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

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Abstract. The interspecific and intrageneric 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. olaichana 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 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, Platycairoideae 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 Juglanaeae (Manchester, 1987; Yong-Ling et al., 1992).

Juglans species 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 center 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 Juglandaceae 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 (Derr 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. olaichana 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 regia 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 Chinese provenance. Another (unidentified) Engelhardia species was collected in Yunnan province, China. The North American Carya illinoensis (Wangh.) 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–tronF region 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 (tronL), the intron it contains, the trnE phenylalnine (GAA) gene (tronF), and the intergenic spacer between trnL (5’ exon) and trnF, was amplified by PCR (Mullis and Foorana, 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 3dNTPs’ (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 Welch and McClelland (1990) was followed. DNA
fragments of *Juglans* and outgroup were amplified using the following primers: OPA5 (AGGGGTATTCG), OPA7 (GAAACGGGTG), OPA8 (GTGACCGTG), OPA9 (CAAACGTCGG), and OPA10 (GTGATCGCAG) (QIAGEN Operon). The reaction mixture for RAPDs consisted of: 2 µL 10× PCR buffer, 2 µL MgCl₂, 25 mm 2 µL dNTP, 4 µL primer (20 mm), 10 µL H₂O, 0.1 µL of Taq polymerase (5 units/µL, Promega), and 2 µL 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 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 amide gel electrophoresis and visualized by silver-staining. Gene Gel Exel 12.5/24 pre-cast 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 \( N_{x} \) is the number of bands shared by specimens x and y, and \( N_{y} \) 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. ailantifolia* 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...
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, sug-
gested that the American sequence was rela-
tively plesiomorphic. Pterocarya has been shown to be the sister
genus of Juglans (Stanford et al., 1999). The
sequence difference between Pterocarya and
Juglans was 0.7%.

RAPD. The RAPD data placed the three
American accessions with J. nigra (100% jackknife support) and separate from
Asian species. Using Pterocarya, Engelhar-
dia, and Carya illinoinsis 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. 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 spe-
cies (Fig. 2).

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

<table>
<thead>
<tr>
<th>Species</th>
<th>J. australis</th>
<th>J. oleana</th>
<th>J. ailantifolia</th>
<th>J. regia</th>
<th>J. sigillata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bark texture</td>
<td>smooth (0); rough (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Bark colour</td>
<td>dark (0); light (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Young branch surface</td>
<td>glabrous (0); hairy (1)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bud</td>
<td>sharp and elongated (0); blunt and short (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foliage</td>
<td>deciduous (0); evergreen (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leaves</td>
<td>aromatic (0); odourless (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rachis</td>
<td>glabrous (0); hairy (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of leaflets</td>
<td>5–9 (0); 11–17 (1); 20–22 (2)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Leaflet lamina</td>
<td>oblong (0); elliptic (1); lanceolate (2); ovate (3); obovate (4); cuneate (5)</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Leaf base: truncate (0); cordate (1); rounded (2); oblique (3)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Leaf attachment: petiolate (0); sessile (1)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leaf apex: acute (0); obtuse (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Leaf margins: entire (0); serrate (1); serrulate (2); dentate (3); sinuate (4)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Upper leaf surface: glabrous (0); pubescent (1)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Leaflet arrangement: distichous (0); opposite (1); spiral (2)</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Leaves: paripinnate (0); imparipinnate (1)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leaflet venation: pinnate simple and craspedodoromous (0) or eucamptodoromous (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fruit: drupe (0); samara (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Indumentum: glabrous (0); pubescent (1)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Husk texture: glutinous/glandular (0), non secretory (1)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Nut apex: blunt (0); sharp (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Nut shape: globose (0); ellipsoid (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nut shape: compressed (0); not compressed (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nut: crested or with a visible suture (0); lacking crest or suture (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Shell thickness: 0.5–1.0 mm (0); 3–5 mm (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fruit arrangement on branches: solitary – 3 (0); &gt;3 (2)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

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

*Molecular Methods*. 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 separation of *Juglandaceae* at 3.36 x 10^-7 per site per year, with *Oreomunnea* and *Pterocarya* as a sister to *Juglans* and *Alnus*, who found no sequence differences according to morphological data. *Juglans* presents ambiguities. The DNA data clearly place this species with its geographically proximate cogners, but the morphological data place it intermediate between Eurasian and American *Juglans*. Contact and genetic introgression between American and Asian 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). Guan 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 *Cleistes*, are closely related to American species, (Cameron and Chase, 1999).

*(Juglandaceae) as determined from chloroplast DNA restriction site variation and morphology syntheses of DNA in vitro via xpolymerase catalysed chain reaction. Methods Enzymol.*

Table 3. Pairwise distances among Juglandaceae.

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<td>0.00</td>
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<td>0.28</td>
<td>0.34</td>
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<td>0.76</td>
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