ALLOZYME VARIATION AND MATING SYSTEM OF BLACK WALNUT (*JUGLANS NIGRA* L.) IN THE CENTRAL HARDWOOD REGION OF THE UNITED STATES

Victor B. Busov[1], George Rink[2] & Keith Woeste[3]

[1)] Research Associate, Department of Forest Science, Oregon State University, Corvallis, OR 97331.
[2)] Research Geneticist, USDA Forest Service, Golden, CO 80401
[3)] Research Geneticist, USDA Forest Service Hardwood Tree Improvement and Regeneration Center, 1159 Forestry Building, Purdue University, West Lafayette, IN, 47907.

Received October 1, 2002; accepted March 4, 2003

ABSTRACT

Allozyme data for 10 polymorphic loci from 7 isozyme systems was used to elucidate the genetic diversity, population structure and mating system of black walnut (*Juglans nigra* L.), in the central hardwood region of the United States. The average expected heterozygosity (0.241), percent polymorphic loci (82 %) and mean number of alleles per locus (2.1) indicate that black walnut is highly variable across the studied range. Observed heterozygosity was higher in mature trees (0.332) than in their progeny (0.241), a result that has become typical for walnuts. Outcrossing rates assessed in all locations were high (0.900 – 1.012), indicating that black walnut is predominantly outcrossed. The greatest proportion of genetic variation was allocated within populations (94 %), and very small part among populations (6 %). Estimated genetic distances among populations did not reveal any major genetic subdivisions among five widely dispersed provenances.

Key words: allozymes, black walnut, *Juglans nigra* L., heterozygosity, outcrossing rates, fixation indices.

INTRODUCTION

Black walnut (*Juglans nigra* L) is a large, deciduous tree native throughout the eastern United States from New England to Texas (FOWELLS 1965). It is one of the most valued tree species in the central hardwood forests of the United States (BRESNAN *et. al.* 1994). Demand for improved plant stock is growing, particularly among private landowners using the species as a component in agro-forestry systems (NOWEG & KURTZ 1987; RULE *et al.* 1994). Efforts to understand the wild black walnut germplasm have been limited to provenance tests (BRESNAN *et al.* 1992; RINK 1997). The associated morphological and phenological data have been important tools for breeders and foresters (GURIES *et al.* 1981), but provenance tests are expensive and time consuming because the species has a long juvenility and mature trees are large.

It has been predicted that the reproductive and ecological characteristics of black walnut tend to increase the relatedness of individuals in natural stands and the differentiation among provenances (BEINEKE 1989). Black walnut usually occurs as scattered single trees or in fragmented groups. In addition, black walnut seeds are heavy and usually are not dispersed far from mother trees. Furthermore, warm spring temperatures can reduce the effectiveness of dichogamy as a barrier against self-pollination (MASTERS 1974).

To devise effective breeding and germplasm management programs for black walnut it is important to understand its reproductive biology and the allocation of genetic variance among populations, families, and within families. If inbreeding is common, phenotypically superior trees from native stands may be the result of matings among close relatives, the sample size required to capture the genetic variance present in a local population will be larger than for a strictly random mating population, and mass selection will have some of the characteristics of family selection.

RINK *et al.* (1989) used eight polymorphic isozyme loci to estimate heterozygosity, fixation indices and outcrossing rates in naturally occurring black walnut trees from southern Illinois. Contrary to what was expected, Rink's study disclosed almost complete outcrossing, and high mean heterozygosity compared to several other tree species (HAMRICK 1989; STREIFF *et al.* 1998). Further studies on trees from this location and a seed orchard confirmed the first results (RINK *et al.* 1994). Rink's studies, however, were confined to trees growing in southern Illinois and/or seed orchards. No information is available for the population structure and mating system of naturally occurring black walnut

in other areas.

In this study, we surveyed ten polymorphic isozyme loci to explore patterns in the genetic variation, population structure, and mating system of black walnut across four states in the central hardwoods region of the United States. We estimated the amount of allelic fixation, heterozygosity and outcrossing rates to assess the levels of genetic differentiation within and among five black walnut populations.

