SAMPLING VARIATION IN BLACK WALNUT *(JUGLANS NIGRA* L.) OUTCROSSING RATES AND FIXATION INDICES

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Abstract: We studied black walnut mating parameters derived from electrophoretic analysis of open-pollinated embryos from local Jackson County, Illinois trees. Due to the periodicity of black walnut bearing, nuts could not be sampled from the same trees in all consecutive years. Thus we had nut collections from mother trees common to several or all years (1984, '87, '89, '91 and 1992) as well as nut collection from mother trees not common to all collection years. Also, there were sampling differences in allozyme patterns; in some years certain allozyme systems did not display variation and were not included in the data analysis. The objective of this study was to detect what effect the use of different open-pollinated families (i.e., mother trees), different years, and allozyme systems had on estimates of outcrossing rate and fixation indices. We applied analysis of variance to estimates of outcrossing rate and fixation indices developed from different subsets of data. The overall average outcrossing rate based on all five collection years was 0.97 (st. dev. = 0.17); the average inbreeding coefficient was -0.09 (st. dev. = 0.26). The implication of these parameters is that black walnut is predominantly an outcrossing species. However, results of the analysis of variance indicated that seed collection years had significant effects on outcrossing rates. Apparently black walnut outcrossing rates fluctuate somewhat from year to year. Outcrossing rates developed from use of seed collections with common mother trees were not significantly different from those developed using collections from trees uncommon to all years. Differences in allozyme systems used from year to year also had no significant effect on outcrossing rates.

Keywords: *Juglans nigra* L., isozymes, outcrossing.

INTRODUCTION

Black walnut *(Juglans nigra* L.) is an economically important species native to most of the eastern and central parts of the United States and southern Ontario, Canada. Black walnut is a monoecious, heterodichogamous, wind-pollinated tree species. It often occurs as a scattered, isolated tree or in small groups in the forest (Funk 1970). Pure natural stands of walnut are rare and usually small. Chief associates include yellow-poplar, white ash, black cherry and oaks (Schlesinger and Funk 1976). Low frequency of occurrence may result in inbreeding depression leading to poor tree quality in future generations. Intense harvesting may aggravate this by removing the best genotypes thus reducing the gene pool (Beineke 1989). Therefore, black

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walnut genetic resources need to be analyzed and conserved.

Population mating systems determine how individuals within a population mate and how the genetic information is transmitted between generations. Species with high outcrossing rates often maintain high genetic diversity with small differences among populations and high within population variation (Adams and Birkes 1991). If the mating system is mixed or if selfing is a part of the mating system, heterozygosity is typically lower. As a result, differentiation among such populations may increase (Loveless and Hamrick 1984; Hamrick and Godt 1990; Schoen and Brown 1991).

The earliest studies of mating systems often relied upon morphological markers. Such research was based on the behavior of pollinators and controlled crossing experiments. The results were often limited to the genotypes tested, the environments experienced, and the mode of measurement. For example, controlled pollination experiments were conducted to study the effects of inbreeding in black walnut (Beineke et al. 1976; 1977). Fortunately, the discovery of abundant and frequently codominant allozyme polymorphisms provided a valuable tool for estimating mating systems in tree species more effieiently. Electrophoresis was used to study allozyme variants (alleles) associated with specific genes to provide direct estimates of genetic variation at the DNA level. Such estimates are relatively free of environmental effects (Brown and Moran 1979). However, sampling variation among experimental units can affect mating parameter estimate. Brown (1990) found that using a larger number of families helped estimate the maternal fixation index accurately and stabilized variation of the inbreeding coefficients among loci. Shaw and Allard (1981) also found that use of different loci provided different results in Douglas-fir *(Pseudotsuga menziesii).* It was suggested that sampling more families and more polymorphic loci per family can increase precision in estimating mating system parameters (Shaw and Allard 1982). However, if alleles of marker loci or genotypes are associated with different flowering time, then heterogeneity in allele frequencies for these loci may result in temporal variation of estimates of mating system parameters (Sampson et al. 1990). In addition, micro geographic differentiation can also lead to inflated estimates of self-fertilization (Brown and Moran 1979).

Due to the periodicity of black walnut nut bearing, nuts could not be collected from the same trees in consecutive years. Thus sampling different families in different seed collection year could potentially affect estimation of mating system parameters. In addition, different enzyme systems were assayed in some years in attempting to find the best suitable isozyme markers for discriminating among black walnut genotypes. In this study we test the effect of different sampling schemes on the estimating mating system parameters.

