were added. This increased resolution seems to be a common result of combining data sets in the literature (Schnabel and Wendel, 1998; Yasui and Osnishi, 1998; Les et al., 1999). The inclusion of multiple data sets provides additional characters, and may help cancel out ''noise'' present in individual data sets. Although it is not always possible, it may be advisable to include two or more data sets in all molecular phylogenetic studies.

Morphology—The phylogenies generated by these analyses are largely in agreement with previous, morphology-based, analyses. The four sections delineated by Dode (1906, 1909a, b) and Manning (1978) coincide with four monophyletic groups generated by parsimony analysis of gene sequence data performed in this study and by parsimony analysis of RFLP data (Fjellstrom and Par-

fitt, 1995). Section *Dioscaryon* typically has leaves with five to nine entire leaflets, dehiscent husks, and smooth nuts with two winged sutures. *Rhysocaryon* is typified by pedicillate staminate flowers and the presence of crystal chains and heterocellular rays in the wood. Notched leaf scars and five rows of scales on embryos and seedlings distinguish *Cardiocaryon* from *Trachycaryon,* which has unnotched leaf scars and scaleless embryos and seedlings. Synapomorphies between *Cardiocaryon* and *Trachycaryon* include downy leaflets, a hairy fringe on leaf scars, nuts two-celled at the base, and flattened ray cells. The butternuts and section *Dioscaryon* share a few characters, including sessile staminate flowers and homocellular rays, but there is no strong morphological evidence for monophyly of these groups.

In agreement with the results of this molecular systematic study is Miller's (1976) division of the *Rhysocaryon* into two groups, the tropical black walnuts and the temperate black walnuts. Miller (1976) found that the tropical walnuts, unlike the temperate walnuts, have wood with diffuse-porous vessel distribution. The tropical walnuts also have longer crystal chains and exclusively heterocellular rays. Temperate black walnuts have semiporous ring vessel distribution, and, unlike any other *Juglans* species, they have reticulate thickenings in the axial parenchyma.

Not in agreement with the results of this molecular study is Dode's (1906, 1909a, b) division of *Rhysocaryon* into three groups and of *Cardiocaryon* into three groups. Dode's (1906, 1909a, b) subsectional divisions were based largely upon nut morphology. *Cardiocaryon* was divided according to the shape of the nuts and the presence of ventral furrows on the nuts. His division of *Rhysocaryon* was based on whether or not the nuts were deeply crested and whether the crests were pointed or dull.

Biogeography—Many events have shaped the distributions of Arcto-Tertiary disjuncts. Asia and America appear to have shared a land connection via the Bering land bridge (and possibly a periodic Aleutian bridge as well) from the Mesozoic (over 70 million years before present) until the late Miocene or early Pliocene, ~10 million years before present (Fujita, 1978; Barron et al., 1981; McKenna, 1983; Briggs, 1987). A north Atlantic European–American land bridge is speculated to have existed from the early Eocene (55 million years before present) until the late Miocene, although it was possibly interrupted during the Oligocene, 30–40 million years before present (Raven and Axelrod, 1974; Tiffney, 1985a, b). However, disagreement about this North Atlantic land bridge abounds. Some geologists and biologists (Kurtén, 1973; Thiede, 1980; McKenna, 1983; Briggs, 1987) have contended that it existed (from the Mesozoic) only until the mid-Eocene (50 million years before present), in which case it would not have been a viable migration route during most of the Tertiary. Hallam (1981) has argued that the Atlantic land bridge lasted until the Oligocene, 38 million years ago. In any case, migration across the north Atlantic via island hopping may still have been possible after the land bridge was submerged. The ability of temperate species in Asia, Europe, and America to exchange genetic information during this time

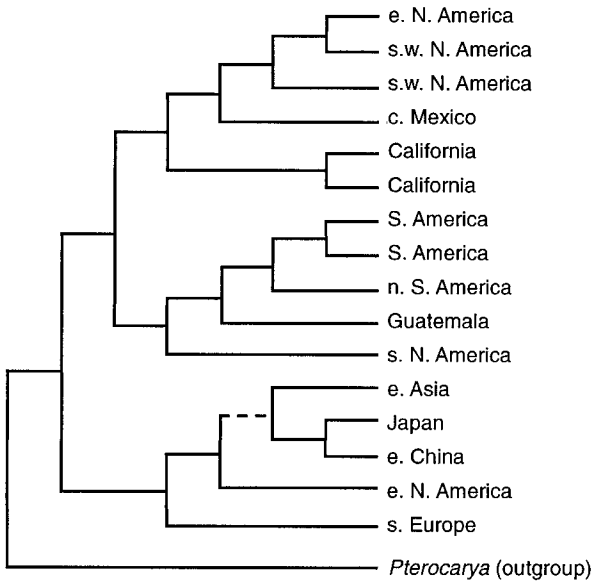

Fig. 6. Area cladogram of *Juglans* based on *matK,* ITS, and RFLP (Fjellstrom and Parfitt, 1995) data.