MATERIALS AND METHODS

Plant material

About 500 seeds were collected from five black walnut populations in Wisconsin, northern Illinois, southern Illinois, Missouri and Tennessee (Table 1). The female parent trees, ten from each population, appeared typical for the location. The parent trees were not sampled. Nuts were dehusked, washed, and stored in a refrigerator until further processing. Seeds were cracked and the embryos excised and kept at –70 °C until analyzed as in RINK (1989). Not all seeds contained embryos that could be analyzed.

Isozyme analysis

Embryos were homogenized in a drop of extraction buffer (MARTY *et al.* 1984). Filter paper wicks (2 × 15 mm) were soaked in the resulting extract and immediately applied to 12 % starch gels for horizontal electrophoresis using a neutral – pH 7.2 running buffer (SOLTIS *et al.* 1983). The electrophoresis tank was held at 4 °C and a 50 mA current was applied until the Bromophenol Blue tracking dye reached the end of the gel. Gels were stained for the following seven enzyme systems: AAT (aspartate amino transferase – E.C. 2.6.1.1.), ACO (aconitase – E.C. 4.2.1.3), ACP (acid phosphatase – E.C.3.1.3.2), ADH (alcohol dehydroge nase – E.C.1.1.1.1), PGI (phospho-glucose isomerase – E.C.5.3.1.9), PGM (phospho-glucomutase – E.C. 2.7.5.1) and 6-PG (6-phosphogluconic dehydrogenase – E.C.1.1.1.44), using standard recipes (MARTY *et al.* 1984). Interpretation and scoring was based on the segregation patterns observed in the progeny and information from previous studies (RINK *et al.* 1989, ARULSEKAR *et al.* 1985, ARULSEKAR *et al.* 1986, ALETA *et al.* 1990).

Data analysis

Data were analyzed using the Generalized Multilocus Estimation software[1] (RITLAND 1988) developed from an outcrossing model by RITLAND and JAIN (1981) based on maximum likelihood equations of BROWN and ALLARD (1970). The program identifies the most likely maternal genotype for each enzyme system based on the segregation patterns and estimates gene frequencies in the pollen and the ovule. It summarizes genotype frequencies and calculates outcrossing rates. Multilocus outcrossing rates are calculated based on a maximum likelihood model taking into account all loci at the same time (RITLAND & JAIN 1981). Single-locus outcrossing rates were calculated by the same model but one locus at a time and then averaged across the ten studied loci. Genetic variability measures, genetic distances and population structure parameters were calculated by BIOSYS-1 (SWOFFORD & SELANDER 1989) and PopGene version 1.31 (from F. C. Yeh, University of Alberta and T. Boyle, Center for International Forestry Research). A comparison of heterozygosities and fixation indices between the progeny and maternal generations was performed using a t-test for unpaired observations with unequal variance. The same test was used to compare the differences in single-locus outcrossing estimates among the five populations. Correspondence analysis was performed using PROC CORRESP in SAS (Cary, NC).

RESULTS

The seven enzyme systems assayed in this study were coded by fourteen loci. Two of three loci coding for ACP displayed blurred and inconsistent staining pattern and were not scored or analyzed. Both 6PG and PGI were coded by two loci, but only the cathodal ones (*6pg-2* and *Pgi-2*) were polymorphic and further analyzed. Of the original fourteen, only ten loci were polymorphic, displayed consistent patterns, and were scored and analyzed in all populations (Table 2). The *Aat-1*b allele was found only in the Wisconsin population location (Table 2).

Black walnut displayed high genetic diversity in all five populations

By sampling a large number of progeny from each mother tree (30 progeny per maternal parent), we were able to deduce the most likely genotype of the maternal

[1] The use of trade names is for the information and convenience of the reader and does not imply official endorsement or approval by the U.S. Department of Agriculture or the Forest Service of any product to the exclusion of others that may be suitable.

Table 1. Sample sizes and genetic variability at ten loci for maternal trees and progeny from five black walnut populations.

