Characterization of 11 Juglandaceae Genotypes Based on Morphology, cpDNA, and RAPD

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Abstract. The interspecific and intergeneric relationships of eight species of *Juglans* (walnuts) and three other members of Juglandaceae were investigated. The following species were included: the American *J. australis* Griseb., *J. neotropica* Diels., *J. olanchana* Standl. et L.O. Williams, *J. nigra* L., and *Carya illinoensis* (Wang.) K. Koch.; two *Juglans* from South China, namely, *J. sigillata* Dode and an unidentified *J. sp.*; an *Engelhardia* also from China and the Asian *J. ailantifolia* Carr., *Pterocarya stenoptera* var. *tonkinensis* Franchet and the Eurasian *J. regia* L. Cladistic analysis of 27 multistate morphological characters showed that the juvenile *J. ailantifolia* possessed similar physical traits to that of the juvenile American *Juglans* species. The chloroplast DNA in the trnL–trnF region indicated a close relationship between *Juglans* species. *Pterocarya* put the root of the cpDNA network among the American species. RAPD analysis was performed using eight primers. A total of 138 fragments were generated but only 78 clearly defined bands were used in the analysis. All the DNA data grouped the tropical/subtropical American *Juglans* with *J. nigra*, and the two new Asian species with *J. ailantifolia* and *J. regia*. The American species were closely related, more so than their Asian counterparts. The closeness of the investigated species predicts interspecific graft compatibility not only within the Asian and American groups, but also between them.

Juglandaceae contains six genera and 60 species (Bhattacharyya and Johri, 1998; Stace, 1996). It has been divided into two subfamilies, Platycaryoideae and Juglandoideae, and these subfamilies have each been divided into two tribes (Manchester, 1987). Of the four genera examined in this study, *Engelhardia* belongs to the tribe Engelhardieae in Platycaryeae, *Carya* to the tribe Hicoreae in Juglandoideae, and *Juglans* and *Pterocarya* to the tribe Juglandieae (Manchester, 1987; Yong-Ling et al., 1992).

Juglandaceae are distributed in two geographically distinct areas—in the Americas, and in Eurasia, from Central Europe to Japan (Bhattacharyya and Johri, 1998; Krussmann, 1985; Leroy, 1953; Manning, 1960). Eastern Asia is considered to be the current center of diversity for the Juglandaceae, but not the cen-

ter of origin (Manchester, 1987). Manchester argued that the Juglandaceae originated in the Northern Hemisphere, with a major diversification occurring during the Paleocene. This view was reinforced with the discovery of the extinct Juglandaceous species, *Polyptera manningii*, in Paleocene deposits in Wyoming and Montana (Manchester and Dilcher, 1997).

Grafts between the varieties of same species, and between different species within the same genus, account for the majority of all grafts performed in horticultural industries (Dirr and Heuser, 1987; Ross, 1997; Thain and Hickman, 1995). Graft compatibility between species in different genera, or even different families, is also known. In commercial horticulture, a particular concern is “delayed graft incompatibility.” In some instances, grafts will take initially, but rejection and consequent death (or at least debility) of the scion, may occur several years after the graft was initially performed. So, having an indication of the likely long-term success of various grafts, which can be provided by an indication of the

genetic similarity of the component elements, can be of practical advantage. Our long term goal is to develop superior rootstocks for *Juglans* trees for timber production. The first step towards that goal was to infer phylogenetic relatedness between our accessions based on morphology, cpDNA, and RAPD analysis presented in this study.

Materials and Methods

Accessions used (Table 1). *Juglans nigra* L. and *J. olanchana* Standl. et L.O. Williams are from temperate and subtropical North America, respectively, and *J. neotropica* Diels. and *J. australis* Griseb. from tropical and temperate South America. *Juglans nigra* is a well-identified cultivated species, while the other three are new germplasm accessions. *Juglans regia* is of Eurasian origin; *J. sigillata* Dode and an undescribed species (“*J. sp.*”) from China, and *J. ailantifolia* Carr. from Japan. *Engelhardia spicata* Leschenault ex Blume is from Royal Botanic Gardens, Sydney, of Vietnamese provenance. Another (unidentified) *Engelhardia* species was collected in Yunnan province, China. The North American *Carya illinoensis* (Wang.) K. Koch. specimen is from the living collection of the Univ. of Western Sydney.

