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CHAPTER 7

Cladistic Biogeography of *Juglans* (Juglandaceae) Based on Chloroplast DNA Intergenic Spacer Sequences

Juglans L. is principally a New World genus within the tribe Juglandeae of the family Juglandaceae, comprising about 21 extant deciduous tree species occurring from North and South America, the West Indies, and southeastern Europe to eastern Asia and Japan (Manning, 1978). It is one of the approximately 65 genera that are known to exhibit a disjunct distributional pattern between eastern Asia and eastern North America (Manchester, 1987; Wen, 1999; Qian, 2002; figure 7.1). Four sections are commonly recognized within *Juglans,* based mainly on fruit morphology, wood anatomy, and leaf architecture (Dode, 1909a, 1909b; Manning, 1978). Section *Rhysocaryon* (black walnuts), which is endemic to the New World, comprises five North American temperate taxa: *J. californica* S. Wats., *J. hindsii* (Jeps.) Rehder, *J. nigra* L., *J. major* (Torr. ex Sitgr.) Heller, and *J. microcarpa* Berl.; three Central American subtropical taxa: *J. mollis* Engelm., *J. olanchana* Stadl. & I. O. Williams, and *J. guatemalensis* Mann.; and two South American tropical taxa, *J. neotropica* Diels and *J. australis* Griesb, mainly occurring in the highlands. They typically bear nuts that are four-chambered with thick nutshells and septa. Section *Cardiocaryon* (Asian butternuts) contains four taxa: *J. hopeiensis* Hu, *J. ailantifolia* Carr., *J. mandshurica* Maxim., and *J. cathayensis* Dode, all native to East Asia, and section *Trachycaryon* consists of the only North American butternut taxon, *J. cinerea* L. Both Asian and

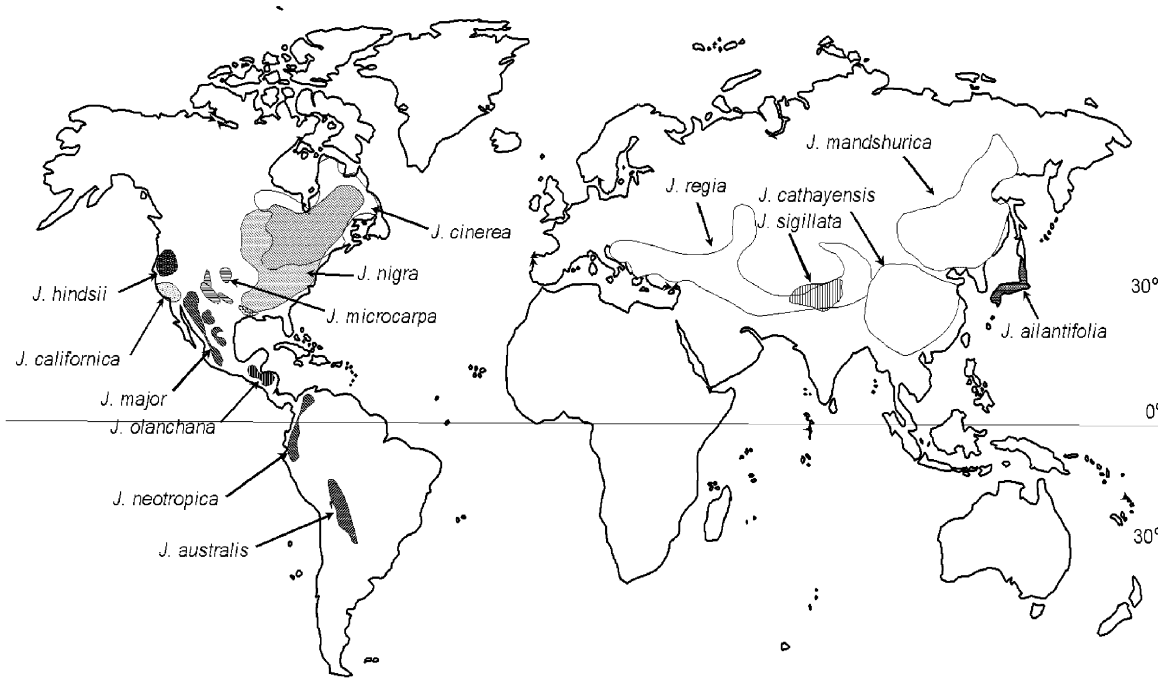

FIGURE 7.1 Geographic distribution of extant taxa of *Juglans* (Juglandaceae). The distribution of cultivated species *J. regia* extends beyond its natural home range.

American butternuts possess two-chambered nuts with thick nutshells and septa. Section *Juglans* includes two taxa: The cultivated Persian or English walnut, *J. regia* L., ranges from southeastern Europe to the Himalayas and China and bears four-chambered nuts with thin nutshells and papery septa, and the iron walnut, *J. sigillata* Dode, ranges from southern China and Tibet and has thick, rough-shelled nuts and characteristic dark-colored kernels (Dode, 1909a). The iron walnut sometimes is considered an ecotype of *J. regia,* but some botanists treat it as a separate species (Kuang et al., 1979). It is known to have been cultivated for a long time in Yunnan province of China for its oil. Complete descriptions of the morphological variation, ecological distribution, and taxonomic treatment of the genus *Juglans* are found in Manning (1957, 1960, 1978).

Plant species disjunctions have been the subject of many taxonomic and biogeographic studies. The most notable among them is the East Asian–North American disjunction, the origin of which has been studied from the paleobotanical, geological, and paleoclimatic perspectives. Various hypotheses have been proposed to explain the origin of these disjunctions, and Asa Gray's (1859, 1878) pioneering accounts in the mid-19th century of the floristic similarities of East Asia and eastern North America serve as the foundation for the modern systematic syntheses of plant species disjunctions. He proposed that many plant taxa were widely distributed throughout the Northern Hemisphere during the early Tertiary, and later disruptions by glaciation led to eastern Asian–eastern North American disjunctions. Subsequently, Chaney (1947) and Axelrod (1960) independently modified Gray's hypothesis to suggest that the floristic similarities originated as the result of range restrictions and southward migration of the homogeneous Arcto-Tertiary geoflora of the Northern Hemisphere caused by climatic changes in the late Tertiary and Quaternary. Recently, additional paleofloristic and geological discoveries have led to more complex alternative hypotheses regarding the mode and time of origin of disjunction patterns (Wolfe, 1975, 1978, 1985; Tiffney, 1985a, 1985b). However, it is now known from fossil records that deciduous woody taxa first appeared in northern latitudes as part of a mostly broad-leaved evergreen, tropical forest in the late Eocene (Wolfe, 1969, 1972). Cooling climates during the Oligocene and Miocene saw diversification and expansion of broad-leaved, deciduous taxa throughout the northern latitudes of Eurasia and North America (Wolfe, 1978, 1985), and taxa were exchanged via the Bering or North Atlantic land bridges throughout the mid-Tertiary. Continued cooling in the Pliocene produced retraction of mixed mesophytic forest

from northern latitudes and greatly reduced the possibility of migration between Eurasia and North America (Wolfe, 1978, 1985; Tiffney, 1985a). Further climatic changes during the Quaternary effectively eliminated the northern mixed mesic forests, leaving eastern North America, eastern Asia, and to a much lesser extent the Balkans and Caucasus as the main refugia of many genera (Graham, 1972; Tiffney, 1985a). Others have implicated convergent adaptation to similar climatic conditions and long-distance dispersal in the development of present-day floristic disjunctions (Raven, 1972; Wolfe, 1975).