Population	N	Alleles per locus	Polymorphic loci[A] (%)	Mean heterozygosity[B]		F^C
				H_{obs}	H_{exp}	
			Maternal[D]			
Missouri	7	1.9	80.0	0.329±0.077	0.282±0.061	-0.167
Northern Illinois	11	1.9	80.0	0.330±0.110	0.234±0.065	-0.410
Southern Illinois	12	2.0	80.0	0.367±0.067	0.293±0.049	-0.253
Tennessee	10	2.0	70.0	0.345±0.091	0.271±0.063	-0.273
Wisconsin	10	1.8	80.0	0.290±0.081	0.230±0.054	-0.261
Mean				0.332	0.262	
			Progeny			
Missouri	208	2.3	90.0	0.305±0.070	0.285±0.053	-0.070
Northern Illinois	297	2.3	60.0	0.173±0.052	0.189±0.053	0.085
Southern Illinois	479	2.3	90.0	0.285±0.078	0.264±0.047	-0.080
Tennessee	290	2.2	60.0	0.256±0.076	0.235±0.060	-0.089
Wisconsin	215	2.2	70.0	0.205±0.048	0.206±0.045	0.005
Mean				0.245	0.236	

[A] Percentage of ten loci. A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95.
[B] ± standard error.
[C] Fixation Index, $F = 1 - (H_o/H_e)$.
[D] Maternal genotypes were deduced from the segregation in their progenies (see also Materials and Methods).

trees from the allozyme segregation patterns of their progeny. This made possible the evaluation of the diversity in both parent trees and their progenies. Both generations displayed high levels of genetic polymorphism at all studied locations (Table 1). The number of alleles per locus ranged from 1.8 to 2.0 among female parents across populations, and from 2.0 to 2.2 among the progeny. The percentage of polymorphic loci was high, from 70 % to 80 % among female parents across populations, and from 60 % to 90 % among the progeny. These estimates are close to the estimates of RINK *et al.* (1994), who found the percentage of polymorphic loci in a seed orchard and in wild trees in Southern Illinois to vary between 75.0 % and 87.5 %. The difference in percent polymorphic loci between the parents and progeny was consistent with the results of RINK *et al.* (1994) for a southern Illinois location. Average observed heterozygosity among progeny in this study (0.24) was close to what was found by RINK *et al.* (1994) (0.224– 0.234) in a survey that included a black walnut seed orchard, progeny test, and natural population. The average observed heterozygosity among the mother trees (0.33) across all populations in our study was close to that estimated by RINK *et al.* (1989) for adult trees (0.31) in a southern Illinois location. The overall average number of alleles per locus for the black walnut populations in this study was about 2.0 (Table 1), lower than the values reported for European oak (*Quercus robur* L. and *Q. petraea* Liebl.) populations (4.3; STREIFF *et al.* 1998), and European populations of Persian walnut (*Juglans regia* L) (2.27; FORNARI *et al.* 1999), but comparable to values reported for butternut (*J. cinerea* L.); 1.3 and 2.3 alleles per locus and per polymorphic locus, respectively (MORIN *et al.* 2000). The average observed heterozygosity was higher for black walnut than for butternut (0.33 versus 0.028) and the oaks (0.25), but slightly lower than that found in Persian walnut (0.39).

Higher heterozygosity in mature trees

The maternal parents had a higher observed heterozygosity than the progeny in all locations (Table 1) and all loci (data not shown). This trend was also evident from the estimated fixation indices, which were higher in the progeny and lower in the parental generation (Table 1). The differences in H_{obs} and F between the maternal and progeny generations were statistically significant, at $P < 0.01$ and $P < 0.001$ respectively, based on an unpaired t-test (unequal variance). Similar differences

Table 2. Allelic frequencies for ten polymorphic loci in five black walnut populations.

