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 Juglands (walnuts) and three other members of Juglandaceae were investigated. The following species were included: the American J. nigra L., J. regia L., and Carya illinoensis (Wang.), K. Koch.; two Juglandaceae 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.

Materials and Methods
Accessions used (Table 1). Juglans nigra L. and J. oalanchana 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 Vietnamese 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 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 (trnM), and the intronic spacer between trnL and trnF, was amplified by PCR (Pullis and Ffioona, 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 ‘dNTP’s’ (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 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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fragments of *Juglans* and outgroup were amplified using the following primers: OPA5 (AGG0GTTCTTG), OPA7 (GAAACCGGTTG), OPA10 (GTGATGCCAG), OPA18 (AGGTTGCGCCGT), OPA19 (CACAACGTCCG), and OPA20 (GTGCGGATCC) (QIAGEN OPERON P/L).

The reaction mixture for RAPDs consisted of: 2 µL Taq polymerase (5 units/µL, Promega), 2 µL of each respective DNA (quantity not stated), 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; 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 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).

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

| Taxon             | Voucher no. | GenBank no. | Wild source | Cultivation site |
|-------------------|-------------|-------------|-------------|------------------|
| *Juglans ailantifolia* Carr. | 1178         | NY231169    | unknown     | Nambucca Heads  |
| *Juglans australis* Griseb. | 1179         | NY231171    | Tucuman, Argentina | Nambucca Heads  |
| *Juglans neotropica* Diels. | 1180         | NY231168    | Loja, Ecuador | Nambucca Heads  |
| *Juglans nigra* L. | 1181         | NY231170    | unknown     | Cowra            |
| *Juglans olanchana* Standl. & L.O. Williams | 1182         | NY231171    | Turrialba, Costa Rica | Nambucca Heads  |
| *Juglans regia* | 1183         | NY231167    | unknown     | Cowra            |
| *Juglans sigillata* Dode | 1184         | NY231170    | Yunnan, China | Nambucca Heads  |
| *Juglans* sp. | 1185         | NY231172    | Quang Xi, China | Nambucca Heads  |
| *Carya illinensis* (Wangh.) K.Koch. | 1186         | NY231167    | unknown     | Mt Irvine        |
| *Pterocarya stenoptera* var. tonkinensis Franchet | 1187         | NY231168    | Sapa, Vietnam | RBG, Sydney      |
| *Engelhardtia* sp. | 1188         | NY231169    | Yunan, China | Nambucca Heads  |

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All of these sites are in New South Wales, Australia.  
Annex of Univ. of Western Sydney, Hawkesbury Campus, Sydney, Australia.  
Collected by Simon Goodwin, RBG, Sydney, Australia; all others collected by Orel and/or Richards.  
Royal Botanic Gardens, Sydney, Australia.*

**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 rRNA 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*, 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 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*, *Engelhardia*, 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 of *Carya illinoensis* and *P. stenoptera*.

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

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

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.
Table 3. Pairwise distances among Juglandaceae.

|       | J. aust | J. neo | J. olan | J. nig | J. reg | J. sp. | J. sig | P. sten | E. sp. | C. ill |
|-------|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| J. aust | 0.00    | 0.10   | 0.15   | 0.28   | 0.34   | 0.75   | 0.77   | 0.76   | 0.76   | 0.93   |
| J. neo  | 0.00    | 0.24   | 0.22   | 0.91   | 0.91   | 0.92   | 0.92   | 1.00   | 1.00   | 0.93   |
| J. olan | 0.00    | 0.18   | 0.86   | 0.72   | 0.74   | 0.73   | 0.73   | 0.82   | 1.00   | 0.82   |
| J. nig  | 0.00    | 0.84   | 0.84   | 0.86   | 0.85   | 0.87   | 0.87   | 1.00   | 0.80   | 0.80   |
| J. reg  | 0.00    | 0.17   | 0.24   | 0.44   | 0.80   | 0.89   | 0.89   | 0.65   | 0.65   | 0.65   |
| J. sp.  | 0.00    | 0.16   | 0.73   | 0.36   | 0.68   | 0.68   | 0.68   | 0.72   | 0.72   | 0.72   |
| J. sig  | 0.00    | 0.34   | 0.73   | 0.65   | 0.60   | 0.79   | 0.79   | 0.76   | 0.76   | 0.76   |
| P. sten | 0.00    | 0.74   | 0.73   | 0.72   | 0.74   | 0.74   | 0.74   | 0.72   | 0.72   | 0.72   |
| E. sp.  | 0.00    | 0.74   | 0.73   | 0.72   | 0.74   | 0.74   | 0.74   | 0.72   | 0.72   | 0.72   |
| C. ill  | 0.00    | 0.74   | 0.73   | 0.72   | 0.74   | 0.74   | 0.74   | 0.72   | 0.72   | 0.72   |

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 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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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 the American accessions tested. This is comparable to the findings of Guo et al. (1998) 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 Juglans at 3.36 x 10^{-10} to 10.7 x 10^{-10} substitution rates per site per year, with Oreamnun Pterocarya rate as high as 10.7 x 10^{-10}. With a substitution rate of 97 x 10^{-10} to 97 x 10^{-10}, which was estimated for many 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.

Summary. Taxonomic analysis of all data showed that the “New World” species formed a separate clade. American species under investigation 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. olandana and the nigellatum, characters 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.
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