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Molecular phylogeny of *Juglans* (Juglandaceae): a biogeographic perspective

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Abstract The eastern Asian and eastern North American disjunction in *Juglans* offers an opportunity to estimate the time since divergence of the Eurasian and American lineages and to compare it with paleobotanical evidence. Five chloroplast DNA noncoding spacer (NCS) sequences: *trn*T−*trn*F, *psb*A−*trn*H, *atp*B−*rbc*L, *trn*V-16S *rRNA*, and *trn*S-*trn*fM and data from earlier studies (*mat*K, ITS, and nuclear RFLP) were used to reconstruct phylogeny and to estimate the divergence time of major lineages. Seventeen taxa from four sections of *Juglans* and two outgroup taxa, *Pterocarya stenoptera* and *Carya illinoiensis* were included. NCS data was congruent only with *mat*K data. Both maximum parsimony (MP) and maximum likelihood (ML) cladograms were concordant at the sectional level and revealed three well-supported monophyletic clades corresponding to sections *Juglans*, *Cardiocaryon*, and *Rhysocaryon* in both NCS and combined analyses. The single extant American butternut, *Juglans cinerea* was placed within the poorly resolved, but well-supported *Rhysocaryon*. Placement of taxa within *Rhysocaryon* and *Cardiocaryon* were inconsistent between NCS and combined analyses. Overall, the results suggest that: (1) the NCS sequence divergence observed within and between sections of *Juglans* is low and the addition of *mat*K data only marginally improved resolution within *Rhysocaryon*; (2) the early divergence of section *Juglans* in both MP and ML analyses of NCS and combined data implies its ancient origin in contrast to fossil evidence, which suggests the earliest divergence of sections *Rhysocaryon* and *Cardiocaryon*; and (3) the extant taxa may not hold the footprints to unravel the evolutionary history of the genus.

Keywords Biogeography · Chloroplast DNA · Cladogenesis · Disjunction · Juglandaceae · *Juglans* · Noncoding spacer sequence · Phylogeny · Walnut

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Introduction

Juglans L. is one of the eight living genera in the family Juglandaceae consisting of ~21 extant taxa divided into four sections mainly based on fruit morphology, wood anatomy, and foliage architecture (Dode 1909a,b; Miller 1976; Manning 1978). Members of the genus *Juglans* are deciduous, monoecious trees distributed from North and South America to southeastern Europe, eastern Asia, and Japan (Manning 1978) exhibiting a discontinuous distribution between eastern Asia and North America (Fig. 1). A fossil species is also reported from the West Indies (Descourtilz 1829; Manning 1960). Section *Rhysocaryon* (black walnuts) is endemic to the Americas and includes six North American taxa: *Juglans californica* S. Wats., *Juglans hindsii* (Jeps.) Rehder, *Juglans major* (Torr. ex Sitsgr.) Heller, *Juglans microcarpa* Berl., *Juglans mollis* Engelm. ex Hemsl., and *Juglans nigra* L.; three Central American taxa: *Juglans olanchana* Standl. & L. O. Williams, *Juglans*

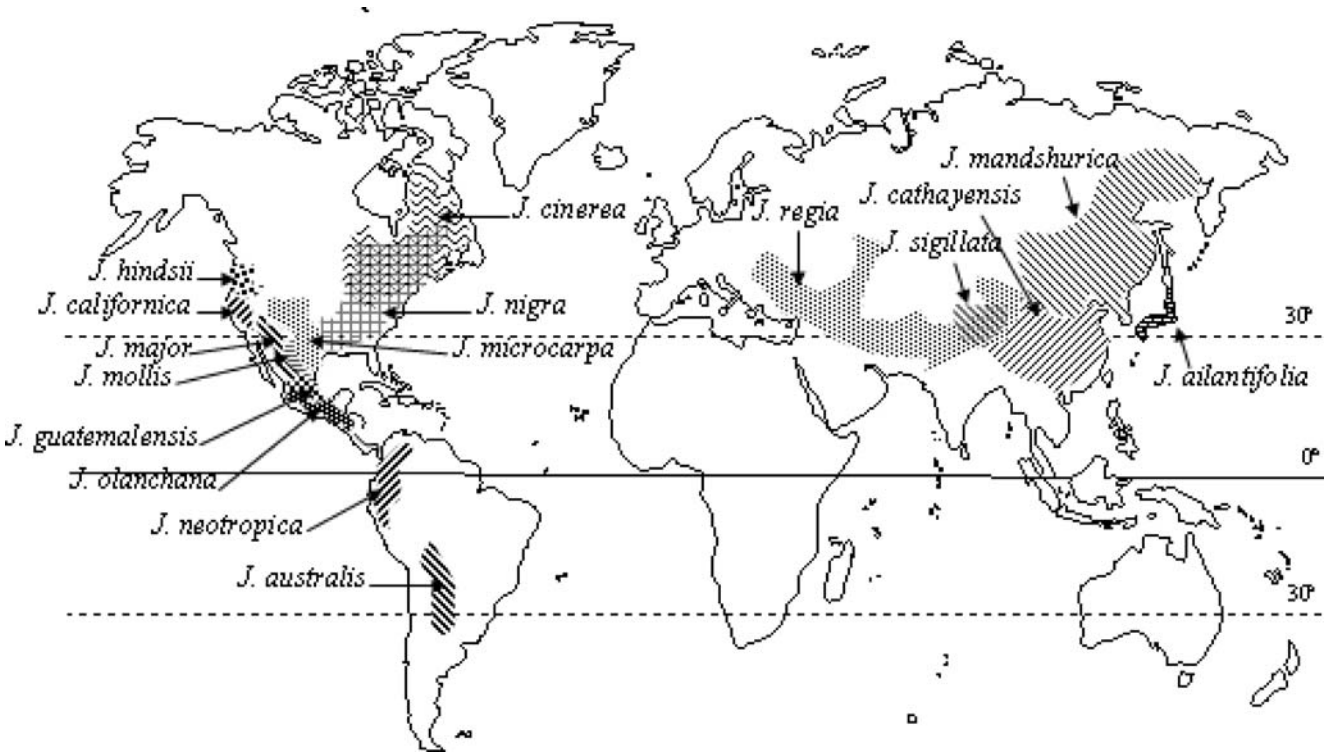

Fig. 1 Modern distribution of *Juglans* (Juglandaceae) taxa used in the study (modified from McGranahan and Leslie 1991). The distribution of the cultivated species, *J. regia* extends beyond its natural range

steyermarkii Mann., and *Juglans guatemalensis* Mann.; and four South American taxa: *Juglans australis* Griesb., *Juglans boliviana* (C.DC.) Dode, *Juglans neotropica* Diels, and *Juglans venezuelensis* Mann., mainly occurring in the highlands. Black walnuts typically bear four-chambered nuts with thick nutshells and septa; thick, indehiscent and adherent husks; and are borne singly or in pairs. Section *Cardiocaryon* (Oriental butternuts) contains three taxa: *Juglans ailantifolia* Carr., *Juglans cathayensis* Dode, and *Juglans mandshurica* Maxim., all native to East Asia, while section *Trachycaryon* consists of a single extant butternut, *Juglans cinerea* L. native to eastern North America. Asian butternuts possess two-chambered nuts with thick nutshells and septa, indehiscent and persistent husk, and are borne in long racemes of up to 20 nuts, while American butternut bears two-chambered nuts with thick, rough shells featuring distinct sharp ridges and furrows on the surface, indehiscent and persistent husk, and are borne in clusters of 2–3 nuts on long stalks. Section *Juglans* includes the cultivated Persian or English walnut, *Juglans regia* L. with a distribution ranging from southeastern Europe to China and the Himalayas. Persian walnut bears four-chambered nuts generally singly or in pairs, occasionally three nuts, smooth, thin nutshells, and papery septa, and a dehiscent husk that separates easily from the nut. Section *Juglans* also includes the iron walnut, *Juglans sigillata* Dode, from the southern Chinese province of Yunnan and Tibet, with thick, rough-shelled nuts, and characteristic dark-colored kernels (Dode 1909a). The iron walnut is sometimes considered as an ecotype of *J. regia*, but some botanists have treated it as a separate species (Kuang et al. 1979). Complete descriptions of the morphological variation, ecological distribution, and taxonomic treatment of the genus *Juglans* are found in Manning (1957, 1960, 1978) and McGranahan and Leslie (1991).