Locus	Alleles	Population				
		Missouri	Northern Illinois	Southern Illinois	Tennessee	Wisconsin
Aat-1	a	1.000	1.000	1.000	1.000	0.887
	b	0.000	0.000	0.000	0.000	0.113
Aat-2	a	0.257	0.272	0.208	0.351	0.277
	b	0.743	0.728	0.793	0.649	0.723
Aco-1	a	0.594	0.849	0.532	0.749	0.686
	b	0.264	0.045	0.463	0.211	0.271
	c	0.142	0.106	0.005	0.040	0.043
Aco-2	a	0.695	0.641	0.730	0.565	0.836
	b	0.305	0.359	0.270	0.435	0.164
Acp-2	a	0.923	0.960	0.767	0.742	0.922
	b	0.077	0.040	0.233	0.258	0.078
Adh-1	a	0.938	0.990	0.937	0.985	0.974
	b	0.055	0.007	0.027	0.015	0.026
	c	0.007	0.003	0.036	0.000	0.000
6pg-2	a	0.745	0.916	0.829	0.850	0.972
	b	0.255	0.084	0.172	0.150	0.028
Pgi-2	a	0.885	0.998	0.930	0.983	0.933
	b	0.115	0.002	0.070	0.017	0.067
Pgm-1	a	0.802	0.898	0.826	0.882	0.954
	b	0.115	0.064	0.115	0.051	0.037
	c	0.083	0.038	0.059	0.067	0.009
Pgm-2	a	0.821	0.801	0.877	0.953	0.846
	b	0.167	0.195	0.119	0.040	0.154
	c	0.012	0.004	0.004	0.007	0.000

Table 3. Multi- and single-locus outcrossing rates in five black walnut provenances.

Provenance	Multi-locus outcrossing rate	Singl-locus outcrossing rate	Difference
Missouri	0.975±0.019	0.934±0.029	0.041±0.022
Southern Illinois	1.012±0.087	1.086±0.047	-0.074±0.047
Tennessee	0.958±0.066	0.900±0.120	0.058±0.059
Northern Illinois	0.991±0.104	0.981±0.059	0.010±0.058
Wisconsin	0.900±0.050	0.777±0.041	0.123±0.018

[A] ± standard error.

between parents and progeny were observed in Persian walnut. by (MALVOLTI *et al.* 1994) and in black walnut from southern Illinois (RINK *et al.* 1989).

Black walnut is predominantly outcrossing

Multi-locus outcrossing rates (t_m) ranged from 0.900 in Wisconsin to 1.012 in southern Illinois (Table 3). Multilocus outcrossing rates in the different popula-

Table 4. Summary of F-statistics for ten loci and five black walnut provenances.

Locus	F_{IS}	F_{IT}	F_{ST}
	Maternal		
Aat-1	-0.111	-0.020	0.082
Aat-2	-0.428	-0.398	0.021
Aco-1	-0.379	-0.215	0.119
Aco-2	-0.574	-0.456	0.075
Acp-2	-0.287	-0.154	0.103
Adh-1	-0.058	-0.044	0.014
6pg-2	-0.233	-0.194	0.032
Pgi-1	-0.104	-0.052	0.047
Pgm-1	-0.175	-0.110	0.056
Pgm-2	-0.307	-0.260	0.036
Mean	-0.338	-0.259	0.059
	Progeny		
Aat-1	-0.080	0.020	0.092
Aat-2	0.071	0.081	0.011
Aco-1	-0.316	-0.223	0.070
Aco-2	-0.213	-0.166	0.039
Acp-2	0.034	0.100	0.069
Adh-1	0.042	0.056	0.014
6pg-2	0.357	0.389	0.050
Pgi-1	-0.020	0.013	0.032
Pgm-1	-0.017	0.033	0.020
Pgm-2	0.079	0.101	0.023
Mean	-0.041	0.002	0.041

tions were not significantly different from each other as determined by an unpaired t-test (unequal variance). Single-locus estimates of outcrossing (t_s) were in the same range as the multi-locus estimates except for the Wisconsin provenance ($t_s = 0.777$), which had a significantly lower t_s than the provenances in southern Illinois, northern Illinois and Missouri (t-test, unpaired observations; unequal variance; $P < 0.05$). The calculated outcrossing rates in all provenances were very high and in close agreement with previous studies (RINK *et al.* 1989; RINK *et al.* 1994), indicating that black walnut is predominantly outcrossed. We observed no clear geographic trends in the variation of outcrossing rates (Table 3).