Based on fossil evidence, Manchester (1987) suggests that the origin of *Juglans,* including the initial split into black walnuts and butternuts, may have occurred sometime during the Middle Eocene in North America. Furthermore, expansion and migration between North America and Eurasia were facilitated by the presence of the Bering land bridge that connected eastern Asia with western North America throughout the mid-Tertiary and by a North Atlantic land bridge during the late Eocene, when there was a favorable climate in upper latitudes for the establishment and dispersal of deciduous and some broad-leaved evergreens. The latter were able to adapt to the Neogene cooler climate (Wolfe, 1978; Tiffney, 1985a), attaining a broad distribution extending farther south into southeastern Europe and Central and South America by the late Miocene. However, the fossil record suggests that black walnuts remained endemic to the Americas, whereas butternuts are represented by members in Asia as well as one in eastern North America. The section *Juglans* is not known in the fossil record.

The usefulness of chloroplast DNA (cpDNA) sequence data to estimate the rate and time of divergence between disjunct taxa is well documented (Crawford et al., 1992), but only a limited number of disjunct taxa have been examined phylogenetically using cpDNA data in order to explore the biogeographic relationships, mode, and tempo of disjunction (Wen, 1999). The eastern Asian–eastern North American Tertiary disjunction in *Juglans* offers an opportunity to estimate the level and time of evolutionary divergence between vicariant groups and to compare this with the time of divergence inferred from paleobotanical evidence. Earlier molecular systematic studies based on nuclear RFLPs (Fjellstrom and Parfitt, 1995) and *matK* and internal transcribed spacer (ITS) sequences (Stanford et al., 2000) support the traditional taxonomic classification of *Juglans* and are consistent with what is known about the geological history of the genus (Dode, 1909a, 1909b; Manning, 1978; Manchester, 1987).

Noncoding intergenic spacer regions of cpDNA, which are presumably under less functional constraint than coding regions, are known to evolve rapidly and provide useful information to examine systematic relationships at lower taxonomic levels (Ogihara et al., 1991; Gielly and Taberlet, 1994). Recently, availability of several universal chloroplast primers to amplify noncoding regions (Taberlet et al., 1991; Demesure et al., 1995) has facilitated this effort to infer phylogenetic relationships at the generic (Gielly and Taberlet, 1994; Small et al., 1998; Cros et al., 1998; Aradhya et al., 1999; Stanford et al., 2000) and even infraspecific levels (Demesure et al., 1996; Petit et al., 1997; Mohanty et al., 2001). In the present study, we examine the utility of some of these cpDNA intergenic spacer sequences for phylogenetic reconstruction and for assessing the level of evolutionary divergence within and between sections of *Juglans*. We also explore the biogeography of the genus *Juglans* based on the phylogenetic inferences and, in particular, the origin, evolution, and domestication history of the section *Juglans,* to which the cultivated walnut *J. regia* belongs.

Materials and Methods

Plant Materials, DNA Isolation, PCR Amplification, and Sequencing

Seventeen taxa representing the four sections of *Juglans* and one outgroup taxon, *Pterocarya stenoptera,* were sampled for this study (table 7.1). *Pterocarya* was chosen as the outgroup taxon because it is closely related to *Juglans* (Smith and Doyle, 1995; Manos and Stone, 2001). Total DNA was isolated using the cetyltrimethylammonium bromide method (Doyle and Doyle, 1987) and further extracted with phenol-chloroform and treated with RNAse to remove protein and RNA contaminants, respectively.

Five cpDNA intergenic spacer regions: *trnT–trnF* (Hodges and Arnold, 1994), *psbA–trnH* (Sang et al., 1997), *atpB–rbcL* (Taberlet et al., 1991), *trnV–16S rRNA* (Al-Janabi et al., 1994), and *trnS–trnfM* (Demesure et al., 1995) were PCR amplified separately in a 100-µL reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ (all included in 10 µL of 10x PCR buffer), 10–20 pmol of each primer, 200 µM of each dNTP, 2 U of Taq polymerase (Perkin Elmer Biosystems, CA, USA), and 50 ng of template DNA. The PCR conditions were as follows: one cycle of 5 min at 94°C, 30 cycles of 45 s to 1 min at 94°C, 45 s to 1 min at 55–62°C, 2–3 min at 72°C, and one cycle of 7 min at 72°C. Amplification products were purified and concentrated using QIAquick PCR purification kit (Qiagen Inc., CA, USA)

Table 7.1 Species list, collection site, geographic origin, and GenBank accession numbers

			GenBank Accession Numbers				
Taxon (ncgr* Accession no.)	**Collection Site**	**Origin**	**atpB–rbcL**	**psbA–trnH**	**trnS–trnfM**	**trnT–trnF**	**trnV–16s RRNA**
Cardiocaryon (Asian Butternut)							
J. ailantifolia (DJUG 91.4)	NCGR, Davis, CA	Japan	AY293314	AY293335	AY293365	AY293398	AY293360
J. cathayensis (DJUG 11.4)	NCGR, Davis, CA	Taiwan	AY293312	AY293334	AY293367	AY293396	AY316200
J. mandshurica (DJUG 13.1))	NCGR, Davis,CA	Korea	AY293315	AY293337	AY293364	AY293397	AY293361
J. hopeiensis (DJUG 462)	NCGR, Davis, CA	China	AY293320	AY293342	AY293371	AY293390	AY293358
Juglans (English Walnut)							
J. regia (DJUG 379.1b)	NCGR, Davis, CA	China	AY293322	AY293344	AY293369	AY293395	AY293356
J. sigillata (DJUG 528)	NCGR, Davis, ca	China	AY293317	AY293346	AY293370	AY293393	AY293357
Rhysocaryon (Black Walnut)							
J. australis (DJUG 429)	NCGR, Davis, CA	Argentina	AY293319	AY293343	AY293379	AY293391	AY293352
J. californica (DJUG 28.5)	NCGR, Davis, CA	USA	AY293323	AY293331	AY293377	AY293384	AY293359
J. microcarpa (DJUG 52.1)	NCGR, Davis, CA	USA	AY293324	AY293332	AY293372	AY293385	AY293349
J. mollis (DJUG 218.3)	NCGR, Davis, CA	USA	AY293329	AY293340	AY293375	AY293388	AY293350
J. neotropica (DJUG 330.2)	NCGR, Davis, CA	Ecuador	AY293321	AY293341	AY293368	AY293389	AY293351
J. nigra (DJUG 57.12)	NCGR, Davis, CA	USA	AY293327	AY293339	AY293366	AY293382	AY293348
J. olanchana (DJUG 212.14)	NCGR, Davis, CA	Mexico	AY293328	AY293333	AY293380	AY293387	AY293353
J. guatemalensis	UC Davis Arboretum	Guatemala	AY293316	AY293345	AY293374	AY293394	AY293354

