

respectively are now already registered for trade in the Federal Republic of Germany.

The research on in vitro-techniques presented in this report is therefore of far-reaching practical significance. We are convinced that the measures already successfully begun or planned for the near future will open up new and hopeful perspectives for the breeding and cultivation of poplars in our country.

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Linkage Disequilibria Among Allozyme Loci in Natural Populations of *Liriodendron tulipifera* L.

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Summary

Linkage disequilibria between pairs of six isozyme loci were studied in natural populations of yellow poplar (*Liriodendron tulipifera* L.) from six locations in North Carolina. Genotypic data were used to estimate the BURROWS composite measure of linkage disequilibrium. In three of the locations, linkage disequilibria were estimated for populations belonging to two different generations. Trees in the older generation were seed parents of seedlings studied in the younger generation. Extensive linkage dise-

quilibrium was found in the seedling populations, whereas in the sexually mature populations only weak evidence for disequilibria was detected. In the seedling populations, disequilibria were found at five of the six locations studied. This result indicates that linkage disequilibria are widespread during the early seedling stage of development in this species.

Key words: Gametic Phase Disequilibrium, BURROWS Measure, Yellow-Poplar, Correlation, Digenic Measure.

Zusammenfassung

Bei natürlichen Populationen von *Liriodendron tulipifera* auf 6 Standorten im nördlichen Carolina wurden Kopplungsgleichgewichte zwischen 6 Isoenzym-Loci-Paaren untersucht.

Um das Kopplungsgleichgewicht der zusammengesetzten Messung nach BURROWS zu schätzen, wurden genotypische

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Daten benutzt. Auf 3 Standorten wurde das Kopplungsgleichgewicht für die zu 2 verschiedenen Generationen gehörenden Populationen geschätzt. Bäume der älteren Generation waren die Eltern der Sämlinge, die in der jüngeren Generation untersucht wurden. In den Sämlingspopulationen wurden ausgedehnte Kopplungsgleichgewichte gefunden, während bei den älteren fruktifizierenden Populationen nur schwache Beweise für Ungleichgewichte zu erkennen waren. In den Sämlingspopulationen wurden Ungleichgewichte an 5 von 6 untersuchten Standorten gefunden.

Dieses Ergebnis zeigt, daß Kopplungsgleichgewichte in der frühen Jugendentwicklung bei dieser Baumart weit verbreitet sind.

Introduction

Questions concerning population structure, mating systems, divergence of populations and the effects of selection have been extensively investigated in natural populations of forest tree species using analyses of allozyme loci. This research generally considers results for a number of loci but tends to emphasize effects measured on a single locus basis. Although findings are usually summarized over loci and comparisons are made between results obtained at different loci, little work has been done in which the joint behavior of two or more loci has been considered. If loci essentially behave as independent units, evolutionary processes can be effectively studied on a single locus basis and results averaged over loci. However, there are substantial reasons to suspect that not all evolutionary processes involve loci acting independently and that in many species multiple-locus associations are important. In such cases, it is inappropriate to use descriptive statistics of single-locus measures to summarize effects over loci. Natural selection, genetic sampling due to small effective population sizes, and restricted migration of gametes among subdivisions can produce linkage disequilibria among loci and, furthermore inbreeding as well as population subdivision are known to impede the rate at which such disequilibria decay (HEDRICK, *et al.*, 1978).

Here we describe results of an investigation on two-locus linkage disequilibrium for pair-wise combinations of six isozyme loci in several natural populations of yellow-poplar (*Liriodendron tulipifera* L.). Evidence in the single-locus analyses for population substructure in newly germinated seedling populations and for a mixed mating system resulting in some inbreeding (BROTSCHOL, 1983) suggested that linkage disequilibria might exist in these populations. Since both population subdivision and inbreeding retard the decline of linkage disequilibrium, any disequilibria that recently became established in these populations would not have fully dissipated at the time of our sampling even in the absence of selection and should be detectable.

