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Different histories but similar genetic diversity and structure for black walnut in Indiana and Missouri

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DIFFERENT HISTORIES BUT SIMILAR GENETIC DIVERSITY AND STRUCTURE FOR BLACK WALNUT IN INDIANA AND MISSOURI

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Abstract.—Missouri and Indiana have markedly different histories of glaciation and recolonization by forest trees. These states also differ in land use patterns and degree of anthropogenic landscape change such as forest fragmentation. To determine the overall effects of these and other demographic differences on the levels of genetic diversity and structure in black walnut (*Juglans nigra* L.) more than 550 total black walnut trees from nine populations in Indiana and 10 in Missouri were sampled and analyzed using 12 nuclear microsatellite loci. Although genetic diversity parameters such as allelic richness and expected heterozygosity were high overall, they varied little among populations and their mean values for the two states were not significantly different. Pairwise genetic distance values between all population pairs ranged from 0.012-0.159, but no significant pattern of isolation by distance was detected. The estimate of the degree of genetic differentiation between states (F_{PT} = 0.0009) was very small and not significant, indicating that differences between states explained an inconsequential portion of the total variance. The observed low levels of local and regional genetic structure indicate that high levels of pollen flow have buffered black walnut from the genetic consequences of founder effects and genetic drift in both geologic and recent time scales.

INTRODUCTION

Black walnut is currently one of the most economically important deciduous species in the Midwest (Hoover and Preston 2004, Jones 2004). Despite its economic importance, little is known about how differing natural histories and forest management regimes affect the genetic diversity and structure of this species.

The results of recent investigations of the genetic structure and the factors that determine patterns of genetic variability for black walnut indicated very little evidence of genetic variance partitioning at large spatial scales (Robichaud and others 2006, Victory and others 2006). The consideration of regional scale partitioning of genetic variance in black walnut might provide a clearer understanding of the impacts of glaciation, habitat quality, habitat loss, fragmentation, and demographic changes on the population genetics of this species. We analyzed a subset of populations from the broad scale analysis to investigate levels of genetic diversity and structure in regional black walnut populations from Indiana and Missouri. These two states were chosen for comparison because, despite their relative proximity: (1) their glacial histories are dissimilar; and (2) the patterns of land-use change and human disturbance over the past century differ considerably between these two states.

Indiana and Missouri have different histories of glaciation and recolonization by forest trees. The northern half of Indiana was glaciated during the last glacial episode, but Missouri was entirely unglaciated. During

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presettlement, the vegetation of the northwestern half of Missouri was mixed prairie and forest, whereas Indiana was covered almost entirely by deciduous forest (Dorale and others 1998). If palyonological records accurately reflect population levels, black walnut population levels have been marked by strong and dynamic fluctuations. Nevertheless, Indiana has almost always been near the center of black walnut's range, and except for the modern period, black walnut has been considerably more abundant in Indiana than Missouri for nearly all of the past 13,000 years (Williams and others 2004). Missouri's climate has been considerably drier than the climate of Indiana since the last glaciation (Thornwaite 1941, Dorale and others 1998), and since Missouri is near the periphery of black walnut's range, black walnut populations in Missouri may have experienced strong pressure for adaptational divergence (Garcia-Ramos and Kirkpatrick 1997).

Indiana and Missouri differ now both in terms of the total amount of forest land and in the proportion of land under agricultural production. According to the U.S. Geological Survey (2003), Indiana has approximately 73 percent of its total area in row crops, small grains, and hay production; by comparison, these crops occupy 54 percent of the land area of Missouri. In 1900, less than 7 percent of the original forestland remained in Indiana, and by 2002 that percentage had increased to only 22 percent, for a total forested area in Indiana of about 2 M hectares (Tormoehlen and others 2000). Although large areas of forest in Missouri also have been cleared, Missouri, unlike Indiana, has large tracts of continuous forest totaling 5.7 M hectares, almost 32 percent of its land area.

The silvics of black walnut differ markedly between the states as well; black walnut is relatively rare in Indiana compared to Missouri. The net volume of live black walnut on Indiana forest land, approximately 7,000 m^3, is less than the volume of nearly all other hardwood species (Moser and others 2004a). About one percent of the 7.3 billion trees in Missouri are black walnut (Vasievich and Kingsley 1995). The net volume for black walnut on Missouri's forest lands is approximately 13,500 m^3, surpassing all other species in the state except red and white oak species and other miscellaneous eastern soft hardwoods (Moser and others 2004b).