(*continued*)

Table 7.1 (*continued*)

Taxon (ncgr* Accession no.)	Collection Site	Origin	atpB–rbcL	psbA–trnH	trnS–trnfM	trnT–trnF	trnV–16s rRNA
J. hindsii (DJUG 91.4)	NCGR, Davis, CA	USA	AY293326	AY293330	AY293373	AY293383	AY293363
J. major (DJUG 78.6)	NCGR, Davis, CA	USA	AY293325	AY293338	AY293378	AY293386	AY316201
Trachycaryon (American Butternut)							
J. cinerea	UC Davis Pomology	USA	AY293318	AY293347	AY293376	AY293392	AY293355
Outgroup (Wingnut)							
Pterocarya stenoptera (DPTE 17.1)	NCGR, Davis, CA	China	AY293313	AY293336	AY293381	AY293399	AY293362

*USDA National Clonal Germplasm Repository, One Shields Avenue, University of California, Davis, CA 95616, USA.

and sequenced using an ABI PRISM 377 automated sequencer with BigDye Terminator Cycle Sequencing Kit (Perkin Elmer Biosystems).

Sequence Analyses

Alignment of DNA sequences was performed using the software Sequencher (GeneCodes Corp., Ann Arbor, MI, USA) and subsequently manually adjusted. Indels were coded as binary characters regardless of length, and all characters were equally weighted and unordered. Congruence of intergenic spacer sequences was examined with the incongruence length difference (ILD; Farris et al., 1994, 1995) test as implemented in PAUP* 4.0b10 (partition homogeneity test) (Swofford, 2002). Invariant sites were removed from the test, and 100 replications were performed.

Phylogenetic analyses were performed with PAUP* using the maximum parsimony (MP), maximum likelihood (ML), and minimum evolution (ME) methods. MP analysis was performed using the branch-and-bound algorithm with MulTrees activated and the addition of sequence set to Furthest (character optimization accelerated transformation and tree bisection and reconnection [TBR] branch swapping options) to find most parsimonious trees. Bootstrap analysis (100 replicates) using a heuristic search with the

TBR branch swapping option was performed to assess relative support for different clades. Decay values (Bremer, 1988), the number of extra steps needed to collapse a clade, were computed by examining trees longer than the MP solutions, in which strict consensus trees for all topologies that were up to five steps longer than the MP trees generated using branch-and-bound approach were evaluated. An ME tree was constructed using the Kimura (1980) two-parameter distance with ML estimates of gamma and proportion of invariable sites, and 100 bootstrap replications were used to estimate the support for different nodes. The ML analysis was performed using the best evolutionary models identified by the hierarchical likelihood ratio test and Akaike information criterion method provided in the program Modeltest version 3.06 (Posada and Crandall, 1998) with a Jukes–Cantor tree as the starting tree and indel characters excluded from the analysis. A heuristic search with 10 replications of random addition sequence and TBR branch-swapping options was used. One hundred bootstrap replications were performed under the same conditions.

The sequence divergence between two sister lineages was estimated as the average of all pairwise divergence values between species from the two different clades (Xiang et al., 2000). Evolutionary rates were estimated based on the fossil record, and the time of evolutionary divergence was estimated by dividing the pairwise sequence divergence by twice the rate of nucleotide substitution. The molecular clock hypothesis (Zuckerkandl and Pauling, 1965) was tested by computing the difference in the log likelihood scores between ML trees with and without a molecular clock assumption ($2\Delta = \log L_{\text{no clock}} - \log L_{\text{clock}}$), which follows a chi-square distribution with $n - 2$ degrees of freedom where n is the number of sequences or taxa. A likelihood ratio test (Muse and Weir, 1992), which allows for different transversion and transition rates, was used to test the equality of evolutionary rates along different paths of descent leading to two species, using *Pterocaya* as the reference taxon.

Results

Sequence Characteristics and Divergence

More than 3.8 kb of cpDNA sequence from five spacer regions was assembled for each of the 17 ingroup and 1 outgroup taxa. Although potentially parsimony informative characters were found in all five regions, the variation within individual regions was insufficient to obtain a reasonable level of

phylogenetic resolution. The ILD test to examine the null hypothesis that the five data sets were homogenous with respect to phylogenetic information suggested that pairwise combinations and combination of all five data partitions did not result in significant incongruence ($p = .01$). The sequence data therefore were combined to obtain a composite data matrix to perform the phylogenetic analyses. There were 112 (2.9%) variable sites among 3834 total characters within *Juglans,* of which 40 (1.04%) were potentially parsimony informative. Eight indels out of a total of 19 observed were potentially informative. Alignment of the *trnT–trnF* region required one 18-bp deletion for the sections *Rhysocaryon* and *Trachycaryon* and a 9-bp insertion for *J. microcarpa* within *Rhysocaryon,* and the rest of the indels, including the remaining four spacer regions, were 1–5 nucleotides long. The GC content ranged from 30.1% for the *atpB–rbcL* region to 47.2% for the *trnV–16S rRNA* region, with an overall average of 31.7%, which is typical for plastomes (Palmer, 1991). The ti/tv ratio for pairwise comparisons between taxa ranged from 0 to 3.0, and, surprisingly, most comparisons showed a bias favoring transversion. In general, pairwise sequence divergence was extremely low within and between the sections of *Juglans* (table 7.2). Within the section *Rhysocaryon,* sequence divergence ranged from 0.08% between the two Central American taxa, *J. mollis* and *J. guatemalensis,* to 0.51% between the Central American walnut, *J. olanchana,* and northern California walnut, *J. hindsii*. Among the four Asian butternuts, divergence ranged from 0.159% between *J. ailantifolia* and *J. mandshurica* to 0.635% between *J. cathayensis* and *J. hopeiensis*. Surprisingly, the degree of divergence between American butternut *J. cinerea* and the black walnuts (0.26%) was lesser than to its Asian counterparts (0.717%). The Persian walnut *J. regia* (section *Juglans*) was found to be more similar to the Asian butternuts (0.773% divergence) than to black walnuts (0.818% divergence).