Digenic Measures of Linkage Disequilibrium

Following the notation of WEIR (1979), let the frequency of genotypes with the i^{th} and k^{th} alleles at the first locus and with j^{th} and l^{th} alleles at the second locus be indicated by $p_{kl}^{ij} = p_{kj}^{il}$. Our analyses include multiple allelic loci so consider that i and k represent alleles 1, 2, . . . , m , and j and l represent alleles 1, 2, . . . , n . Gametic frequencies with alleles i and j at the two loci can be represented by *Formula 1*:

$$p_{..}^{ij} = \sum_{k1} p_{k1}^{ij} \quad (1)$$

Likewise, the frequency of the nongametic combination of alleles i and j is given by *Form. 2*:

$$p_{.j}^i = \sum_{k1} p_{kj}^{i1} \quad (2)$$

Allele frequencies are represented by p_i for the first locus and q_j for the second.

The usual coefficient of linkage disequilibrium is a measure of gametic disequilibrium and for gametes with the i^{th} and j^{th} alleles can be written as in *Form. 3*:

$$D_{..}^{ij} = p_{..}^{ij} - p_i q_j \quad (3)$$

This measure can be decomposed into two disequilibrium components (COCKERHAM and WEIR, 1977, and WEIR, 1979),

$$D_{..}^{ij} = D_w^{ij} + D_b^{ij} \quad (4)$$

which are referred to as the within individuals and the between individual components respectively. Here, $D_w^{ij} = p_{..}^{ij} - p_{ij}^i - p_{ij}^j$ and is equal to half the difference in frequency of coupling and repulsion double heterozygotes, while $D_b^{ij} = p_{..}^{ij} - p_i q_j$ is a measure of nonrandom union of gametes (WEIR and COCKERHAM, 1979).

If gametic data are available, or if repulsion and coupling double heterozygotes can be distinguished, $D_{..}^{ij}$ can be estimated without difficulty. However, for genotypic data in which the two types of double heterozygotes cannot be distinguished, $D_{..}^{ij}$ can only be measured if gametes unite at random, i.e., $D_b^{ij} = 0$. For situations in which the two types of double heterozygotes cannot be identified and random union of gametes cannot be assumed, the Burrows composite measure (*Form. 5*):

$$\Delta_{ij} = p_{..}^{ij} + p_{.j}^i - 2p_i q_j \quad (5)$$

can be used (COCKERHAM and WEIR, 1977). An equivalent expression of this measure is given in *Form. 6*:

$$\Delta_{ij} = D_w^{ij} + 2D_b^{ij} \quad (6)$$

It is apparent that with random union of gametes $\Delta_{ij} = D_{..}^{ij}$.

Linkage disequilibria for pairs of loci can also be measured in terms of correlation coefficients. Such a measure based upon $D_{..}^{ij}$ (*Form. 7*) is:

$$R_{ij} = D_{..}^{ij} / [p_i(1 - p_i) q_j(1 - q_j)]^{1/2} \quad (7)$$

A similar correlation coefficient based upon Δ_{ij} is given by *Form. 8*:

$$R_{ij}^* = \Delta_{ij} / [(p_i(1 - p_i) + D_{..}^{ij})(q_j(1 - q_j) + D_{..}^{ij})]^{1/2} \quad (8)$$

$$\text{where } D_{..}^{ij} = p_{..}^{ij} - p_i^2$$

$$\text{and } D_{..}^{ij} = p_{..}^{ij} - q_j^2$$

Both coefficients are bounded by -1 and 1 , and are useful for comparing disequilibria for nonallelic combinations involving different allele frequencies. A more detailed description of these measures is found in WEIR (1979).

Materials and Methods

Populations and Loci Studied

The populations sampled were from widely separated locations in North Carolina. In three of the locations —

Bernardsville, Poplar Cove, and Duke Forest — seeds were collected from the ground or from felled trees (hereafter referred to as ground collections). Isozyme assays were made from 100 newly germinated seedlings chosen at random to represent each population. The Bernardsville and Poplar Cove sites are in the mountains of western North Carolina, whereas the Duke Forest site is in the central North Carolina Piedmont.