appears to have depended largely upon climate: only during warm periods would the land bridges have been forested with temperate species (Wolfe, 1978; Frakes, 1979; McKenna, 1983). Warm periods with mean temperatures in northern latitudes ~20°C occurred at ~63 million years before present, 55 million years before present, and 30 million years before present. Less extreme warming periods, with mean temperatures around 10°C, occurred more recently, ~25, 18, and 4 million years before present (Savin, 1977; Vail, Mitchum, and Thompson, 1977; Wolfe, 1978; Frakes, 1979). The current distributions of Arcto-Tertiary disjunct plants have probably resulted from periodic contact (among the taxa of northern temperate forests) during warmer climate periods when northern land bridges were present.

The *Juglans* area cladogram (Fig. 6) does not support any particularly theory of the origin of the genus. However, it certainly does not conflict with the North American origin of the genus suggested by the fossil record. The earliest *Juglans* fossils, dating from the Eocene (50 million years before present) were found in several North American sites. It is conceivable that this early North American lineage diversified into the two main lineages, the North American lineage *Rhysocaryon* Dode and the primarily Eurasian butternut-*Dioscaryon* Dode lineage, during the Eocene (40-50 million years before present). Certainly *Rhysocaryon* had evolved by the mid-Eocene (45 million years before present)–fossil black walnut species *J. clarensis* Scott was found in the Middle Eocene Clarno Formation in Oregon (Manchester, 1987). However, the earliest butternut fossil, extinct species *J. lacunosa* Manchester, came from the early Oligocene (~35 million years before present) Blakely Formation of Washington. While the phylogenies generated by this study and by Fjellstrom and Parfitt (1995) imply that *Dioscaryon* Dode evolved from a common ancestor with *Cardiocaryon,* the absence of *Dioscaryon* from the fossil record makes it difficult to even speculate as to the group's

origins. The history of the butternuts and *Rhysocaryon* seems clearer.

The earliest known walnut fossils from Asia (Miocene *J. megacinerea* Miki ex Chaney from ~25 million years before present) and Europe (Oligocene *J. tephrodes* Unger from ~30 million years before present) are butternuts. The butternuts, which had evolved by 35 million years before present (Oligocene) in North America, probably migrated to Eurasia during the warming trend underway until ~30 million years before present. The genus would have migrated to Europe, where the fossil record dates from the Oligocene (30 million years before present). However, it is probable that the north Atlantic land bridge, which connected Europe and North America during much of the Tertiary, was submerged during the Oligocene (McKenna, 1983; Briggs, 1987), when the migration would have taken place. Another possibility is that *Juglans* arrived in Europe during the Eocene warming trend (45 million years before present), when the north Atlantic land bridge was almost certainly extant, although no European *Juglans* fossils are known from the Eocene time period (Manchester, 1987). Thereafter, *Juglans* would have migrated to Asia, where the fossil record dates from the Miocene, ~25 million years before present (Manchester, 1987). This migration could have been from Europe, as Yurtsev (1972) asserts that almost all migrations over the Beringian land bridge were from Asia to America. Based on the cladogram, however, it seems more likely the migration to Asia was from North America via the Beringian land bridge during the Oligocene warming trend (30 million years before present).

CONCLUSIONS

Based on *matK*, ITS, and nuclear RFLP data, *Juglans* is a monophyletic group, and *Pterocarya* is the sister group to *Juglans*. Within the *Juglans* clade are two subclades, one consisting of section *Rhysocaryon* and one consisting of sections *Cardiocaryon*, *Dioscaryon*, and *Trachycaryon*. Within *Rhysocaryon* (the black walnuts) are two groups, also suggested by Miller's (1976) study of wood anatomy in walnuts, the temperate black walnuts and the tropical black walnuts. Within the second subclade, *Dioscaryon* is the sister group to *Cardiocaryon* and *Trachycaryon* (the butternuts), but phylogenetic relationships within the butternuts remain unclear. Three of the previously proposed sections (Dode, 1908, 1909; Manning, 1978), *Dioscaryon*, *Rhysocaryon*, and *Trachycaryon*, are monophyletic groups. It seems likely that *Cardiocaryon* is also monophyletic. Although combined *matK* and ITS sequence data generated for this study do not clearly support monophyly, both the individual ITS and *matK* data and Fjellstrom and Parfitt's (1995) RFLP data indicate that *Cardiocaryon* is monophyletic.

A complex history of climate changes and continental movement has shaped the current biogeography of the group. The evolution of the butternut clade may have occurred as a result of the cooling trend ~40 million years ago, since the first known fossils of the group date from 35 million years ago. This group could have migrated to Europe, where the oldest fossils date from 30 million years ago, during the warming trend which occurred ~30 million years ago. Overall, the well-resolved phylogenies generated for *Juglans*, combined with the well-studied fossil record of the group, present a clear picture of the history of *Juglans*.

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