Comparison of outcrossing estimates obtained by single and multi-locus methods can provide evidence for forms of inbreeding other than selfing (SHAW *et al.* 1981). Single-locus estimates are biased downward by homozygosity. Matings between closely related individuals increase homozygosity and consequently decrease the single-locus estimate of outcrossing rate. The multi-locus estimate of outcrossing is based on a composite of many loci and is not as downwardly biased by homozygosity. We observed indications of matings between relatives based on differences in multi-and single-locus outcrossing rates in all provenances except southern Illinois (Table 3). However, the outcrossing rate differences were low and within the ranges of the standard deviations, and because the multi- and single-locus outcrossing rates were not independently determined, differences between them could not be tested.

Black walnut population structure in the central US is homogenous

We calculated Wright's F-statistics to estimate the partitioning of genetic variance (WRIGHT 1965). F_{ST} values, calculated using data from both adult and progeny trees, indicated that most of the variation is located within provenances (Table 4). Considerable variation of F_{ST} values was observed among studied loci, ranging from 0.014 to 0.119 in the maternal parents (Table 4). Multi-locus genetic distance coefficients were low, varying from 0.010 to 0.027, consistent with low levels of genetic differentiation (Table 5). This conclusion was supported by the results of a correspondence analysis that showed non-significant chi-square values in all dimensions (data not shown), indicating no significant association between populations and allele frequencies. The populations under study were characterized by genetic similarity, rather than dissimilarity.

DISCUSSION

Our study demonstrates that black walnut in the central US maintains high genetic variability. Average heterozygosities and percent polymorphic loci are higher or comparable to other species with similar mating systems and geographic distribution (MORIN *et al.* 2000), although the diversity measures for walnut were biased upward by the exclusion of monomorphic loci from the analysis. As in many trees, most of the genetic diversity of black walnut was allocated within populations. As pointed out earlier (BEINEKE 1989), population size, habitat fragmentation, seed dispersal, and mating system in black walnut would seem to promote local genetic differentiation, inbreeding, and loss of diversity. However, previous work and our study unanimously disclose population-level genetic diversity estimates comparable to or higher than tree species with continuous distribution and long-range pollen flow and seed dispersal. Long generation time is one of several possible explanations of this result (AUSTERLITZ *et al.*, 2000).

Table 5. Nei (1978) unbiased genetic distances for ten loci in five black walnut populations.

Population	Missouri	Northern Illinois	Southern Illinois	Tennessee	Wisconsin
Missouri	0.000				
Northern Illinois	0.013	0.000			
Southern Illinois	0.010	0.027	0.000		
Tennessee	0.016	0.013	0.016	0.000	
Wisconsin	0.014	0.013	0.015	0.021	0.000

A few studies, including this one, demonstrated higher heterozygosity in walnut parents compared to their progeny (MALVOLTI *et al.* 1994; RINK *et al.* 1989). We sampled only seeds and so could not evaluate selection against inbreeding throughout the black walnut life cycle, but our observation that some of the black walnut embryos in this study were not viable may indicate that selective forces acted during seed formation. BEINEKE (1989) found that germination and early survival rates differ between inbred and outcrossed black walnut seeds, and MITTON (1992) showed that conifer seed viability could be associated with heterozygosity. RINK *et al.* (1989) and MALVOLTI *et al.* (1995) suggested that differences in the observed heterozygosity between parents and progeny might be due to the failure of inbreds to compete over time. An association of heterozygosity with fitness has been demonstrated in some pine species (FARRIS & MITTON 1984; STRAUSS 1986; BUSH *et al.* 1987), but not in others (SAVOLAINEN & HEDRICK 1995). Levels of heterozygosity in *Cyclolanopsis championii* seem to be associated with adaptation to different ecological factors (CHEN *et al.* 2001). Thus, it is possible that selective forces favor heterozygosity throughout the life cycle of black walnut, but sampling errors, differences in sampling methods (parents were arbitrarily selected individuals comprising a small population, progeny were large half-sib arrays), or dissasortive mating may have also been responsible for some of the observed differences in heterozygosity between parents and progeny. The variability of F_{ST} values among different loci indicates the possibility of assortative mating at the studied loci. It may also be a manifestation of diversifying selection acting directly upon some of the studied genes or tightly linked ones, or simple sampling error.

