

Origin, Evolution, and Biogeography of *Juglans*: a Phylogenetic Perspective

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Abstract

Phylogenetic analyses of extant *Juglans* (Juglandaceae) using five cpDNA intergenic spacer (IGS) sequences (*trnT-trnF*, *psbA-trnH*, *atpB-rbcL*, *trnV-16S rRNA*, and *trnS-trnfM*) were performed to elucidate the origin, diversification, historical biogeography, and evolutionary relationships within the genus. Overall, 3848 characters were sampled, yielding 150 (3.8%) variable sites, of which, 45 (1.03%) were potentially parsimony informative. In all, seventeen in-group (*Juglans*) and two out-group (*Carya* and *Pterocarya*) taxa were analyzed using the parsimony and maximum likelihood methods, which produced concordant trees with three well-supported clades corresponding to the sections *Juglans*, *Cardiocaryon* (butternuts), and *Rhysocaryon* (black walnuts). *Juglans cinerea* from the section *Trachycaryon* was placed within the black walnut clade, probably suggesting historical introgression of black walnut chloroplast during its evolution in North America. Black walnuts were not well resolved, perhaps suggestive of their relatively recent origin and diversification or reticulate evolution within the section. Within butternuts, *J. hopeiensis* is moderately supported as a sister to *J. ailantifolia*, *J. cathayensis*, and *J. mandshurica*, which were well supported as a clade in all three analyses. The Persian walnut, *J. regia* and its sister taxon, *J. sigillata* formed a distinct clade sister to both butternuts and black walnuts. The cladogenesis within the genus *Juglans* appears to deviate from the evolutionary hypothesis based on the fossil history and biogeographic evidence. The results suggest that: (1) the IGS sequence divergence levels observed within and between different sections of *Juglans* are low; (2) basal placement of the section *Juglans* in both the analyses suggests its ancient origin in contrast to fossil evidence, which suggests the earliest divergence of sections *Rhysocaryon* and *Cardiocaryon*; (3) the Persian walnuts and butternuts have evolved at different rates as compared to black walnuts; and finally (4) the extant taxa may not represent the entire evolutionary history of the genus.

INTRODUCTION

Juglans L. is principally a New World genus in the family Juglandaceae comprising about 21 extant deciduous tree species occurring over North and South America, the West Indies, and southeastern Europe to eastern Asia and Japan (Manning, 1978), and exhibits disjunct distributional pattern between eastern Asia and eastern North America (Manchester, 1987). The genus is divided into four sections, based mainly on the morphology (Dode, 1909ab; 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, *J. guatemalensis* Mann.; and two South American tropical taxa, *J. neotropica* Diels, and *J. australis* Griesb, mainly occurring in the highlands. They typically bear four-chambered nuts 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, while section *Trachycaryon* consists of the only North American butternut taxon, *J. cinerea* L. Butternuts possess two-chambered nuts with thick nutshells and septa. Section *Juglans* includes two taxa: the cultivated Persian or English walnut, *J. regia* L., occurring

naturally in the Balkan, north Iran, Turkey, the south Caspian region, central Asia, the Himalayas, and China, and bears four-chambered nuts with thin nutshells and papery septa, and cultivated throughout the subtropical regions of the World. The second one is called iron walnut, *J. sigillata* Dode, which is restricted to southern China and Tibetan regions bears thick, rough-shelled nuts and the characteristic dark-colored kernels (Dode, 1909a), and often is 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 (1978).

