Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms

R.G. FJELLSTROM AND D.E. PARFITT
Department of Pomology, University of California, Davis, CA 95616, U.S.A.

Corresponding Editor: R.S. Singh
Received September 16, 1993
Accepted May 24, 1994

FJELLSTROM, R.G., and PARFITT, D.E. 1994. Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms. Genome, **37**: 690–700.

The genetic diversity of 13 *Juglans* species was characterized using nuclear RFLPs. Allelic frequencies among 41 *Juglans* populations were determined at 19 RFLP loci by hybridizing single locus probes to walnut DNAs digested with the restriction endonuclease *Eco*RI or *Hin*dIII. A 10-fold difference in species heterozygosity levels was seen among species in different sections of the genus. Differentiation among conspecific populations varied over threefold between species. Genetic differentiation among conspecific east Asian populations was larger than that seen among east Asian species, while the opposite trend was seen for Western Hemisphere species. Taxonomic affinities were also indicated by these results, suggesting that *J. cinerea* should be included as part of section *Cardiocaryon* rather than as a unique section, *Trachycaryon*. *Juglans hindsii* is classified as a distinct species and not a subspecies of *J. californica*. Strategies for germplasm preservation and species requiring marked collection efforts are given.

Key words: *Juglans*, RFLP, genetic diversity, walnut.

FJELLSTROM, R.G., et PARFITT, D.E. 1994. Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms. Genome, **37** : 690–700.

La diversité génétique de 13 espèces de *Juglans* a été caractérisée à l'aide du polymorphisme des longueurs de fragments nucléaires de restriction (PLFR). Dans une population de 41 *Juglans*, les fréquences alléliques ont été déterminées dans 19 locus de PLFR, en hybridant les sondes d'un seul locus aux ADN des noyers digérés par les endonucléases de restriction *Eco*RI ou *Hin*dIII. Des différences égales à 10 fois ont été observées dans les niveaux d'hétérozygosité entre les espèces des différentes sections de ce genre. Chez les populations conspécifiques, la différenciation entre les espèces a varié de trois fois. La différenciation génétique entre les populations conspécifiques de l'Asie de l'est a été plus importante qu'entre les espèces de l'Asie de l'est, alors que la tendance inverse a été observée chez les espèces de l'hémisphère occidental. Des affinités taxonomiques ont aussi été indiquées par ces résultats; ceux-ci suggèrent que le *J. cinerea* devrait être inclus comme partie intégrante de la section *Cardiocaryon* plutôt que de constituer une section unique, dite *Trachycaryon*. Le *J. hindsii* est classé comme une espèce distincte et non comme une sous-espèce de *J. californica*. Les stratégies pour la conservation des plasmas germinaux ainsi que des espèces exigeant des efforts de collection marqués sont proposées.

Mots clés : *Juglans*, PLFR, diversité génétique, noyer.

[Traduit par la rédaction]

Introduction

The genus *Juglans* L. is made up of 21 species of long-lived deciduous trees that produce large woody-shelled nuts. *Juglans* has been divided into four taxonomic sections as described by Manning (1978): (*i*) section *Cardiocaryon* Dode (the east Asian heartnuts) with three species, *J. ailantifolia* Carr., *J. mandshurica* Maxim., and *J. cathayensis* Dode; (*ii*) section *Rhysocaryon* Dode (black walnuts) of North, Central, and South America and the West Indies with 16 species; (*iii*) section *Dioscaryon* Dode with one species, *J. regia* L. (Persian walnut) distributed from southeastern Europe to the Himalayan mountains; and (*iv*) section *Trachycaryon* Dode ex Mann. with one species, *J. cinerea* L. (butternut) in eastern North America. *Juglans* species are monoecious, with separate male and female inflorescences, and outcrossing owing to heterodichogamy with wind pollination (Manning 1978; Gleeson 1982).

The majority of *Juglans* species are represented only by native populations, but selected cultivars and breeding lines utilized for commercial production have been developed from *J. regia*, *J. nigra* L., and *J. cinerea*. *Juglans regia* cultivars are grown primarily for nut production as varietal clones (McGranahan and Leslie 1990). *Juglans nigra* is grown for timber and nut production from both clones and selected populations (Beinecke 1983; Funk 1970). Selected cultivars have also been developed from *J. ailantifolia*, *J. cinerea*, *J. cathayensis*, and *J. mandshurica* (McDaniel 1979). Wild walnut species are valuable sources of nuts and shelter for wildlife (Vines 1960; Elias 1980).

Despite their importance in native habitats and widespread commercial utilization, there has been limited information developed about the extent and nature of genetic variation in these species. Loci involved in the expression of heterodichogamy (Gleeson 1982) and virus resistance (McGranahan and Leslie 1990) have been described, but the majority of phenotypically scored traits have unknown genetic and environmental components. Isozyme loci for four enzyme systems (glucose phosphate isomerase, aspartate amino transferase, phosphoglucomutase, and esterase) were described by Arulsekar et al. (1985, 1986). Rink et al. (1989) studied outcrossing in *J. nigra* using isozyme loci for six enzyme systems (aconitase, alcohol dehydrogenase, aspartate amino transferase, 6-phosphogluconate dehydrogenase, fluorescent esterase, and phosphoglucomutase).

Prior research by Arulsekar et al. (1985, 1986; S. Arulsekar personal communication, D.E. Parfitt, personal observation)

Printed in Canada / Imprimé au Canada

TABLE 1. Description of *Juglans* spp. populations analyzed for genetic diversity

Species	Population name	Population size	Location	Environment
Section *Cardiocaryon*				
J. ailantifolia	NCGR 3	12	Japan, Kusajima (Jinzu River)	Temperate river valley
	NCGR 4	11	Japan, Nagano	Urban area
	NCGR 6	9	Japan, Myoga-bashi	Temperate woodland
	NCGR 9	12	Japan, Nagano	Urban area
J. cathayensis	NCGR 11	4	Taiwan, Taichung	Tropical coast
	GM 85-5	2	China, Zhe Jiang	Semi-tropical mountains
J. mandshurica	NCGR 13	2	Korea	Arboretum
	NCGR 87	2	China	Unknown
	NCGR 194	12	Korea, Jonnan	Temperate
Section *Trachycaryon*				
J. cinerea	IL	12	Illinois, Pike Co. (Mississippi River)	Temperate, riparian
	NC	12	North Carolina, Brevard (Appalachian highland)	Warm temperate mountain forest
	VT	12	Vermont, Lamoille Co.	Cold temperate mountain forest
Section *Rhysocaryon*				
J. californica	CC	12	California, Carbon Canyon	Semi-desert – mountain spring
	WC	11	California, West Covina	Semi-desert mountainside
	GP	12	California, Griffith Park	Ravine, urban parkland
	NCGR 17	12	California, Ventura Co.	Streamside canyon, semi-desert
J. hindsii	NCGR 33	11	California, Napa Co.	Mountain spring, mediterranean
	NCGR 37	12	California, Yolo Co. (Cache Creek)	Mediterranean, riparian
	NCGR 41	12	California, Contra Costa Co.	Mediterranean woodland
	NCGR 44	12	California, Yolo Co. (Sacramento R.)	Mediterranian, riparian
J. major	NCGR 47	10	Arizona, Pine	Semi-desert mountains
	NCGR 78	12	Arizona, Sedona (Oak Creek Canyon)	Semi-desert mountain river
	NCGR 80	12	Arizona, Wilhoit	Desert mountains
	NCGR 82	12	Arizona, Salt River	Desert basin, dry wash
J. microcarpa	OK	8	Oklahoma, Caddo Co.	Prairie, temperate grass–woodlands
	NCGR 29	11	Texas, Edwards Co.	Dry temperate grasslands
	NCGR 31	11	Texas, Ft. Stockton	Dry temperate grasslands
	NCGR 52	2	New Mexico, Carlsbad	Desert mountains
	NCGR 53	3	New Mexico, Carlsbad	Desert mountains
J. mollis	NCGR 218	4	Mexico, Zimapan	Semi-tropical, dry mountains
J. neotropica	NCGR 285	6	Ecuador, Otavalo	Unknown
	NCGR 330	2	Ecuador, Ambato (Andes highlands)	Mountain highland
J. nigra	GA	11	Georgia, Byron	Warm temperate
	MO	11	Missouri, Boone Co.	Temperate
	PA	11	Pennsylvania, Bradford Co.	Temperate
	TX	12	Texas, Shelby Co. (Biggar Creek)	Warm temperate
	WI	12	Wisconsin, Dane Co.	Temperate woodland
J. olanchana	NCGR 212	2	Mexico, San Antonio	Humid tropical mountains
	NCGR 213	2	Mexico, San Antonio	Humid tropical mountains
Section *Dioscaryon*				
J. regia	NCGR 122	12	Pakistan	Unknown
	NCGR 254	6	Pakistan, Gilgit	Wild grove, desert mountain valley