Phylogenetic Reconstruction

Parsimony analysis of the combined data matrix using a branch-and-bound search generated three equally most parsimonious trees of 146 steps (including autapomorphies) with a consistency index of 0.795 (0.595 excluding autapomorphies) and retention index of 0.762. The trees differ only in relative positions of *J. microcarpa* and *J. guatemalensis*. Three major clades are apparent in the strict consensus tree corresponding to the sections *Juglans* (J clade), *Cardiocaryon* (C clade), and *Rhysocaryon–Trachycaryon* (RT clade) (figure 7.2). The single butternut species, *J. cinerea,* native to eastern

Table 7.2 Estimates of pairwise distance between taxa: absolute distance (above diagonal) and Kimura 2-parameter distance (below diagonal)

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *J. nigra*	11	13	12	7	14	6	11	9	6	8	30	22	20	36	27	24	38	
2 *J. hindsii*	0.0016		14	15	8	19	11	14	12	11	11	35	25	23	41	32	29	41
3 *J. californica*	0.0019	0.0030		16	10	17	13	12	12	11	13	34	23	19	37	28	27	39
4 *J. microcarpa*	0.0019	0.0030	0.0029		13	17	8	13	13	7	10	31	24	23	38	29	28	39
5 *J. major*	0.0005	0.0016	0.0019	0.0019		15	7	8	8	7	9	31	21	19	37	28	25	39
6 *J. olanchana*	0.0024	0.0035	0.0038	0.0035	0.0024		12	15	13	10	14	32	26	25	41	31	32	40
7 *J. mollis*	0.0011	0.0021	0.0024	0.0013	0.0011	0.0024		9	9	3	8	26	21	20	35	26	23	37
8 *J. neotropica*	0.0011	0.0021	0.0024	0.0019	0.0011	0.0030	0.0011		8	7	11	33	24	21	38	29	28	40
9 *J. australis*	0.0011	0.0021	0.0024	0.0024	0.0011	0.0030	0.0016	0.0005	7	9	32	19	21	37	26	27	40	
10 *J. guatemalensis*	0.0008	0.0019	0.0021	0.0013	0.0008	0.0022	0.0005	0.0008	0.0013	5	24	18	18	31	23	23		33
11 *J. cinerea*	0.0011	0.0021	0.0024	0.0024	0.0011	0.0030	0.0016	0.0016	0.0016	0.0011		30	22	20	35	27	26	34
12 *J. regia*	0.0062	0.0073	0.0070	0.0065	0.0062	0.0070	0.0056	0.0064	0.0070	0.0051	0.0064		13	30	35	25	27	40
13 *J. sigillata*	0.0040	0.0051	0.0049	0.0048	0.0040	0.0060	0.0049	0.0043	0.0046	0.0038	0.0046	0.0024		18	27	17	18	34
14 *J. hopeiensis*	0.0038	0.0048	0.0040	0.0048	0.0038	0.0054	0.0043	0.0043	0.0043	0.0040	0.0043	0.0064	0.004		24	15	12	37
15 *J. cathayensis*	0.0083	0.0094	0.0086	0.0091	0.0083	0.0100	0.0086	0.0086	0.0089	0.0078	0.0086	0.0086	0.0067	0.0056		8	14	46
16 *J. mandshurica*	0.0057	0.0067	0.0059	0.0065	0.0057	0.0076	0.0059	0.0059	0.0062	0.0054	0.0062	0.0059	0.0043	0.0029	0.0019		6	38
17 *J. ailantifolia*	0.0051	0.0062	0.0054	0.0059	0.0051	0.0070	0.0054	0.0054	0.0056	0.0051	0.0056	0.0062	0.0037	0.0024	0.0032	0.0008	39	
18 *Pterocarya*	0.0078	0.0089	0.0081	0.0089	0.0078	0.0087	0.0081	0.0081	0.0086	0.0073	0.0078	0.0083	0.0070	0.0080	0.0107	0.0083	0.0083	

North America, representing the section *Trachycaryon,* is placed within the black walnut (*Rhysocaryon*) clade. Clades J and RT are strongly supported (bootstrap > 90%, decay = 4), whereas support for C clade, including *J. hopeiensis,* is somewhat lower (bootstrap = 69%), and the sister relationship between the C and RT clades is only weakly supported. However, there is strong support for *J. ailantifolia, J. mandshurica,* and *J. cathayensis* (bootstrap = 83%, decay = 3) within the C clade. The J clade, weakly supported as a sister group to the C and RT clades, is itself strongly supported (bootstrap = 97%, decay = 4) with four unique synapomorphies. Within the clade, the English walnut has seven unique autapomorphies, whereas its sister taxon, *J. sigillata,* possesses one unique mutation. The ME tree (figure 7.2) is basically concordant with the MP analysis, and there is strong bootstrap support for all three major clades.

Modeltest found two optimum models of sequence evolution: the F81+I+G model (I = 0.8957; α = 0.9144; base frequencies: A = 0.3491, C = 0.1465, G = 0.1722, T = 0.3322; Felsenstein, 1981) based on the likelihood ratio test, and the K81uf+I model (R[A↔C] = 1, R[A↔G] = 0.8245, R[A↔T] = 0.1759, R[C↔G] = 0.9378, R[C↔T] = 0.8245, R[G↔T] = 1; I = 0.9378; Kimura, 1981) based on the Akaike information criterion. However, both F81+I+G (–Ln = 5760.87) and K81uf+I (–Ln = 5748.94) models resolved trees with a topology identical to the MP and ME analyses (figure 7.2) and strong bootstrap support, estimated based on the analysis using K81uf+I model, to the sections *Juglans, Cardiocaryon,* and *Rhysocaryon–Trachycaryon*.

There is some evidence for differentiation within the black walnut clade in all three analyses (MP, ME, and ML), indicating biogeographic assemblages representing North American temperate, Central American subtropical, and South American tropical highland black walnuts. However, these affinities are weakly supported except for the South American group comprising *J. neotropica* and *J. australis,* which is supported by two unique synapomorphies. Surprisingly, southern California black walnut, *J. californica,* which is considered a conspecific variant of *J. hindsii,* is placed as sister to the rest of the section, *Rhysocaryon*.

Rate of Divergence

The cpDNA intergenic spacer sequence divergence rates for *Juglans* are unknown. However, one can use estimates of time since divergence based on fossil records to compute the rates of sequence evolution. The average overall rate was calculated by dividing the Kimura 2-parameter distances by twice the