The East Asian-North American disjunction has been studied from the paleobotanical, geological, and paleoclimatic perspectives, and various hypotheses have been proposed to explain the origin of these disjunctions (Gray, 1878; Chaney, 1947; Axelrod, 1960; Wolfe, 1969, 1972, 1978, 1985; Tiffney, 1985ab, Graham, 1972), including convergent adaptation to similar climatic conditions and long-distance dispersal in the development of present-day floristic disjunctions (Raven, 1972). Based on fossil evidence, Manchester (1987) suggested 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. Further, expansion and migration between North America and Eurasia were facilitated by the presence of the Bering and the North Atlantic land bridges during the late Eocene, when there was a favorable climate in upper latitudes for the establishment and dispersal of deciduous elements. 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 disjunct distribution of *Juglans* offers an opportunity to examine the level and time of evolutionary divergence between vicariant groups and to compare with the time of divergence inferred from paleobotanical evidence. Earlier molecular systematic studies based on nuclear RFLPs (Fjellstrom and Parfitt, 1995) and *matK* and ITS sequences (Stanford et al., 2000) supported the traditional taxonomic classification of *Juglans* and are consistent with what is known about the geological history of the genus (Dode, 1909ab; Manning, 1978; Manchester, 1987). In the present study, we examine phylogenetic relationships within the genus *Juglans* using the cpDNA intergenic spacer sequences and further explore the biogeography based on the phylogenetic inferences.

MATERIALS AND METHODS

Seventeen taxa representing the four sections of *Juglans* and two outgroup taxa, *Pterocarya stenoptera* and *Carya illinoensis* were sampled for this study (Table 1). 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 and sequenced using an ABI PRISM 377 automated sequencer. The sequences (over 3.8 kb) were aligned region-wise using the software Sequencher™ (GeneCodes Corp. Ann Arbor, Michigan) and later adjusted manually. Phylogenetic analyses were performed with PAUP* 4.0b10 (Swofford, 2002) using the maximum parsimony (MP) and maximum likelihood (ML) methods. MODELTEST version 3.06 (Posada and Crandall, 1998) was used to select appropriate model of evolution for ML analysis. Bootstrap and Decay analyses were performed to estimate the support for different nodes. 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 the chi-square distribution with (n-2) degrees of freedom where n is the number of sequences or taxa.

The age of nodes representing the divergence between the sections *Juglans* and *Rhysocaryon* (JR) and between *Rhysocaryon* and *Cardiocaryon* (RC) was estimated with the molecular clock assumption as well as using the nonparametric rate smoothing (NPRS; Sanderson, 1997) and the penalized likelihood (PL; Sanderson, 2002) methods.

RESULTS AND DISCUSSION

There were 150 (3.8%) variable sites among 3848 total characters within *Juglans*, of which 45 (1.03%) were potentially parsimony-informative. Alignment of the *trnT-trnF* region required one 18 bp long deletion for the sections *Rhysocaryon* and *Trachycaryon* and a 9 bp long insertion for *J. microcarpa* within *Rhysocaryon*, and the rest of the indels, including the remaining four spacer regions, consisted of 1-5 nucleotides in length. Parsimony analysis using a branch-and-bound search generated a single equally most parsimonious tree (MPT) of 199 steps (including autapomorphies) with a consistency index of 0.799 (0.556 excluding autapomorphies) and retention index of 0.728. Three well-supported clades are apparent corresponding to the sections *Juglans* (J), *Cardiocaryon* (C), and *Rhysocaryon-Trachycaryon* (RT) (Fig. 1). The single butternut species, *J. cinerea*, native to eastern North America representing the section *Trachycaryon* is placed within the black walnut (*Rhysocaryon*) clade. The RT clade was strongly supported (bootstrap = 90 %, decay >5), whereas support for C clade including *J. hopeiensis* is somewhat lower (bootstrap = 67%), and the sister relationship between the C and RT clades was weakly supported. However, there was strong support for *J. ailantifolia*, *J. mandshurica*, and *J. cathayensis* (bootstrap = 87%, decay >5) within the C clade. The section *Juglans* was weakly supported as a sister group to the C and RT clades, while itself strongly supported (bootstrap = 86, decay = 5).

The ML analysis based on the transversion (TVM+I+G) model of sequence evolution, identified by the Model test, generated a tree with the best log-likelihood score (- 6502.568) topologically identical to the MP tree (Fig. 1). There was some evidence for differentiation within the black walnut clade in both MP and ML analyses, 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*.