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 among them Asa Gray's pioneering accounts in the mid-nineteenth century of the floristic similarities of East Asia and eastern North America serves as the foundation for the modern syntheses of plant species disjunctions (Gray 1859, 1878). Subsequently, Chaney (1947) and Axelrod (1960) have independently modified Gray's hypothesis to suggest that the floristic similarities originated as a result of range restrictions and southward migration of the largely unchanging Arcto-Tertiary Geoflora of the Northern Hemisphere due to climatic changes in the late Tertiary and Quaternary. More 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,b). These discoveries suggest 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) and later cooling during the Oligocene and Miocene resulted in diversification and expansion of broad-leaved, deciduous taxa throughout the northern latitudes of Eurasia and North America (Wolfe 1978, 1985). Floristic exchanges across the Bering land bridge throughout the mid-Tertiary and the North Atlantic land bridge during the late Eocene (McKenna 1983; Tiffney 1985b) produced diverse mixed mesophytic forests of eastern Asia and North America. Continued cooling in the Pliocene produced southward movement of the mixed mesophytic forests from northern latitudes and greatly reduced the possibility of migration between Eurasia and North America (Wolfe 1978, 1985; Tiffney 1985b). Further climatic changes and glaciations during the Quaternary effectively eliminated the northern mixed mesic forests, leaving North America, eastern Asia, and, to a much lesser extent, the Balkans and Caucasus, as refugia for many genera (Graham 1972; Tiffney 1985b). Conversely, convergent adaptation to similar climatic conditions and long-distance dispersal has also been implicated for the development of present-day floristic disjunctions (Raven 1972; Wolfe 1975). Overall, biogeographical disjunctions originated as a result of complex interactions of many processes such as migration/dispersal, extinction, speciation, and vicariance. Alternatively, similarities among many congeneric disjunct taxa may have resulted from stasis or low rates of morphological evolution (Wen 1999, 2001).

Phylogenetic studies with the application of molecular clock and fossil evidence have been used to estimate the time since divergence between eastern Asian and eastern North American disjunct taxa (Wen and Stuessy 1993; Schnabel and Wendel 1998; see review Wen 1999; Xiang et al. 2000; Azuma et al. 2001; Donoghue and Smith 2004). These studies suggest middle to late Tertiary as the period of formation of the disjunct pattern for many temperate taxa (Xiang et al. 2000; Wen 2001). The eastern Asian–eastern North American disjunction in *Juglans* and the rich fossil history (Manchester 1987) from the Tertiary of the Northern Hemisphere offer an opportunity to estimate the time since divergence between Eurasian and North American lineages and compare it with the paleobotanical evidence. Earlier molecular systematic studies based on nuclear RFLPs (Fjellstrom and Parfitt 1995) and *mat*K and ITS sequences (Stanford et al. 2000) support the traditional taxonomic classification of *Juglans* (Dode 1909a,b; Manning 1978) and are consistent with what is known about the geological history of the genus (Manchester 1987). The present study differs from earlier studies in that it attempts to estimate the rate and time of divergence among taxa within and between different clades representing the three major sections, *Juglans*, *Cardiocaryon*, and *Rhysocaryon*, and examine the contentious placement of the only extant New World butternut, *J. cinerea*.

Noncoding intergenic spacer (NCS) 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 efforts to infer phylogenetic relationships both 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 at infraspecific levels (Demesure et al. 1996; Petit et al. 1997; Mohanty et al. 2001).

The objectives of this study are to: (1) examine the phylogenetic utility of cpDNA noncoding spacer (NCS) sequences and assess whether NCS sequences are congruent with data from earlier studies, ITS and *mat*K sequences (Stanford et al. 2000), and nuclear RFLP data (Fjellstrom and Parfitt 1995); (2) determine whether combined data supports previous phylogenetic hypothesis and provide additional support and resolution; (3) estimate divergence time of major lineages in the NCS and combined analyses; and (4) discuss the biogeography of eastern Asian and eastern North American disjuncts of *Juglans* based on phylogeny, fossil evidence, and modern distribution.

Materials and methods

Taxa examined and molecular methods

Seventeen taxa representing the four sections of *Juglans* and two outgroup taxa, *Pterocarya stenoptera* and *Carya illinoiensis* were sampled for this study (Table 1). *Pterocarya* and *Carya* were chosen as outgroup taxa because of their close affinity to *Juglans* (Manos and Stone 2001; Smith and Doyle 1995). The ingroup taxa includes two taxa of uncertain taxonomic status: Guatemalan walnut, *J. guatemalensis*, which was considered a separate species (Manning 1948) is now treated as synonymous to *J. olanchana* (Manning 1978), although the species differ in foliage characteristics. *Juglans hopeiensis* a species native to Hebei province in northeastern China (McGranahan and Leslie 1991) exhibits leaf morphology and fruiting structures similar to *J. regia* and nut characteristics resembling *J. mandshurica*. Classification of *J. hopeiensis* as a species is not recognized by Manning (1978).

Total DNA was isolated using the CTAB method (Doyle and Doyle 1987), further extracted with phenol-chloroform,

Table 1 Species list, collection site, geographic origin, and GenBank accession numbers

Taxon (NCGR[a] accession no.)	Collection site	Origin	NCS region (GenBank accession numbers)				
			*atp*B-*rbc*L	*psb*A-*trn*H	*trn*S-*trn*fM	*trn*T−*trn*F	*trn*V-16S *rRNA*
Cardiocaryon (Asian butternut)							
Juglans ailantifolia (DJUG 91.4)	NCGR, Davis, CA	Japan	AY293314	AY293335	AY293365	AY293398	AY293360
Juglans cathayensis (DJUG 11.4)	NCGR, Davis, CA	Taiwan	AY293312	AY293334	AY293367	AY293396	AY316200
Juglans mandshurica (DJUG 13.1)	NCGR, Davis, CA	Korea	AY293315	AY293337	AY293364	AY293397	AY293361
Juglans hopeiensis (DJUG 462)	NCGR, Davis, CA	China	AY293320	AY293342	AY293371	AY293390	AY293358
Juglans (English walnut)							
Juglans regia (DJUG 379.1b)	NCGR, Davis, CA	China	AY293322	AY293344	AY293369	AY293395	AY293356
Juglans sigillata (DJUG 528)	NCGR, Davis, CA	China	AY293317	AY293346	AY293370	AY293393	AY293357
Rhysocaryon (Black walnut)							
Juglans australis (DJUG 429)	NCGR, Davis, CA	Argentina	AY293319	AY293343	AY293379	AY293391	AY293352
Juglans californica (DJUG 28.5)	NCGR, Davis, CA	USA	AY293323	AY293331	AY293377	AY293384	AY293359
Juglans microcarpa (DJUG 52.1)	NCGR, Davis, CA	USA	AY293324	AY293332	AY293372	AY293385	AY293349
Juglans mollis (DJUG 218.3)	NCGR, Davis, CA	USA	AY293329	AY293340	AY293375	AY293388	AY293350
Juglans neotropica (DJUG 330.2)	NCGR, Davis, CA	Ecuador	AY293321	AY293341	AY293368	AY293389	AY293351
Juglans nigra (DJUG 57.12)	NCGR, Davis, CA	USA	AY293327	AY293339	AY293366	AY293382	AY293348
Juglans olanchana (DJUG 212.14)	NCGR, Davis, CA	Mexico	AY293328	AY293333	AY293380	AY293387	AY293353
Juglans guatemalensis	Arboretum, UC Davis	Guatemala	AY293316	AY293345	AY293374	AY293394	AY293354
Juglans hindsii (DJUG 91.4)	NCGR, Davis, CA	USA	AY293326	AY293330	AY293373	AY293383	AY293363
Juglans major (DJUG 78.6)	NCGR, Davis, CA	USA	AY293325	AY293338	AY293378	AY293386	AY316201
Trachycaryon (American butternut)							
Juglans cinerea	Pomology, UC Davis	USA	AY293318	AY293347	AY293376	AY293392	AY293355
Outgroup							
Pterocarya stenoptera (DPTE 17.1)	NCGR, Davis, CA	China	AY293313	AY293336	AY293381	AY293399	AY293362
Carya illinoiensis	Pomology, UC Davis	USA	Can be obtained from the corresponding author				

[a] National Clonal Germplasm Repository, USDA-ARS, One Shields Avenue, University of California, Davis, CA 95616, USA
NCS cpDNA non-coding spacer

and treated with RNase to remove protein and RNA contaminants, respectively.