indicated that only a limited number of polymorphisms can be provided by isozyme markers in walnuts. With the potential of restriction fragment length polymorphism (RFLP) markers to detect numerous polymorphisms (Kesseli et al. 1991, Lubbers et al. 1991), RFLP markers identifying codominant single copy walnut loci (Fjellstrom and Parfitt 1994) were constructed to study the genetic variability in walnut species. Nineteen RFLP loci were used to characterize the genetic variability among 41 populations of walnuts comprising 13 *Juglans* species. RFLPs have been successfully employed to study genetic diversity among cultivated and native species accessions in a broad range of herbaceous, primarily annual, plant species (Miller and Tanksley 1990; Brummer et al. 1991; Chase et al. 1991; Kesseli et al. 1991; Wang et al. 1992).

Materials and methods

Plant materials

Leaves were collected from 41 populations of 13 walnut species (Table 1) as well as from 16 cultivated clonal selections of *J. cinerea* and *J. nigra* (cvs. Booth, Craxezy, Henderson#2, Johnson, Kenney Glen, and Weschke from *J. cinerea* and cvs. Crantz, Farrington, Horton, Ohio, Thomas, Todd, Beinecke#160, Rink#344, Rink#631, and Rink#585 from *J. nigra*). Leaves were obtained from mature trees at the USDA National Clonal Germplasm Repository (NCGR) walnut collection in Winters, California. The NCGR collection consists of clonally propagated cultivars, plant introductions, and seedling populations of walnut species collected worldwide. Species from North America were collected to represent the diversity of natural populations by D. Parfitt. Asian species were collected by several individuals with a focused emphasis on germplasm for breeding. The collection has

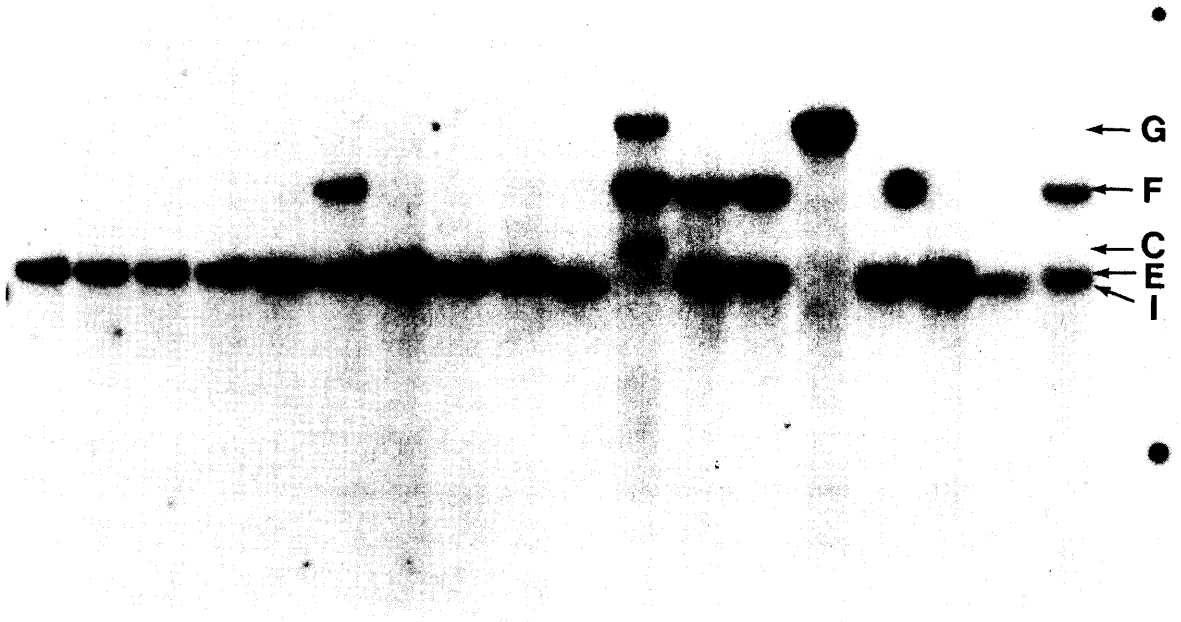

FIG. 1. Autoradiograph of *Juglans cinerea* cultivars and populations digested with *Hin*dIII and probed with pFP48. Lanes 1–6, cvs. Weschke, Kenney Glen, Johnson, Henderson #2, Craxezy, and Booth; lane 7, bulked VT population; lanes 8–10, individuals from VT population; lane 11, bulked NC population; lanes 12–14, individuals from VT population; lane 15, bulked IL population; lanes 16–18, individuals from IL population. Alleles are listed on right. Bulked DNA allele frequencies using BIOSYS image analysis (see Materials and methods) were determined to be as follows. (*i*) VT population: E, 0.89; I, 0.11; (*ii*) NC population: C, 0.17; E, 0.11; F, 0.44; G, 0.28; (*iii*) IL population: E, 0.78; I, 0.22.

roughly 13 populations each of *J. ailantifolia*, *J. californica* S. Wats., *J. hindsii* (Jeps.) Rehder, and *J. major* (Torr. ex Sitsgr.) Heller. It has only one population each of *J. cathayensis* and *J. mollis* Englm. ex Hemsl., two populations of *J. olanchana* Standl. & L.O. Williams and *J. neotropica* Diels, three populations of *J. mandshurica*, and five populations of *J. microcarpa* Berl. The *J. mollis* and especially the *J. olanchana* accessions probably represent the available variation for these species since only a few populations of limited distribution exist. Additional collections were also obtained from *J. nigra* and *J. cinerea* seedlings at NCGR greenhouses in Davis, California; as fresh leaves from NCGR Corvallis, Oregon (*J. cinerea* cultivars); and as fresh leaves collected directly from Southern California field sites for *J. californica*.