Despite significant rate differences among lineages observed in the study, we proceeded to estimate node ages with ML under clock assumption so as to compare them to ages obtained by PL and NPRS methods. Early Eocene time frame corresponding to the divergence between *Pterocarya* and *Juglans* about 54 million years ago (Mya) was used to compute the age of nodes representing the *Juglans-Rhysocaryon* (JR node) and *Rhysocaryon-Cardiocaryon* (RC node) splits in the evolution of *Juglans*. The time since divergence computed for both JR and RC nodes based on molecular clock assumption matched closely with the time frame computed based on PL and NPRS methods. The clock-based estimates (47.68 and 41.48 Mya for JR and RC nodes, respectively) were marginally smaller than NPRS (48.49±3.51 and 43.25±3.76 Mya, respectively) and PL (49.95±3.76 and 44.21±4.03 Mya, respectively) estimates (Fig. 2).

Phylogeny

The cladograms from both the MP and ML analyses were 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 trees could not be rooted with *Carya* as outgroup in both analyses. 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 due to 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). The single North American butternut species, *J. cinerea*, with nut characteristics (two-chambered nuts) 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 chloroplast *matK* sequences, whereas the phylogeny based on nuclear internal transcribed spacer (ITS) sequences, nuclear genome RFLPs, and the combined data set placed *J. cinerea* sister to *Cardiocaryon* (Fjellstrom and Parfitt, 1995; Stanford et al., 2000). This controversial placement of butternut into the black walnut clade by cpDNA, with a strong bootstrap support (90%) and decay index >5, suggests historical introgression of *Rhysocaryon* chloroplast into an ancestral member of section *Cardiocaryon*, which may have later 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 sometime 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 reported in several plant groups, perhaps the best studied is 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 members of *Rhysocaryon*.

Members of the section *Rhysocaryon* are not well resolved; however, in the MP and ML analyses, they are somewhat segregated into three biogeographic groups reflecting specific adaptations to the temperate, sub-tropical and tropical highland environments in which they are found. The clade as a whole is well supported with a bootstrap value and decay index of 90% and >5, respectively. Many of these taxa have accumulated a number of autapomorphic mutations along with some homoplasious ones shared mostly within and, to a lesser extent 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*, is 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* is probably due to two substitutions that it shares with the section *Cardiocaryon*, which may represent convergence. Lower resolution within the black walnut section is probably indicative of: (1) relatively recent diversification, possibly in the Pliocene; (2) reticulate evolution within the section; and (3) persistence of ancestral polymorphisms through speciation. On the contrary, the fossil evidence 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 inter- 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 which 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 both MP and ML analyses. This is in contrast to earlier studies, which placed the cultivated walnut, *J. regia* within either *Cardiocaryon* (Fjellstrom and Parfitt, 1995; Stanford et al., 2000) or *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 sometime in the middle Eocene (Manchester, 1987). Within the section *Juglans*, the cultivated species, *J. regia*, with thin-shelled four chambered nuts, has differentiated from its sister taxon *J. sigillata*, which retains many primitive nut characteristics such as thick rough-shelled nuts with dark colored kernels (Dode, 1909a) and may represent a semi-domesticated form within the section.

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 because of the large fruit size in *Juglans*, which does not appear to have great dispersal ability.

Based on fossil evidence, Manchester (1987) proposed that the divergence of *Petrocarya* 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, Hills et al., (1974) based on extensive analysis of nut specimens of a fossil walnut, *J. eocinerea*, from the Beaufort Formation (Tertiary), southwestern Banks Island, arctic Canada, concluded that it is closely related and probably ancestral to fossil *J. tephrodes* from the Early Pliocene Germany and the extant *J. cinerea* from the eastern United States. Further, 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 (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 the relic in the south of the glacial limit in North America to evolve into its present form, *J. cinerea*.

