

## GENETIC HOMOGENEITY IN *JUGLANS NIGRA* (JUGLANDACEAE) AT NUCLEAR MICROSATELLITES<sup>1</sup>

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Broad-scale studies of genetic structure and diversity are indicative of the recent evolutionary history of a species and are relevant to conservation efforts. We have estimated current levels of genetic diversity and population structure for black walnut (*Juglans nigra* L.), a highly valuable timber species, in the central hardwood region of the United States. Black walnut trees from 43 populations across this region were genotyped at 12 highly polymorphic microsatellite loci. Genetic diversity was high and populations only slightly deviated from Hardy-Weinberg proportions ( $F_{IS} = 0.017$ ). Considering the scale of our sampling, the species was remarkably genetically homogenous:  $F_{ST}$  was quite low (0.017), and in a Bayesian analysis the optimal higher-order partition was into a single group comprised of all 43 populations. Although black walnut is predominantly a bottomland species, very little genetic variance was partitioned among broad-scale hydrologic regions ( $F_{PT} = 0.002$ ). However, a weak, but statistically significant pattern of isolation by distance was detected. The results are consistent with a scenario in which black walnut recolonized its current range from a single glacial refugium, and where subsequent genetic effects associated with deforestation and habitat fragmentation have been mitigated by high levels of pollen flow. Nuclear microsatellites alone may be insufficient to identify hotspots for black walnut conservation.

**Key words:** black walnut; central hardwood region; genetic distance; Juglandaceae; microsatellites; Midwestern flora.

Black walnut (*Juglans nigra* L.) is widely distributed in the deciduous forests of eastern North America. It grows in scattered pockets on favorable sites as far north as Ontario, Canada, as far west and east, respectively, as Kansas and New Jersey (with scattered occurrences up into Vermont), and south to northern Florida (Williams, 1990). The current number of walnut stems greater than 12.7 cm in diameter growing on timberland (native stands in which timber harvest is allowed) in the USA has been estimated at 200 million trees (Shifley, 2004), but the size of the entire black walnut population is undoubtedly much larger. Veneer-grade black walnut lumber is more valuable on a per volume basis than that of any other Midwestern timber species (Illinois Agricultural Statistics Service, 2004). Hence, in most parts of its natural range, walnut has been heavily exploited by humans (Elias, 1980; McGuire et al., 1999). Walnuts also are an important food source for rodents, especially squirrels (Vander Wall, 2001). The population genetic structure of black walnut is of interest because of the economic, cultural, and ecological value of this species.

The silvics and reproductive biology of black walnut are important determinants of its population genetics. Walnut is a pioneering, shade-intolerant species that, under natural conditions, typically lives up to 250 years. Black walnut is monoecious, heterodichogamous (the male and female functions are temporally separated, there are two morphs where flowering is synchronous, reciprocal, and individuals are either protandrous or protogynous [Gustafson and Morrissey, 1990;

Renner, 2001; Kimura et al., 2003]), and trees attain reproductive maturity at roughly 20–30 years of age (Schlesinger and Funk, 1977). Although selfing is possible in black walnut, the mating system is predominantly outcrossing (Rink et al., 1989, 1994; Busov et al., 2002). Pollen dispersal is by wind; seed dispersal is by gravity, rodents, and more recently by humans (Talalay, 1984; Williams, 1990). Black walnut is often found near flowing water and is most abundant on the well-drained, deep, and fertile soils of wet-mesic stream terraces, and on north- and east-facing slopes (Williams, 1990; Hicks, 1998). Because fruits with viable nuts float in water (J. Glaubitz, personal observation), hydrochory (the dispersal of diaspores by water) may act as an agent of long-distance seed dispersal for black walnut. Hydrochory can lead to an association between watersheds and patterns of genetic diversity (Kudoh and Whigham, 2001; Barrett et al., 2004; Kitamoto et al., 2005).

Current population genetic structures of temperate forest tree species reflect the combined influence of a variety of evolutionary and anthropogenic factors operating at contemporary, historical, and prehistorical time scales (Hedrick, 2000). Broad-scale studies of population genetic structure with molecular markers have the potential to shed light on the relative influence of factors such as selection (Namkoong et al., 2000), mutation, and genetic drift (Rhodes et al., 1996), with the caveat that the footprints of these factors are often inextricably intertwined (Felsenstein, 1982; Waples, 1998; Whitlock and McCauley, 1999; Excoffier, 2001).

Considering the wide availability of nuclear microsatellite markers in tree species and genera, there have been remarkably few studies of broad-scale genetic structure in temperate tree species using these markers (Al-Rabab'ah and Williams, 2002, 2004; Derory et al., 2002; Jones et al., 2002; Heuertz et al., 2004; Muir et al., 2004). The vast majority of DNA-level studies to date have employed chloroplast or mitochondrial DNA markers (e.g., Mitton et al., 2000; Liepelt et al., 2002; Petit et

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al., 2002, 2003; Magni et al., 2005); however, nuclear markers can also contribute to our understanding of postglacial range expansion and regional diversity (Kitamura and Kawano, 2001; Jorgensen et al., 2002; Byrne et al., 2003; Lascoux et al., 2003; Petit et al., 2004). Black walnut probably recolonized the Midwest as a single, large population from a glacial refugium in the Lower Mississippi Valley between 14 000 yr BP and 12 000 yr BP (Delcourt and Delcourt, 1987; Williams et al., 2004; see also pollen viewer 3.2 <http://www.ngdc.noaa.gov/paleo/pollen/viewer/webviewer.html>). The population size of the genus *Juglans* appears to have reached a distinct peak in the Midwest between 8000 and 5000 yr BP, corresponding to the warm and wet climatic period after the collapse of the Laurentide ice sheet (Prentice, 1991). However, interpretation of the paleoecological evidence is far from unequivocal (e.g., see McLachlan and Clark, 2004).