Genotypes within species were sampled with two different methods. Populations with one to four plants were sampled individually. Populations with 6–12 plants were initially sampled individually (2 or 3 plants) and a bulk sample was collected from the remaining population (4–9 plants), with each plant contributing equal amounts of fresh weight to the total sample. Samples were bulked to assay as many population members as possible, while selected plants were analyzed separately to identify typical RFLP genotypes within a species.

Leaves were ground in liquid nitrogen and stored frozen at −70°C before isolation of DNA by a modification of the method of Doyle and Doyle (1987). Five grams of frozen leaves were added to 20 mL of 60°C preheated 2× CTAB buffer (2% CTAB, 1% PVP, 1% β-mercaptoethanol, 0.1% sodium bisulfite, 1.4 M NaCl, 50 mM Tris, 20 mM Na EDTA, pH 8.0), and incubated at 60°C for 30 min. The aqueous solution was extracted with 20 mL 24:1 chloroform – isoamyl alcohol, centrifuged 10 min at 1800 rpm in a bench-top centrifuge at 25°C, and the aqueous layer retained. Fifteen millilitres isopropanol was added to precipitate the nucleic acids. The precipitate was spooled and washed in 76% ethanol with 10 mM ammonium acetate. The nucleic acid precipitate was air-dried overnight, rehydrated in 1 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), digested with 10 μg RNase at 37°C for 1 h, and ethanol precipitated. The DNA precipitate was washed in 75% ethanol and dried overnight before rehydration in 200 μL TE (pH 8.0). The DNA was quantified spectrophotometrically at 260 nm and visually in a 0.8% agarose gel stained with ethidium bromide containing lambda phage standards, with approximately 25 μg/mL DNA (1.0 Å at 260 nm; 1 Å = 0.1 nm).

RFLP detection

Six micrograms of walnut DNA was digested with 30 U of *Eco*RI or *Hin*dIII for 6 h and electrophoresed in 0.8% agarose with 1× TAE (45 mM Tris–acetate, 1 mM EDTA, pH 8.0) buffer for 18 h at 0.7 V/cm. Gels were stained with ethidium bromide to visualize the DNA and transferred (Southern 1975) to 150 × 150 mm nylon membranes (Nytran, Schleicher & Schuell). Membranes were rinsed in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), dried 2 h in a 65°C oven, and stored dry at room temperature. Membranes were trimmed to 140 × 150 mm, loaded into 30 × 300 mm hybridization bottles, prehybridized for 1 h, and hybridized for 16–20 h at 65°C according to the method of Church and Gilbert (1984). Thirty nanograms of insert DNA from 19 single locus walnut probes, pFP01, 02, 03, 04, 06, 11, 15, 17, 18, 24, 25, 26, 32, 34, 43, 45, 48, and 67 (Fjellstrom and Parfitt 1994), were ^{32}P radiolabelled by random priming (Feinberg and Vogelstein 1983), denatured by boiling and rapid cooling, and added directly to each hybridization tube. Following hybridization, membranes were washed 2 × 15 min in 2× SSC, 0.1% SDS at room temperature, 2 × 15 min in 1× SSC, 0.1% SDS at 45°C, and 1 × 30 min in 0.5 × SSC, 0.1% SDS at 65°C, rinsed in 2× SSC, blotted on paper towels, and autoradiographed on X-OMATAR film (Kodak) at −70°C using Cronex (DuPont) intensifying screens for one to 5 days. Blots were stripped by soaking in 5 mM Tris (pH 8.0), 0.5 mM EDTA (pH 8.0), 0.01× Denhardts solution, 0.05% Na pyrophosphate at 65°C for 30–60 min, rinsed in 2× SSC, and stored dry. Blots were reprobed 5–10 times.

Genetic analysis

Allelic genotypes were scored for the codominant single-copy loci identified by probes pFP02, 03, 06, 15, 17, 18, 24, 26, and

TABLE 2. Summary statistics for populations (combining bulked and individual data) of 13 *Juglans* species

Species	No. of populations	Alleles/ locus	% Polymorphic loci	H_I[a]	F_{ST}[b]
J. ailantifolia	4	1.16	36.8	0.121 (0.011)	0.108 (0.013)
J. cathayensis	2	1.21	26.3	0.094 (0.010)	0.314 (0.069)
J. mandshurica	3	1.63	42.1	0.130 (0.010)	0.280 (0.018)
J. cinerea	3	2.11	73.7	0.224 (0.011)	0.384 (0.014)
J. californica	4	1.53	26.3	0.106 (0.011)	0.060 (0.007)
J. hindsii	4	1.26	21.1	0.035 (0.007)	0.080 (0.050)
J. major	4	1.63	36.8	0.095 (0.010)	0.139 (0.017)
J. microcarpa	5	1.53	36.8	0.096 (0.010)	0.157 (0.015)
J. mollis	1	1.42	21.1	0.105 (0.012)	—
J. neotropica	2	1.11	10.5	0.023 (.004)	0.000 (0.0)
J. nigra	5	1.47	31.6	0.100 (0.009)	0.104 (0.006)
J. olanchana	2	1.11	10.5	0.049 (0.008)	0.065 (0.0)
J. regia	2	1.68	52.6	0.214 (0.012)	0.108 (0.008)

NOTE: H_T, total species diversity (Nei 1987) with standard errors from jacknife analysis across loci in parentheses; F_{ST}, Wrights (1969) statistic of population differentiation with standard errors from jacknife analysis across loci in parentheses.

67 hybridized to *Eco*RI-digested DNA and probes pFP01, 04, 11, 12, 25, 32, 34, 43, 45, and 48 hybridized to *Hin*dIII-digested DNA. Individual allelic genotypes were directly converted into allele frequencies. Allele frequencies were also estimated from bulked population members by analyzing autoradiograms from bulked samples with BIOIMAGE (Millipore) software (Fig. 1). The percentage of total integrated optical density for each RFLP band was determined and converted into estimated allele frequencies. Allelic bands frequently did not demonstrate equal autoradiographic intensity, as determined from heterozygous population members isolated individually within populations. Therefore, the average autoradiographic intensity for each band was determined by analyzing single individuals and used to convert bulked population RFLP band intensities into population allele frequencies. Allele frequencies were rounded off to values representative of the population being analyzed (e.g., the possible allele frequencies for a population of five individuals range from 0 to 100% in increments of 10%). Allele frequencies for larger populations were computed using individual genotype data combined with bulked gene frequency data, while only individual genotype data was used for smaller populations where population sizes did not justify using bulked samples. Allelic frequency analysis of RFLP loci was performed using BIOSYS-1 (Swofford and Selander 1989) to generate genetic identity matrices, perform average linkage UPGMA (Sneath and Sokal 1973) cluster analysis based on Nei's genetic identity (Nei 1972), and calculate Wright's population structure *F* statistics (Wright 1969).