The geographic and stratigraphic fossil distribution strongly supports the above 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 throughout the Eocene, when there was still a favorable climate in upper latitudes for the establishment and dispersal of broad-leaved deciduous taxa (Wolfe, 1972, 1978; Tiffney, 1985a). 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 or it may not be represented among the extant taxa.

The estimates of time since divergence for JR and RC nodes using PL and NPRS methods suggest that the *Juglans* is the oldest lineage followed by the split between black walnuts and Asian butternut clades, and they closely matched with clock-based estimates. Overall, most diversification within the genus occurred sometime in the middle of Eocene.

The cladogenesis and estimates of time since divergence for JR and RC nodes suggest that the diversification within the genus must have occurred sometime in middle of the Eocene and ancestors of the section *Juglans* were the first to split following the divergence between *Rhysocaryon* and *Cardiocaryon*. On the contrary, the fossil evidence suggests the split between *Rhysocaryon* and *Cardiocaryon* occurring almost simultaneously or soon after the origin of the genus itself. However, the low resolution within *Rhysocaryon* suggests either relatively recent diversification sometime during the late Pliocene and early Pleistocene, or extinction of many ancestral taxa at the base of the clade. The extant taxa within the clade exhibit some level of differentiation into temperate, subtropical and tropical groups. Finally, the evolutionary history of *Juglans* is riddled with range reduction, geographic isolation, local and regional extinctions within and between clades, and as a consequence, the extant taxa may not adequately represent the entire evolutionary history of the genus.

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Tables

Table 1. Species used in the study, their collection site, and geographic origin.

| Taxon (NCGR* Accession no.) | Collection site | Origin |
|---|--------------------|-----------|
| <i>Cardiocaryon</i> (Asian butternut) | | |
| <i>J. ailantifolia</i> (DJUG 91.4) | NCGR, Davis, CA | Japan |
| <i>J. cathayensis</i> (DJUG 11.4) | NCGR, Davis, CA | Taiwan |
| <i>J. mandshurica</i> (DJUG 13.1) | NCGR, Davis, CA | Korea |
| <i>J. hopeiensis</i> (DJUG 462) | NCGR, Davis, CA | China |
| <i>Juglans</i> (English walnut) | | |
| <i>J. regia</i> (DJUG 379.1b) | NCGR, Davis, CA | China |
| <i>J. sigillata</i> (DJUG 528) | NCGR, Davis, CA | China |
| <i>Rhysocaryon</i> (Black walnut) | | |
| <i>J. australis</i> (DJUG 429) | NCGR, Davis, CA | Argentina |
| <i>J. californica</i> (DJUG 28.5) | NCGR, Davis, CA | USA |
| <i>J. microcarpa</i> (DJUG 52.1) | NCGR, Davis, CA | USA |
| <i>J. mollis</i> (DJUG 218.3) | NCGR, Davis, CA | USA |
| <i>J. neotropica</i> (DJUG 330.2) | NCGR, Davis, CA | Ecuador |
| <i>J. nigra</i> (DJUG 57.12) | NCGR, Davis, CA | USA |
| <i>J. olanchana</i> (DJUG 212.14) | NCGR, Davis, CA | Mexico |
| <i>J. guatemalensis</i> | UC Davis Arboretum | Guatemala |
| <i>J. hindsii</i> (DJUG 91.4) | NCGR, Davis, CA | USA |
| <i>J. major</i> (DJUG 78.6) | NCGR, Davis, CA | USA |
| <i>Trachycaryon</i> (American butternut) | | |
| <i>J. cinerea</i> | UC Davis Pomology | USA |
| Outgroup | | |
| <i>Pterocarya stenoptera</i> (DPTE 17.1) | NCGR, Davis, CA | China |
| <i>Carya illinoensis</i> | NCGR, Davis, CA | USA |