Studies of the population genetic structure of black walnut have been limited in geographical scope (Rink et al., 1989; Busov et al., 2002) and sample size (Fjellstrom and Parfitt, 1994). On the other hand, there have been numerous common garden progeny and provenance trials in black walnut (Bey, 1979; Beineke, 1989; Bresnan et al., 1994; Miller, 1995; Victory et al., 2004; Woeste and McKenna, 2004). Such quantitative genetic analyses have demonstrated clear adaptational and performance differences among different sources (Bey, 1980; Bresnan et al., 1994). Results from broad-scale studies of the genetic structure at the DNA level supplement those from common garden quantitative genetic studies; these two types of information, taken together, can lead to the formulation of optimal gene conservation strategies (Rhodes and Chesser, 1994; Eriksson, 1995; Petit et al., 1998).

We present a broad-scale study of the genetic structure of black walnut based on analysis of nuclear microsatellite markers. This study is one of the largest of its kind in terms of the sample size (1250 trees in total sampled from 43 populations in 10 states) and the number of loci (12 microsatellites). Our study objectives were (1) to draw inferences regarding the postglacial recolonization history of black walnut and the effect of contemporary and historical gene flow on the distribution of genetic variance at neutral markers, (2) to explore how much genetic variation is partitioned at the level of major watersheds, and (3) to identify populations or regions of potential high priority for gene conservation based upon their genetic uniqueness or level of allelic richness.

## MATERIALS AND METHODS

**Sampling**—With the aid of numerous volunteers and district foresters, black walnut trees ( $N = 1250$ ) were sampled from 43 indigenous populations across the central hardwood portion of the species' range over three summers (2001–2003) (Fig. 1). Three populations per state were sampled from Illinois (IL), Iowa (IA), Kansas (KS), Kentucky (KY), Ohio (OH), Pennsylvania (PA), Tennessee (TN), and West Virginia (WV). From Indiana (IN) and Missouri (MO), nine and 10 populations were sampled, respectively. Sampled populations were located at least 1.6 km from any known black walnut plantation and were located in private woodlots, state parks, and forests. In four cases (IA-A, IN-F, PA-A, and WV-D), the samples were from trees dispersed throughout fragmented forests, while in another (MO-G), the samples were from a region spanning three adjoining counties (Table 1). The average distance between a population and its nearest sampled neighbor was 106 km. Sampled trees within a population were spaced ca. 100 m apart or more. Leaf tissue (4–6 leaflets per tree) was collected from each sampled tree using a variety of techniques including a throwbag and rope, pole pruner, slingshot and fishing line, and shotgun. Upon collection, leaves were placed into re-

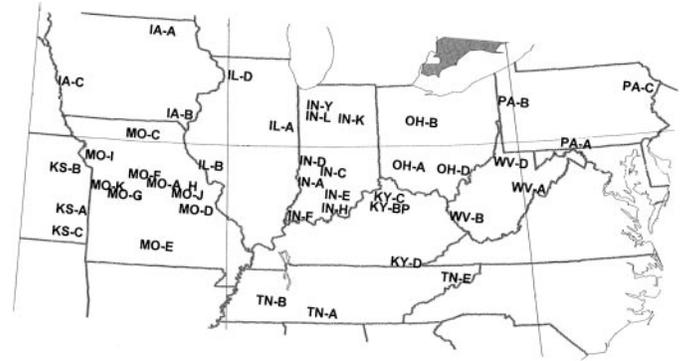


Fig. 1. Names and locations of 43 indigenous populations of *Juglans nigra* sampled from across the central hardwood region during the summers of 2001–2003.

sealable plastic bags and mailed to Purdue University where they were stored at  $-80^{\circ}\text{C}$  or freeze-dried until the DNA could be extracted.

**DNA extraction**—DNA was extracted from leaf tissue for up to 30 samples per population by placing approximately 100 mg of freeze-dried leaf tissue and a 0.64 cm ceramic bead (Bio 101-Savant, Carlsbad, California, USA) into a 2-mL screw-cap tube, then adding 1 mL of hexadecyltrimethylammonium bromide (CTAB) buffer (Lefort and Douglas, 1999; modified with  $2\times$  polyvinylpyrrolidone [(PVP),  $2\times$  CTAB, and 2.0%  $\beta$ -mercaptoethanol). Samples then were ground in a Fast Prep 120 (Bio 101-Savant) machine for three 40-s cycles and cooled on ice between each cycle. Next, samples were incubated in a  $65^{\circ}\text{C}$  water bath for at least 30 min and periodically shaken by hand. Samples then were spun at  $12500 \times g$  for 5 min using a tabletop centrifuge, and DNA was extracted from 500  $\mu\text{L}$  of the supernatant using an Autogen NA-2000 automatic nucleic acid extractor following the manufacturer's standard extraction protocol for plant tissue (Autogen, Framingham, Massachusetts, USA). The DNA was resuspended in 100  $\mu\text{L}$  TE and stored at  $-20^{\circ}\text{C}$ . All samples were quantified using an FL-600 fluorometer (Bio-Tek Instruments, Winooski, Vermont, USA) and were brought to a working concentration of 5 ng/ $\mu\text{L}$ .