OBJECTIVES

Given that Missouri has more forested land, a higher density of black walnut in suitable habitat, more contiguous forests, and a different legacy of post-glacial recolonization than Indiana, we expected the genetic diversity and structure of black walnut to differ markedly between the two states. Thus, the goal of this study was to examine genetic differentiation within and among populations of black walnut in Indiana and Missouri, in order to understand the role of ecological and anthropogenic change in forming patterns of regional genetic structure.

STUDY AREAS

We sampled black walnut trees (n=552) from nine and ten natural populations in Indiana and Missouri, respectively, in 2001-2003 (Table 1). Sampled populations were located at least 1.6 km from any known black walnut plantation, and occurred in diverse ownerships (Table 1). The average distance between a population and its nearest sampled neighbor was 73.18 (± 40.2) km. Sampled trees within a population were spaced about 100 m apart or more. Leaf tissue (4 to 6 leaflets per tree) was collected from each sampled tree, placed into a resealable plastic bag, and mailed to Purdue University, where it was stored at -80 °C or freeze-dried until the DNA could be extracted.

Table 1.—Sampling locations, sample sizes, population sizes, and population descriptions for the sampled Indiana and Missouri black walnut populations

Population	County	Sample size	Description	Stand size (hectares)
Indiana				
IN-A	Sullivan	19	Private	180
IN-C	Morgan	30	Private	20
IN-D	Parke	30	Shades State Park	20+
IN-E	Jennings	30	State Forest	140
IN-F	Posey	29	Private	81+
IN-H	Harrison	29	Blue Spring Wildlife area	16
IN-K	Grant	29	Public	49[a]
IN-L	Carroll	30	Private	12[b]
IN-Y	Pulaski	30	Private	140
Missouri				
MO-A	Boone	30	Private	8
MO-C	Adair	30	Private	14
MO-D	Jefferson	30	Private	160
MO-E	Texas	30	Private	41
MO-F	Howard	30	Private	8
MO-G	Johnson/Pettis/Henry	29	Private	16[c]
MO-H	Boone	30	Private	16
MO-I	Clinton	30	Private	13
MO-J	Callaway	30	Private	12
MO-K	Cass	28	Private	10

[a]Three tracts totaling 49 ha
[b]Two fragments totaling 12 ha
[c]Size for population where majority of leaves were sampled

MATERIALS AND METHODS

DNA extraction, quantification, and genotyping were performed according to Victory and others (2006), using the same set of 12 microsatellite loci reported therein. For quality control, a suite of DNA samples with known genotypes were amplified with each Polymerase Chain Reaction (PCR) as a positive control, and one set was run with each of the three populations per gel as allele size standards. A negative control was included in each amplification set. As a further control, 30 individuals were selected at random and genotyped independently. Allele calls for this positive control group matched the previously determined alleles more than 95 percent of the time (data not shown).

Data analysis was as described in Victory and others (2006) except hierarchical F-statistics for a three-level hierarchy (state, population, individual) were calculated with GDA (Lewis and Zaykin 2001). Estimates were obtained for F_{IS} (=f), F_{IT} (=F), F_{SP} (=θ_S) and F_{PT} (=θ_P), where the subscript *I* stands for individual, *S* for subpopulation (local population or county within each state), *P* for population (state), and *T* for total. Confidence intervals (α = 0.05) around the hierarchical *F*-statistics were obtained by bootstrapping 20,000 times over the 12 loci. Analysis of a two-level hierarchy also was performed in GDA so that F_{ST} also could be estimated. An alternate test for differences in genetic parameters (e.g., rarefacted allelic richness, observed heterozygosity, and expected heterozygosity) among the 19 sampled populations – in which

Table 2.—Genetic diversity measures[a] for each microsatellite locus across the entire sample of 552 black walnut trees in 19 populations