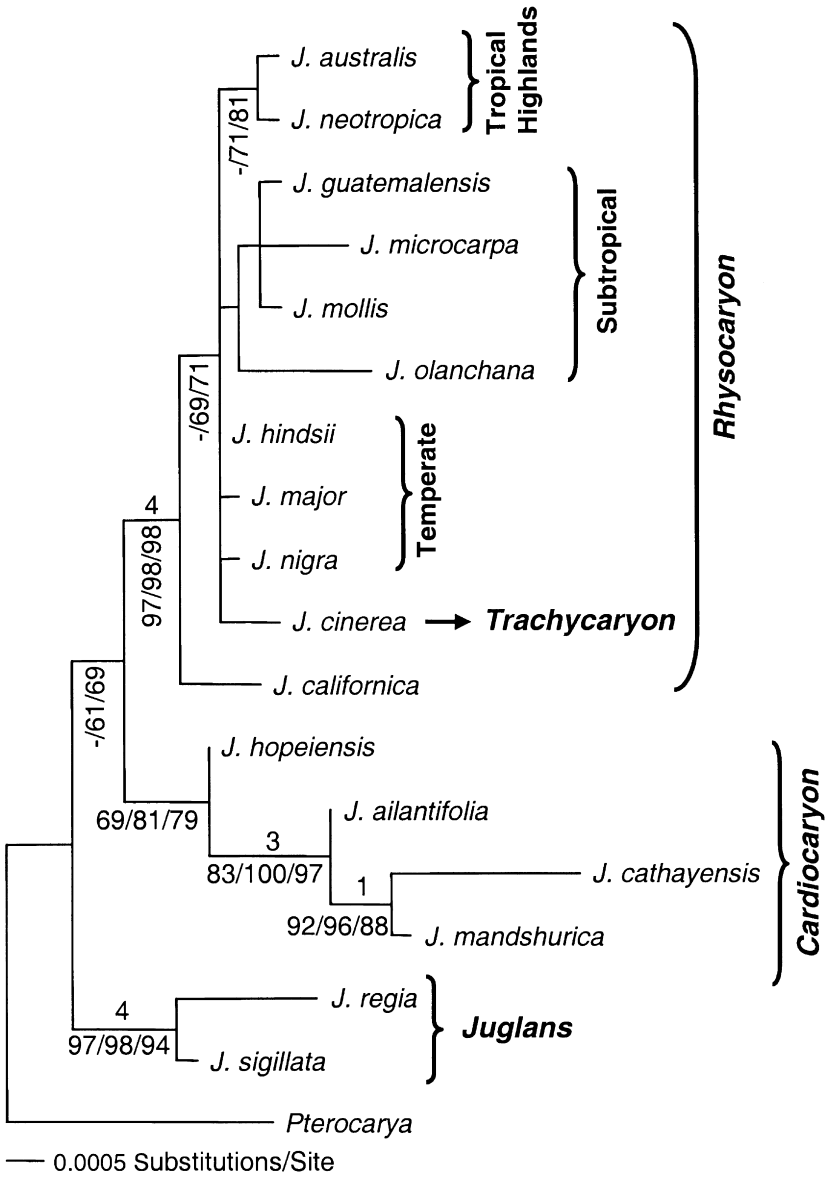

FIGURE 7.2 Phylogram of the genus *Juglans* inferred from maximum likelihood method using K81uf+I model of sequence evolution. Numbers above branches are decay indices, and numbers below are bootstrap support (>50%) based on the following analyses: MP/ME/ML (K81uf+I).

time since divergence. The two landmark divergence events in the evolutionary history of *Juglans* documented in the fossil record were used to compute overall nucleotide substitution rates: the late Paleocene/early Eocene time frame for the divergence of *Pterocarya* and *Juglans* (~54 mya), which yields an average overall rate of sequence divergence of 0.772×10^{-10} substitutions per site per year; and the middle Eocene time frame for the divergence of *Rhysocaryon* and *Cardiocaryon* (~45 mya), as proposed by Manchester (1987), which yields a divergence rate of 0.69×10^{-10} substitutions per site per year. If one of these rates or the average rate (0.731×10^{-10}) is used to compute the time since divergence of different sections within *Juglans,* the results contradict the evolutionary hypothesis based on the fossil history. To address this discrepancy, the test of relative overall nucleotide substitution rates (Muse and Weir, 1992) was used. Using *Pterocarya* as the reference taxon, rates along different paths of descent leading to two ingroup taxa indicated that the section *Juglans,* especially the cultivated walnut *J. regia,* and some taxa in the section *Cardiocaryon* seem to have evolved at significantly different rates than the taxa in the section *Rhysocaryon* (table 7.3). This rate heterogeneity demonstrates that either the ~50-million-year-old *Juglans* lineage is not adequately represented by the extant taxa included in the study, or many taxa at the basal and intermediate nodes might have undergone extinction.

Discussion

Sequence Evolution

Noncoding regions of the chloroplast genome have been suggested to be potentially informative in reconstructing phylogenetic relationships at lower taxonomic levels (Taberlet et al., 1991; Demesure et al., 1995). Nevertheless, the five intergenic spacer sequences (*trnT–trnF, psbA–trnH, atpB–rbcL, trnV–16S* rRNA, and *trnS–trnfM*) used in our study provided little resolution within the major clades, especially among the New World black walnuts and butternut (RT clade). Such low resolution often is seen among taxa that have undergone radiation recently, or it may be result from reticulate evolution within the clade. Despite variation in the information content between different intergenic spacers, the region-specific analysis indicated that the overall phylogenetic structure is conserved across the spacer regions, which was further confirmed by the ILD test. Among the substitutions, transversions were more prevalent than transitions except for the region *psbA–trnH* located within the inverted repeat region of the cpDNA. Although intergenic

Table 7.3 Likelihood ratio between taxa pairs for comparing rates of evolutionary change, with Pterocarya used as a reference taxon

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
J. nigra												***			*		
J. hindsii	4.473									*		***			*		
J. californica	1.390	3.107										***					
J. microcarpa	3.612	2.347	0.547							*		***					
J. major	0.000	4.472	1.389	3.610								***			*		
J. olanchana	3.326	0.289	1.934	0.995	3.325							***			*		
J. mollis	1.352	1.289	1.800	4.291	1.351	1.025						***			*		
J. neotropica	1.357	1.278	1.803	2.080	1.357	0.758	0.000					***			*		
J. australis	2.766	0.484	2.869	2.752	2.769	0.475	0.417	1.852				***			*		*
J. guatemalensis	2.197	7.554	5.363	7.216	2.197	3.715	2.816	4.141	5.087			***			**		
J. cinerea	2.766	0.671	3.214	3.386	2.765	0.797	0.510	0.505	0.017	3.340		***			**		*
J. regia	3307.380	3272.150	3299.350	3277.770	3307.410	3280.030	3300.140	3298.290	3283.570	3320.240	3306.360		***	***	***	***	***
J. sigillata	0.926	2.858	0.600	1.955	0.925	2.257	0.887	0.888	2.139	0.506	2.252	3345.540			**		
J. hopeiensis	1.558	2.243	0.117	0.482	1.557	1.135	1.729	1.729	2.413	5.790	2.773	3309.320	0.501		*		
J. cathayensis	8.757	7.739	5.175	2.607	8.755	6.497	6.814	6.816	8.708	11.182	12.330	3238.370	12.816	6.479			**
J. mandshurica	4.349	4.742	1.554	0.537	4.348	3.107	2.922	2.923	5.197	4.246	6.127	3295.320	4.248	2.056	5.645		
J. ailantifolia	4.816	5.812	1.987	1.100	4.814	4.156	3.476	3.477	6.076	5.144	6.868	3303.260	3.775	4.082	10.045	3.883	

Above diagonal: Taxa pair, with significance at *p < .001, **p < .01, and ***p < .05.

spacers are considered to be under fewer functional constraints and expected to evolve more rapidly than coding sequences (Wolfe et al., 1987; Zurawski and Clegg, 1987), surprisingly, the level of within-clade resolution observed is far lower than the divergence levels reported for the cpDNA *matK* gene and nuclear ITS spacer sequences for the genus *Juglans* (Stanford et al., 2000). Two possibilities could explain the low rate: Either the rate of substitution is inherently low for *Juglans,* or the extant species may not represent the entire ~50 million years of evolutionary history but represent a more recent divergence or a part of it, indicating past extinctions.

Molecular Phylogeny and Cladogenesis

The cladograms from the three analyses (MP, ML, and ME) are concordant with each other and contain three well-supported, monophyletic clades corresponding to the sections *Juglans, Cardiocaryon,* and *Rhysocaryon–Trachycaryon* described within the genus *Juglans*. The clades exhibit a high degree of differentiation and differ significantly in leaf architecture, wood anatomy, and pollen and fruit morphology (Manchester, 1987). However, monophyly of the genus was not evident, probably because of past extinctions obscuring the evolutionary history.