Figures

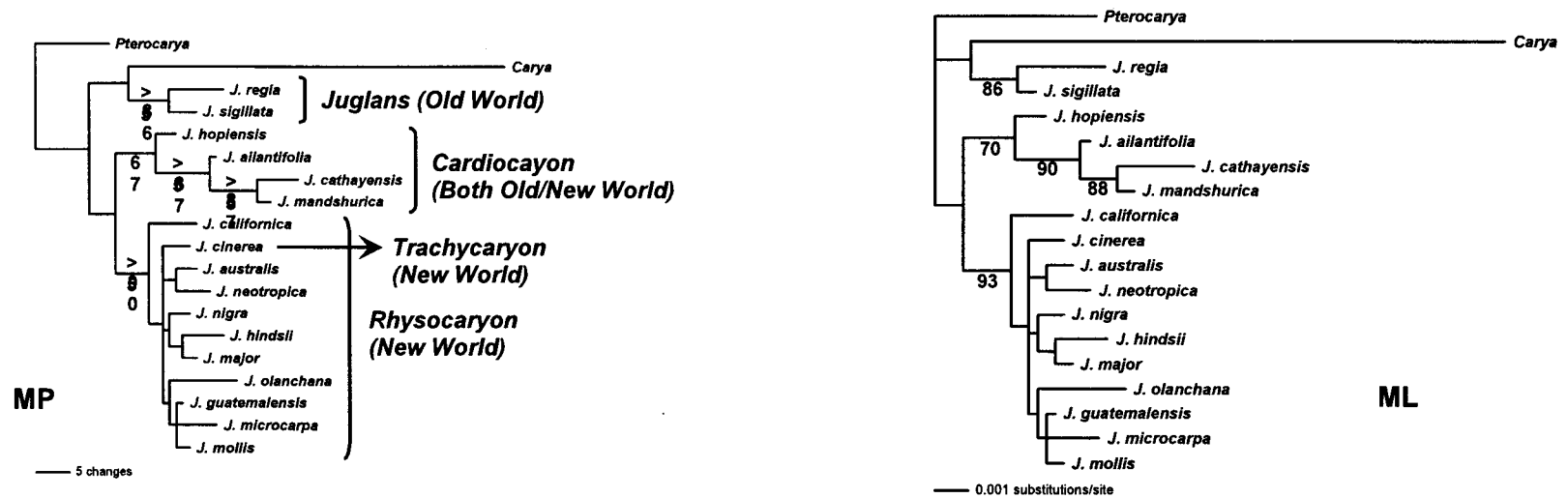


Fig. 1. Phylograms of *Juglans* inferred using maximum parsimony (MP) and maximum likelihood (ML) methods. Numbers above and below branches indicate decay and bootstrap values, respectively.

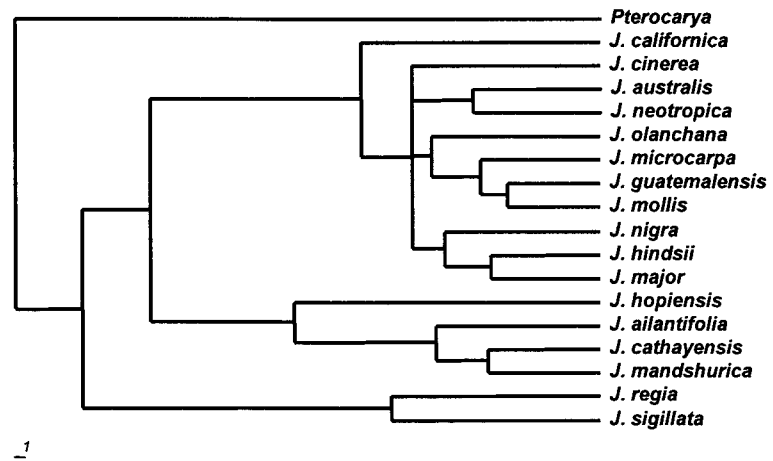


Fig. 2. An ultrametric tree with time since divergence in Mya between major lineages, estimated based on molecular clock, penalized likelihood, and Nonparametric rate smoothing methods.