Five cpDNA NCS regions: *trn*T−*trn*F, *psb*A−*trn*H, *atp*B−*rbc*L, *trn*V-16S *rRNA*, and *trn*S−*trn*fM were PCR-amplified in 100-µl reaction mixtures containing 10 µl of 10X PCR buffer containing 2 mM $MgCl_2$, 10–20 pM 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: 1 cycle of 5 min at 94°C; 30 cycles of 1 min at 94°C, except for *trn*S-*trn*fM which required 45 s at 94°C; 1 min at 50°C for *trn*T−*trn*L and *trn*L−*trn*F, 55°C for *atp*B−*rbc*L, *psb*A−*trn*H, and *trn*V-16S *rRNA*, and 45 s at 62°C for *trn*S−*trn*fM, 2 min at 72°C; and finally 1 cycle of 7 min at 72°C. Amplified products were purified and concentrated using QIAquick PCR purification kit (Qiagen, CA, USA) and sequenced using an ABI PRISM 377 automated sequencer with BigDye Terminator Cycle Sequencing Kit (PE Biosystems) and sequences deposited in GenBank (Table 1).

Sequence analyses

Sequences from each of the five NCS regions were aligned using default settings in Clustal X (Thompson et al. 1997), adjusted manually, and merged to obtain a combined data matrix comprising 3,808 characters. Two informative indels with identical 5′ and 3′ termini, one being 7 bp long within the *atpB-rbcL* region and the other 18 bp long within the *trnT-trnF* region, were binary coded following the simple indel coding method suggested by Simmons and Ochoterena (2000), and the remaining indels were treated as missing data, and all characters were equally weighted and unordered. Congruence among sequences from different NCS regions and with data from two previous phylogenetic studies in *Juglans* (*mat*K and ITS data from Stanford et al. 2000 and the nuclear RFLP data from Fjellstrom and Parfitt 1995) was examined with the partition homogeneity test (PHT; Farris et al. 1994, 1995) as implemented in PAUP* 4.0b10 (Swofford 2002). Invariant sites were excluded from

the test and 1,000 replications were performed with maxTree set at 1,000. The criterion $P \leq 0.05$ was used to reject the null hypothesis, as PHT is supposed to be a conservative test of data combinability (Cunningham 1997; Baker and Lutzoni 2002). A second approach to test data congruence suggested by Johnson and Soltis (1998), which includes computation of three topological and two character congruence measures, was also implemented. Only pairwise combinations involving NCS data (NCS-ITS, NCS-*mat*K, and NCS-RFLP) were subjected to congruence tests, and taxa common to all data sets were used in the assessment of congruence, which resulted in the retention of 12 taxa representative of all the four sections of *Juglans*.

Phylogenetic analyses were performed with PAUP* using the maximum parsimony (MP) and maximum likelihood (ML) methods. MP analysis was performed using the branch-and-bound algorithm with MulTrees activated and addition of sequence set to Furthest (character optimization ACCTRAN and TBR branch swapping options) to find maximally parsimonious trees (MPTs). Bootstrap analysis (500 replicates) using heuristic search was performed to assess relative support for different clades with simple addition sequence and TBR, and MaxTrees set at 200. Decay values (Bremer 1988), the number of extra steps required to collapse a clade, were computed with the program TreeRot, version 2.0 (Sorenson 1999) using the heuristic search option with 1,000 replications of random addition sequence and MaxTrees set at 2,000. The ML analysis was performed using the best evolutionary model and parameter values estimated by the hierarchical likelihood ratio test as implemented in the program MODELTEST version 3.06 (Posada and Crandall 1998) with a Jukes–Cantor tree as the starting tree. Heuristic search with 10 replications of random addition sequence and TBR branch-swapping options was used, and 100 bootstrap replications were performed under the same conditions. Gaps were treated as missing characters and indels were obviously excluded from the analyses. The MP and ML trees were rooted with outgroup taxa constrained to be monophyletic, whenever possible.

Hypothesis testing and molecular dating

The molecular clock hypothesis (Zuckerkandl and Pauling 1965) was tested by computing the difference in the log likelihood scores between ML trees generated with and without a molecular clock assumption ($2\Delta = \log L_{\text{no clock}} - \log L_{\text{clock}}$), which is distributed as χ^2 with $(n-2)$ degrees of freedom (Felsenstein 1981; Huelsenbeck and Rannala 1997), where n is the number of sequences or taxa. Because of the apparent rate heterogeneity among taxa, we implemented three different tests to further evaluate the relative rates of substitution within and between lineages and to identify taxa contributing to the heterogeneity. First, the likelihood ratio test (LRT; Muse and Weir 1992) and the relative rate test (RRT; Tajima 1993) were used in pairwise comparison of taxa to a reference outgroup. Both these methods check for equality of rates of change along the paths of descent leading to two taxa in a phylogeny, but they are known to suffer from lack of power when the substitution rates are low. They may however provide valuable clues concerning the sources of heterogeneity. A sequential Bonferroni correction (Bonferroni 1936) was applied because of multiple tests, which renders LRT and RRT more conservative. Second, the two-cluster test (TCT), which examines the equality of the average substitution rates for two clusters that are created by a node in a given tree, and the branch-length test (BLT), which examine the deviation of the sum of branch lengths from the root to each sequence from the average of all sequences except for the outgroup sequence(s), as implemented in the software LINTREE (Takezaki et al. 1995), were used to evaluate different lineages simultaneously to identify taxa/lineages that are deviating significantly from the overall average rate of evolution based on a neighbor-joining (NJ) tree topology generated by the program based on Kimura two-parameter (K2P) distance matrices. The hypothesis of rate constancy for all sequences is tested simultaneously by Q-values of the U-statistic, which approximately follows the χ^2 distribution with $n-1$ degrees of freedom, where n is the number of taxa (Rao 1973). The deviations were considered significant only at $P \leq 0.05$ level because good time estimates are known to be obtained even with considerably large deviations (Nei and Kumar 2000). After removing the taxa which showed significant deviation, the rate constancy tests were repeated to make sure that the rates conform to clock-like evolution. A linearized tree for a given topology and model of evolution was subsequently constructed for the remaining taxa using LINTREE. To convert branch lengths on the linearized tree into times we fixed the basal node, representing the most recent common ancestor (MRCA) of *Juglans* corresponding to the divergence of *Pterocarya* and *Juglans*, at 50 Ma based on the earliest confirmed fruit fossil of *Juglans*, presumably of *J. clarnensis* (see pages 117 and 122 of Manchester 1987). Time since divergence was computed based on the mean heights and respective standard errors for the nodes of interest.

Additionally, we used both the clock-dependent Langley–Fitch (LF; Langley and Fitch 1974) method, which uses maximum likelihood to reconstruct divergence times and the clock-independent nonparametric rate smoothing (NPRS; Sanderson 1997) and penalized likelihood (PL; Sanderson 2002) methods as implemented in r8s, version 1.7 (Sanderson 2003) to date the nodes of interest. The NPRS estimates divergence rates and times using a least squares smoothing of local estimates, whereas PL is a semiparametric rate smoothing method that combines a

parametric model in which rate heterogeneity among branches is combined with a nonparametric roughness penalty that attempts to minimize rate changes among lineages. The relative contribution of the two components is determined by a smoothing factor estimated based on a data driven cross-validation procedure (Sanderson 2002). Both MP and ML trees with branch lengths from NCS and combined analyses were transformed into ultrametric trees using all three methods (LF, NPRS, and PL). The oldest confirmed fossil fruit of *Juglans* (50 Ma) was used as the MRCA of the genus (see pages 117 and 122 of Manchester 1987) to constrain the basal node of trees in all the analyses. We generated 100 bootstrap replicated trees by enforcing topological constraint and input them to r8s to compute the variance associated with the mean minimum ages estimated for the nodes of interest.

Results

Data incongruence

The PHT results indicated no significant heterogeneity among the NCS regions, and therefore the sequences from all five regions were combined into one data set. Assessment of combinability of the NCS data with *mat*K and ITS data from Stanford et al. 2000 and the nuclear RFLP data from Fjellstrom and Parfitt 1995 suggested varied levels of incongruence between the data sets. Among the three data set comparisons (NCS-ITS, NCS-*mat*K, and NCS-RFLP), the PHT indicated no significant heterogeneity for NCS-*mat*K (P=0.132), while the NCS-ITS comparison was marginally significant (P=0.031) and the NCS-RFLP comparison resulted in significant heterogeneity (P=0.004) based on the criterion ($P \leq 0.05$) for rejecting the null hypothesis of data set homogeneity. The second approach, using the three topological congruence measures: the partition metric (PM; Robinson and Foulds 1981), the explicitly agree quartet similarity measure (EA; Estabrook et al. 1985), and the greatest agreement subtree metric (D_1; Finden and Gordon 1985); and two character congruence measures: the incongruence metric (I_{MF}; Mickevich and Farris 1981) and the incongruence metric of Miyamoto (I_M; in Swofford 1991) as outlined in Johnson and Soltis (1998) produced results similar to that of the PHT (results not presented in this study, but available from the communicating author). Furthermore, phylogenies generated from individual data sets are resolved and congruent at the sectional level, except for the section *Trachycaryon* represented by *J. cinerea*, which was placed within *Rhysocaryon* when the analyses were based on cpDNA sequences (NCS and *mat*K), but within *Cardiocaryon* when the analysis was based on nuclear DNA data (ITS and RFLP, trees are not shown in this study). Apparent conflicts in the placement of taxa within clades confirm the presence of incongruent phylogenetic signals in the data sets.