In black walnut, temporal separation of male and female flowering (dichogamy) is not complete and seems to be modulated by spring temperatures (MASTERS 1974). MCDANIELS (1956) and STROMBERG (1990) also found substantial shifts in dichogamy patterns of other walnut species in response to environmental changes. These shifts could potentially affect local levels of inbreeding over time. Although estimates of outcrossing rates based on a single year's data can be misleading (MORAN & BROWN 1980), in part because heterodichogamy can lead to a non-random sampling of the pollen pool and an over-estimation of the outcrossing rate (MITTON 1992), our study discloses almost complete outcrossing, and agrees with previous estimates in one wild population and two seed orchards (RINK *et al.* 1994). It has not yet been determined if black walnut has higher rates of consanguineous mating in years when dichogamy is less pronounced.

Walnut is heavy seeded, and close range seed dispersal might tend to increase the frequency of matings among related trees. We observed lower multilocus versus single- locus outcrossing rates that may have been indicative of close relative matings. However single-locus estimates are sensitive to violations in the assumption of pollen pool homogeneity (RITLAND & JAIN 1981) and other factors not controlled in this experiment. Self-pollination is more common in black walnut when dichogamy is reduced by rapid warming in the spring (MASTERS 1974). This type of abrupt temperature change is typical in Wisconsin, the most northerly provenance we collected, and it may have contributed to the observation that the t_m and t_s from the Wisconsin population were the lowest in the study. Finally, pistillate flower abscission caused by excess pollen load may reduce the frequency of self-pollinations or even matings with (nearby) close relatives. Pistillate flower abscission has been best documented in *J. regia* (MCGRANAHAN *et al.* 1994) but is suspected in black walnut as well (Beineke, personal communication). When all the evidence is weighed, it seems unlikely that close relative mating is common in black walnut.

The *Aat-1*b allele of the *Aat-1* locus was detected only in Wisconsin. More intensive sampling will be needed to determine the distribution of this allele throughout the range of black walnut. If the allele has a very limited distribution, it may be possible to use it for provenance-specific assignment of genotypes and/or for verifying seed source identity.

Older black walnut trees are significantly more heterozygous than younger ones. Thus, for a given amount of sampling effort, more genetic diversity will likely be captured by sampling older trees. The need for

range-wide sampling to understand the overall diversity of black walnut and for detecting alleles related to local adaptation is indicated by the presence of a rare allele in Wisconsin and by black walnut provenance trials (BRESNAN *et al.* 1994). Microsatellites (WOESTE *et al.* 2001), RAPDs and other DNA-based markers could potentially detect such adaptive alleles by providing more complete marker coverage of the black walnut genome than is possible with isozymes.

LITERATURE CITED

ALETA, N., C. OLARTE, M., TROCO, J. & ARUS, P. 1990: Identification of walnut cultivars by isozyme analysis. *Acta Hort.* **284**: 91–96.

ARULSEKAR, S., PARFITT, D. E. & MCGRANAHAN, G. H. 1985: Isozyme gene markers in *Juglans* species. *J.Hered.* **76**: 103–106.

ARULSEKAR, S., MCGRANAHAN, G. H. &. PARFITT, D. E. 1986: Inheritance of phosphoglucomutase and esterase isozymes in Persian walnut. *J. Hered.* **77**: 220–221.

AUSTERLITZ, F., MARIETTE, S., MACHON, N., GOUYON, P. & GODELLE, B. 2000: Effects of colonization processes on genetic diversity: Differences between annual plants and tree species. *Genetics* **154**: 1309–1321.