The low consistency index apparently indicates that the spacer regions have been subjected to a moderate level of homoplasy across the lineages during the evolution and diversification of *Juglans*. Previous molecular systematic studies generally supported two major groups, one corresponding to section *Rhysocaryon* (black walnuts) and the second including the members of sections *Cardiocaryon* (Asian butternuts), *Trachycaryon* (North American butternut), and *Juglans* (Fjellstrom and Parfitt, 1995; Stanford et al., 2000). In a recent study, Manos and Stone (2001) found section *Juglans* as the sister group to the black walnuts, suggesting a second biogeographic disjunction within the genus *Juglans*. The single North American butternut species, *J. cinerea,* with nut characteristics (two-chambered nuts with four-ribbed husks) resembling the members of section *Cardiocaryon,* is placed within the *Rhysocaryon* clade, members of which are characterized by four-chambered nuts with indehiscent hulls. The placement of *J. cinerea* within *Rhysocaryon* was supported in a recent phylogenetic study based on the chloroplast *matK* sequences, whereas the phylogeny based on the nuclear ITS sequences, nuclear genome RFLPs, and the combined data set placed *J. cinera* sister to *Cardiocaryon* (Fjellstrom and Parfitt, 1995; Stanford et al., 2000). This controversial placement of butternut into the black walnut clade by cpDNA,

with five unique synapomorphies, strong bootstrap support, and decay index = 4, suggests historical introgression of *Rhysocaryon* chloroplast into an ancestral member of section *Cardiocaryon,* which later may have given rise to the North American butternut, *Trachycaryon.* The introgression may have occurred during range reduction and selective extinction of juglandaceous taxa in general and of *Juglans* in particular in northern latitudes, including some of the ancestral butternuts in North America in the early Neogene. Fossil records indicate that butternuts were widely distributed throughout the northern latitudes during the late Eocene and Oligocene. Chloroplast capturing has been documented in several plant groups, perhaps the best studied of which are in cotton (Wendel et al., 1991). The present-day *Trachycaryon* is represented by a single taxon, *J. cinerea,* found only in eastern North America and sympatric with *Rhysocaryon*.

Members of the section *Rhysocaryon* are not well resolved; however, in the MP and ML analyses, they are segregated into three biogeographic groups reflecting specific adaptations to the temperate, subtropical, and tropical highland environments in which they are found (figure 7.2). The clade as a whole is well supported, with five unique synapomorphies and a bootstrap value and decay index of 97% and 4, respectively. Many of these taxa have accumulated a number of autapomorphic mutations along with some homoplasious ones shared mostly within and to a less extant between different clades. The basal placement of southern California black walnut, *J. californica,* within the RT clade, well separated from its putative close relatives *J. hindsii* and *J. major,* was surprising because *J. hindsii* has often been treated as a conspecific variant within *J. californica* (Wilken, 1993), and a sister relationship between these two taxa has been reported in other studies (Fjellstrom and Parfitt, 1995; Stanford et al., 2000). The basal placement of *J. californica* probably results from two substitutions that it shares with the section *Cardiocaryon,* which may represent convergence. Lower resolution within the black walnut section probably indicates recent diversification, possibly in the upper Miocene; reticulate evolution within the section; and persistence of ancestral polymorphisms through speciation. This is contrary to the fossil evidence that suggests that the earliest evolutionary split within *Juglans* during the middle Eocene involved the origin of black walnut and butternut sections and thus these two sections would have had enough time for intersectional and intrasectional diversification.

Section *Cardiocaryon* is well supported and resolved as a monophyletic lineage. Within *Cardiocaryon, J. hopeiensis* is moderately supported as sister to the remaining three Asian butternuts, *J. ailantifolia, J. cathayensis,* and

J. mandshurica, which are well supported as a clade in all three analyses. In overall tree morphology, *J. hopeiensis* closely resembles the Persian walnut, *J. regia,* but the nut characters are similar to *J. mandshurica,* and it has been considered as either an interspecific hybrid between *J. regia* and *J. mandshurica* (Rehder, 1940) or as a subspecies of *J. mandshurica* (Kuang et al., 1979). In contrast to earlier studies that placed *J. mandshurica* as sister to *J. ailantifolia* and *J. cathayensis* (Stanford et al., 2000; Fjellstrom and Parfitt, 1995), in our study *J. cathayensis* and *J. mandshurica* are closely united with five unique synapomorphies.

The Persian walnut, *J. regia,* and its sister taxon, *J. sigillata* (section *Juglans*), form a distinct clade sister to both *Cardiocaryon* and *Rhysocaryon–Trachycaryon* in all three of our analyses. This was in contrast to earlier studies, which placed the cultivated walnut *J. regia* either within *Cardiocaryon* (Fjellstrom and Parfitt, 1995; Stanford et al., 2000) or within *Rhysocaryon* (Manos and Stone, 2001). The early evolutionary split of this clade within the genus *Juglans* contradicts the traditional taxonomic treatments and fossil evidence, both of which supported the almost simultaneous ancient divergence of sections *Cardiocaryon* and *Rhysocaryon,* and the origin of the genus in the middle Eocene (Manchester, 1987). Within the section *Juglans,* the cultivated species *J. regia* accumulated seven unique autapomorphies with unique nut characteristics (thin-shelled four chambered nuts) and is differentiated from its sister taxon *J. sigillata,* which contains one unique mutation and retains many primitive nut characteristics such as thick rough-shelled nuts with dark kernels (Dode, 1909a). *J. sigillata* may represent a semidomesticated form within the section. It is known to have been cultivated in southern China for its oil and wood. Furthermore, early Chinese records suggest that domestication and selection of walnut occurred in the southern Tibetan and Yunnan regions, and better varieties were brought to the north during the Han dynasty (de Candolle, 1967).

Biogeography

The extant species of *Juglans* show an intercontinental disjunction with the modern distributions of sections *Juglans* and *Cardiocaryon* limited to Eurasia and section *Rhysocaryon* endemic to the Americas. A single butternut species, *J. cinerea,* with modern distribution in eastern North America, is generally considered to be a disjunct of *Cardiocaryon* (Asian butternuts) (Manchester, 1987). Recently, Manos and Stone (2001) proposed a sister group relationship between the cultivated walnut, *J. regia,* and section *Rhysocaryon,*

suggesting the possibility of a second disjunction within *Juglans*. These disjunctions could have arisen as a result of either a vicariance event disrupting the geographic continuity of ancestral populations that once spanned from Eurasia to North America or a long-distance dispersal from one region to the other. The vicariance hypothesis is favored over the long-distance dispersal theory because of the large fruit size in *Juglans,* which does not appear to have great dispersal ability.