Locus	n	Allele size range (bp)	Number of alleles	H_O	H_T	F_{IS}[b]
AAG 01	550	148 - 172	9	0.656	0.697	0.045
WGA 06	550	134 - 170	18	0.606	0.609	-0.002
WGA 24	530	221 – 253	16	0.860	0.884	0.010
WGA 27	539	200 - 248	24	0.857	0.886	0.013
WGA 32	546	163 - 231	33	0.908	0.935	0.011
WGA 69	542	162 - 186	11	0.576	0.595	0.021
WGA 72	542	134 - 160	13	0.620	0.594	-0.055
WGA 76	547	224 - 252	13	0.733	0.742	-0.005
WGA 82	540	154 - 238	41	0.919	0.966	0.031*
WGA 89	526	181 - 235	28	0.922	0.922	-0.019
WGA 90	550	138 - 190	25	0.904	0.909	-0.011
WGA 97	543	148 - 194	22	0.864	0.900	0.031
Mean	542	---	21.08	0.785	0.803	0.007

[a]n, sample size for locus; HO, observed heterozygosity; HT, total expected heterozygosity; FIS, within population inbreeding coefficient (index of panmixia).
[b]Asterisks indicate FIS estimates significantly greater than zero after sequential Bonferroni correction (alpha = 0.05).

multilocus genotypes were permuted among the populations 10,000 times, contingency tables constructed, and the log-likelihood statistic *G* used as a test statistic – was carried out with the aid of FSTAT (Goudet 1995). A matrix of pairwise genetic distances (*D*; Nei 1978) among all population pairs was generated using GDA. Because spatial coordinates were not taken for all sampled trees or populations, pairwise geographic distance values were calculated based upon the distance between the county seats of the counties in which the populations were sampled. The only exception to this method of calculating geographic distance was for population MO-A, for which geospatially referenced global positioning system (GPS) coordinates specific to that population were used; this step was necessary since population MO-H was sampled in the same county and thus shared the same county seat. A Mantel test of association between the pairwise genetic and geographic distances was performed using the ISOLDE program of GENEPOP (version 3.4, de Genetique et Environment, Montpellier, France) (Raymond and Rousset 1995, Rousset 1997); 10,000 permutations were employed to obtain the *p*-value.

RESULTS

Complete genotypes at all 12 microsatellite loci were obtained for 98 percent of all sampled individuals. Based upon the overall sample of 552 individuals, the average genetic diversity across loci was high (Table 2), as measured by observed heterozygosity (H_O = 0.785), the average number of alleles per locus (A_N = 21.1), and the total number of alleles per locus, which ranged from nine (AAG 01) to 41 (WGA 82). Hardy-Weinberg genotypic proportions were rejected for only one of the 12 loci (WGA 82), on the basis of the permutation test (using F_{IS} as a test statistic) and after sequential Bonferroni correction (Table 2). The very large number of alleles at this locus (41 alleles) was likely a contributing factor. Low-frequency null alleles also may have been present at WGA 82.

Table 3.—Comparison of genetic diversity measuresa between Indiana and Missouri

Population	n	A_N	$A_{R(18)}$	$A_{R(23)}$	H_O	H_S	F_{IS}	F_{ST}
Indiana								
IN-A	18.8	10.0	9.87	------	0.787	0.782	-0.006	
IN-C	28.8	11.8	10.19	11.03	0.769	0.794	0.033	
IN-D	29.6	11.8	9.93	10.86	0.756	0.758	0.002	
IN-E	28.5	11.8	10.45	11.27	0.792	0.810	0.023	
IN-F	28.1	11.9	10.39	11.25	0.791	0.805	0.017	
IN-H	28.3	12.5	10.78	11.76	0.783	0.805	0.027	
IN-K	29.0	12.8	11.06	11.98	0.792	0.802	0.015	
IN-L	29.7	11.7	10.19	10.97	0.801	0.797	-0.006	
IN-Y	29.9	12.1	10.23	11.13	0.769	0.794	0.032	
IN mean	27.8	11.8	10.34	11.28	0.782	0.794	0.015	0.0128
Missouri								
MO-A	29.8	11.7	10.15	10.92	0.791	0.795	0.005	
MO-C	29.8	12.1	10.17	11.10	0.813	0.789	-0.031	
MO-D	29.3	12.1	10.29	11.21	0.794	0.799	0.006	
MO-E	29.1	11.3	10.03	10.72	0.785	0.797	0.015	
MO-F	29.8	12.8	10.58	11.63	0.782	0.803	0.027	
MO-G	28.8	12.3	10.48	11.40	0.805	0.798	-0.009	
MO-H	29.5	11.2	9.25	10.19	0.767	0.731	-0.050	
MO-I	28.7	11.7	10.07	10.92	0.763	0.777	0.018	
MO-J	29.3	12.7	10.83	11.81	0.782	0.791	0.011	
MO-K	27.4	12.1	10.36	11.36	0.805	0.800	-0.006	
MO mean	29.2	12.0	10.22	11.13	0.789	0.788	-0.001	0.0182
p-value[b]	----	----	0.259	0.480	0.163	0.256	0.032	0.198
Overall mean	28.5	11.9	10.28	11.20	0.786	0.791	0.007	0.0155