All sequences were generated during this study except for *Juglans cathayensis* Dode. The trnL intron sequence of *J. cathayensis*, a Chinese species, was imported from GenBank (Accession No. AF 200936; Wu et al., 1999).

DNA extraction and purification. Genomic DNA was extracted from fresh leaves, using a method similar to that of DellaPorta et al. (1983, as described by Wilkie, 1997), followed by purification using diatomaceous earth binding, adapted from the technique described by Gilmore et al. (1993).

Polymerase chain reaction (PCR). A region of chloroplast DNA comprising the tRNA leucine (UAA) gene (trnL), the intron it contains, the tRNA phenylalanine (GAA) gene (trnF), and the intergenic spacer between trnL (5' exon) and trnF, was amplified by PCR (Mullis and Faloona, 1987), using the primers A50272 and B49317 of Taberlet et al. (1991). Reactions were performed in a HYBAID OMN-E thermocycler, using the following program: 5 min at 94 °C; 30 cycles of 30 s at 94 °C; 30 s at 60 °C and 1 min at 72 °C.

The reaction mixture contained 2.5 µL 10× PCR buffer (Promega #M190G), 1.5 µL 25 mM MgCl₂, 2 L 'dNTPs' (2.5 mM each of dATP, dCTP, dGTP, and dTTP), 5 µL of each of the two primers at a concentration of 20 µM, 8 µL H₂O, and 0.1 µL Taq Polymerase (5 units/µL, Promega). PCR products were purified using the CONCERT PCR Purification Kit (GibcoBRL Co.). DNA sequences were ascertained by the Univ. of Sydney and Prince Alfred Molecular Analysis Centre, Sydney, Australia, using the ABI 'Prism' fluorescent dye-terminator system (Applied Biosystems, Foster City, Calif.).

Random Amplification of Polymorphic DNA (RAPD). The protocol of Welsh and McClelland (1990) was followed. DNA

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Table 1. Provenance details of *Juglandaceae* specimens used in this study.

Taxon	Voucher no. ^z	GenBank no.	Wild source	Cultivation site ^y
<i>Juglans ailantifolia</i> Carr.	NSW 476462	AY231169	unknown	Mt Irvine
<i>Juglans australis</i> Griseb.	NSW 476464	AY231171	Tucuman, Argentina	Nambucca Heads ^x
<i>Juglans neotropica</i> Diels.	NSW 476473	AY231168	Loja, Ecuador	Nambucca Heads
<i>Juglans nigra</i> L.	NSW 476485	AY231170	unknown	Cowra
<i>Juglans olanchana</i> Standl. & L.O. Williams	NSW 476481		Turialba, Costa Rica	Nambucca Heads
<i>Juglans regia</i>	NSW 476488	AY231167	unknown	Cowra
<i>Juglans sigillata</i> Dode	NSW 476484	AY231170	Yunnan, China	Nambucca Heads
<i>Juglans</i> sp.	NSW 476489	AY231172	Quang Xi, China	Nambucca Heads
<i>Carya illinoensis</i> (Wangh.) K.Koch.	NSW 476493		unknown	Nambucca Heads
<i>Pterocarya stenoptera</i> var. <i>tonkinensis</i> Franchet	NSW 476491 ^w	AY231174	Sapa, Vietnam	RBG ^z , Sydney
<i>Engelhardia</i> sp.	NSW 476494		Yunan, China	Nambucca Heads

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fragments of *Juglans* and outgroup were amplified using the following primers: OPA5 (AGGGGTCTTG), OPA7 (GAAACGGGTG), OPA10 (GTGATCGCAG), OPA18 (AGGTGACCGT), OPA19 (CAAACGTCCG), and OPA 20 (GTTGCGATCC) (QIAGEN OPERON P/L).