Results

A total of 117 alleles were detected at the 19 RFLP loci for an average 6.16 alleles per locus (Appendix). Every locus displayed at least one polymorphism among the 13 species. Two loci, *pFP01* and *pFP26*, were monomorphic within individual species, each having only two alleles for the 13 species. The maximum number of alleles for one locus was 15 at *pFP12*. Individual species had an average of 1.45 alleles per locus, ranging from 1.11 for *J. neotropica* and *J. olanchana* to 2.11 for *J. cinerea* (Table 2). The number of alleles per locus was generally proportional to the percentage of polymorphic loci, ranging from 10.5% for *J. neotropica* and *J. olanchana* to 73.7% for *J. cinerea*, and averaging 32.8% for all species (Table 2). Estimates of species heterozygosity levels (H_T), based on gene frequencies in Hardy–Weinberg equilibrium, ranged from 0.023 for *J. neotropica* to 0.224 for *J. cinerea* with an average value of 0.108 (Table 2).

F_{ST} measurements of the differentiation among populations within a species had values from 0.000 for *J. neotropica* and *J. olanchana* to 0.384 for *J. cinerea*. Among the North American species, *J. californica*, *J. major*, *J. microcarpa*, and *J. nigra* (representing the most completely sampled species) have similar heterozygosity levels but have different degrees of genetic variation among populations within species (F_{ST} = 0.060, 0.104, 0.0139, and 0.157, respectively). Even though *J. cinerea* appears to be the most polymorphic species, a substantial proportion of the variation is found only in the North Carolina (NC) population. This population has the highest heterozygosity levels of any population (H_S = 0.289) and its variability accounts for the high degree of population differentiation in this species. When this population is excluded from the analysis, and the remaining *J. cinerea* populations are analyzed, dramatic decreases in

heterozygosity (H_T = 0.068) and population differentiation (F_{ST} = 0.071) levels are seen, making *J. cinerea* appear less variable than average. Since total sample size for the species was decreased by one-third, this result was not primarily a function of sample size difference.

Differentiation can also be analyzed among species within two sections of *Juglans*. The Asian heartnut species of section *Cardiocaryon* have a large proportion of genetic differentiation at the population level ($F_{pop/species}$ = 0.287) and little at the species level ($F_{species/section}$ = 0.022). The black walnut species of section *Rhysocaryon* have a different pattern of differentiation, lower at the population level ($F_{pop/species}$ = 0.135) and higher at the species ($F_{species/section}$ = 0.696).

Cultivated selections of *J. cinerea* and *J. nigra* were also analyzed for their genetic variability. The six *J. cinerea* cultivars had relatively low heterozygosity (H_S = 0.088) and genetic differentiation (F_{ST} = 0.048) among cultivars, while the 10 *J. nigra* cultivars had moderate levels of heterozygosity (H_S = 0.111) and genetic differentiation (F_{ST} = 0.118).

Genetic identity values (Nei 1972) among the 41 *Juglans* populations were subjected to UPGMA cluster analysis (Sneath and Sokal 1973), generating the tree in Fig. 2. The separation of *Juglans* into three major groups, made up of sections *Cardiocaryon/Trachycaryon*, *Rhysocaryon*, and *Dioscaryon*, is readily seen. The species of section *Rhysocaryon* are well defined, as seen by the large proportion of conspecific populations found clustered together. One population of *J. microcarpa* was clustered with *J. nigra* populations rather than the other populations of *J. microcarpa*. This was the smallest population of *J. microcarpa* (with n = 2) and random sampling could have selected more of the predominant *J. nigra* alleles rather than *J. microcarpa* alleles when the proportions of shared alleles at a locus was different for these two closely related species. The species of section *Cardiocaryon* were poorly delineated, with many populations from different species found more closely associated with each other than with their own conspecific populations. Although small population sample size could explain some of these results, even the larger-sized populations were found intermixed among these Asian species. *Juglans cinerea* of section *Trachycaryon* clustered with the section *Cardiocaryon* species, with the NC *J. cinerea* population clustering closer to section *Cardiocaryon* than its other conspecific populations. Section *Dioscaryon*, with *J. regia*, is separated from the other sections.

There is certainly a loss of information when individuals are bulked to estimate gene frequencies, as had been done here for four to nine members of larger populations. The percentage of heterozygous individuals in bulked populations can only be estimated using allelic frequencies by assuming Hardy–Weinberg equilibrium exists within the population sampled, since heterozygotes can not be directly detected. In the 141 cases where polymorphism was detected in either a bulked population or one of its individual members sampled separately, 46.1% of the cases showed the expected number of heterozygotes to be equal to the number of observed individual heterozygotes, 28.4% had more observed heterozygotes, and 25.5% had less observed heterozygotes would be expected from a normal distribution. There was no evidence for directional selection. Variability in the amount of DNA yielded by different members bulked together could also alter the estimated gene frequencies. Observations on the variability of DNA yield from individual members of the same population show that the 34.8% coefficient of variation (CV) for DNA yield is relatively high. Additional error in allele frequency estimation could result from RFLP band autoradiographic signal intensity variation. Despite the lack of precision involved in estimating allele frequencies, similar patterns of genetic diversity and identical cluster analysis groupings resulted when we analyzed individual data alone as compared with analyzing the combined bulk and individual population data (results not shown). Uniformly larger values of population differentiation were seen across the majority of the species when only individual data was analyzed, a result consistent with the findings of Gorman and Renzi (1979).

Discussion

This research demonstrated that RFLPs are suitable characters for analyzing the genetic diversity within and among populations and species of this widely distributed woody plant genus. As expected in outcrossing species, heterozygosity within populations was common and intraspecific population differentiation was reduced. Although the levels of heterozygosity appear low compared with other studies based on isozyme data, the comparative degree of heterozygosity can be adjusted to that of isozyme studies where diversity is reported from data on polymorphic loci alone (as opposed to both monomorphic and polymorphic loci). When this adjustment is made (by dividing H_T by the proportion of polymorphic loci), these walnut species have an average heterozygosity of 0.338, which is actually greater than the heterozygosity values cited by Loveless and Hamrick (1984) for species classified as wind pollinated, monoecious, and long lived, with heterozygosities of 0.250, 0.224, and 0.221, respectively, for isozyme loci. The high level of RFLP heterozygosity in walnut could well be attributed to RFLP loci detecting more variation than isozyme loci, which has been demonstrated in both outcrossing and inbreeding species (Kesseli and Michelmore 1986; Kesseli et al. 1991; McGrath and Quiros 1992). Diversity among populations (measured by F_{ST}) only accounted for 13.9% of individual species diversities, which is typical for the range seen in species with similar reproductive biology (Loveless and Hamrick 1984). This measure of diversity is generally similar for isozyme and RFLP data (Kesseli and Michelmore 1986; Kesseli et al. 1991; McGrath and Quiros 1992), which is expected since this statistic is unaffected by the proportion of polymorphic loci detected. The range in variation among F_{ST} values in this study can be ascribed to small sample sizes, ecological parameters, or extremes in genetic diversity. Small population size most likely gave rise to the extreme F_{ST} values for *J. cathayensis*, *J. mollis*, *J. mandshurica*, *J. neotropica*, and *J. olanchana*. *Juglans mandshurica* and *J. cathayensis* could also have uncharacteristic F_{ST} values because of artificial selection forces (e.g., as would be seen in progeny from botanical garden introductions). Certainly, greater population sizes and more populations would be needed to obtain more accurate diversity estimates for these species.