Phylogenetic reconstruction

Chloroplast DNA noncoding spacer data analyses 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. Because PHT suggested congruence among the five NCS regions, the combined data was used to perform phylogenetic analyses. There were 80 (2.1%) variable sites among the ingroup taxa, of which 33 (0.87%) were potentially parsimony informative. Parsimony analysis using branch-and-bound search generated 197 equally most parsimonious trees (MPTs) of 168 steps long (including autapomorphies) with a consistency index (CI) of 0.863 (0.635 excluding autapomorphies) and retention index (RI) of 0.810, a strict consensus of which is shown in Fig. 2a. Three monophyletic clades are apparent corresponding to sections *Juglans*, *Cardiocaryon*, and *Rhysocaryon–Trachycaryon*. The single North American butternut, *J. cinerea* (*Trachycaryon*), is placed within the well-supported black walnut (*Rhysocaryon*) clade (bootstrap=93% and decay value=4). Section *Juglans*, which contained the cultivated walnut *J. regia* and its close relative *J. sigillata*, is strongly supported as monophyletic (bootstrap=97% and decay value=4) sister to *Cardiocaryon* and *Rhysocaryon*. The *Cardiocaryon* including *J. hopeiensis* is not supported as a monophyletic clade. However, there is strong support for *J. ailantifolia*, *J. mandshurica*, and *J. cathayensis* (bootstrap=93%, decay value=3) within the *Cardiocaryon*. Furthermore, the Chinese (*J. cathayensis*) and the Manchurian (*J. mandshurica*) butternuts are closely aligned (bootstrap=89% and decay value=2), with *J. ailantifolia*, mainly distributed in Japan as a sister. There is not much resolution within *Rhysocaryon* except for some subtropical taxa mainly from southern North America and Central America (*J. mollis*, *J. olanchana*, and *J. guatemalensis*) and a North American taxon (*J. microcarpa*) showing a marginal differentiation.

The ML analysis was performed using the TVM + I + G model (base frequencies: A=0.3508, C=0.1448, G=0.1696, T=0.3348; $R[A\leftrightarrow C]$=2.0605, $R[A\leftrightarrow G]$=1.3694, $R[A\leftrightarrow T]$=0.3568, $R[C\leftrightarrow G]$=0.3641, $R[C\leftrightarrow T]$=1.3694, $R[G\leftrightarrow T]$=1; I=0.8393; α=0.8471) selected based on the hierarchical likelihood ratio test as implemented in the model test. The analysis resolved a tree with the best log-likelihood score (−6,251.1768), which is identical to the consensus MP tree (Fig. 2b) with strong bootstrap support to the sections *Juglans* (92%), *Cardiocaryon* including *J. hopeiensis* (81%), and *Rhysocaryon–Trachycaryon* (91%), with only marginal support for the monophyly of the genus (bootstrap=55%). As in MP analysis, the resolution within *Rhysocaryon* is

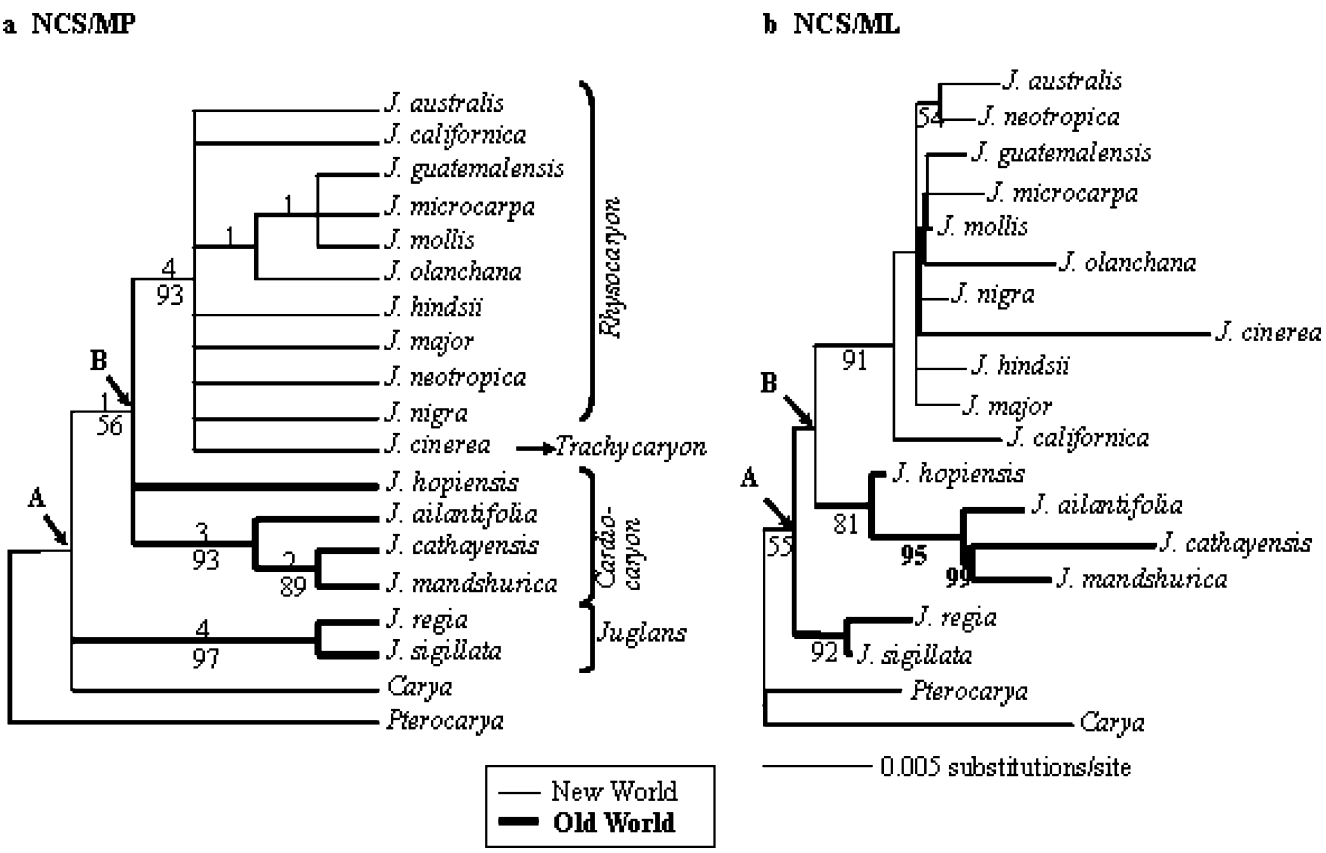

Fig. 2 Phylogenetic analyses of NCS data. **a** Strict consensus tree of 197 equally most parsimonious trees, length=168 steps, CI=0.635 (excluding autapomorphies), RI=0.810. *Numbers above branches* are decay values; *numbers below branches* are bootstrap values. **b** Maximum likelihood tree based on TVM + I + G model of sequence evolution with the best log-likelihood score (−6,251.1768)

poor and the two South American tropical taxa, *J. australis* and *J. neotropica*, were marginally supported as a subclade.