**DNA amplification**—DNA was amplified using primers flanking 11 dinucleotide microsatellite loci, and one trinucleotide microsatellite locus previously screened for polymorphism and the absence of null alleles in black walnut (Woeste et al., 2002) (Table 2). Forward primers for each locus were modified with a 5' fluorescent molecule [hexachlorofluorescein (HEX), 6-carboxyfluorescein (6-FAM), or (NED) Applied Biosystems, Foster City, California, USA] consistent with matrix D on an ABI 377XL automated sequencer (Applied Biosystems). Polymerase chain reaction (PCR) was carried out for each locus in 10- $\mu\text{L}$  reaction volumes containing 10 ng DNA, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP,  $1\times$  PCR buffer (1 mM Tris-HCl [pH 9.0 at  $25^{\circ}\text{C}$ ], 5 mM KCl, 0.01% Triton X-100), 0.4  $\mu\text{g}/\mu\text{L}$  BSA, 0.75 U *Taq* polymerase (Promega, Madison, Wisconsin, USA; or New England Biolabs, Beverly, Massachusetts, USA), and 0.8 pmol/ $\mu\text{L}$  primer. PCR conditions were as follows: an initial 1-min incubation at  $94^{\circ}\text{C}$ ; followed by 30 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at the annealing temperature for the primer  $T_a$  (Table 2) and 30 s at  $72^{\circ}\text{C}$ ; and then a final incubation at  $60^{\circ}\text{C}$  for 45 min.

**Genotyping**—After PCR amplification, samples were diluted up to 1 : 15 in water and 1  $\mu\text{L}$  of the diluted PCR product was combined with 0.37  $\mu\text{L}$  of a 400-bp internal lane standard (CXR, Promega) and 1.5  $\mu\text{L}$  blue dextran (Promega) and denatured for 2 min at  $94^{\circ}\text{C}$ . Samples were loaded onto a CAL 96 paper comb (Gel Co., San Francisco, California, USA) and run in a 5% polyacrylamide gel (Long Ranger Single packs, BMA, Rockland, Maine, USA) for 3 h at 3000 V, 60 mA, 200 W,  $51^{\circ}\text{C}$  on an ABI 377XL automated sequencer. Loci were combined into sets of four (Table 2) and multiloaded to run in a single gel lane. Fragment length data were collected using Gene Scan

TABLE 1. Geographic and population information for all *Juglans nigra* populations sampled in the central hardwood region of the USA.

State	County	Population code	Hydrologic region <sup>a</sup>	Sample size	Description	Stand size (hectares)
Illinois	Vermilion	IL-A	05	29	Public	20–25
Illinois	Greene	IL-B	07	28	Private	20
Illinois	Carroll	IL-D	07	30	State park	1020
Indiana	Sullivan	IN-A	05	19	Private	416
Indiana	Morgan	IN-C	05	30	Public	20
Indiana	Parke	IN-D	05	30	State park	20+
Indiana	Jennings	IN-E	05	30	State forest	140
Indiana	Posey	IN-F	05	29	Private	80+
Indiana	Harrison	IN-H	05	29	Wildlife area	16
Indiana	Grant	IN-K	05	29	Public	48 <sup>b</sup>
Indiana	Carroll	IN-L	05	30	Private	12 <sup>c</sup>
Indiana	Pulaski	IN-Y	05	30	State park	140
Iowa	Allamakee	IA-A	07	30	Private	13
Iowa	Des Moines	IA-B	07	29	Private	28
Iowa	Harrison	IA-C	10	30	State forest	2493
Kansas	Linn	KS-A	10	27	Private	48
Kansas	Jefferson	KS-B	10	29	State park	320
Kansas	Crawford	KS-C	11	30	State park	120
Kentucky	Grant	KY-BP	05	27	Private	247
Kentucky	Campbell	KY-C	05	29	Private	4
Kentucky	Bell	KY-D	05	30	State forest	4545
Missouri	Boone	MO-A	10	30	Private	8
Missouri	Adair	MO-C	07	30	Private	14
Missouri	Jefferson	MO-D	07	30	Private	160
Missouri	Texas	MO-E	10	30	Private	40
Missouri	Howard	MO-F	10	30	Private	8
Missouri	Johnson/Pettis/Henry	MO-G	10	29	Private	16
Missouri	Boone	MO-H	10	30	Private	16
Missouri	Clinton	MO-I	10	30	Private	12
Missouri	Callaway	MO-J	10	30	Private	12
Missouri	Cass	MO-K	10	28	Private	10
Ohio	Greene	OH-A	05	30	Private	18
Ohio	Crawford	OH-B	04	30	Private	12–16
Ohio	Morgan	OH-D	05	29	Private	—
Pennsylvania	Franklin	PA-A	02	30	Public	20
Pennsylvania	Mercer	PA-B	05	29	State park	398
Pennsylvania	Pike	PA-C	02	30	National recreation area	31
Tennessee	Marshall	TN-A	06	30	State park	456
Tennessee	Henderson	TN-B	06	30	State forest	14 800
Tennessee	Washington	TN-E	06	30	Private	20
West Virginia	Hardy	WV-A	02	30	Private	20
West Virginia	Wayne	WV-B	05	24	State forest	3249
West Virginia	Monongalia	WV-D	05	28	Public	—

<sup>a</sup> Major hydrologic regions of the United States Geological Survey.

<sup>b</sup> Three tracts totaling 49 hectares.

<sup>c</sup> Two fragments totaling 12 hectares.

Analysis v. 3.1 (Perkin Elmer) software, and genotypes were assigned with the aid of Genotyper v. 2.5 (Perkin Elmer) software.