The difference in F_{ST} values among *J. californica*, *J. major*, and *J. microcarpa* and *J. nigra* can be understood from a consideration of the geographic ranges and distribution of these species. *Juglans californica* occupies the smallest geographic range among these species. Lower F_{ST} values may be due to a lack of differentiation occurring within its small

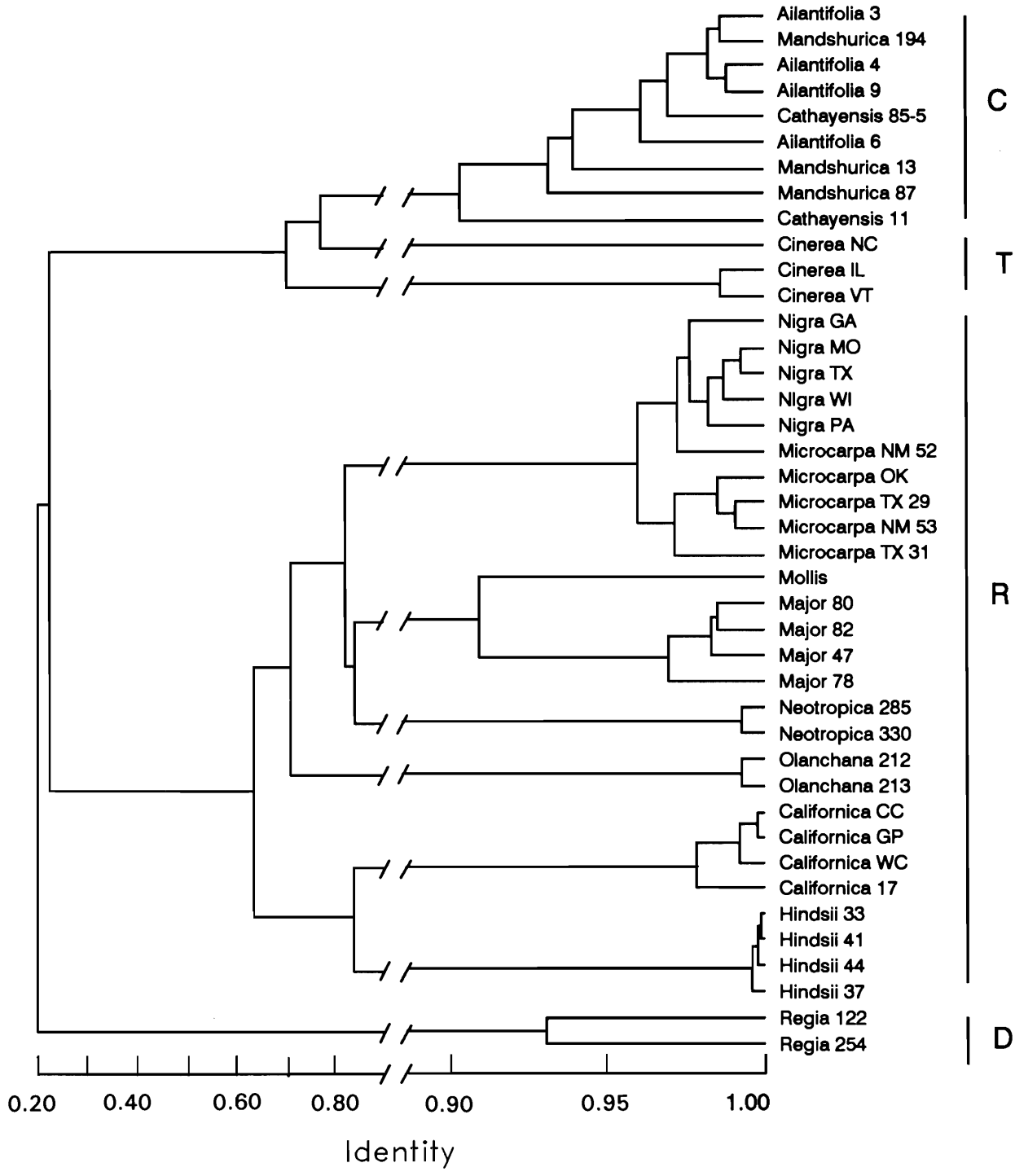

FIG. 2. UPGMA cluster analysis of RFLP genetic identity (Nei 1972) among 41 *Juglans* populations from 13 species. To the right are population species/number designations (from Table 1) and the taxonomic *Juglans* sections to which they belong (C, section *Cardiocaryon*; T, section *Trachycaryon*; R, section *Rhysocaryon*; D, section *Dioscaryon*).

habitat range. However, *Juglans californica* is also found in geographically isolated regions within its geologically differentiated range, which were not sampled in this study. Much higher F_{ST} values might have been obtained with more extensive sampling. The largest geographic range is found for *J. nigra*, but this species has only moderate levels of differentiation. Since *J. nigra* is a common tree found spread throughout Eastern North America and no major geographic or climatic barriers are present within its range, gene flow between populations by wind pollination has probably reduced genetic differentiation among populations of this species. The southwestern species of the United States, *J. major* and *J. microcarpa*, have the largest F_{ST} values, which can be explained by the disjunct distribution of these species. These species exist as isolated populations separated by mountains, desert, or prairie, which resulted in greater differentiation occurring among populations in separate habitats with limited gene flow between them.

The high degree of differentiation in *J. cinerea* is attributable to the highly variable North Carolina population.

Sampling more *J. cinerea* populations throughout its range will indicate whether this population or the less diverse Illinois and Vermont populations, on the extreme north and west margins of the range, are more typical of this species. *Juglans cinerea* has been devastated throughout all but its northernmost range from butternut canker disease, caused by *Sirococcus clavigignenti-juglandacearum* (Orchard et al. 1982). If *J. cinerea* has minimal diversity it could be highly genetically vulnerable and could become extinct. Cultivated selections of *J. cinerea* had notably low levels of genetic differentiation among themselves. Cultivars had moderate levels of heterozygosity, below that of the total species but greater than that seen in the Illinois and Vermont populations. Low diversity could explain why *J. cinerea* cultivars are uniformly susceptible to butternut canker.

The low genetic differentiation within *J. hindsii*, as well as its low heterozygosity levels, could be due to a lack of genetic diversity resulting from a genetic bottleneck if the entire species was founded by one or a few small populations (McGranahan et al. 1988). The cultivated selections of *J. nigra* have higher heterozygosity and differentiation levels than the wild accessions tested. This increased heterozygosity could be the result of selection for increased vigor, which could have also created greater genetic differentiation or a disjunct population structure.

The two *J. regia* populations had high levels of heterozygosity and moderate levels of genetic differentiation, as is characteristic for cultivated genotypes of this species (Fjellstrom et al. 1994). Only two *J. regia* populations were included as these were the only ones available that could be considered "wild" populations comparable with the other *Juglans* undomesticated populations studied.