Combined (NCS + matK) data analyses Based on the results of congruence tests, we combined the NCS and *mat*K data. Altogether, 6,185 characters were included in the combined analyses. Sequences for three ingroup taxa (*J. mollis*, *J. hopeiensis*, and *J. sigillata*), and one outgroup taxon (*C. illinoiensis*) were not available for *mat*K data set and hence were treated as missing in the combined data set. There were 286 variable sites (4.6%) of which 92 (1.5%) were parsimony informative. Parsimony analysis resulted in six equally most parsimonious trees of 480 steps long, with a CI of 0.9 (0.522 excluding autapomorphies) and a RI of 0.666, a strict consensus of which is shown in Fig. 3. The trees were topologically similar to MPTs from NCS analysis, except for some minor changes in placement of taxa within major lineages. Unlike the NCS analysis, the *Cardiocaryon* including *J. hopeiensis* was supported as a monophyletic clade (bootstrap value=75% and decay index=2) in the combined analysis. Surprisingly, the two butternuts (*J. cathayensis* and *J. mandshurica*) from mainland China that were well supported as a subgroup with *J. ailantifolia* as a sister in the NCS analysis were replaced by an allopatric species combination of the Chinese butternut *J. cathayensis* and the Japanese walnut *J. ailantifolia* with distribution restricted to Japan (bootstrap=98% and decay index=4), with *J. mandshurica* as a sister (Bootstrap=94% and decay=3) in the combined analysis suggesting incongruence between the NCS and *mat*K data sets. Section *Rhysocaryon* is moderately supported (bootstrap=60% and decay index=2), but the *Rhysocaryon–Trachycaryon* lineage with the American butternut *J. cinerea* is strongly supported (bootstrap=91% and decay index=4) as monophyletic clade. The resolution within *Rhysocaryon* was not improved by the addition of *mat*K data except for marginal differentiation of *J. microcarpa* and *J. mollis* (bootstrap=56% and decay index=1). Overall, there was a minor improvement in the support for the three major monophyletic lineages in the combined analysis, including monophyly of the genus (bootstrap=53% and decay index=1).

The ML analysis using the K81uf+I+G model (base frequencies: A=0.3410, C=0.1501, G=0.1670, T=0.3418; $R[A\leftrightarrow C\ \&\ G\leftrightarrow T]$=1, $R[A\leftrightarrow G\ \&\ C\leftrightarrow T]$=1.3170, $R[A\leftrightarrow T\ \&\ C\leftrightarrow G]$=0.4418; I=0.8281; α=0.8359; Kimura 1981) of sequence evolution selected by the likelihood ratio test resolved a tree with the best log-likelihood score

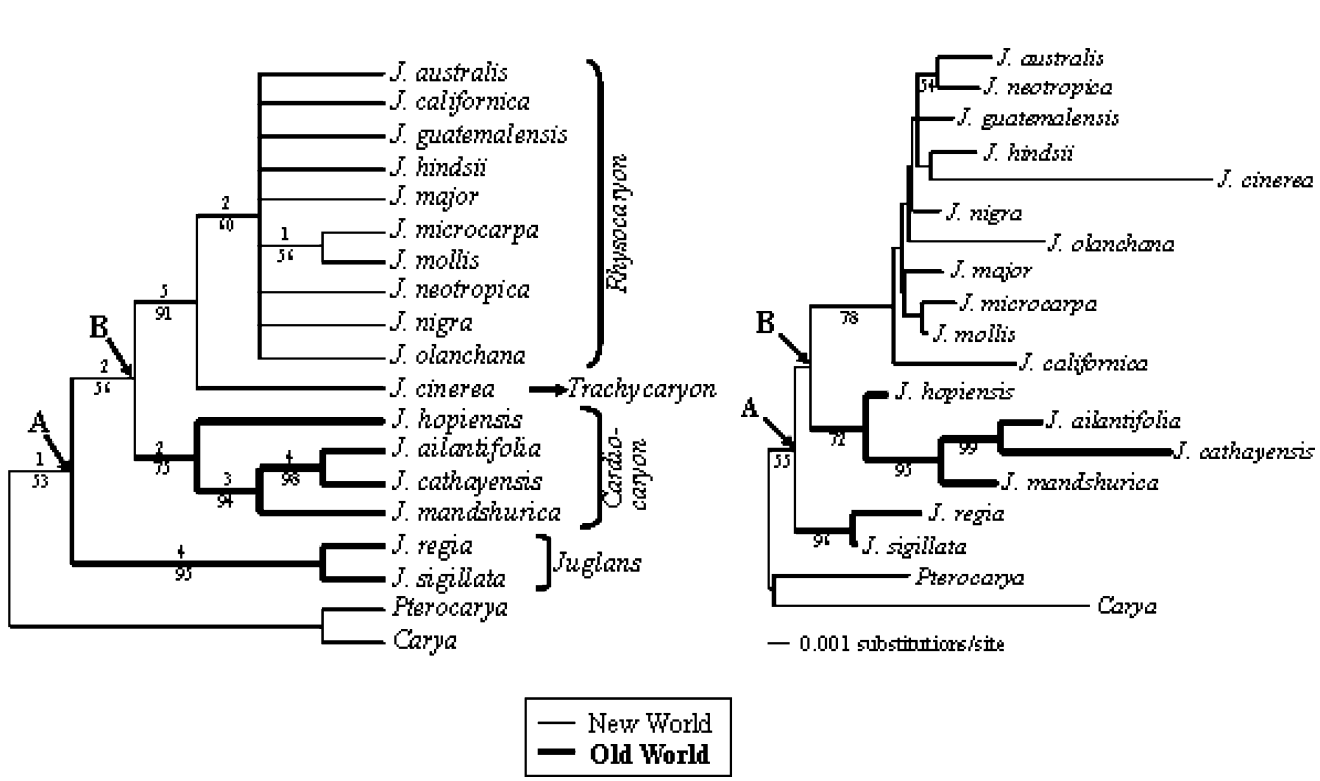

Fig. 3 Phylogenetic analyses of combined (NCS + *mat*K) data. **a** Strict consensus tree of six equally most parsimonious trees, length = 480 steps, CI=0.522 (excluding autapomorphies), RI=0.666. *Numbers above branches* are decay values; *numbers below branches* are bootstrap values. **b** Maximum likelihood tree based on K81uf+I+G model of sequence evolution with the best log-likelihood score (−11,518.1736)

(−11,518.1736) (Fig. 3b). The tree is concordant with the ML analysis of NCS data as well as MP analysis of the combined data at the sectional level, except for some minor shuffling within the *Rhysocaryon* lineage. The two South American tropical taxa, *J. australis* and *J. neotropica* were marginally supported as a subclade. The overall support for different lineages remained roughly the same as ML analysis of NCS data.

Rate heterogeneity and estimation of divergence times

Since comparison of clock and nonclock based trees by applying the likelihood ratio test (Felsenstein 1981; Huelsenbeck and Rannala 1997) rejected the clock-like evolution for both NCS [−InL=2(−6251.18−(−6274.44))=46.52, *df*=17, *P*<0.001] and combined [−In=2(−11,518.17-(−11,595.45))=154.56, *df*=17, *P*<0.001] data sets, we attempted to examine the rate heterogeneity within and between different lineages to identify taxa contributing to the heterogeneity. The pair-wise LRT and RRT used to examine the rate heterogeneity produced similar results indicating that the Asian butternut, *J. cathayensis*, and American butternut, *J. cinerea*, accounted for most of the rate heterogeneity in the combined data. A few other pair-wise combinations involving the black walnut species, *J. olanchana*, *J. guatemalensis*, and *J. nigra*, showed deviation from the average rate of evolution (Table 2). Neither test detected rate heterogeneity in the NCS data. Members of the *Cardiocaryon* and some members of *Rhysocaryon* were implicated for rate heterogeneity by the TCT in both the NCS and combined data sets (*Q*=35.73, *P*<0.01 and 34.41, *P*<0.01 for NCS and combined data sets, respectively), but unlike LRT and RRT, *J. cinerea* was not included (Table 3). On the contrary, BLT identified one Asian butternut, *J. cathayensis* and one each of North and Central American black walnuts, *J. hindsii* and *J. guatemalensis*, respectively for the NCS data set, while it suggested *J. guatemalensis* as a single contributor to heterogeneity in the combined data set (Table 2). We used K2P distance-based NJ tree topologies generated by the LINTREE program for both TCT and BLT instead of trees based on the evolutionary models identified as appropriate for the data sets because those models were not available in the LINTREE program. It is known that most evolutionary distances converge to a simple model of evolution when species pair-wise distances are less than 0.25, as in the present case, and distances based on sophisticated models of evolution are not necessarily efficient for obtaining correct topology (Nei and Kumar 2000). Two taxa, *J. cathayensis* and *J. guatemalensis* from both NCS and combined data sets