It is likely that the ancestral populations of *Juglans* were widely distributed throughout the middle and upper latitudes of the Northern Hemisphere during the early Tertiary up until the late Miocene, when the climate was generally warm enough (Wolfe and Upchurch, 1987) for the successful establishment and periodic exchange of broad-leaved deciduous taxa across the Bering and North Atlantic land bridges connecting Asia, North America, and Europe (McKenna, 1983; Tiffney, 1985b; Ziegler, 1988). The gradual cooling during the Neogene produced range contraction and greatly reduced the migration between Eurasian and North American floras by the mid-Pliocene (Wolfe, 1978; Tiffney, 1985a). Further climatic changes during the Quaternary eliminated mixed mesophytic forests in the northern latitudes, leaving eastern North America, eastern Asia, and to a much lesser extent the Balkans and Caucasus as the main refugia of many genera (Tiffney, 1985a).

Based on fossil evidence, Manchester (1987) proposed that the divergence of *Pterocarya* and *Juglans* may have occurred sometime during the late Paleocene or early Eocene (~54 mya) and that the initial split of sections *Rhysocaryon* and *Cardiocaryon* probably occurred during the middle Eocene (45 mya) in North America, but the two sections were clearly resolved only in the early Oligocene (38 mya). However, based on extensive analysis of nut specimens of a fossil walnut, *J. eocinerea* from the Beaufort Formation (Tertiary), southwestern Banks Island, arctic Canada, Hills et al. (1974) concluded that it is closely related and probably ancestral to fossil *J. tephrodes* from early Pliocene Germany and the extant *J. cinerea* from the eastern United States. Furthermore, they argued that butternuts may have evolved independently in the Arctic, attaining a broad distribution in the upper latitudes of the Northern Hemisphere by the Miocene, and that subsequent geoclimatic changes (Wolfe and Leopold, 1967; Axelrod and Bailey, 1969; Wolfe, 1971) resulted in the southward movement of the floras across the Bering Strait. However, the early Pleistocene glaciations have completely eliminated butternuts from Europe and northwestern parts of North America, leaving small disjunct populations in eastern Asia to evolve into three major present-day taxa, *J. cathayensis, J. mandshurica,*

and *J. ailantifolia,* and one south of the glacial limit in North America to evolve to its present form, *J. cinerea*. The geographic and stratigraphic fossil distribution strongly supports the hypothesis that butternuts may have originated and radiated from high northern latitudes. At about the same time, black walnuts spanned throughout North America and extended into the Southern Hemisphere, reaching Ecuador by the late Neogene, and remained endemic to the Americas throughout their evolutionary history.

One can argue that if butternuts and black walnuts diverged from a common ancestor in North America during the middle Eocene, as suggested by Manchester (1987), there would have been ample opportunity for both groups to become established in both Asia and North America because both the Bering and North Atlantic land bridges were in continuous existence from the middle Eocene through the late Miocene, when there was a favorable climate in upper latitudes for the establishment and dispersal of broad-leaved deciduous taxa (Wolfe, 1972, 1978; Tiffney, 1985b). However, the distributional range of the Tertiary fossils of butternuts and black walnuts does not overlap except in the northwestern parts of the United States around 40°N latitude, strongly suggesting that they may have evolved independently, as suggested by Hills et al. (1974). The weak support for the sister relationship between these two groups observed in our phylogenetic analysis further substantiates this point and also suggests that they may not share an immediate common ancestor.

An analysis of the comparative rates of molecular evolution along the branches of the cladogram indicated that the rates did not conform to the expectation of the molecular clock hypothesis (Zuckerkandl and Pauling, 1965). Relative rates of sequence evolution based on overall substitutions, estimated using *Pterocarya* (outgroup) as the reference, indicated that the differences between species pairs are mostly insignificant except for combinations involving *J. regia* and a few members of *Cardiocaryon,* especially *J. cathayensis* (table 7.3). The differential rates of divergence associated with these Eurasian taxa and their basal placement in the cladograms could indicate their ancient and distinct origin or the fact that extant taxa may not reflect the entire evolutionary history of *Juglans*. The range reduction, local extinctions, and geographic isolation during the late Tertiary and early quaternary glaciations and the subsequent expansion into central Asia and southeastern Europe might have played an important role in the evolution and diversification of sections *Juglans* and *Cardiocaryon*. Influence of both natural and human selection and introgression during domestication may have further altered the rate and direction of evolution of the cultivated walnut.

Estimates of time since divergence may be obtained from fossil evidence or from computations assuming a molecular clock. For *Juglans,* the sequence divergence rates for the five intergenic cpDNA regions used in this study are unknown, and the estimation of divergence times relies strictly on fossil records. Therefore, the accuracy of fossil records and the variation of molecular evolutionary rate and patterns of extinction in a clade affect the estimations. Nevertheless, the estimations of nucleotide substitution rates or time since divergence using the molecular clock hypothesis, although based on uncertain assumptions and approximate values, are helpful in understanding the tempos of evolution and plant historical geographies (Parks and Wendel, 1990; Crawford et al., 1992; Wendel and Albert, 1992).

Paleobotanical evidence suggests two major landmarks in the evolution and diversification of *Juglans,* the first corresponding to the divergence of *Pterocarya* and *Juglans* (early Eocene, ~54 mya) and the second corresponding to the early split between sections *Rhysocaryon* and *Cardiocaryon* (mid-Eocene, ~45 mya) (Manchester, 1987). Based on these events, the rates of divergence between the outgroup taxon *Pterocarya* and the ingroup *Juglans,* and between the sections *Rhysocaryon* and *Cardiocaryon* within *Juglans,* were estimated to be approximately 0.772×10^{-10} and 0.69×10^{-10} nucleotide sites per year, respectively. These estimates were much lower than the earlier reports between *Pterocarya* and *Juglans* (3.36×10^{-10}) based on the cpDNA RFLPs (Smith and Doyle, 1995) and between the sections *Cardiocaryon* and *Rhysocaryon* (1.17×10^{-9}) based on nuclear genome RFLPs (Fjellstrom and Parfitt, 1995). The nonparametric rate smoothing method (Sanderson, 1997), which combines likelihood and the nonparametric penalty function to estimate ages for different nodes based on fossil calibration, has resulted in inconsistent estimation of age for different nodes with large variances.

Given the many caveats mentioned earlier, we proceeded with caution in calculating the time since divergence for some of the other major bifurcations observed in the phylogenetic analyses. The time since divergence between clades provides a rough estimate of the time since isolation between them. If an overall divergence rate of 0.772×10^{-10} substitutions per site per year, estimated from the time since divergence between the outgroup taxon, *Pterocarya,* and the ingroup *Juglans* (54 mya) as a whole, is used, then the divergence times between sections *Rhysocaryon* and *Juglans, Rhysocaryon* and *Cardiocaryon,* and *Cardiocaryon* and *Juglans* are estimated to be 41.6, 40.2, and 43.8 mya, respectively. However, if it is based on 0.69×10^{-10} nucleotide sites per year, estimated using the Middle

Eocene as the time frame for divergence between sections *Rhysocaryon* and *Cardiocaryon* (45 mya) (Manchester, 1987), the divergence times between section *Rhysocaryon* and *Juglans* and section *Cardiocaryon* and *Juglans* are estimated to be 46.5 and 50 mya. Based on sequence data, estimated divergence times for different lineages within *Juglans* range from the early to late Eocene, which coincide roughly with the divergence times proposed by Manchester (1987), but the sequence of divergence events contradicts the fossil evidence. Contrary to fossil evidence, which suggests the split between black walnuts and butternuts as the earliest evolutionary event, our analyses suggest that the divergence of section *Juglans* is the first splitting of the lineage to have occurred within the genus *Juglans*.