The cluster analysis of genetic identity estimates (Nei 1972) show that the Asian species of section *Cardiocaryon* are more closely related to each other than the American species of section *Rhysocaryon*. Although morphological differences are seen between *J. ailantifolia*, *J. cathayensis*, and *J. mandshurica*, these results indicate that little genetic differentiation has occurred among these species. The contention that *J. cathayensis* may be a subspecies of *J. mandshurica* (McGranahan and Leslie 1990) may be justified since populations of *J. mandshurica* show more differentiation among each other than they do to other populations of *J. cathayensis*. Although populations of *J. ailantifolia* are physically separated from the other Asian heartnuts, distributed solely on the islands of Japan, they have not differentiated much from their congeneric mainland relatives. Perhaps gene flow by nut dissemination has reduced the rate of differentiation and allowed these species to remain similar. Most of the species of section *Rhysocaryon*, on the other hand, commonly showed pronounced genetic differentiation. This research supports the position that *J. hindsii* and *J. californica* are distinct species (McGranahan et al. 1988), a view that is not shared among all botanists (Wilken 1993). Also because the species of sections *Cardiocaryon* and *Trachycaryon* display similarity to the degree seen among species within section *Rhysocaryon*, the inclusion of *J. cinerea* in section *Cardiocaryon* appears appropriate, as had been supported by Manchester (1987) and at one time by Manning (1957). However, Stone and Broome (1975) and later Manning (1978) placed *J. cinerea* in a separate section, a position not supported by this study. The third distinct section, *Dioscaryon*, comprised solely of *J. regia*, appears almost equally distant from the other two walnut sections.

Bulking plant samples within populations, and isolating and analyzing their DNA en masse, had both positive and negative consequences. The primary benefit of bulking was significantly reduced labor and material costs (by over 60%) compared with sampling all plants individually. More allelic information was gained per Southern blot utilized but with a concomitant loss of genotype frequency information. Estimating gene frequencies from these bulked plants probably introduced some errors into the allele frequency determination. Despite the variation seen in DNA yield within populations, which could change allelic frequency estimations for specific loci, these effects should be random and should not unidirectionally affect mean heterozygosity estimates at the population or species level. Heterozygosity at the loci tested is independent of relative DNA yields. The use of bulked samples proved particularly useful when one notes that extreme diversity values were common for populations based on a few individuals.

The number of individuals studied are far fewer than those typically analyzed in isozyme studies. Such small sample sizes increase the variance of diversity measures. Using Nei's (1978) method, the expected standard error owing to sample size is 0.034 when $n = 2$ and 0.012 when $n = 12$. The variance owing to sample size is approximately 43.1 and 8.8% of the total variance when $n = 2$ and 12, respectively, with the remainder of the variance owing to the number of loci examined. The empirical results of Gorman and Renzi (1979) indicate that a sample size of $n = 2$ and $n = 8$–12 yield a heterozygosity estimate within 0.025 and 0.010, respectively, of that obtained from larger samples. Therefore, our diversity estimates probably provide an adequate overall characterization of *Juglans* species diversity, although further analysis would provide greater resolution of this diversity. Unfortunately, except for the North American species, we have sampled the entire available germplasm base collected for these species, many of which are rare or difficult to locate. Still, even for these small sample sizes, increasing the number of loci studied will be more valuable than increasing the number of individuals sampled, as noted by Nei (1978) and Gorman and Renzi (1979).

An important component of this genetic diversity study was to answer the important questions of how to conserve diversity in *Juglans* collections. We attempted to determine the extent of diversity within the NCGR walnut collection and the genus *Juglans* as a whole. This research suggests that additional collections of *J. cathayensis*, *J. mandshurica*, *J. mollis*, *J. neotropica*, *J. olanchana*, and wild *J. cinerea* germplasm are needed. Less emphasis may be required for less diverse species, e.g., *J. hindsii*, or relatively plentiful species, e.g., *J. nigra*. Collection strategies would be similar to those used for most outcrossed species. Collection should be equally divided between sampling within locations (i.e., populations) and at different locations. Most of the variation detected in these species will be present within a single population, but additional variation exists between populations and will be greater where geographic or climatic isolation mechanisms are present. Collection strategies should be implemented quickly before disease, in the case of *J. cinerea*, or land development, in the case of Latin American species, erodes the remaining genetic variability within these species. The importance of prompt collection efforts is of particular consequence in species such as *J. australis*, *J. boliviana*,

J. hirsuta, *J. jamaicensis*, *J. pyriformis*, and *J. venezuelensis* (McGranahan and Leslie 1990) for which no collections have been made.

Acknowledgements

We appreciate the assistance of (*i*) Dr. Gale McGranahan for providing background information and plant materials; (*ii*) Dr. Katie Rigert USDA–ARS NCGR—Davis, California, for providing collections of *Juglans* spp. and greenhouse space for seedling walnuts; and (*iii*) Dr. Jerry Payne USDA–ARS Byron, Georgia, Dr. David Funk USDA–USFS Durham, New Hampshire, Dr. Raymond Guries, Department of Forestry, University of Wisconsin, Madison, Wisconsin, Dr. Dan Millikan, Department of Plant Pathology, University of Missouri, Columbia, Missouri, Dr. Ken Hunt, Department of Horticulture, University of Missouri, Columbia, Missouri, Dr. Dan Creech, Department of Agriculture, Stephen F. Austin, University of Nacogdoches, Texas, Dr. Marvin Prits, Department of Pomology, Cornell University Ithaca, New York, and Mr. Al Miyat, Forestry Division, Oklahoma Department of Agriculture, Washington, Oklahoma, for shipments of *J. nigra*, *J. cinerea*, and *J. microcarpa* seed. This research was supported in part by the USDA–CSRS, UC—Davis Genetic Resources Conservation Program, and the California State Agricultural Experiment Station.

Arulsekar, S., Parfitt, D.E., and McGranahan, G.H. 1985. Isozyme gene markers in *Juglans* species. J. Hered. **76**: 103–106.

Arulsekar, S., McGranahan, G.H., and Parfitt, D.E. 1986. Inheritance of phosphoglucomutase and esterase isozymes in Persian walnut. J. Hered. **77**: 220–221.

Beineke, W.F. 1983. The genetic improvement of black walnut for timber production. Plant Breed. Rev. **1**: 236–266.

Brummer, E.C., Kochert, G., and Bouton, J.H. 1991. RFLP variation in diploid and tetraploid alfalfa. Theor. Appl. Genet. **83**: 89–96.

Chase, C.D., Ortega, V.M., and Vallejos, C.E. 1991. DNA restriction fragment length polymorphisms correlate with isozyme diversity in *Phaseolus vulgaris* L. Theor. Appl. Genet. **81**: 806–811.

Church, G.M., and Gilbert, W. 1984 Genomic sequencing. Proc. Natl. Acad. Sci. U.S.A. **81**: 1991–1995.

Doyle, J.J., and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11–15.

Elias, T.S. 1980. The complete trees of North America. VanNostrand Reinhold, New York.

Feinberg, A.P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction fragments to a high specific activity. Anal. Biochem. **132**: 6–13.

Fjellstrom, R.G., and Parfitt, D.E. 1994. RFLP inheritance and linkage in walnut. Theor. Appl. Genet. In press.

Fjellstrom, R.G., Parfitt, D.E., and McGranahan, G.H. 1994. Genetic relationships and characterization of Persian walnut (*Juglans regia* L.) cultivars using restriction fragment length polymorphisms. J. Am. Soc. Hort. Sci. **119**: 833–839.

Funk, D.T. 1970. Genetics of black walnut. USDA For. Ser. Res. Pap. WO-10.

Gleeson, S.K. 1982. Heterodichogamy in walnuts: inheritance and stable ratios. Evolution, **36**: 892–902.

Gorman, G.C., and Renzi, J., Jr. 1979. Genetic distance and heterozygosity estimates in electrophoretic studies: effect of sample size. Copeia, **2**: 242–249.