Table 2 Tests for rate heterogeneity

Number	Taxon	LRT		RRT		BLT; *P*-value	
		NCS	NCS + *mat*K	NCS	NCS + *mat*K	NCS	NCS + *mat*K
1	*J. australis*	NS	11, 14	NS	11, 14	NS	NS
2	*J. californica*	NS	NS	NS		NS	NS
3	*J. guatemalensis*	NS	10, 11, 14	NS	11, 14	<0.05	<0.05
4	*J. hindsii*	NS	11, 14	NS	11, 14	<0.05	NS
5	*J. major*	NS	11, 14	NS	11, 14	NS	NS
6	*J. microcarpa*	NS	11, 14	NS	11, 14	NS	NS
7	*J. mollis*	NS	14	NS		NS	NS
8	*J. neotropica*	NS	11, 14	NS	11, 14	NS	NS
9	*J. nigra*	NS	10, 11, 14	NS	11, 14	NS	NS
10	*J. olanchana*	NS	3, 9	NS	3, 9	NS	NS
11	*J. cinerea*	NS	1, 3–6, 8, 9, 15, 16	NS	1, 3–6, 8, 9, 15, 16	NS	NS
12	*J. hopeiensis*	NS	14	NS	NS	NS	NS
13	*J. ailantifolia*	NS	14	NS	14	NS	NS
14	*J. cathayensis*	NS	1, 3–9, 12, 13, 15–17	NS	1, 3–6, 8, 13, 15, 16	<0.05	NS
15	*J. mandshurica*	NS	11, 14	NS	11, 14	NS	NS
16	*J. regia*	NS	11, 14	NS	11, 14	NS	NS
17	*J. sigillata*	NS	14	NS	NS	NS	NS

Numbers for LRT and RRT correspond to numbers in column 1 identifying taxa/lineages with significantly different rates of evolution ($P<0.05$) after Bonferroni correction for multiple testing. *Pterocarya* was used as outgroup for both LRT and RRT.
NCS cpDNA noncoding spacer, *LRT* likelihood ratio test (Muse and Weir 1992), *RRT* relative rate test (Tajima 1993), *BLT* branch-length test (Takezaki et al. 1995) with K2P distance measure, *NS* denotes nonsignificant result

and one additional taxon, *J. hindsii* from the combined data set, which deviated significantly from the average evolutionary rates, were pruned and data reanalyzed. The TCT still showed that the cultivated species *J. regia* evolved significantly ($P<0.05$) faster than its sister taxon, *J. sigillata* for the combined data, but overall, the sequences conformed to the rate constancy as indicated by *Q*-values of the *U*-statistic, which was insignificant ($P>0.05$) for both NCS and combined data sets. With the sequences conforming to rate constancy, we constructed linearized trees for both NCS and combined data sets using the K2P distance-based NJ topology as implemented in LINTREE and reestimated branch lengths at nodes representing divergence of the major lineages, *Rhysocaryon*, *Cardiocaryon*, and *Juglans*.

Table 3 Overall rate heterogeneity and detection of taxa/clades contributing for heterogeneity

Data	TCT[a]	Clades contributing for rate heterogeneity and their rate relationships[b]
NCS	*Q*=35.73**	15>16 (**) 14<15, 16 (**) 13<14, 15, 16 (**) 6, 10, 12>5 (**) 4<7, 8 (**)
NCS + *mat*K	*Q*=34.41**	15>16 (**) 14<15, 16 (**) 13<14, 15, 16 (*) 4<7, 8 (**)

[a] Two cluster test identifies overall rate heterogeneity and clades with significant rate differences (Takezaki et al. 1995)
[b] Numbers correspond to column 1 of Table 2
*Indicate significance at $P=0.05$
**Indicate significance at $P=0.01$

Despite narrow rejection of molecular clock hypotheses, we estimated age using both clock-dependent LF and clock independent NPRS and PL methods as implemented in the program r8s. The earliest confirmed fruit fossil of *Juglans* (50 Ma) was used as the MRCA of the genus to constrain the trees at the root to estimate the ages for nodes. Smoothing parameter (λ) values selected through a cross-validation procedure for the PL method of age estimations were identical for both NCS and combined data sets, but they differed between MP and ML topologies used in the estimation (1.78 and 1 for MP and ML based topologies used in the estimation, respectively). Estimates of age since divergence at node A for the sections *Juglans* and node B for *Cardiocaryon* and *Rhysocaryon* gave comparable results (Figs. 2 and 3; Table 4). Generally, age estimates using PL, NPRS, and LF methods based on MP topology tended to be higher than that of the estimates based on ML topology for both data sets. The MP topology-based age estimates were higher generally for NCS than the combined data whereas this trend was opposite when estimates were based on ML topologies. The estimated ages based on the linearized NJ trees generated by LINTREE program after deleting taxa which deviated significantly from the average rate of evolution, compared well with PL, NPRS, and LF

Table 4 Divergence time estimates (Ma±standard error) for nodes A and B based on NCS and combined data

Divergence event (node)	Method	NCS				NCS + *mat*K			
		LINTREE	PL	NPRS	LF	LINTREE	PL	NPRS	LF
Pterocarya/*Juglans* (constrained)		50	50	50	50	50	50	50	50
Optimal smoothing parameter (λ)	CV		1.78				1.78		
Juglans/ *Rhysocarya* (A)	MP		47.0±0.11	47.1±0.10	46.4±0.14		44.6±0.12	44.3±0.12	45.3±0.12
Cardiocaryon/ *Rhysocarya* (B)	MP		43.7±0.19	43.9±0.18	42.9±0.23		33.9±0.17	33.5±0.15	35.2±0.19
Optimal smoothing parameter (λ)	CV		1				1		
Juglans/ *Rhysocarya* (A)	ML	31.4±6.2	30.7±0.35	33.7±0.29	28.3±0.39	35.7±6.3	35.4±0.19	35.1±0.20	32.0±0.23
Cardiocaryon/ *Rhysocarya* (B)	ML	29.9±6.6	25.2±0.29	28.7±0.24	22.9±0.32	35.5±6.3	33.8±0.23	33.6±0.23	30.2±0.29

NCS cpDNA noncoding spacer, *LINTREE* age estimation based on linearized trees after elimination of taxa deviating from average rate of substitution (Takezaki et al. 1995), *LF* Langley–Fitch, *NPRS* nonparametric rate smoothing, *PL* penalized likelihood (methods implemented in r8s; Sanderson 2003), *CV* Cross-validation, *MP* maximum parsimony, *ML* maximum likelihood, *A* and *B* refer to nodes in Figs. 2 and 3

estimates using ML topology for both A and B nodes, but the standard error was considerably bigger than that of the other estimates. The estimated age difference between the nodes A and B using ML topology across different methods is wider for NCS as compared to estimates based on the combined data. Overall, node B is younger than the node A in all the estimates irrespective of methods of estimation and topology used.

Discussion

Chloroplast DNA noncoding spacer sequence characteristics and evolution

Over 3.8 kb of cpDNA sequence from five spacer regions were assembled for each of the seventeen ingroup and two outgroup taxa. Although potentially phylogenetically informative characters were found in all five regions, the variation within individual regions was insufficient to obtain a reasonable level of phylogenetic resolution. Sequence data were therefore merged across all the five cpDNA regions to obtain a composite data matrix to perform the final phylogenetic analyses. There were 80 (2.1%) variable sites among 3,808 total characters within *Juglans* of which 33 (0.87%) were potentially phylogenetically informative. Alignment of *trnT*−*trnF* and *atpB*−*rbcL* regions required an 18 and 7 bp long indels, respectively, and remaining indels were of 1–5 bp long and not informative. 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 in general (Palmer 1991). The transition/tranversion ratio for pairwise comparisons between taxa ranged from zero to 3.0 and surprisingly most comparisons showed a bias favoring transversion.

Noncoding spacers are selectively neutral, under less functional constraints, and expected to evolve more rapidly than coding sequences (Wolfe et al. 1987; Zurawski and Clegg 1987), and potentially phylogenetically informative at the lower taxonomic levels (Taberlet et al. 1991; Demesure et al. 1995). However, the level of divergence observed in our study is surprisingly lower than the divergence levels reported for *mat*K gene and ITS spacer sequences for the genus *Juglans* (Stanford et al. 2000). Despite low variation within and among NCS sequences, the region-wise analyses indicated that the overall phylogenetic structure is conserved across the spacers at the sectional level and there was no significant incongruence among the data partitions. The NCS was however congruent only with *mat*K data from earlier studies. Among the substitutions, transversions were more prevalent than transitions except for the region *psb*A−*trn*H from within the inverted repeat region. The following possibilities could explain the low levels of variation observed in the NCS regions: (1) the rate of substitution is inherently low for *Juglans*; (2) the presence of some regulatory sequences or secondary structures with functional constraints not apparent in sequence alignments; and (3) the extant species may not embody the entire evolutionary history of *Juglans*, but may represent a more recent divergence indicating past extinctions.