MATERIALS AND METHODS

In the fall of 1984, 1987, 1989, 1991 and 1992, about 900 nuts per year were collected from the ground under the crowns of 24 to 37 trees that seemed representative of naturally growing walnut trees. No other selection criteria were imposed on the trees from which collections were made. Trees included are located in Jackson County, Illinois. To reduce the possibility of sampling closely related individuals, the distance between sampled trees was set at a minimum of 10 m, but most trees were between 0.5 and 1 km apart. Nuts collected from

these trees were dehusked and stratified at 2-5° C in the refrigerator. Embryos were removed and frozen at -80° C until used.

Before electrophoresis, embryos were individually ground and homogenized with an extraction buffer. The extraction buffer chosen was Marty et al. (1984). After homogenization, filter paper wicks were used to soak up the resulting liquid. A 2 mm x 15 mm wide wick per embryo was used to load samples into the gel with 30 samples per gel. The gels were placed into a refrigerator and 50 milliamperes/gel maximum electric current was applied for about 4.5 hours. After the first 20 minutes, the sampling wicks were removed from the gels to improve quality of enzyme migration.

When the indicator dye marker arrived at the anodal end of the gel (4.5 hours), the electric power was turned off and gels removed from the refrigerator. Each gel was marked in the upper right-hand corner of the gel to indicate its identity. Plexiglass guides (20mm x 240mm x lmm) and nylon sewing thread were used to slice the gel. Each gel was sliced into 8 to 10 horizontal slices with the top slice being discarded.

The gel slices were stained and then rinsed with distilled water three to four times and placed onto the light table to score the allozyme bands. Eight enzymes with enzyme activity were analyzed: aconitase (ACO); alcohol dehydrogenase (ADH); aspartate aminotransferase (AAT); 6-phosphogluconic dehydrogenase (6PG); phosphoglucose isomerase (PGI); fluorescent esterase (FEST); acid phosphatase (ACP); and phosphoglucomutase (PGM). These eight enzymes gave eleven isozyme loci detectable variation: ACO-1, ACO-2, ADH, AAT-1, 6PG-2, PGI-2, FEST, ACP-2, PGM-1, PGM-2, PGM-3.

We partitioned the complete data set according to four control variables: (1) two levels of family identity (common between years as yes and no); (2) four levels of repeated seed collection (repeated 2, 3, 4, or 5 times); (3) five levels of sampling year (sampled in 84, 87, 89, 91, or 92); and (4) three levels of enzyme identity (alike loci, different loci, or all loci).

Each data subset was processed by the Multilocus Estimation Program (Ritland and Jain 1981) to obtain one estimate each for multilocus outcrossing rate (MT) single locus outcrossing rate (ST) and fixation indices (f). We had a total 179 subsets of data to estimate the mating parameters.

The three mating parameters (MT, ST, and f) were used as dependent variables and the four factors (CYON, NYR, YR and EM) were used as classification variables in a general linear model to test the null hypothesis that there are no differences among main effects and no differences among two-factor interactions. The F-values for testing each effect were obtained from PROC GLM (SAS 1988).

RESULTS AND DISCUSSION

The linear models for the three mating parameters are all significant. More than 60% of the total variance in outcrossing rate and 37% in inbreeding coefficient were explained by the linear model. The population mean outcrossing rate in black walnut was high in comparison with

its root mean square error (RMSE). Results for the multilocus estimate are similar to that for the single locus estimate (mean MT = 0.969 and mean ST = .965, RMSE MT = 0.128 and RMSE ST = .146). The implication of high outcrossing rate and low RMSE indicate that we can accept the hypothesis MT=ST=1 and reject the hypothesis MT=ST=0. Furthermore, the population inbreeding coefficient was not significantly different from zero (mean =-0.094, RMSE = 0.234). Thus, black walnut is predominantly an outcrossing species.