At the remaining three locations, Bent Creek and the two coastal plain sources (organic and mineral) seeds were collected and kept separate by individual tree seed lots. Twenty seedlings per parent tree were assayed for isozymes. The Bent Creek site is in the mountains of western North Carolina and the two coastal plain sites, one on organic soil and the other on mineral soil, are about 15 miles apart in southeastern North Carolina. In these populations, genotypes for each parental tree were determined from the genotypic composition of its progeny sample using the maximum likelihood method of BROWN and ALLARD (1970). Since genotypic determinations were available for parents and progeny, linkage disequilibria could be studied for both.

Four enzyme systems were analyzed using starch gel electrophoresis. Two polymorphic loci were studied for acid phosphatase (ACPH1 and ACPH2) and glutamate oxaloacetate transaminase (GOT1 and GOT2). One polymorphic locus was investigated for both the peroxidase (PER) and esterase (EST) systems. Two alleles per locus were detected for ACPH1, ACPH2, PER, and GOT2. Four alleles were detected for GOT1 and five alleles were detected at the EST locus.

Further information about the populations, method of sampling, electrophoretic techniques, and banding patterns for the isozymes studied can be found in BRORSCHOL (1983).

Estimation and Hypothesis Testing

The statistical procedures used are suitable for codominant loci and are based on genotypic frequency data in which coupling and repulsion double heterozygotes cannot be distinguished. A method for obtaining maximum likelihood (ML) estimates for d_{ij}^{ij} using genotypic data was introduced by HILL (1974). However, this procedure is only appropriate when random association of gametes, i.e., $d_{ij}^{ij} = 0$, has occurred (WEIR and COCKERHAM, 1979). Single-locus results for our progeny populations indicate the presence of population subdivision and some inbreeding (BRORSCHOL, 1983). Clearly then, an assumption of random association of gametes is not justified for these populations and Hill's method cannot be relied upon to provide proper estimates of linkage disequilibria. However, the BURROWS composite measure of linkage disequilibrium (Δ_{ij}) is estimable under conditions of nonrandom union of gametes and incomplete identification of genotypes and is appropriate for our data.

ML estimates of Δ_{ij} can be computed directly from genotypic data (WEIR 1979) and are given by Form. 9:

$$\tilde{\Delta}_{ij} = \tilde{p}_{ij}^{ij} + \tilde{p}_{ij}^i - 2\tilde{p}_i\tilde{q}_j \quad (9)$$

where the tildes represent ML estimates. Although the ML estimator is not unbiased for finite sample sizes, the unbiased estimator is the following function of the ML estimator (Form. 10):

$$\hat{\Delta}_{ij} = N\tilde{\Delta}_{ij}/(N-1) \quad (10)$$

where N represents the size of the sample. When gametes unite at random, $d_{ij}^{ij} = 0$ and estimates of Δ_{ij} reduce to estimates of d_{ij}^{ij} . In this paper we report values for the correlation coefficient (r_{ij}^{ij}) computed from estimates of Δ_{ij} . An estimator for this correlation (WEIR, 1979) is shown in Form. 11:

$$r_{ij}^{ij} = \tilde{\Delta}_{ij} / [\tilde{p}_i(1-\tilde{p}_i) + \tilde{D}_{ij}^i(\tilde{q}_j(1-\tilde{q}_j) + \tilde{D}_{ij}^j)]^{1/2} \quad (11)$$

Here:

$$\tilde{D}_{ij}^i = \tilde{p}_{ij}^i - (\tilde{p}_i)^2 \quad \text{and} \quad \tilde{D}_{ij}^j = \tilde{p}_{ij}^j - (\tilde{q}_j)^2$$

If d_{ij}^{ij} is the only disequilibrium present, r_{ij}^{ij} is then clearly based only on d_{ij}^{ij} . Several investigators have used this estimator to study linkage disequilibria in other organisms (LANGLEY *et al.*, 1978; LAURIE-AHLBERG and WEIR, 1979).

A chi square test can be used to test the hypothesis H_0 : all $\Delta_{ij} = 0$. The test statistic (Form. 12):

$$\chi^2 = N \sum_{ij} (\tilde{\Delta}_{ij})^2 / \tilde{p}_i\tilde{q}_j \quad (12)$$

is distributed as χ^2 with $(m-1)(n-1)$ degrees of freedom under this null hypothesis (WEIR 1979). If random union of gametes occurs, this test actually provides a test of the hypothesis $d_{ij}^{ij} = 0$.