The reaction mixture for RAPDs consisted of: 2 μ L 10 $\times$ PCR buffer, 2 μ L MgCl₂ 25 mM, 2 μ L dNTP, 4 μ L primer (20 mM), 10 μ L H₂O, 0.1 μ L of Taq polymerase (5 units/ μ L, Promega), and 2 μ L of each respective DNA (quantity not estimated). The PCR was performed using a Corbett FTS 4000 Thermal Sequencer and the following program: 94 °C for 3 min; 40 cycles of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 1.5 min; followed by final extension at 72 °C for 5 min.

PCR products were analyzed by polyacrylamide gel electrophoresis and visualized by silver-staining. Gene Gel Exel 12.5/24 pre-cast gels were run on a GenePhor electrophoresis apparatus, and stained with the PlusOne kit (all from Pharmacia, 100V, 2 h). Images of the silver-stained gels were scanned directly into a computer and enlarged and printed for visual analysis.

Only 78 clearly defined DNA bands were recognized. These bands were the product of repeated PCR (Fig. 1). Molecular sizes of identified bands were estimated by comparison with Promega "pGem DNA markers" (#G174). A binary number data matrix was constructed in which the absence of a band was denoted 0 and the presence 1. The matrix was analyzed using PAUP version 4.0b10 for Macintosh software package (Swofford, 2000) and MacClade (Maddison and Maddison, 1992).

Cladistic analysis of morphological characters. Twenty-seven multiple-state characters were scored from in situ observations of all accessions grown under field conditions. The investigated plants were seed-grown and had been established for a period of 6 years and were still in the juvenile stage and lacking in adult characters, like size, branch ramification, bark texture, flowers, and fruit. Ten additional multiple-state characters were scored from a recently collected quantity of fruit (infructescences) (Table 2).

Genetic distances. Pairwise genetic distances were calculated on the basis of the

proportion of fragments, using the formula:

$$1 - 2N_{xy}/(N_x + N_y)$$

where N_{xy} is the number of bands shared by specimens x and y, and N_x is the number of bands from specimen x (based on Upholt, 1977; as cited by Avise, 1994).

Results

Morphology. Cladistic analysis of the morphological data (Fig. 2) separated the accessions into distinct American and Old World clades, with the exception of the Japanese *J. ailantifolia*, the position of which was ambiguous. The three Old World species, i.e., the two Chinese species and the western Eurasian *J. regia*, were placed in a clade with bootstrap support of 64%, but relationships among them were unresolved. The American clade was strongly supported (96%) and within this clade *J. australis* and *J. olanchana* were robustly grouped (74%).

The position of the Japanese *J. ailantifolia*, according to the morphological data, was ambiguous, not fitting within the American or European/Asian relatives. We propose cladistic

state changes of some relevant morphological characters (Fig. 3). The characters designated 13, 21, and 27 are plesiomorphic in *J. ailantifolia*. Character 22 reverts in *J. ailantifolia*, uniquely among *Juglans* species. A parallel reversion is apparent in *J. ailantifolia* and *J. neotropica* (character 20). A parallel change in leaf surface texture is shown in *J. ailantifolia* and *J. neotropica* (character number 14) and another parallel change of indumentum texture (19) in *J. ailantifolia* and *J. australis*. Character 23 (nut shape compressed/not compressed) shows a parallel change in *J. ailantifolia* and all American species (that is, if the evidence from the DNA is accepted as confirming the affinity of *J. ailantifolia* with the other Eurasian species).

cpDNA. The information gained from sequencing of the *Juglans* chloroplast DNA (1013 base pairs), and from the data of Wu et al. (1999) for *J. cathayensis*, indicated a very close relationship among all species. No sequence differences were detected in the tRNA coding regions, nor in the trnL–trnF spacer of these species. The trnL intron had three informative nucleotide substitutions in *Juglans*, one of which grouped the four American species

Fig. 1. A typical gel using OPA10 (GTGATCGCAG) primer. From left to right: M = marker (1 μ L Promega "pGem DNA markers" #G174); 1 = *J. australis*; 2 = *J. neotropica*; 3 = *J. olanchana*; 4 = failed reaction; 5 = *J. ailantifolia*; 6 = failed reaction; 7 = *J. sp.*; 8 = *J. regia* $\times$ *J. nigra*; 9 = *P. stenoptera* var. *tonkinensis*; 10 = *E. sp.*; 11 = *J. regia* $\times$ *J. neotropica*; 12 = *J. sigillata*; 13 = *C. illinoensis*; M = marker (as above).