For quality control, a suite of DNA samples with known genotypes were amplified with each PCR as a positive control, and one set was run with each of the three populations per gel as a set of allele size standards. A negative control was included in each amplification set. Those samples that could not be definitively scored were re-amplified and genotyped until the allele sizes could be definitively determined. Nine samples that could only be amplified at one or two loci, even after multiple DNA extractions, were discarded as unreliable.

**Genetic diversity analysis**—The software program Genetic Data Analysis (GDA, v. 1.1, Lewis and Zaykin, 2001) was used to estimate expected and observed heterozygosities, the number of alleles per locus, and to identify private alleles. The program FSTAT (Goudet, 2001) was used to estimate allelic richness standardized to a common sample size of 22 diploid individuals via rarefaction; the population IN-A was excluded from this analysis, due to its small sample size ( $N = 19$ ). The relationship between latitude and

standardized allelic richness was examined via linear regression using Microsoft Office Excel 2003 (Microsoft, Redmond, Washington, USA). The program FSTAT was also used to test the conformation of each locus to Hardy-Weinberg genotypic proportions by permutation of alleles among individuals within populations, using the global  $F_{IS}$  estimate across populations as a test statistic. Sequential Bonferroni correction (Rice, 1989) was used to assess statistical significance at each locus, so that Type I error rate ( $\alpha$ ) of 5% was achieved across the 12 tests.

**Genetic structure analysis**—Black walnut grows along bottomlands and is associated with river systems (Williams, 1990). Thus, hydrochory may be an important mechanism of long distance seed dispersal. To investigate the degree to which genetic variance in black walnut is partitioned among regional watersheds, we performed a three-level hierarchical analysis of population genetic structure (Weir and Cockerham, 1984) using GDA. All populations were classified according to regional scale hydrologic units of the U.S. Geological Survey (Seaber et al., 1987; <http://water.usgs.gov/GIS/huc.html>). The 43 populations comprised seven hydrologic regions, containing 1–18 popu-

TABLE 2. Characteristics of the 11 dinucleotide microsatellite loci and one trinucleotide microsatellite locus from *Juglans nigra* used in this study.

Locus	Motif	Median PCR product size (bp)	GenBank accession number	T <sub>s</sub> (°C)	Label <sup>a</sup>	Primer sequence (5' → 3') <sup>b</sup>
AAG 01	(CTT) <sub>9</sub>	160	AY694137	57	NED	F: GCTTTTGTATCAATCGCCCAA R: ACCCCATTTGTAGCTTGGA
WGA 06	(AG) <sub>5</sub> AA(AG) <sub>19</sub> AT(AG) <sub>3</sub>	154	AY333949	57	HEX	F: CCATGAAACTTCATGCGTTG R: CATCCCAAGCGAAGGTTG
WGA 24	(T) <sub>8</sub> N <sub>29</sub> (CT) <sub>17</sub> N <sub>24</sub> (TC) <sub>5</sub>	239	AY333950	55	NED	F: TCCCCCTGAAATCTTCTCCT R: TTCTCGTGGTGTGTTGAG
WGA 27	(GT) <sub>3</sub> TT(GA) <sub>29</sub>	226	AY333951	55	HEX	F: AACCTACAACGCCTTGATG R: TGCTCAGGCTCCACTTCC
WGA 32	(TC) <sub>3</sub> CG(TC) <sub>19</sub>	195	AY333952	58	6-FAM	F: CTCGGTAAGCCACACCAATT R: ACGGCAGTGTATGCATGTA
WGA 69	(AG) <sub>4</sub> N <sub>6</sub> (AG) <sub>17</sub>	172	AY333953	55	NED	F: TTAGTTAGCAAACCCACCCG R: AGATGCACAGACCAACCCTC
WGA 72	(TC) <sub>15</sub>	148	AY333954	55	NED	F: AAACCACCTAAACCCCTGCA R: ACCCATCCATGATCTTCCAA
WGA 76	(GA) <sub>12</sub>	236	AY688266	58	HEX	F: AGGGCACTCCCTTATGAGGT R: CAGTCTCATTCCCTTTTTC
WGA 82	(CT) <sub>20</sub>	194	AY333956	57	6-FAM	F: TGCCGACACTCCTCACTTC R: CGTGATGTACGACGGCTG
WGA 89	(TG) <sub>9</sub> (GA) <sub>21</sub>	209	AY352440	57	HEX	F: ACCCATCTTTCACGTGTGTG R: TGCCTAATTAGCAATTTCCA
WGA 90	(CT) <sub>4</sub> T(TC) <sub>14</sub>	162	AY352441	55	6-FAM	F: CTGTAAATCGCCCTCTGCTC R: TACCTGCAACCCGTTACACA
WGA 97	(GA) <sub>26</sub>	170	AY352442	57	HEX	F: GGAGAGGAAAGGAATCCAAA R: TTGAACAAAAGGCCGTTTTC

<sup>a</sup> Forward primers were modified at the 5' end with a fluorescent label: HEX (green), 6-FAM (blue), or NED (yellow) (see Materials and Methods, DNA amplification).