None of the main factors were significant for the fixation index. Outcrossing rates and fixation indexes developed from use of seed collections with common mother trees were not significantly different from those developed using collections from trees not represented in all years. This is in agreement with random sampling theory where sampling with replacement, sampling with partial replacement, and sampling without replacement all result in essentially the same estimate of population mean (Cochran 1977). Also, there was no significant effect of using various numbers of years in repeated seed collection. Among the four main factors, the effect of collection year was significant on multilocus and single locus outcrossing rates (Table 1). Apparently black walnut outcrossing rates fluctuate somewhat from year to year. Outcrossing rates were lower in the early years (1984 and 87) than that in the later years (1989, '91 and '92) The use of the same or different enzyme systems did not seem to have an effect on estimating the multilocus outcrossing rate, but did affect the single locus outcrossing rate. Using noncommon loci data resulted in lower values for the single locus outcrossing rates than when only common loci were used.

Table 1. F-Value and level of Significance for the four main effects and for the six interactions. The dependent variables in the general linear model were multilocus (MT) and single (ST) locus outcrossing rate, and inbreeding coefficient (f).

Source	DF	F-Value for		
		MT	ST	f
CYON	1	2.23	0.14	3.82
NYR	3	0.13	0.03	1.12
YR	4	18.29***	24.92***	1.92
EM	2	1.32	5.79***	0.40
CYON*NYR	3	0.65	0.46	0.74
CYON*YR	4	5.03***	3.92**	4.69**
CYON*EM	2	2.36	1.52	0.41
NYR*YR	12	0.61	1.04	1.09
NYR*EM	6	1.27	0.33	0.24
YR*EM	8	6.68***	10.30***	3.39**

*** Significant at the 0.001 level.
** Significant at the 0.01 level.

The interaction between family replacement and collection year was significant (Table 1). Outcrossing rates peaked at 1987 for common families, but for the noncommon families the

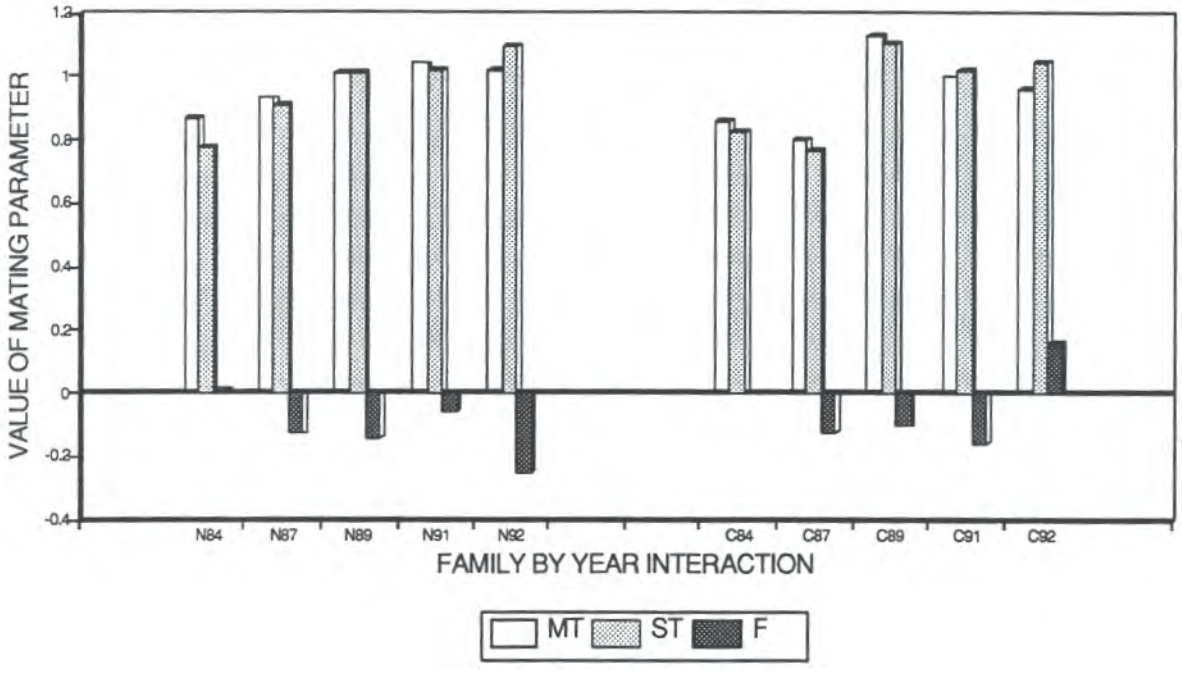

Figure 1. Multilocus (MT), single locus (ST) outcrossing rate and fixation index (F) between common (C) and noncommon (N) families in five years.