Table 2. Morphological characters and matrix derived from observations of 11 Juglandaceae genera.

Morphological characters	Species										
	<i>J. aust.</i>	<i>J. neo.</i>	<i>J. olan.</i>	<i>J. nig.</i>	<i>J. ail.</i>	<i>J. reg.</i>	<i>J. sp.</i>	<i>J. sig.</i>	<i>C. ill.</i>	<i>P. sten.</i>	<i>E. sp.</i>
Bark texture: smooth (0); rough (1)	0	0	0	0	0	0	0	0	1	1	1
Bark colour: dark (0); light (1)	0	1	0	0	1	1	1	1	1	1	1
Young branch surface: glabrous (0); hairy (1)	1	1	1	1	0	0	0	0	0	0	0
Bud: sharp and elongated (0); blunt and short (1)	0	0	0	1	1	1	1	1	0	1	1
Foliage: deciduous (0); evergreen (1)	0	0	0	0	0	0	0	0	0	1	0
Leaves: aromatic (0); odourless (1)	1	0	1	0	0	0	0	0	1	1	1
Rachis: glabrous (0); hairy (1)	1	1	1	1	0	0	0	0	0	1	0
Number of leaflets: 5–9 (0); 11–17 (1); 20–22 (2)	2	2	2	2	1	0	1	0	1	1	1
Leaflet lamina: oblong (0); elliptic (1); lanceolate (2); ovate (3); obovate (4); cuneate (5)	0	0	5	0	1	4	3	2	2	2	2
Leaf base: truncate (0); cordate (1); rounded (2); oblique (3)	2	2	0	2	1	3	3	3	3	3	3
Leaf attachment: petiolate (0); sessile (1)	0	1	0	0	0	0	0	0	0	1	0
Leaf apex: acute (0); obtuse (1)	0	0	0	0	0	0	1	0	0	0	1
Leaf margins: entire (0); serrate (1); serrulate (2); dentate (3); sinuate (4)	1	1	1	1	1	2	4	0	3	1	3
Upper leaf surface: glabrous (0); pubescent (1); glandular (2)	0	2	0	0	1	0	0	0	1	0	1
Leaflet arrangement: distichous (0); opposite (1); spiral (2)	2	1	0	1	1	1	1	1	1	1	1
Leaves: paripinnate (0); imparipinnate (1)	1	0	0	0	1	1	1	0	0	0	0
Leaflet venation: pinnate simple and craspedodromous (0) or eucamptodromous (1)	0	0	0	0	0	1	0	0	1	1	1
Fruit: drupe (0); samara (1)	0	0	0	0	0	0	0	0	0	1	1
Indumentum: glabrous (0); pubescent (1)	1	0	0	0	1	0	0	0	0	N	N
Husk texture: glutinous/glandular (0), non secretory (1)	1	0	1	1	0	1	1	1	0	N	N
Nut apex: blunt (0); sharp (1)	1	1	1	1	1	0	0	0	1	N	N
Nut shape: globose (0); ellipsoid (1)	0	0	0	0	1	0	0	0	1	N	N
Nut shape: compressed (0); not compressed (1)	0	0	0	0	0	0	1	1	1	N	N
Surface sculpturing: ridged (0); pitted (1); smooth (2)	0	0	0	0	2	2	1	2	2	N	N
Nut: crested or with a visible suture (0); lacking crest or suture (1)	1	1	1	1	0	0	0	0	0	N	N
Shell thickness: 0.5–1.0 mm (0); 3–5 mm (1)	0	0	0	0	1	1	1	1	1	N	N
Fruit arrangement on branches: solitary – 3 (0); >3 (2)	1	1	1	0	1	0	0	0	1	1	1

Fig. 2. Fifty percent majority rule bootstrap consensus tree calculated from morphological data, with percentage support indicated for clades.

together (Fig. 4). Another substitution was shared by *J. regia* and *J. sigillata*, and a third by *J. ailantifolia* and *J. cathayensis*. Comparison of the trnL from the outgroup, *Pterocarya stenoptera* var. *tonkinensis*, suggested that the American sequence was relatively plesiomorphic. *Pterocarya* has been shown to be the sister genus of *Juglans* (Stanford et al., 1999). The sequence difference between *Pterocarya* and *Juglans* was $\approx 0.7\%$.