[a]n, avg. sample size per locus (based on 12 loci); A_N, avg. number of alleles per locus; $A_{R(18)}$, allelic richness standardized to a sample size of 18 trees per population; $A_{R(23)}$, allelic richness standardized to a sample size of 23 trees per population (IN-A omitted); H_O, within-population observed heterozygosity; H_S, within-population expected heterozygosity; F_{IS}, inbreeding coeffcient (or 'index of panmixia'); F_{ST}, fixation index for each state (measure of population differentiation).

[b]For the comparison of IN mean vs. MO mean, based upon 10,000 permutations of populations among states (one-tailed test).

All of the genetic diversity measures were markedly similar across populations, both within and between states (Table 3). The mean number of alleles per population ranged from 10.0 to 12.8 for Indiana and 11.2 to 12.8 for Missouri. Rarefaction to common sample sizes of 18 (with all populations) or 23 (excluding IN-A) reduced these ranges. Within-population observed heterozygosties ranged from 0.756 to 0.801 in Indiana and from 0.763 to 0.813 in Missouri. Permutation tests for differences in genetic diversity measures between Indiana and Missouri were not significant for all measures except mean F_{IS} (0.015 in Indiana vs. -0.001 in Missouri; $p = 0.032$). This result could be interpreted as indicating that in recent generations, matings between relatives (and/or selfing) have occurred at an elevated rate in Indiana populations relative to Missouri populations, possibly due to more severe fragmentation in Indiana. However, the effect is not very strong (F_{IS} in Indiana = 0.015); the global value of F_{IS} (across loci) was

Table 4.—Hierarchical F-statistics[a] across 12 loci for black walnut subpopulations grouped by state (Indiana versus Missouri) and associated confidence intervals (based on 20,000 bootstraps)

	F_{IS}	F_{IT}	F_{SP}	F_{PT}
Estimate	0.007	0.023	0.017	0.0009
Lower Bound[b]	-0.007	0.010	0.014	-0.0002
Upper Bound	0.019	0.035	0.019	0.0019

[a]Subscripts: I, individual; S, subpopulation (local population or county within a state); P, population (state); T, total.
[b]Lower and upper bounds for confidence intervals indicate statistical significance (alpha = 0.05) if zero is not included.

significantly different from zero (p = 0.011). However, both of these results were due to a single locus, WGA 82, so we caution against over-interpreting the somewhat elevated mean FIS in Indiana.

Twenty-three private alleles were observed (i.e., alleles occurring in only a single population sample; data not shown). In general, these private alleles were evenly distributed across the populations. All of the private alleles were present at low frequencies in the overall sample (P< 0.005) and all occurred at or very near the ends of the allele size range for each locus, indicating that they may have been recent mutations. Most of the private alleles were rare in the population where they were found, usually at a frequency of less than 0.05. Overall, the distribution of private alleles among the populations suggests that they are the products of sampling error rather than limited gene flow.

Hierarchical F-statistics (Table 4) provided little evidence of genetic structure between states: F_{PT} was estimated as 0.0009 and was not significantly different from zero (based upon bootstrapping loci). Hence, the additional hierarchical level to evaluate differences between states explained an inconsequential portion of the total variance. The estimate of the amount of differentiation among populations within states (F_{SP}) was small (0.017) but significant. The estimate of the amount of differentiation among populations regardless of their state of origin (F_{ST} = 0.016; obtained from a two-level analysis) was significant ($p < 0.05$). The estimate of F_{IS} was low (0.007) and not significantly different from zero (Table 4), reflecting the fact that most loci displayed Hardy-Weinberg genotypic proportions within populations (Table 2). An essentially identical result was obtained when the data were analyzed using Peakall and Smouse's R statistics (Peakall and Smouse 2006).