Kesseli, R., and Michelmore, R. 1986. Genetic variation and phylogenies detected from isozyme markers in species of *Lactuca*. J. Hered. **77**: 324–331.

Kesseli, R., Ochoa, O., and Michelmore, R. 1991. Variation at RFLP loci in *Lactuca* spp. and origin of cultivated lettuce (*L. sativa*). Genome, **34**: 430–436.

Loveless, M.D., and Hamrick, J.L. 1984. Ecological determinants of genetic structure in plant populations. Annu. Rev. Ecol. Syst. **15**: 65–95.

Lubbers, E.L., Gill, K.S., Cox, T.S., and Gill, B.S. 1991. Variation of molecular markers among geographically diverse accessions of *Triticum tauschii*. Genome, **34**: 354–361.

Manchester, S.R. 1987. The fossil history of the *Juglandaceae*. Monogr. Syst. Bot. Mo. Bot. Gard. **21**: 1–137.

Manning, W.E. 1957. The genus *Juglans* in Mexico and Central America. J. Arnold Arbor. **38**: 121–150.

Manning, W.E. 1978. The classification within the *Juglandaceae*. Ann. Mo. Bot. Gard. **65**: 1058–1087.

McDaniel, J.C. 1979. Other walnuts including butternut, heartnut, and hybrids. *In* Nut tree culture in North America. *Edited by* R.A. Jaynes. North American Nut Growers Association, Hamden, Conn. pp. 98–110.

McGranahan, G., and Leslie, C. 1990. Walnuts (*Juglans*). *In* Genetic resources of temperate fruit and nut crops. Vol. 2. *Edited by* J.N. Moore and J.R. Ballington. International Society for Horticultural Science, Wageningen. pp. 907–951.

McGranahan, G.H., Hansen, J., and Shaw, D.V. 1988. Inter- and intraspecific variation in California black walnuts. J. Am. Soc. Hort. Sci. **113**: 760–765.

McGrath, J.M., and Quiros, C.F. 1992. Genetic diversity at isozyme and RFLP loci in *Brassica campestris* as related to crop type and geographic origin. Theor. Appl. Genet. **83**: 783–790.

Miller, J.C., and Tanksley, S.D. 1990. RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. Theor. Appl. Genet. **80**: 437–448.

Nei, M. 1972. Genetic distance between populations. Am. Nat. **106**: 283–292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, **89**: 583–590.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Orchard, L.P., Kunz, J.E., and Kessler, K.J. 1982. Reactions of *Juglans* species to butternut canker and implications for disease resistance. *In* Black walnut for the future. US For. Serv. Gen. Tech. Rep. NC-74. pp. 127–131.

Rink, G., Carroll, E.R., and Kung, F.H. 1989. Estimation of *Juglans nigra* L. mating system parameters. For. Sci. **35**: 623–627.

Sneath, P.H.A., and Sokal, R.R. 1973. Numerical taxonomy. W.H. Freeman, San Francisco.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–517.

Stone, D.E., and Broome, C.E. 1975. Juglandaceae A. Rich. ex Kunth. World Pollen Spore Flora, **4**: 1–35.

Swofford, D.L., and Selander, R.B. 1989. BIOSYS-1: a computer program for the analysis of allelic variation in population genetics and biochemical systematics, version 1.7. Natural History Survey, Champaign, Ill.

Vines, R.A. 1960. Trees, shrubs, and woody vines of the Southwest. University of Texas Press, Austin.

Wang, Z.Y., Second, G., and Tanksley, S.D. 1992. Polymorphism and phylogenetic relationships in the genus *Oryza* as determined by analysis of nuclear RFLPs. Theor. Appl. Genet. **83**: 565–581.

Wilken, D.H. 1993. *Juglandaceae*. *In* The Jepson manual of higher plants of California. *Edited by* J.C. Hickman. University of California Press, Berkeley.

Wright, S. 1969. Evolution and the genetics of populations. Vol. 2. The theory of gene frequencies. University of Chicago Press, Chicago.

Appendix

TABLE A1. Allelic frequencies at 19 loci for 13 *Juglans* species

Locus	Allele	*ailantifolia*	*cathayensis*	*mandshurica*	*cinerea*	*nigra*	*olanchana*	*mollis*	*neotropica*	*microcarpa*	*major*	*californica*	*hindsii*	*regia*
01	*a*	0	0	0	0	0	0	0	0	0	0	0	0	1.00
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
02	*a*	0	0	0	0	0	0	0	0	0	0	0	0	0.92
	b	0	0	0	0.75	0.07	0	0	0	0	0	0	0.23	0.08
	c	1.00	1.00	1.00	0.25	0	0	0	0	0	0	0	0	0
	e	0	0	0	0	0.84	1.00	1.00	1.00	1.00	1.00	0.22	0	0
	f	0	0	0	0	0.09	0	0	0	0	0	0	0	0
	g	0	0	0	0	0	0	0	0	0	0	0.27	0.76	0
	h	0	0	0	0	0	0	0	0	0	0	0.49	0.01	0
	i	0	0	0	0	0	0	0	0	0	0	0.02	0	0
03	*a*	0	0	0	0	0	0	0	0	0	0	0	0	1.00
	b	0	0	0	0.10	1.00	0.38	1.00	1.00	1.00	1.00	1.00	0.52	0
	c	0.52	0.58	0.31	0.90	0	0	0	0	0	0	0	0	0
	e	0.43	0	0.69	0	0	0	0	0	0	0	0	0	0
	f	0	0.42	0	0	0	0	0	0	0	0	0	0	0
	g	0	0	0	0	0	0.63	0	0	0	0	0	0	0
	h	0	0	0	0	0	0	0	0	0	0	0	0.05	0
	i	0	0	0	0	0	0	0	0	0	0	0	0.43	0
	m	0.05	0	0	0	0	0	0	0	0	0	0	0	0
04	*a*	0	0	0	0	0	0	0	0	0	0	0	0	1.00
	b	0	0	0	0	0.52	0	0.50	0	0.81	0.96	0	0.03	0
	c	0.95	1.00	0.75	0.89	0	0	0	0	0	0	0	0	0
	d	0.25	0	0.25	0.11	0	0	0	0	0	0	0	0	0
	g	0	0	0	0	0.11	0.63	0.37	1.00	0.04	0.01	0	0	0
	h	0	0	0	0	0.37	0.38	0	0	0.15	0	0.41	0.97	0
	i	0	0	0	0	0	0	0.13	0	0	0.03	0	0	0
	k	0	0	0	0	0	0	0	0	0	0	0.59	0	0
06	*a*	0	0	0	0	0	0	0	0	0	0	0	0	1.00
	c	0	0	0	0	1.00	0	0	0	0.73	0.28	0	0	0
	d	1.00	1.00	1.00	1.00	0	1.00	1.00	1.00	0.04	0.72	0	0.02	0
	e	0	0	0	0	0	0	0	0	0	0	0.85	0	0
	f	0	0	0	0	0	0	0	0	0.23	0	0	0	0
	g	0	0	0	0	0	0	0	0	0	0	0.15	0.98	0
11	*b*	0	0	0	0	0	0	0	0	0	0	0	0	0.61
	c	0	0	0	0	0	0	0	0	0	0	0	0	0.37
	d	0	0	0	0	0.84	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
	e	0.38	0.83	0.60	0	0	0	0	0	0	0	0	0	0
	f	0	0	0	0	0	0	0	0	0	0	0	0	0.03
	g	0.10	0.17	0.19	0.14	0	0	0	0	0	0	0	0	0
	h	0.52	0	0.22	0.12	0	0	0	0	0	0	0	0	0
	i	0	0	0	0.74	0	0	0	0	0	0	0	0	0
	j	0	0	0	0	0.16	0	0	0	0	0	0	0	0