RAPD. The RAPD data placed the three American *Juglans* accessions with *J. nigra* (100% jackknife support) and separate from the Asian species. Using *Pterocarya*, *Engelhar-*

dia, and *Carya illinoensis* as an outgroup, the monophyly of the American clade had 100% support, but there was no resolution among the four species. The four Old World species formed a clade with 67% support. *Juglans ailantifolia*, *J. regia*, and *J. sp.* formed an unresolved clade within this, with 81% support and *J. sigillata* as its sister (Fig. 5).

Genetic distances were calculated from the RAPD data (Table 3). The average distance between American and Asian species pairs was 0.826. The average distance between pairs of American species was 0.195, whereas the average distance between the

four Asian species was 0.285. UPGMA analysis (not shown) of genetic distance data clearly separated the American from the Asian taxa.

Discussion

Morphology. Morphological data showed the existence of a relationship among the American accessions, separate from their European/Asian relatives, with an unresolved trichotomy within the European/Asian species (Fig. 2). Some of the morphological characteristics that we observed differed from those

Fig. 3. Proposed state changes of homoplasious morphological characters.

Fig. 4. (right) Relationship of sampled *Juglans* species as indicated by nucleotide changes within the chloroplast trnL intron. The base changes are marked on the branches. The outgroup, *Pterocarya stenoptera* var. *tonkinensis* (which is from Asia), puts the root of *Juglans* among the American species. The trnL intron sequence of the Asian *J. cathayensis* is from Wu et al., 1999 (GenBank acc. no. AF 200936). All trnL, intron, and trnL-F spacer sequences were deposited in GenBank.

Fig. 5. Fifty percent majority rule parsimony jackknife consensus tree (100 replicates) calculated from RAPD data, with percentage support indicated. The tree was rooted by defining (*Pterocarya*, *Engelhardia* and *Carya*) as the outgroup to *Juglans*. Jackknife replicates: 100; 37% percentage of character deletion in each replicate (with "emulate 'Jac' resampling" option); starting trees obtained by stepwise addition with random addition sequence. Tree length = 73; Consistency Index (CI) = 0.6438; CI excluding uninformative characters = 0.5439; Retention Index = 0.6438; Rescaled CI = 0.4145.

Table 3. Pairwise distances among Juglandaceae.

	<i>J. aust</i>	<i>J. neo</i>	<i>J. olan</i>	<i>J. nig</i>	<i>J. ail</i>	<i>J. reg</i>	<i>J. sp.</i>	<i>J. sig</i>	<i>P. sten</i>	<i>E. sp.</i>	<i>C. ill</i>
<i>J. aust</i>	0.00	0.10	0.15	0.28	0.84	0.75	0.77	0.76	0.94	1.00	0.93
<i>J. neo</i>		0.00	0.24	0.22	0.91	0.91	0.92	0.92	1.00	1.00	0.93
<i>J. olan</i>			0.00	0.18	0.86	0.72	0.74	0.73	0.82	1.00	0.82
<i>J. nig</i>				0.00	0.84	0.84	0.86	0.85	0.87	1.00	0.80
<i>J. ail</i>					0.00	0.17	0.24	0.44	0.80	0.89	0.72
<i>J. reg</i>						0.00	0.16	0.36	0.73	0.89	0.65
<i>J. spp.</i>							0.00	0.34	0.68	0.80	0.60
<i>J. sig</i>								0.00	0.74	0.79	0.66
<i>P. sten</i>									0.00	0.57	0.52
<i>E. spp.</i>										0.00	0.64
<i>C. ill</i>											0.00

described by Krussmann (1985) and Manning (1960) for the same species. This may be because only juvenile plants (6 years old) were available for morphological observations, and specially selected cultivars of species and varieties (which may not be representative of their "types") were used. The molecular methods give "identities" to these accessions, whose histories and sources were unknown.