Pairwise genetic distance values varied from 0.012 to 0.159, indicating a broad range in the level of differentiation between population pairs. However, the Mantel test for isolation by distance indicated no significant association between geographical and genetic distance ($p = 0.29$).

DISCUSSION

Black walnut in Indiana and Missouri contains high levels of genetic diversity at nuclear microsatellite loci and is remarkably genetically homogenous. The overall level of genetic variance partitioned among all populations in the sample was quite small (F_{ST} = 0.016)—though statistically significant ($P < 0.01$). The data were devoid of any clear pattern of geographical structure. We found very little evidence of genetic variance partitioning among populations either within states or between states, confirming that the lack of genetic differentiation exhibited among black walnut populations at broad spatial scales (Victory and others 2006) also extends to smaller regional populations, despite local differences in glaciation history,

fragmentation, and forest management practices. Also, we found no evidence for differences in mean population genetic diversity parameters between the two states (with the exception of mean F_{IS}, a result that was due solely to the somewhat aberrant locus WGA 82). Given the divergent natural histories of black walnut in Indiana and Missouri, the overall lack of contrast between the two states was surprising.

A number of factors might individually or collectively explain the observed lack of structure, including: (1) post-glacial recolonization of the current geographical distribution of black walnut from a single, large, homogenous glacial refugium; (2) the homogenization of black walnut gene frequencies (a) subsequent to post glacial recolonization and (b) subsequent to the anthropogenic bottlenecks of the last century; (3) high rates of size homoplasy (the potential to share alleles that are identical in size but not identical by descent) among microsatellite alleles in this species; and (4) large effective population size and high rates of gene flow in this species. Almost certainly each of these factors contributed in some way to the current population structure of black walnut in Indiana and Missouri.

High rates of pollen flow among neighboring refugial populations would have homogenized their gene frequencies and reduced overall genetic structure (effectively creating a single, large refugial population). The recolonization process for black walnut after the Last Glacial Maximum (LGM) may have been different for Indiana and Missouri, but whatever the differences may have been, they left no mark that we could detect using our data. Such a mark, if it once existed, may have been erased by extensive pollen flow subsequent to recolonization.

Differing regimes of deforestation and fragmentation that occurred in Missouri and Indiana after European settlement apparently have had little if any effect on the current neutral genetic diversity of black walnut in these states. Since deforestation was most extensive in portions of the species range that were highly suitable for agriculture (e.g., northern Indiana), numerous rare and localized alleles undoubtedly have been lost. Although it is likely that a high percentage of black walnut greater than 150 years of age has been harvested, it is probable that an insufficient number of generations have passed for the effects of forest fragmentation and anthropogenic selection to be discernable (Collevatti and others 2001).

In black walnut, as in other species, the presence of size homoplasy could homogenize the allelic diversity of populations (Estoup 2002). Our data exhibited two characteristics that may indicate the presence of size homoplasy. First, the allele distributions at each locus were relatively uniform across populations (not shown), indicating that most populations had retained or obtained through mutation a common set of allele sizes. Second, all of the private alleles detected occurred at the ends of the distribution of allele sizes, probably because novel mutations at each locus were detected only at the most extreme allele size classes. Homoplasy may have disrupted any traces of allele loss caused by founder effects present in either Indiana or Missouri black walnut populations (see Young and others 1993). Size homoplasy, however, does not fully explain the lack of allele frequency differentiation observed in this study, as studies in other tree species have uncovered pronounced genetic structure (Jones and others 2002, Heuertz and others 2004a).

The reproductive biology of walnut may best explain the current homogeneity within and between the black walnut populations in Indiana and Missouri. High levels of post-glacial gene flow can obscure, at the nuclear level, evidence of ancient population differentiation (Finkeldey and Matyas 2003, Heuertz and others 2004b). Black walnut seems to possess the necessary biological attributes to create and maintain large homogenous complexes of interacting populations and large effective population sizes even in the face of substantial vicariance.

The results reported here indicate that recent landscape changes in the Midwest, while significant, have not yet led to forest islands where the consequences of founder effects and genetic drift dominate the observed diversity of forest trees, at least not for black walnut. Now that there are unusually low population densities of black walnut in the center of its range (in Indiana), gene flow into populations at the periphery (e.g., Missouri) may be reduced, and peripheral populations may become less constrained in their adaptive evolution (Garcia-Ramos and Kirkpatrick 1997).

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