BEINEKE, W. F. 1989: The effects of inbreeding in black walnut. *Proc. Indiana Acad. Sci.* **97**: 105–108.

BRESNAN F., GEYER, W., LYNCH, K.D., & RINK, G. 1992: Black walnut provenance performance in Kansas. North. *J. Appl. For.* **9**:41–43.

BRESNAN, D. F., RINK, G., DIEBEL, K. E. & GEYER, W. A. 1994: Black walnut provenance performance in seven 22 year-old plantations. *Silvae Genet.* **43**: 246–252.

BROWN, A. H. D. & ALLARD, R. W. 1970: Estimation of mating system in open pollinated maize populations using isozyme polymorphism. *Genetics* **66**: 133–145.

BUSH, R. M., SMOUSE, P. E. & LEDIG, F. T. 1987: The fitness consequences of multiple locus heterozygosity: The relationship between heterozygosity and growth rate in pitch pine (*Pinus rigida* Mill.). *Evolution* **38**: 1151–1154.

CHEN, Y. P., CHIEN, C. T. & LIN, T. P. 2001: Allozyme variation of *Cyclobalanopsis championii* (Fagaceae), a narrowly distributed species in southern Taiwan. *J. Hered.* **92**: 65–70.

FARRIS, M. A. & MITTON, J. B. 1984: Population density, outcrossing rate and heterozygote superiority in ponderosa pine. *Evolution* **38**: 1151–1154.

FORNARI, B., CANNATA, F., SPADA, M. & MALVOLTI, M. E. 1999: Allozyme analysis of genetic diversity and differentiation in European and Asiatic walnut (*Juglans regia* L.) populations. *Forest Genetics* **6**: 115–127.

FOWELLS, H. A. 1965: Silvics of Forest Trees of the United States. USDA Agriculture Handbook. 71 p.

GURIES, R. P., BROWN, S., & KRESS, J. 1981: A Guide to Forest Tree Collections of Known Source or Parentage in Northeastern and North Central U.S. and Adjacent Canadian Provinces. University of Wisconsin – Madison, Agricultural. Experiment Station Research Bulletin R–3142. 72 p.

HAMRICK, J. L. 1989: Isozymes in the analysis of the genetic structure of plant populations. *In*: Isozymes in plant biology. (eds. P. S. Soltis & P. E. Soltis). pp. 87–105. Dioscorides Press, Portland, OR, USA.

MALVOLTI, M. E., FINESCHI, S. & PIGLIUCCI, M. 1994: Morphological integration and genetic variability in *Juglans regia* L. *J. Hered.* **85**: 389–394.

MALVOLTI, M.E., FINESCHI, S., MORGANTE, M. & VENDRAMIN, G. G. 1995: Mating system of a naturalized *Juglans regia* L. population in Italy. *In:* Population genetics and genetic conservation of forest trees (eds. PH. BARADAT, W.T. ADAMS & G. MUELLER-STARCK). pp. 305–308. SPB Academic Publishing, Amsterdam, The Netherlands.

MARTY, T. L., O'MALLEY, D. M. & GURIES, R. P. 1984: A manual for starch gel electrophoresis: new microwave edition. University of Wisconsin Staff Papers Series No. 20, 23 p.

MASTERS, C. J. 1974: The controlled pollination techniques and analyses of interspecific hybrids for black walnut. Ph.D. Dissertation, Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN, 122 p.

MCDANIELS, J. C. 1956. The pollination of Juglandaceae varieties – Illinois observations and review of earlier studies. 47th Annual Report of Northern Nut Growers Association. pp. 118–137.

MITTON, J. B. 1992: The dynamic mating systems of conifers. *New Forests* **6**: 197–216.

MCGRANAHAN, G. H., VOYIATZIS, D. G., CATLIN, P.B. & POLITO, V. S. 1994: High pollen loads can cause pistillate flower abscission in walnut. *J. American Soc. Hort. Sci.* **119**: 505– 509.