A test of the composite null hypothesis H_0 : $\Delta_{ij} = 0$ is also equivalently a test of (Form. 13):

$$D_{ij}^{ij} = -D_b^{ij} \quad (13)$$

If this hypothesis is true, either of the two following indistinguishable conditions (Form. 14) holds:

$$D_{ij}^{ij} = D_b^{ij} = 0, \quad \text{or} \quad (14)$$

$$D_{ij}^{ij} \neq 0, \quad D_b^{ij} \neq 0 \quad \text{and} \quad D_{ij}^{ij} = -D_b^{ij}$$

If the null hypothesis is false, then $d_{ij}^{ij} \neq -d_b^{ij}$ and one of three conditions (see Form. 15) must hold:

$$1.) \quad D_{ij}^{ij} \neq 0 \quad \text{and} \quad D_b^{ij} = 0, \quad \text{or} \quad (15)$$

$$2.) \quad D_{ij}^{ij} = 0 \quad \text{and} \quad D_b^{ij} \neq 0, \quad \text{or}$$

$$3.) \quad D_{ij}^{ij} \neq 0, \quad D_b^{ij} \neq 0 \quad \text{and} \quad D_{ij}^{ij} \neq -D_b^{ij}$$

If the frequency of coupling heterozygotes equals the frequency of repulsion phase heterozygotes $d_{ij}^{ij} = d_b^{ij}$ and only the third condition is possible when the null hypothesis is false. These two conditions then imply that $d_{ij}^{ij} = d_b^{ij} > 0$ and the disequilibrium is entirely due to nonrandom union of gametes.

Results and Discussion

Considerable linkage disequilibria were present in the seedling populations we studied. In the three populations in which seed was collected by individual trees, 30 locus pairs out of 39, or 76%, had one or more nonallelic combinations that were significantly different from zero (Table 1). While the number of locus pairs with significant disequilibria was not as great in the populations sampled by ground collections, substantial numbers of significant disequilibria still were found. Seven locus pairs out of the 33 analyzed, or 21%, had significant disequilibria (Table 2).

The difference in the number of significant disequilibria observed in these two populations is probably, for the most part, a reflection of differences in sample size.

Evidence for linkage disequilibria was also found in the mature tree populations, but it was not as strong as in the seedling populations. Significant disequilibria were detected for 3 of the 25 locus pairs investigated, or 12% (Table 3). Such an incidence of significant disequilibria, though low, is still greater than the level expected from random causes if the locus combinations acted independently. Because of the disparity in sample sizes for the two types of populations, it is not clear whether the lower incidence of significant disequilibria observed in the mature tree populations indicates a true increase in disequilibria in the seedling populations.

The existence of linkage disequilibria in yellow-poplar populations focuses attention on the amount of variability in disequilibria over populations. Although a formal analysis of variability was not undertaken, evidence for differences in levels of disequilibria was found among the seedling populations sampled by individual parent tree collections. More specifically, r_{ij} values were compared over populations for nonallelic combinations with significant linkage disequilibria. A chi square test of linkage disequilibrium for a nonallelic combination is $\chi^2 = n r_{ij}^2$ (Weir, 1979).

Nonallelic combinations with significant disequilibria in two or more populations but also with r_{ij} values that differ in sign across populations indicate differences in linkage disequilibria among populations. Disequilibrium patterns of this type were only studied in the seedling populations sampled by collections from individual parent trees. In these populations, sign changes of this type were found for at least one nonallelic combination at 4 out of the 15

Table 1. — Values of r_{ij} for the three seedling populations in which seed were collected by individual parent trees. Values are presented for the nonallelic combinations with maximum absolute values for $\tilde{\Delta}_{ij}$.