<sup>b</sup> F = forward primer, R = reverse primer.

lations each (Table 1). Forty-one of the 43 sampled populations, falling into five hydrologic regions, were used in the hierarchical analysis (populations OH-B and KS-C were the only representatives of their hydrologic region and thus were not included). We obtained estimates for  $F_{IS}$  ( $=f$ ),  $F_{IT}$  ( $=F$ ),  $F_{ST}$  ( $=\theta_s$ ) and  $F_{PT}$  ( $=\theta_p$ ), where  $S$  is the subpopulation (population),  $P$  is the population (hydrologic region or watershed), and  $T$  is the total. We estimated the confidence intervals ( $\alpha = 0.05$ ) around the  $F$  statistics by bootstrapping 20 000 times over loci. To supplement our  $F_{ST}$  estimate, we also calculated  $R_{ST}$  (an analog of  $F_{ST}$  specific to the stepwise mutation model) according to the method of Goodman (1997) using the program FSTAT.

Matrices of pairwise genetic distance ( $D_S$ ; Nei, 1978) and pairwise  $F_{ST}$  values involving all 43 populations were generated using GDA, and a neighbor-joining tree was produced in GDA based upon the  $D_S$  estimates. The tree

was visualized with the aid of TREEVIEW software (v. 1.6.6, Page, 1996). A Mantel test of the association between  $F_{ST}/(1 - F_{ST})$  and the natural logarithm of geographic distance was performed using the ISOLDE program of GENEPOP (v. 3.4, Raymond and Rousset, 1995; Rousset, 1997). Geographic distances were based on the distance between the county seats of the counties in which the populations were sampled. The only exceptions to this method of calculating geographic distance were for all distances to population MO-A, where we used more precise coordinates from a global positioning system (GPS) specific to that population, since MO-A shared the same county seat with MO-H. The test for isolation by distance was based on 10 000 permutations of spatial locations among the sampled populations. A scatter plot of  $F_{ST}/(1 - F_{ST})$  vs. the natural logarithm of geographical distance was obtained in Microsoft Office Excel 2003.

We also applied a Bayesian approach to find the optimal partition of the 43 populations into groups consisting of populations with essentially identical allele frequencies, using the software BAPS (Bayesian Analysis of Population Structure, version 2; Corander et al., 2003, 2004). Rather than relying on a priori grouping by the investigator of the sampled populations into a higher level structure (e.g., into hydrologic regions), use of BAPS allows determination of the grouping into which the populations most naturally fall. We ran three parallel Markov chain Monte Carlo analyses on our data, each with a burn-in period of 500 iterations, with a sampled length (after thinning) of 20 000 iterations, and with a thin parameter of 3 (i.e., every third iteration after the burn-in was sampled for inclusion in the posterior probability distribution). The starting value for the number of groups ( $K$ ) was set at the maximum possible value of 43 (the number of geographical samples).

RESULTS

**Genetic diversity**—Data was obtained for 98.4%, or 14 761 of the 15 000 single locus genotypes attempted in this study (12 loci × 1250 samples). The overall multilocus total heterozygosity ( $H_T = 0.807$ ) and average number of alleles per locus ( $A_R = 22.9$ , Table 3) indicated that black walnut in the central hardwood region is highly genetically diverse, as is typical of

TABLE 3. Total numbers of genotypes ( $N$ ), allele size ranges, total numbers of alleles observed, and genetic parameter estimates for each microsatellite locus surveyed in 43 *Juglans nigra* populations.

Locus	$N$	Allele size range (bp)	Number of alleles	$H_E$	$H_O$	$F_{IS}$
AAG 01	1234	148–172	9	0.700	0.662	0.031
WGA 06	1232	130–176	21	0.614	0.590	0.025
WGA 24	1214	223–253	17	0.876	0.836	0.031 <sup>a</sup>
WGA 27	1227	200–248	25	0.892	0.868	0.007
WGA 32	1237	163–235	34	0.935	0.909	0.008
WGA 69	1234	160–186	14	0.618	0.598	0.021
WGA 72	1234	138–162	14	0.606	0.600	0.000
WGA 76	1235	228–254	15	0.745	0.722	0.017
WGA 82	1231	144–256	46	0.966	0.916	0.035 <sup>a</sup>
WGA 89	1209	181–239	29	0.925	0.909	–0.005
WGA 90	1239	132–190	28	0.907	0.898	–0.004
WGA 97	1225	148–194	23	0.901	0.861	0.033 <sup>a</sup>
Mean	1229	—	23	0.793	0.781	0.017

<sup>a</sup>  $F_{IS}$  estimates significantly greater than zero after sequential Bonferroni correction ( $\alpha = 0.05$ ).

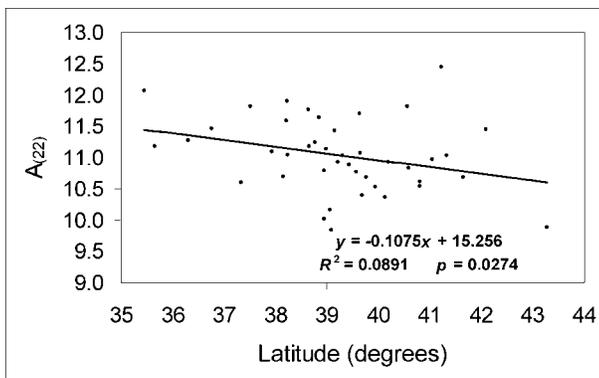


Fig. 2. Linear regression of standardized allelic richness for a sample size of 22 diploid individuals ( $A_{(22)}$ ) versus latitude, for 42 populations of *Juglans nigra*.