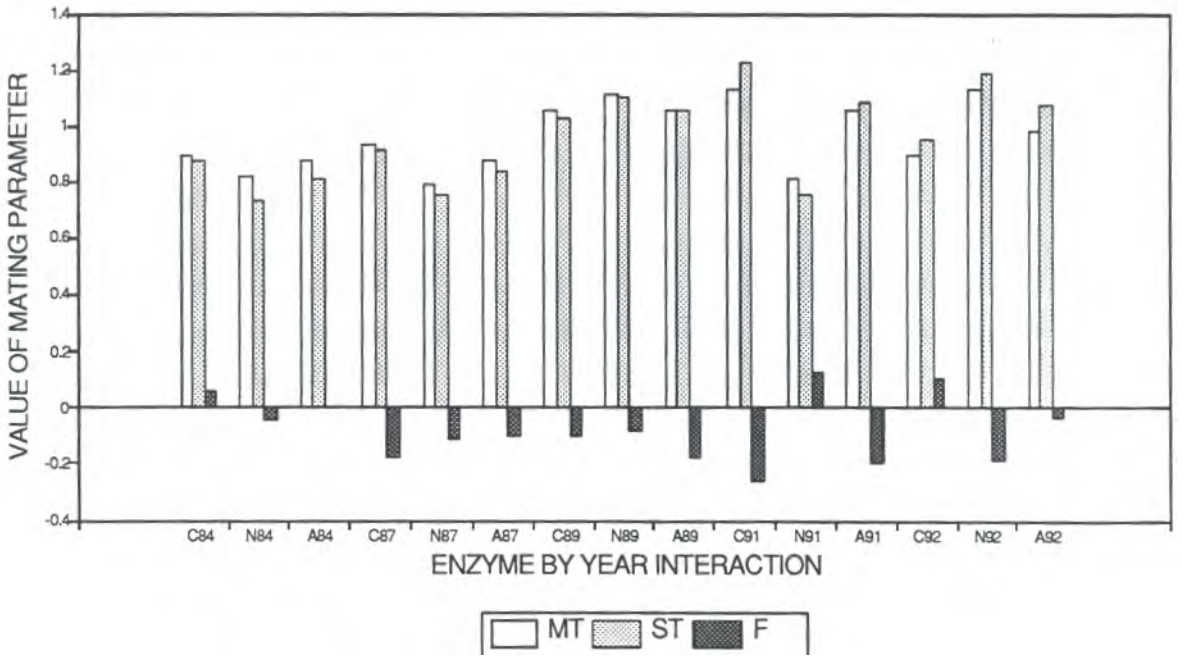

Figure 2. Multilocus (MT), single locus (ST) outcrossing rate and fixation index (F) among alike (C), different (N) and all (A) loci in five years.

outcrossing rate remained average. The trend was different between common and noncommon families; both multilocus and single locus outcrossing rates increased from 1984 to 1987 when data from the noncommon families were used, but with common families, outcrossing rates decreased (Figure 1). The fixation indeces from '87 to '92 were all negative for the noncommon families, but for common families the fixation index became positive in 1992.

Another significant interaction was observed with enzyme and year effects (Figure 2). In 1989 and in '92, the use of different loci provided higher estimates for outcrossing rates than the use of identical loci. However, in 1987 and in '91, outcrossing rates were lower if different loci were used. The trend was dissimilar between alike loci and different loci. The fixation index in 1991 was negative and in 1992 was positive for using alike loci, while using different loci the estimate in 1991 was positive and in 1992 was negative (Figure 2)

CONCLUSION

The choice of using alike or different loci will affect the estimate of single locus outcrossing rate appears to have a lessen impact on the multilocus outcrossing rate. The error variance for the multilocus outcrossing rate is smaller than that for the single locus outcrossing rate. Thus, using multilocus outcrossing rate for estimating mating parameters is preferred.

Mating parameters estimated from single year data may not be representative of the population mean. The choice of loci and the choice of families will affect the single year estimates. However, the choice of loci and the choice of families may have no effect on the mating parameters if more samples were collected in many years.

For the study of outcrossing rates, it is recommended that seeds should be collected for several years. When the same mother trees were not producing adequate number of nuts during the lean years, additional nuts may be collected from additional different mother trees. It may not be necessary to use the identical allozyme systems throughout the course of study.

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