MORAN, G. F. & BROWN, A. H. D. 1980: Temporal heterogeneity of outcrossing rates in alpine ash *(Eucalyptus delegatensis* R. T. Bak). *Theor. Appl. Genet.* **71**: 201–207.

MORIN, R., BEAULIEU, J., DESLAURIERS, M., DAOUST, G. & BOUSQUET, J. 2000: Low genetic diversity at allozyme loci in *Juglans cinerea*. *Can. J. Bot.* **78**: 1238–1243.

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

NOWEG, T. A. & KURTZ, W. B. 1987: Eastern black walnut plantations: an economically viable option for conservation reserve lands within the corn belt. *North. J. Appl. For.* **4**: 158–160.

RINK, G. 1997: Genetic variation and selection potential for black walnut timber and nut production. *In:* Knowledge for the future of black walnut: Proceedings of the fifth black walnut symposium, Gen. Tech. Rep. NC–191. (ed. J. VAN SAMBEEK). pp. 58–62. USDA Forest Service, North Central Forest Experiment Station, St. Paul, MN.

RINK, G., CARROLL, E. & KUNG, F. 1989: Estimation of *J. nigra* L. mating system parameters. *For. Sci.* **35**: 623–627.

RINK, G., ZHANG, G., ZUO, J., KUNG, F. & CARROLL, E. 1994: Mating parameters in *J. nigra* L. seed orchard similar to natural population estimates. *Silvae Genet.* **43**: 261 –263.

RITLAND, K. 1988: Generalized Multilocus Estimation Program MTLF. Department of Botany, University of Toronto, Canada, Accompanied with documentation.

RITLAND, K. & JAIN, S. 1981: A model for estimation of outcrossing rate and gene frequencies, using independent

loci. *Heredity* **47**: 35–52.

RULE, L. C., COLLETTI, J. P., LIU, T. P., JUNGST, S. E., MIZE, C. W. & SHULTZ, R. C. 1994: Agroforestry and forestry related practices in the Midwestern United States. *Agrofor. Syst.* **27**: 79–88.

SAVOLAINEN, O. & HEDRICK, P. 1995: Heterozygosity and fitness: No association in Scots pine. *Genetics* **140**: 755–766.

SHAW, D. V., KAHLER, A. L. & ALLARD, R. W. 1981: A multilocus estimator of mating system parameters in plant populations. *Proc. Natl. Acad. Sci.* USA **78**: 1298–1302.

SOLTIS, D. E., HAUFLER, C. H., DARROW, D. C. & GASTONY, G J. 1983: Starch-gel electrophoresis of ferns: A compilation of grinding buffers, gel, electrode buffers and staining schedules. *Am. Fern J.* **73**: 9–27.

STRAUSS, S. H. 1986: Heterosis at allozyme loci under inbreeding and crossbreeding in (*Pinus attenuata*). *Genetics* **113**:115–134.

STREIFF, R., LABBE, T., BACILIERI, R., STEINKELLNER, H., GLOSSL, J. & KREMER, A. 1998: Within-population genetic structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. assessed with isozymes and microsatellites. *Mol. Ecol.* **7**: 317–328.

STROMBERG, J. C. & PATTEN, D. T. 1990: Flower production and floral ratios in southwestern riparian tree Arizona walnut (*Juglans major*). *Am. Midland Nat.* **124**: 278–288.

SWOFFORD, D. L. & SELANDER, R. B. 1989: Biosys-1, Release 1.7. A computer program for the analysis of allelic variation in population genetics and biochemical systematics. http://www.bio.net//hypermail/BIOSOFTWARE/bio-software.199212/0094.html accessed June 12, 2002.

WOESTE, K., BURNS, R., RHODES, O. & MICHLER, C. 2002: Thirty polymorphic nuclear microsatellite loci from black walnut. *J. Hered.* **93**: 58–60.

WRIGHT, S. 1965: The interpretation of population structure by F-statistics with special regard to mating. *Evolution* **19**: 395–420.