most broadly distributed, temperate forest tree species surveyed at microsatellite loci (Al-Rabab'ah and Williams, 2002; Derory et al., 2002; Jones et al., 2002; Heuert et al., 2004; Muir et al., 2004; Muir and Schlötterer, 2005). The number of alleles per locus ranged from nine (AAG 01) to 46 (WGA 82). We found 28 alleles that had an overall frequency greater than 0.1, and 28 private alleles in 21 populations (not shown). Of the 23 alleles per locus on average, about 20.7 were "rare," having a frequency of less than 0.1, but only 2.3 of the 20.7 rare alleles per locus, on average, were private. Thus, 89% of the rare alleles were found in at least two populations. Private alleles were always rare in the population in which they were detected (frequency of  $<0.054$ , or from 1–3 copies) and always occurred at the ends of the allele size distribution for each locus. Northern populations tended to have fewer alleles per locus than southern ones; i.e., there was a significant negative association between population latitude and allelic richness for a standardized sample size of 22 trees ( $A_{(22)}$ ; Fig. 2). Latitude accounted for roughly 9% of the variance in  $A_{(22)}$  ( $P = 0.027$ ; one-tailed test for the expected negative correlation). The overall picture is that black walnut populations are characterized by a large number of alleles at neutral genetic markers with most alleles at low frequency. Because nearly all the rare genetic variation is dispersed among several populations, this data provides little evidence for local or even regional population differentiation.

Three loci (WGA 24, WGA 82, WGA 97) deviated significantly from Hardy-Weinberg genotypic proportions (HWE), as evidenced by global  $F_{IS}$  estimates that were significantly greater than zero after sequential Bonferroni correction (Table 3). However, the  $F_{IS}$  estimates at the three loci (0.031, 0.035, and 0.033, respectively) were not very large, reflecting the power of the tests to detect effects of small magnitude when using such a large sample of genotypes. Nevertheless, the significant departures from HWE may reflect the presence of low frequency null alleles at these loci.

**Genetic structure**—Hierarchical  $F$  statistics indicated a low but statistically significant level of genetic structure among hydrological regions ( $F_{PT} = 0.002$ ; Table 4). Clearly, black walnut partitions very little of its genetic variance among recognized regional hydrologic units. The overall  $F_{ST}$  was only 0.017, indicating a low but nonzero amount of genetic variance was partitioned among the 41 populations.  $F_{IS}$  (0.017) and  $F_{IT}$  (0.034) were both positive and significantly greater

TABLE 4. Hierarchical  $F$  statistics and associated 95% confidence intervals (based upon 20000 bootstraps) across 12 loci for 41 *Juglans nigra* populations nested within regional scale hydrological units.

Statistic	$F_{IS}$	$F_{IT}$	$F_{ST}$	$F_{PT}$
Estimate	0.017	0.034	0.017	0.002
Lower Bound	0.008	0.025	0.015	0.001
Upper Bound	0.025	0.042	0.019	0.003

Note: I, individual; T, total; S, subpopulation; P, population (hydrological unit).

than zero, indicating a slight heterozygote deficit within populations and overall, relative to HWE. Our estimate of  $R_{ST}$ , an analog of  $F_{ST}$  derived specifically for microsatellites under the assumption that the stepwise mutation model is valid (Slatkin, 1995), was very close to our  $F_{ST}$  estimate ( $R_{ST} = 0.0187$ ).

There was a weak but significant association between the pairwise genetic and geographic distances between the sampled populations ( $R^2 = 0.0342$ ,  $P = 0.016$  based on a Mantel test, not shown). The plot of  $F_{ST}/(1 - F_{ST})$  against  $\ln(\text{geographic distance})$  (Fig. 3) indicates a weak but statistically significant positive relationship between the geographic proximity of black walnut populations and their genetic similarity at neutral markers. The relationship shown in Fig. 3 can be used to infer the relationship between migration and genetic drift (changes that decrease and increase local genetic differentiation, respectively). Because the Mantel test of this relationship for our black walnut populations was weakly significant ( $P = 0.0485$ , 10000 permutations) it appears possible that equilibrium between migration and drift is just emerging for black walnut in the central hardwood region (Slatkin, 1993). At mutation-drift equilibrium under the island model of migration,  $F_{ST}$  is numerically related to the number of migrants per generation ( $Nm$ ), which we estimated to be 14.5. This is another indicator of substantial gene flow among populations; however, the interpretation of  $Nm$  in black walnut is limited by our poor understanding of neighborhood size and by the many unrealistic assumptions of the island model (Whitlock and McCauley, 1999).

All pairwise analyses indicated little genetic divergence between populations. Pairwise genetic distance and  $F_{ST}$  values were low for all combinations of the 43 populations. Pairwise  $F_{ST}$  values among the forty-three populations ranged from

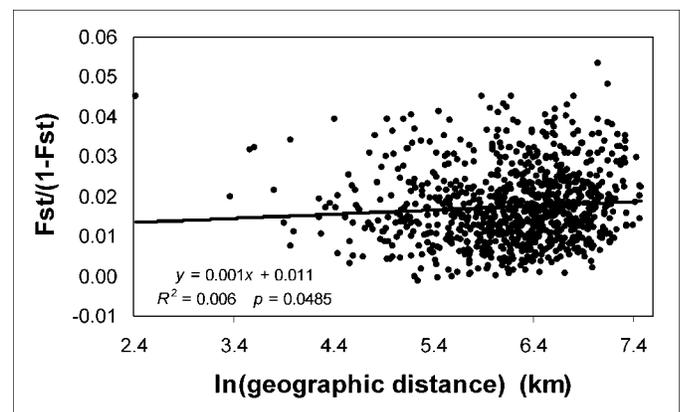


Fig. 3. Scatter plot of  $F_{ST}/(1 - F_{ST})$  vs.  $\ln(\text{geographic distance})$  for all 43 *Juglans nigra* populations. The statistical significance of the linear regression was determined via a Mantel test.