LOCUS PAIR	POPULATION		
	BENT CREEK	COASTAL PLAIN ORGANIC	COASTAL PLAIN MINERAL
ACPH1 x ACPH2	.1210***	-.0322	.1144***
ACPH1 x PER	-.1079***	-.0990**	-.0696*
ACPH1 x EST	.0331	.1955***	-.1111***
ACPH1 x GOT1	-.1366***	.1264***	-.0713**
ACPH1 x GOT2	—	.0628*	-.0575*
ACPH2 x PER	.0756*	—	-.0055
ACPH2 x EST	-.1901***	.1127***	-.0959***
ACPH2 x GOT1	.1394***	.1802***	-.0854***
ACPH2 x GOT2	—	-.0479	.0461
PER x EST	.0337	.0772	-.1655***
PER x GOT1	-.0514*	-.0592	.0595*
PER x GOT2	—	-.0141	.1327***
EST x GOT1	.1224***	-.1664***	-.1511***
EST x GOT2	—	-.1471***	.1379***
GOT1 x GOT2	—	-.3689***	.2001***
N	629	699	826

*** indicates significance at 0.5% level for test of H_0 : all $\Delta_{ij} = 0$.

** indicates significance at 1% level for the above H_0 .

* indicates significance at 5% level for the above H_0 .

Table 2. — Values of r_{ij} for seedling populations sampled from the ground or from felled trees. Values are presented for the nonallelic combinations with maximum absolute values for $\tilde{\Delta}_{ij}$.

LOCUS PAIR	POPULATION		
	BERNARDSVILLE	POPLAR COVE	DUKE FOREST
ACPH1 x ACPH2	—	.2557***	-.0327
ACPH1 x PER	-.0058	.1184	.0167
ACPH1 x EST	.0937	.1256	.0975
ACPH1 x GOT1	-.0056	-.2082*	.1302
ACPH1 x GOT2	—	-.0069	-.1588
ACPH2 x PER	—	—	.2184*
ACPH2 x EST	—	-.0804	-.3056***
ACPH2 x GOT1	—	-.1359	.2228*
ACPH2 x GOT2	—	-.0619	.1365
PER x EST	—	-.1809	.1298
PER x GOT1	—	.1179	.0215
PER x GOT2	—	.0723	-.0169
EST x GOT1	-.1724	.1476	.1325
EST x GOT2	—	-.1481	.3167***
GOT1 x GOT2	—	-.2560*	-.1345
N	100	100	100

*** indicates significance at .5% level for test of H_0 : all $\Delta_{ij} = 0$.

** indicates significance at 1% level for the above H_0 .

* indicates significance at 5% level for the above H_0 .

Table 3. — Values of r_{ij} for mature tree populations. Values given are for the nonallelic combinations with maximum absolute values for $\tilde{\Delta}_{ij}$.

LOCUS PAIR	POPULATION		
	BENT CREEK	COASTAL PLAIN ORGANIC	COASTAL PLAIN MINERAL
ACPH1 x ACPH2	.0863	—	.0141
ACPH1 x PER	-.2368	—	—
ACPH1 x EST	-.0919	.2412	-.1874
ACPH1 x GOT1	.3299	.1034	.0068
ACPH1 x GOT2	—	.2236	-.2067
ACPH2 x PER	—	—	—
ACPH2 x EST	-.5241**	—	.1580
ACPH2 x GOT1	-.0485	—	.1446
ACPH2 x GOT2	—	—	.0529
PER x EST	-.1921	—	—
PER x GOT1	-.1921	—	—
EST x GOT1	-.1983	.2813	.2547
EST x GOT2	—	.2474	-.1540
GOT1 x GOT2	—	-.3647*	-.2879**
N	33	37	44

** Indicates significance at 1% level for test of H_0 : all $\Delta_{ij} = 0$.

* Indicates significance at 5% level for the above H_0 .

locus pairs studied. This is only a conservative indication of differences in disequilibria and thus suggests that there is substantial variation in linkage disequilibria among seedling populations. This method, however, does not provide information about variation in disequilibria when the criterion for detecting variation is not observed. Therefore in the group of populations sampled by ground collections (Bernardsville, Poplar Cove and Duke Forest) no

information about variability in disequilibria was obtained. Within this group of populations, none of the locus pairs showed significant disequilibria in more than one population (Table 2).