The placement of *C. illinoensis* within the outgroup—surprisingly grouping with *Engelhardia*, with *Pterocarya* as a sister to these two—may have been influenced by two other genera having samarae rather than nuts. Their nut character states were scored as "missing."

cpDNA. Chloroplast DNA sequence comparison showed the closeness of all *Juglans* species tested. This is comparable to the findings of Gielly and Taberlet (1994) in *Fraxinus* and *Alnus*, who found no sequence differences between certain groups of species in these tree genera, over the same distributional range.

Manos and Stone (2001), on the basis of their ITS, cpDNA, and morphology/chemistry studies, refer to the Juglandaceae as a "closely knit" family of trees. Smith and Doyle (1995) estimated a moderate to intermediate rate of evolution for the chloroplast genome of Juglandaceae at 3.36×10^{-10} to 10.7×10^{-10} substitution rates per site per year, with *Oreomunneal* *Pterocarya* rate as high as 10.7×10^{-10} . With a substitution rate of $\approx 7 \times 10^{-10}$ per site per year (the average of Smith and Doyle's range estimate boundaries), our data would put the separation of *Juglans* and *Pterocarya* (0.7% sequence difference) at 10 million years.

The similarity of the chloroplast sequence among the *Juglans* taxa makes it of limited value in determining relationships within the genus.

RAPD. The RAPD data agreed with that from cpDNA in showing the monophyly and genetic closeness of all the American accessions, perhaps caused by rapidly progressing process of speciation. The two Asian species, *J. sigillata* and *J. sp.*, were grouped with *J. ailantifolia* and *J. regia*. The relationship within the Asian species was not as well supported as the American. The firm position of *J. ailantifolia* within the Asian clade agreed with the results of RFLP analyses commented on by Fjellstrom and Parfitt (1995).

Summary. Taxonomic analysis of all data showed that the "New World" species formed a separate clade. American species under in-

vestigation were very similar. Phylogenetic analysis clearly showed the division between the 'New World' species and the species of European/Asian origin. The American accessions, namely *J. australis*, *J. neotropica*, *J. olanchana* and *J. nigra*, possessed characteristics which were distinctly different from the characteristics of the European/Asian *J. regia* and the Asian *J. ailantifolia*, *J. sigillata* and the unidentified *J. spp.* from South China. Therefore, the disjunct distribution of *Juglans* between the American and Eurasian continents was mirrored in the data.

Notes regarding the position of *J. ailantifolia*. The juvenile *J. ailantifolia* possessed some physical traits of the American *Juglans* species according to morphological data. *J. ailantifolia* presents ambiguities. The DNA data clearly place this species with its geographically proximate congeners, but the morphological data place it intermediate between Eurasian and American *Juglans*. Contact and genetic introgression between Asian and American forms may have contributed to the origin of *J. ailantifolia*, perhaps involving the geologically recent Bering Strait landbridge (Wen et al., 1998; Marincovich and Gladenkov, 1999). Guo et al. (1998) assert that plant diversity patterns may reflect the historical position of continents relative to the centre of origin. For example, the tree genus *Aesculus*, originated in Eastern Asia and later spread into North America (Xiang et al., 1998). Other Asian plants, such as the orchid genera *Pogonia*, *Isotria* and *Cleistis*, are closely related to American species, (Cameron and Chase, 1999). Wen (1999) writes of some 65 different plant genera with disjunct distributions of species occurring in Eastern Asia and eastern North America. Selection and cultivation by native peoples may have occurred, and seeds may also have been introduced from America via the Aleutian Islands.

Horticultural implications. Plants used in this study had been collected and assessed to develop a breeding programme, which will study interspecific and intergeneric compatibility of selected materials by creating hybrids, and by conducting grafting trials. The data presented could predict likelihood of compatibility between the species and varieties.

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