−0.001–0.051, and Nei's  $D_s$  values ranged from −0.003–50.167. The neighbor-joining tree, based upon Nei's genetic distances, visually represents relationships among the sampled populations (Fig. 4). There was no apparent geographical pattern in the neighbor-joining tree.

Analysis of the higher order genetic structure with BAPS also supported the finding of genetic homogeneity of black walnut in the Midwest. The optimal partition from this analysis was into a single group consisting of all 43 populations, supported by a posterior likelihood of one. All three parallel Markov chains quickly converged upon this optimal solution.

DISCUSSION

Though far from definitive, the pollen and macrofossil evidence for black walnut suggests that it survived the last glacial maximum (LGM) at a low population density in a glacial refugium in the Lower Mississippi Valley (Delcourt and Delcourt, 1987; Williams et al., 2004). High levels of gene flow via pollen may have allowed refugial walnut stands in the Lower Mississippi Valley, likely occurring in scattered pockets of favorable habitat (Delcourt and Delcourt, 1987), to be genetically connected with little genetic differentiation. Weak founder effects associated with northward recolonization, as evidenced by the weak negative correlation between latitude and allelic richness, could have resulted from the maintenance of large effective population sizes as black walnut moved northward. The high level of genetic diversity observed in black walnut indicates that this species maintained a large overall effective population size ( $N_E$ ) throughout the recurrent glacial episodes of the Quaternary, during postglacial recolonization, and in the face of widespread deforestation and fragmentation after European settlement of Eastern North America. Extensive pollen flow during plant migration (Petit et al., 1997; Liepelt et al., 2002) may have led to the present day genetic homogeneity and weak pattern of isolation by distance.

Walnut pollen is dispersed by wind, and likely travels far greater distances, on average, than walnut seed which is dispersed by gravity and rodents. Although we did not directly investigate relative rates of pollen and seed flow, the  $F_{ST}$  data from our study of black walnut are comparable to results from species where there is evidence for a pollen flow : seed flow ratio of nearly 200 (Ennos, 1994). The combination of high diversity and low genetic differentiation suggests that pollen flow is extensive in black walnut, in contrast with earlier speculation that it is limited (Beineke, 1974). Extensive pollen flow would allow the maintenance of interbreeding populations of vast size ( $N_E$ ) occupying broad geographic areas with no distinct genetic boundaries. With populations or genetic neighborhoods (Wright, 1943) of such massive size, genetic drift should be very weak, and isolation by distance should occur only over an expansive geographical scale. This is precisely the pattern we observed here. Although hydrochory may be a source of long distance seed dispersal in black walnut, it is unlikely that it played a significant role in northward post-glacial recolonization, since the major drainage patterns were in the opposite direction (Knox, 1983). Hence, we posit a system where black walnut advanced only short distances by seed each year, with any initial leading edge founder effect at nuclear loci being quickly compensated for by pollen flow from the middle and trailing edge of the advancing population. Overlapping generations and the long reproductive life span of black walnut would contribute to the maintenance of large