The pattern of disequilibria at the GOT1 and GOT2 locus pair was particularly striking. Significant disequilibria were found in five of the six populations in which this combination of loci was studied (Tables 1—3). Pairs of nonalleles that showed significant disequilibria in several populations were consistent with respect to the sign of the disequilibria across these populations. Such a uniform pattern of disequilibria suggests that a strongly expressed control factor is responsible, possibly tight linkage.

Since analyses on a single-locus basis revealed that seedling populations have large departures from Hardy-Weinberg expected frequencies (BROTSCHOL, 1983), nonrandom union of gametes might be responsible for sizeable two-locus disequilibria components. However the Burrows measure cannot be used to distinguish components of disequilibria due to nonrandom union of gametes (D_{ij}^{ij}) from differences in frequency for coupling and repulsion double heterozygotes. Populations may have sizeable components for D_{ij}^{ij} and D_{ij}^{ij} but these may differ in sign resulting in a small Δ_{ij} .

Estimates of D_{ij}^{ij} can be obtained directly from samples that have no double heterozygotes (see Form. 16). In these cases,

$$\tilde{D}_w^{ij} = 0 \text{ and } \tilde{D}_b^{ij} = \tilde{D}_{..}^{ij} = \tilde{\Delta}_{ij}/2. \quad (16)$$

Also population samples in which double heterozygotes are rare, provide opportunities for estimating D_{ij}^{ij} . Under this condition, frequencies for both coupling and repulsion double heterozygotes can be arbitrarily set equal to one-half the observed frequency of double heterozygotes without causing substantial error in estimating linkage disequilibrium (WEIR *et al.* 1972). Estimates of D_{ij}^{ij} and D_{ij}^{ij} using this method (indicated by D_{ij}^{ij} and D_{ij}^{ij}) are equal since estimates of coupling and repulsion heterozygote frequencies are equal. Hence $D_{ij}^{ij} = D_{ij}^{ij} = \tilde{\Delta}_{ij}/2$. Values for D_{ij}^{ij} were computed for locus pairs with one or two double heterozygotes in the population samples. It can be shown that maximum estimation error directly attributable to this technique is $\pm 1/2N$. Clearly, when there are no double heterozygotes in the sample, $D_{ij}^{ij} = D_{ij}^{ij}$. Ten locus pairs in the mature tree populations and 21 in seedling populations had two or fewer double heterozygotes in the samples taken and thus meet our criterion for using the estimator D_{ij}^{ij} .

In the mature tree populations only 1 locus pair out of the 10 investigated had a significant D_{ij}^{ij} , providing little evidence for nonrandom union of gametes. However, since the number of locus pairs studied was small, these results are probably best interpreted as being inconclusive.

In the seedling populations, D_{ij}^{ij} was significant for one or more nonallelic combinations at 6 locus pairs, or 28.6%, of 21 investigated. These results indicate that nonrandom union of gametes contributes to the linkage disequilibria in the seedling populations. In this respect, results for the two-locus analyses appear to corroborate the conclusion of nonrandom union of gametes drawn from the single-locus analyses.

Evidence for linkage disequilibria in mature tree populations is not strong although our results indicate that some disequilibria are present. Additional studies with larger sample sizes are needed to establish conclusively the extent to which linkage disequilibria exist in these populations. Of particular interest is whether disequilibria present in the seedling stage persist into the mature tree phase of the same population or is either reduced or increased.

Undoubtedly, several of the factors that were found to contribute to the departure from Hardy-Weinberg proportions at the single-locus level in the seedling populations also contributed to the linkage disequilibrium observed. For example, amalgamation of subdivisions as well as differences in gene frequencies in the male and female gamete populations are known to cause linkage disequilibrium (COCKERHAM and WEIR, 1977). Indeed, differences were found in allele frequencies between pollen and maternal tree populations and evidence of population substructure was found in the seedling populations (BROTSCHOL, 1983). Other processes such as zygotic selection also can influence linkage disequilibria and may have contributed to the disequilibria observed. However, we do not yet have information about the importance of such processes in natural populations of yellow-poplar.

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