Molecular phylogeny and cladogenesis

Three monophyletic clades corresponding to sections *Juglans*, *Cardiocaryon*, and *Rhysocaryon* were evident in both MP and ML analyses of both NCS and combined data. Addition of *mat*K data neither significantly changed the support for different clades nor resolution within *Rhysocaryon* except for the two South American taxa, *J. neotropica* and *J. australis*, which were marginally supported as a tropical subgroup. The moderate CI for NCS and combined phylogenies apparently indicate that both spacer regions and the *mat*K gene were subjected to some level of homoplasy within and among the lineages during the evolution and diversification of *Juglans* and/or persistence of ancestral polymorphisms across lineages. In contrast to our study, previous molecular systematic studies of genus *Juglans* generally supported two major lineages, 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). The single North American butternut species, *J. cinerea*, classified under a separate section *Trachycaryon* sharing nut characteristics similar to the section *Cardiocaryon*, was placed within the *Rhysocaryon* clade, members of which are characterized by four-chambered nuts. Such a placement of *J. cinerea* was supported by a recent phylogenetic study based on the chloroplast gene *mat*K sequences whereas the phylogeny based on ITS sequences and nuclear RFLPs, and the combined (*mat*K + ITS) data placed it along with *Cardiocaryon* and *Juglans* (Fjellstrom and Parfitt 1995; Stanford et al. 2000). The controversial placement of American butternut into the black walnut clade in the NCS phylogeny, with five unique synapomorphies, including two deletions (bootstrap support >90%, and decay index=4) strongly indicates historical introgression of *Rhysocaryon* chloroplast into an ancestral member of section *Cardiocaryon* from which the modern North American butternut section *Trachycaryon* must have evolved after the Pleistocene glaciations. Fossil records indicate that butternuts were widely distributed throughout the northern latitudes during the Oligocene and gradually underwent range reduction and selective extinction during the late Neogene, leaving behind isolated remnant populations both in the New and Old World. The modern New World butternut, *J. cinerea*, might have evolved sometime during the later part of the Neogene from some of these isolated remnant ancestral butternuts found sympatrically with black walnuts. Chloroplast capturing has been documented in several plant groups such as *Helianthus*, *Gossypium*, *Quercus*, *Pinus*, and *Eucalyptus* (Rieseberg and Soltis 1991), perhaps the best studied of which are in cotton relatives (Wendel et al. 1991).

The relative placement of taxa within *Cardiocaryon* and *Rhysocaryon* differed between the NCS and combined analyses, indicating conflict in phylogenetic signals between the NCS and *mat*K data sets. The MP and ML analyses differed in supporting *Cardiocaryon* as a monophyletic clade. In the MP analysis, *J. hopeiensis* was not supported as a member of the monophyletic clade, whereas ML analysis strongly supported it as a member of the lineage. However, both MP and ML analyses of the combined data strongly supported *Cardiocaryon*, including *J. hopeiensis* as a monophyletic lineage. Morphologically, *J. hopeiensis* closely resembles Persian walnut, *J. regia*, except that the nut characters are similar to *J. mandshurica*, and it is considered as an interspecific hybrid between *J. regia* and *J. mandshurica* (Rehder 1940) or a subspecies of *J. mandshurica* (Kuang et al. 1979). Surprisingly, there is conflict between NCS and combined analyses in the relative placement of taxa within *Cardiocaryon*. The close affinity between the Manchurian walnut, *J. mandshurica*, from northeastern China, and the Chinese walnut, *J. cathayensis*, from central and eastern China, with the Japanese walnut, *J. ailantifolia*, observed in the NCS analyses was changed in the combined analysis to a close affinity between the two geographically isolated taxa, *J. cathayensis* and *J. ailantifolia*, with *J. mandshurica* placed as a sister. The discordance in the placement of taxa within *Rhysocaryon* and *Cardiocaryon* between NCS and *mat*K phylogeny indicates some level of incongruence. However, the earlier study based on *mat*K and ITS sequences (Stanford et al. 2000), from which the *mat*K data was obtained for the present study, reported closer relationships between the geographically isolated species, *J. ailantifolia*, from Japan and the species (*J. cathayensis* and *J. mandshurica*) from mainland China.

The three well-resolved clades exhibit a high degree of morphological differentiation and differ significantly in leaf architecture, wood anatomy, and pollen and fruit morphology (Manchester 1987). Both MP and ML analyses support section *Juglans* as the ancient lineage and the section *Rhysocaryon* as the youngest, confirming the results of an earlier phylogenetic study based on nuclear RFLPs (Fjellstrom and Parfitt 1995). The early divergence of section *Juglans* implies a long period of evolutionary history, perhaps dating back to the mid-Eocene, almost simultaneously with the origin of the genus, and a likelihood the lineage has a rich fossil history. The absence of ancestral forms of section *Juglans* in the fossil flora (Manchester 1987), and the modern distribution pattern of section *Juglans*, imply that (1) the entire lineage of the section *Juglans*, perhaps evolved in isolation in Eurasia from ancestral forms that spanned across the land bridges during the mid-Eocene period coinciding with the early diversification of the genus and/

or (2) there is a paucity of stratigraphic data from Central Asia including the Carpathian and the Caucasus regions extending into southern Europe, West Asia, and east into China and northern Himalayas where the historical distribution of ancestral forms and domestication were known to have existed.

Section *Juglans*, containing the cultivated species *J. regia* and its wild relative *J. sigillata*, is supported as a monophyletic clade sister to both *Cardiocaryon* and *Rhysocaryon–Trachycaryon* in all the analyses. The early divergence of ancestors of section *Juglans* in both MP and ML analyses conflicts with earlier studies that placed *J. regia* sister to *Cardiocaryon* (Fjellstrom and Parfitt 1995; Stanford et al. 2000). In a recent study of Juglandaceae, Manos and Stone (2001) found the section *Juglans* as a sister group to the section *Rhysocaryon*, suggesting a second biogeographic disjunction within *Juglans*. The early evolutionary divergence of section *Juglans* contradicts the fossil evidence that supports the ancient divergence of sections *Cardiocaryon* and *Rhysocaryon* almost simultaneously with the origin of the genus sometime in the middle Eocene (Manchester 1987).

Members of the *Rhysocaryon* are not well resolved. However, in both MP and ML analyses of the NCS data, the subtropical walnuts *J. olanchana*, *J. mollis*, *J. guatemalensis*, and *J. microcarpa*, showed modest affinity with narrow support in the MP analysis. The ML trees from both NCS and combined data marginally supported a subgroup consisting of two South American tropical highland taxa, *J. neotropica* and *J. australis*. The clade as a whole is well supported, and many of the black walnut taxa showed a number of autapomorphic mutations, some of which was homoplasious, shared mostly within and some between different clades. The basal placement of southern California black walnut, *J. californica* within the *Rhysocaryon–Trachycaryon* clade in ML analysis of both NCS and combined data, well separated from its putatively close relative, *J. hindsii* was surprising because *J. hindsii* is treated as a conspecific variant of *J. californica* (Wilken 1993), and a sister relationship between these two taxa has been reported in earlier studies (Fjellstrom and Parfitt 1995; Stanford et al. 2000). The basal placement of *J. californica* is probably due to two substitutions that it shares with the section *Cardiocaryon*, which may represent convergence. Poor resolution within the black walnut section probably indicates that black walnuts are of relatively recent origin and/or probably involved intermixing of chloroplasts due to reticulate evolution within the section. Fossil evidence on the contrary, suggests that the earliest evolutionary split within *Juglans* during the middle Eocene involved black walnut and butternut sections, and consequently these two sections would have had enough time for intrasectional diversification.

Biogeography and fossil history

Modern distribution of *Juglans* shows intercontinental disjunction, with the distribution of sections *Juglans* and *Cardiocaryon* limited to Eurasia, and section *Rhysocaryon* endemic to the Americas. The enigmatic butternut, *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 *J. regia*, and section *Rhysocaryon*, suggesting the possibility of a second disjunction within *Juglans*. The disjunctions in *Juglans* have most likely arisen as a result of either a vicariance event disrupting the geographic continuity of ancestral populations that once spanned across northern latitudes of Eurasia and North America, or intercontinental dispersal, or both. The vicariance hypothesis is favored over the long-distance dispersal theory because of the large fruit size of *Juglans*, which does not appear to have great dispersal ability.