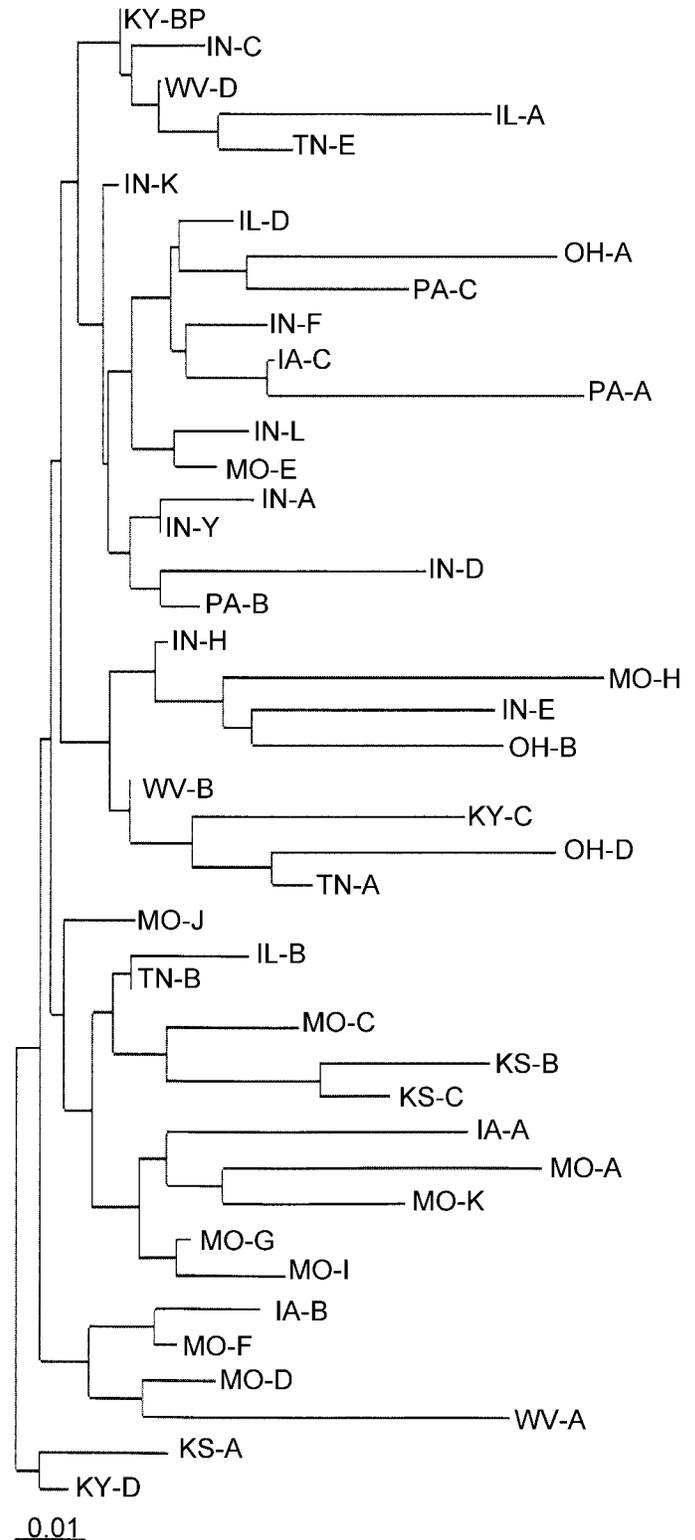


Fig. 4. Neighbor-joining tree based upon Nei's (1978) genetic distance for 12 microsatellite loci surveyed in 43 *Juglans nigra* populations. Population codes are defined in Table 1.

effective population sizes during such advances (Austerlitz et al., 2000; Austerlitz and Garnier-Géré, 2003). How heavy-seeded trees such as walnut were able to disperse into their current range in the time since the LGM remains an active area of research (Clark et al., 1998).

The static snapshot of genetic structure that we have established with nuclear microsatellites does not allow us to determine exactly how and when the current genetic homogeneity was achieved, but it seems unlikely to be the result of recent historical changes. Was the black walnut nuclear genome homogenized during the LGM, during recolonization, or after recolonization? Additional sampling of populations at the northern and southern extremes of the species distribution, and the addition of data from the maternally inherited chloroplast genome could shed further light on this question (Kitamoto et al., 2005).

The mutational mechanisms under which microsatellites evolve likely have contributed, at least in some degree, to the genetic homogeneity we observed in black walnut. Microsatellites are prone to size homoplasy, the origination of alleles that are identical in state (i.e., equal in length) but not identical by descent (i.e., derived from independent mutational events). Size homoplasy at microsatellite loci can lead to significant underestimation of genetic differentiation (Estoup et al., 2002). Although size homoplasy must have had some influence on our results, it is unlikely to be the main factor. In several similar, broad-scale studies with nuclear microsatellites in temperate forest species, significant regional patterns of genetic differentiation and higher order population groupings were detected, in accordance with the hypothesized post-glacial recolonization histories of the study species (Derory et al., 2002; Jones et al., 2002; Heuertz et al., 2004). There is no a priori reason to expect that homoplasy would occur at a greater rate in black walnut than in these other temperate forest species.

Our results indicate that conservation of a single black walnut population would capture more than 98% of the neutral genetic variance present in the central hardwood region. In light of the differences in adaptive traits such as growth and survival observed in provenance tests performed in black walnut (e.g., Bey, 1980; Bresnan et al., 1994), the genetic homogeneity observed in this study appears paradoxical, but the absence of an association between patterns of phenotypic diversity for quantitative or adaptive traits vs. the genetic diversity of putatively neutral genetic markers has been noted by numerous researchers (e.g., Milligan et al., 1994; Karhu et al., 1996; Lynch, 1996; Reed and Frankham, 2001; McKay and Latta, 2002; Bekessy et al., 2003). Strong adaptive differences can persist in the face of high levels of gene flow, and will not be reflected at neutral markers unless they are very tightly linked to the loci under selection (McKay and Latta, 2002). Furthermore, Latta (1998) has shown that, for a polygenic quantitative trait, strong differences at the trait level can accrue even when the allelic frequencies at the underlying QTLs differ only slightly. In light of these considerations, it is preferable to base gene conservation strategies and seed zone designations on ecological criteria rather than on presumably neutral genetic markers (Hu and Li, 2001).

It would seem from our data that the large-scale deforestation and fragmentation that occurred across the range of black walnut after European settlement has had little if any effect on the neutral genetic diversity of black walnut. These recent changes would have tended to increase genetic differentiation among populations. Numerous rare and localized alleles were

undoubtedly lost to deforestation in the portions of the species' range that were highly suitable for agriculture (e.g., northern Indiana). However, if as our results suggest, the genetic structure of black walnut was already highly homogenous prior to deforestation, the anthropogenic bottleneck was probably not severe enough to have much impact upon genetic variance. Furthermore, given that walnut trees can live to greater than 200 years of age, and given that most of our sampling was focused on older trees, it is likely that an insufficient number of generations have passed for the effects of recent forest fragmentation to be discernable (Young et al., 1993; Collevatti et al., 2001). Hence, there is reason for optimism that with proper management (Glaubitz et al., 2004) the genetic resources of black walnut can be successfully conserved for future generations.

In summary, we have observed high genetic diversity combined with high genetic homogeneity at nuclear microsatellite loci in black walnut. Isolation by distance, though present at a broad scale, was extremely weak, as was the genetic similarity of walnut populations sharing the same watershed. There were no detectable patterns in the presence or concentration of rare alleles in populations and so the data do not support the identification of particular regions or populations as hot-spots for conservation. Finally, there was a weak, but statistically significant northward trend of diminishing allelic richness, a result expected when post-glacial recolonization results in serial founder effects. These observations are all consistent with the occurrence of extensive gene flow via pollen in this species, probably both during and after the last glacial maximum.

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