Origin and Domestication of Cultivated Walnut, *J. regia*

One of the puzzling biogeographic questions in *Juglans* is the presence of a Eurasian section comprising two taxa, *J. regia* and *J. sigillata,* with four-chambered nuts similar to *Rhysocaryon,* which is endemic to the New World. The nutshell thickness of these taxa may vary from extremely thick, as in black walnuts in the case of *J. sigillata,* to paper-thin, as in *J. regia,* whereas the other Asian section, *Cardiocaryon,* strictly possesses two-chambered, thick-shelled nuts. The placement of the cultivated species *J. regia* has been problematic in earlier phylogenetic studies, and recent studies place it as sister to either butternuts (Stanford et al., 2000) or black walnuts (Manos and Stone, 2001). Our data strongly support the section *Juglans* as an independent clade basal to the remaining three sections within the genus *Juglans*. It evolved at a significantly higher rate than section *Rhysocaryon* and some taxa of section *Cardiocaryon*. However, the evolutionary history of the section *Juglans* may have been confounded by widespread extinctions, geographic isolation, and bottlenecks during the Pleistocene glaciations, when the ancestral forms were in refugia in central Asia and southeastern Europe. Subsequent expansion, human selection, and introgression among isolated diverse populations during the post-Pleistocene glaciations may have rapidly changed the genetic structure and differentiation patterns within the section *Juglans* (Popov, 1929; Beug, 1975; Huntley and Birks, 1983). *J. regia* is a highly domesticated and economically important walnut species, occurring mostly under cultivation in both the Old and New World, whereas its sister taxon, *J. sigillata,* with primitive nut characteristics, may represent a semidomesticated or primitive form within the section restricted to parts of southern China.

It is appropriate here to provide some details on the domestication history and development of cultivated walnut. All walnut species bear edible nuts, but the Persian or English walnut (*J. regia*) is the most delicious, economically important, and successfully cultivated throughout the temperate regions of the world. Although its origin is obscure, it has been thought to be indigenous to the mountainous regions of central Asia extending from the Balkan region across Turkey, the Caucasus, Iraq, Iran, and Afghanistan, parts of Kazakhstan, Uzbekistan, and southern Russia to northern India (Dode, 1909b; Forde, 1975; McGranahan and Leslie, 1991). However, the pollen data (Bottema, 1980) suggest that *J. regia* went into extinction in southeastern Europe and southwestern Turkey during the glacial period but survived in the Pontic and Hyrcanic refugia and reappeared there around 2000 BC (Zohary and Hopf, 1993). If true, this evidence strongly points to the Caucasus and northern Iran as the most plausible area of walnut domestication. The walnuts have been found in prehistoric deposits in Europe dating back to the Iron Age and were also prevalent in Palestine and Lebanon during that period (Rosengarten, 1984). At present, natural populations of Persian walnut, some as good as modern cultivars, exist in many parts of Central Asia from the Caucasus to the mountains of Tien-Shan. They represent the natural range of diversity, probably as a consequence of complex interactions of natural and human selection after postglacial expansion and domestication (Takhtajan, 1986; Vavilov, 1992). However, *J. regia* found in the flora of the Khasi-Manipur province belong to the eastern Asiatic elements tied to floras of the eastern Himalayas, upper Burma, and eastern China. This region represents one of the most important centers of the Tertiary flora of eastern Asia (Bor, 1942). Furthermore, it is suggested that the mountainous regions of central and western China and adjacent lowlands along with west Asia and Asia Minor are areas of diversity for walnut. The Chinese center of diversity is further supported by the ancient walnut fossils and archaeological material found in the ruins at Cishan Hebei and the walnut pollen dating back to 4000–5000 BC found in the spore pollen analysis of Banpo Xian (Rong-Ting, 1990).

Further support for the Eurasian origin of cultivated walnuts comes from the fact that the Tertiary relict flora comprising mostly deciduous and some evergreen woody taxa survived in the regions of equable climate in southeastern Europe, the Caucasus, and southwestern and eastern Asian refugia during the late Miocene to Pliocene cooling and Quaternary glaciations (Tiffney, 1985a, 1985b; Wen, 1999; Xiang et al., 2000), where perhaps

small remnant populations of ancestral walnuts may have survived. Expansion of these relict floras into the central European regions comprising Balkan, Carpathian, and Euxinian provinces and south into Asia Minor, northern parts of Iran, Afghanistan, Turkmenistan, Uzbekistan, north into Tien-Shan mountains, and the Himalayas started at the end of the glacial period and the beginning of the Holocene (Beug, 1975; Davis, 1982; Takhtajan, 1978). There is evidence of a floristic connection between some Tertiary relict species from the south central European refugia, which migrated via the southern route of the North Atlantic land bridge, and the East Asian relicts including eastern China and some regions in the Himalayas, derived predominantly through migration across the Bering land bridge. The East Asian refugia may have included some of the ancestral forms of butternuts and cultivated walnut, *J. regia,* which may have gradually evolved into the modern Asian butternut clade (Wen, 1999, 2001; Milne and Abbott, 2002). According to Rong-Ting (1990), the native populations of walnut in China exhibit a wide range of variation for all discernible characters, with 6000–7000 years of evolutionary and domestication history, extending across a wide range of environments. Dode (1909b) described the section *Juglans* by recognizing six species in addition to *J. regia* with distribution extending from central to East Asia including China and the Himalayan region, which others have not accepted but which could be treated as ecotypes within *J. regia*.

In summary, the cladogenesis within *Juglans* based on cpDNA intergenic sequence analyses does not fully corroborate the evolutionary hypothesis based on the fossil history and biogeographic evidence. Neither the fossil nor molecular phylogenetic evidence strongly supports the monophyletic origin of *Juglans*. If Eocene North America is considered the center of origin and diversification of *Juglans,* as suggested by Manchester (1987), there would have been sufficient opportunity for members of different sections to become distributed in both North America and Eurasia because land bridges across the Bering Sea and North Atlantic Ocean were in continuous existence from the middle Eocene through the late Miocene (Tiffney, 1985b). On the contrary, the Tertiary fossil evidence suggests that section *Rhysocaryon* remained endemic to the Americas throughout its evolutionary history, and the section *Juglans* was not represented in the fossil records from North America. Furthermore, the results allow for some generalizations on the origin and evolution of the genus *Juglans*: The cpDNA intergenic spacer sequence divergence levels observed within and between different sections of *Juglans* are low; basal placement of the section *Juglans* in the phylogenetic analyses suggests its ancient origin contrary to fossil

evidence, which suggests the earliest origin of sections *Rhysocaryon* and *Cardiocaryon;* the two Asian sections, *Juglans* and *Cardiocaryon*, evolved at different rates than *Rhysocaryon*; and the extant taxa may not adequately represent the entire evolutionary history of the genus.

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