Fossil evidence suggests that the divergence of *Pterocarya* and *Juglans* may have occurred sometime during the Early Eocene (~50 Ma) followed by an initial split into sections *Rhysocaryon* and *Cardiocaryon*, probably during the middle Eocene (~45 Ma) in North America, but the two sections were clearly resolved only in the early Oligocene (38 Ma) (Manchester 1987). Further range expansion probably occurred during the Oligocene and by the Miocene, black walnut spanned from the west to east coast of North America extending into the Southern Hemisphere reaching Ecuador by late Neogene, but remained endemic to the Americas throughout their evolutionary history. At about the same period, members of *Cardiocaryon* spread beyond North America into Eurasia across the Bering and North Atlantic land bridges and attained much broader distribution. By the late Miocene, *Juglans* had attained a broad distribution both in the New and Old Worlds. Hills et al. (1974) based on extensive analysis of nut specimens of a fossil walnut, *J. eocinerea*, from the Tertiary Beaufort Formation, southwestern Banks Island, Arctic Canada, concluded that it is closely related and probably ancestral to the fossil species *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 to the stage of development exhibited by *J. tephrodes* and attained a broad distribution in the upper latitudes of the Northern Hemisphere by the Miocene. Subsequent geoclimatic changes (Wolfe and Leopold 1967; Axelrod and Bailey 1969; Wolfe 1971) resulted in southward migration of the floras, splitting the continuous distribution into Eurasian and North American disjunct floras. The early Pleistocene glaciations have completely eliminated *J. tephrodes* type butternuts from Europe and northwestern parts of North

America, leaving small refuges of disjunct populations in eastern Asia to evolve into three present day butternuts, *J. cathayensis*, *J. mandshurica*, and *J. ailantifolia*, and one to the south of the glacial limit in North America to evolve gradually into its present form, *J. cinerea*. The geographic distribution of fossils strongly supports the above hypothesis that butternuts may have originated and radiated independently from high northern latitudes.

One can argue that if sections *Cardiocaryon* and *Rhysocaryon* diverged from a common ancestor in North America during the mid-Eocene, there would have been ample opportunity for both groups to have become established in both Asia and North America, as 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 1985a). Furthermore, the distributional ranges of the Tertiary fossils of butternuts and black walnuts do 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 them observed in our phylogenetic analyses further substantiates this point, and suggests that they may not share a recent common ancestor.

Furthermore, monophyly of the ancient stem lineage is not apparent in the analyses, but the crown groups comprising the living descendents of the most recent common ancestors in different clades are well supported suggesting a widespread extinction of stem taxa followed by rapid diversification of the genus. The basal lineages may not truly represent the most immediate common ancestors of modern taxa/sections, but may represent common ancestors of Eurasian and North American lineages that evolved in isolation. Therefore, the vicariance hypothesis for the origin of stem lineage provides the most parsimonious explanation for the current geographic distribution pattern of *Juglans*. Furthermore, the genus is characterized by animal-dispersed fruits and it is unlikely that long distance dispersal has played a major role in the biogeography of the genus.

Divergence time

The hypothesis of rate constancy within and among lineages, tested using LRT, RRT, and TCT, was rejected for both NCS and combined data sets. The differential rates of divergence among Eurasian taxa and their basal placement in the cladograms could reflect their distinct and ancient origin and/or the extant taxa may not reveal the entire evolutionary history of different lineages in *Juglans*.

Estimates of time since divergence may be obtained from the fossil record and/or from computations assuming a molecular clock. For *Juglans*, the sequence divergence rates for the five noncoding cpDNA regions used in this study are unknown and the estimation of divergence times strictly relies on fossil records. Therefore, the accuracy of fossil records as well as variations of molecular evolutionary rates and patterns of extinction in different clades will affect the estimates. Nevertheless, it has been shown that the estimations of nucleotide substitution rates or time since divergence using the molecular clock hypothesis, although based on uncertain assumptions and approximate values, are still helpful to understand the tempos of evolution and plant historical geographies (Parks and Wendel 1990; Crawford et al. 1992; Wendel and Albert 1992). Paleobotanical evidence suggests two landmark events in the evolution and diversification of *Juglans*, the first corresponding to divergence of the MRCA of *Juglans* (early Eocene, ~50 Ma) and the second to the early split of sections *Rhysocaryon* and *Cardiocaryon* (mid-Eocene, ~45 Ma) as indicated by nodes A and B in Figs. 2 and 3. These two events are well documented in the fossil record (Manchester 1987) that could be used to date the evolutionary divergence of major sections of *Juglans*.

Comparisons of age estimates for the nodes A and B on MP and ML phylogenies by the three dating methods (clock-dependent LF and clock-independent NPRS and PL methods) as implemented in the program r8s showed remarkable similarities. The age estimates based on MP trees were significantly older than the estimates based on ML trees for all three methods and suggested the mid-Eocene time frame, but the estimates from the combined data indicated a much later time frame (late Eocene to early Oligocene). However, both data sets suggested section *Juglans* as the oldest of the three major lineages. The same estimates using the ML topologies were more recent for NCS data (mid- to late Oligocene) than for the combined data (early to mid-Oligocene). The linearized trees from LINTREE, generated after eliminating the taxa deviating significantly from the average rate and calibrated at the basal node representing the MRCA of the genus *Juglans* produced estimates that matched closely the ML estimates from PL, NPRS, and LF methods, but with bigger standard errors.

Overall, the section *Juglans* represents the oldest lineage within the genus *Juglans*, which contradicts the fossil evidence that the origin of the genus, and the almost simultaneous split of sections *Cardiocaryon* and *Rhysocaryon* was sometime during the mid-Eocene of North America. However, the lack of fossil evidence for the origin and historical distribution of section *Juglans* either from Eurasia or from North America leaves us with no option to further corroborate the age inferred from phylogenetic and

biogeographic analyses. The presence in the Eurasian section *Juglans* of four-chambered nuts similar to section *Rhysocaryon*, which remained strictly American in distribution throughout its history, probably represents evolutionary convergence within the genus. The butternut section *Cardiocaryon* with modern distribution in East Asia, possesses two-chambered and thick-shelled nuts, but historically had both Eurasian and North American distribution. The present study strongly supports section *Juglans* as an independent, monophyletic clade sister to sections *Cardiocaryon* and *Rhysocaryon*. However, the evolutionary history of the section *Juglans* may have been confounded by widespread extinctions, geographic isolation, and bottlenecks when the ancestral forms went into refugia in the Carpathian and Caucuses regions during the Pleistocene glaciations. 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). While *J. regia* was domesticated into a cultivated species of economic importance, its sister taxon, *J. sigillata*, with many primitive nut characteristics, may represent a semidomesticated or primitive form within the section. Generally, the section *Juglans* is considered monotypic with the cultivated species *J. regia*, but it is highly variable in it native range, especially in China and the Himalayas where six additional species were described to accommodate the variation (Dode 1909a), but not considered in the recent treatment (Manning 1978). Widespread extinctions and isolation during mid-Tertiary climatic changes in the upper latitudes of the Northern Hemisphere and subsequently in Eurasia and North America during the late Tertiary and early Quaternary glaciations within and between Asian and American lineages have contributed to the modern disjunction within *Juglans*.

In conclusion, the cladogenesis within *Juglans*, based on NCS and combined analyses, does not fully corroborate the evolutionary hypotheses based on the fossil history and biogeographic evidence. If Eocene North America is considered the center of origin and diversification of *Juglans* (Manchester 1987), there would have been sufficient opportunity for members of the different sections to have become established in both North America and Asia during the mid- and late Tertiary periods because land bridges across the Bering Sea and North Atlantic Ocean were in continuous existence from the middle Eocene through the late Miocene (Tiffney 1985a). On the contrary, the Tertiary fossil evidence suggests section *Rhysocaryon* remained endemic to the Americas throughout its evolutionary history, and section *Juglans* was not represented in the fossil flora of North America. Furthermore, the results allow for some generalizations on the origin and evolution of the genus *Juglans*: (1) the NCS sequence divergence levels observed within and between different sections of *Juglans* are low, and addition of *mat*K data did not improve resolution within *Rhysocaryon*; (2) early divergence of ancestors of section *Juglans* in the phylogenetic analyses suggests its ancient origin in contrast to fossil evidence, which suggests the split between sections *Rhysocaryon* and *Cardiocaryon* as the earliest in the diversification of *Juglans*; (3) the two Asian sections, *Juglans* and *Cardiocaryon*, show close affinity and may have evolved from a distant common ancestor after isolation in Eurasia; and finally, (4) the extant taxa may not hold footprints of the entire evolutionary history of the genus.

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