TABLE A1 (*continued*)

Locus	Allele	*ailantifolia*	*cathayensis*	*mandshurica*	*cinerea*	*nigra*	*olanchana*	*mollis*	*neotropica*	*microcarpa*	*major*	*californica*	*hindsii*	*regia*
12	*a*	0	0	0	0	0	0	0	0	0	0	0	0	0.46
	b	0	0	0	0	0	0	0	0	0	0	0	0	0.21
	c	0	0	0	0.72	0	0	0	0	0	0	1.00	1.00	0
	d	1.00	1.00	1.00	0.28	0	0	0	0	0	0	0	0	0
	e	0	0	0	0	0	0	0	0	0	0.65	0	0	0
	f	0	0	0	0	1.00	0	0.25	0	0.80	0	0	0	0
	g	0	0	0	0	0	1.00	0	0	0	0	0	0	0
	h	0	0	0	0	0	0	0.13	0	0	0	0	0	0
	m	0	0	0	0	0	0	0	0	0	0	0	0	0.32
	n	0	0	0	0	0	0	0	0.94	0	0	0	0	0
	o	0	0	0	0	0	0	0	0.06	0	0	0	0	0
	q	0	0	0	0	0	0	0	0	0.19	0	0	0	0
	r	0	0	0	0	0	0	0.50	0	0.01	0.16	0	0	0
	s	0	0	0	0	0	0	0	0	0	0.04	0	0	0
	t	0	0	0	0	0	0	0	0	0	0.14	0	0	0
	u	0	0	0	0	0	0	0.13	0	0	0	0	0	0
15	*a*	0	0	0	0	0	0	0	0	0	0	0	0	0.83
	b	0	0	0	0	0	0	0	0	0	0	0	0	0.17
	c	0	0	0	0.71	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
	d	0	0	0	0.19	0	0	0	0	0	0	0	0	0
	e	1.00	1.00	1.00	0.10	0	0	0	0	0	0	0	0	0
17	*a*	0	0	0	0	0	0	0	0	0	0	0	0	0.73
	b	0	0	0	0	0	0	0	0	0	0	0	0	0.27
	c	0	0	0	0	1.00	0	0	0	0.57	0	0	0	0
	d	0.73	0.17	0.74	0	0	0	0	0	0	0	0	0	0
	e	0.20	0.83	0.15	0	0	0	0	0	0	0	0	0	0
	f	0.06	0	0.11	0	0	0	0	0	0	0	0	0	0
	h	0	0	0	0.46	0	0	0	0	0	0	0	0	0
	i	0	0	0	0.51	0	0	0	0	0	0	0	0	0
	j	0	0	0	0.03	0	0	0	0	0	0	0	0	0
	k	0	0	0	0	0	1.00	0	0	0	0	0	0	0
	l	0	0	0	0	0	0	1.00	1.00	0.43	1.00	0.83	1.00	0
	m	0	0	0	0	0	0	0	0	0	0	0.14	0	0
	r	0	0	0	0	0	0	0	0	0	0	0.03	0	0
18	*a*	0.15	1.00	0.13	0.14	0	0	0	0	0	0	0	0	0.14
	b	0.85	0	0.87	0.86	1.00	1.00	1.00	1.00	1.00	1.00	0	0	0.83
	c	0	0	0	0	0	0	0	0	0	0	1.00	1.00	0
	f	0	0	0	0	0	0	0	0	0	0	0	0	0.03
24	*a*	0.94	1.00	0.94	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.30
	b	0	0	0	0	0	0	0	0	0	0	0	0	0.70
	c	0.06	0	0.06	0	0	0	0	0	0	0	0	0	0
25	*a*	1.00	1.00	1.00	0.97	0	0	0	0	0	0	0	0	0.57
	b	0	0	0	0.03	0.92	1.00	1.00	1.00	0.99	0.99	0	0	0
	c	0	0	0	0	0	0	0	0	0	0.01	1.00	1.00	0
	d	0	0	0	0	0	0	0	0	0	0	0	0	0.43
	e	0	0	0	0	0.08	0	0	0	0.01	0	0	0	0

TABLE A1 (*concluded*)

Locus	Allele	*ailantifolia*	*cathayensis*	*mandshurica*	*cinerea*	*nigra*	*olanchana*	*mollis*	*neotropica*	*microcarpa*	*major*	*californica*	*hindsii*	*regia*
26	*a*	0	0	0	0	1.00	1.00	1.00	0.75	1.00	1.00	1.00	1.00	1.00
	f	1.00	1.00	1.00	1.00	0	0	0	0	0	0	0	0	0
32	*a*	0	0	0	0	0	0	0	0	0	0	0	0	0.86
	b	1.00	1.00	1.00	1.00	0	0	0	0	0	0	0	0	0.14
	c	0	0	0	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0
34	*a*	0	0	0	0	0	0	0	0	0	0	0	0	1.00
	f	1.00	0.91	1.00	0.97	0	0	0	0	0	0.01	0	0	0
	g	0	0.09	0	0.03	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	0
43	*a*	0	0	0	0	0	0	0	0	0	0	0	0	1.00
	b	0	0	0	0.79	0	0	0	0	0	0	0	0	0
	c	0	0	0	0	0	0	0	0	0	0	0.10	0	0
	d	0	0	0	0	0.78	1.00	0.75	0.06	0.90	1.00	0.90	1.00	0
	e	0	0	0	0	0.22	0	0	0	0.10	0	0	0	0
	f	0	0	0	0	0	0	0.25	0.94	0	0	0	0	0
	g	1.00	1.00	1.00	0.21	0	0	0	0	0	0	0	0	0
45	*a*	1.00	1.00	0.97	1.00	1.00	0	0	1.00	0.99	0.73	1.00	1.00	1.00
	b	0	0	0.03	0	0	0	0	0	0	0	0	0	0
	c	0	0	0	0	0	1.00	1.00	0	0.01	0.27	0	0	0
48	*a*	0	0	0	0	0	0	0	0	0	0	0	0	0.06
	b	0	0	0	0	0	0	0	0	0	0	0	0	0.73
	c	0	0	0	0	0.86	0	0.25	1.00	1.00	0.80	0	1.00	0
	d	0	0	0	0	0	0	0	0	0	0	0	0	0.21
	e	0	0	0	0.79	0	1.00	0.75	0	0	0.14	1.00	0	0
	f	0.76	0.33	0.78	0.01	0	0	0	0	0	0	0	0	0
	g	0.24	0.67	0.22	0	0	0	0	0	0	0.05	0	0	0
	h	0	0	0	0	0.14	0	0	0	0	0	0	0	0
	i	0	0	0	0.20	0	0	0	0	0	0	0	0	0
67	*a*	0	0	0	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	1.00	1.00	1.00	0.41	0	0	0	0	0	0	0	0	0
	c	0	0	0	0.59	0	0	0	0	0	0	0	0	0