Linkage studies in Pinus sylvestris L. – using macro gametophyte allozymes mes

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Summary

Linkage studies between 12 allozyme (allelic isozyme) loci from macrogametophytes of Pinus sylvestris are reported. Seeds for isozyme analyses were collected mainly from single trees in one seed tree stand in northern Sweden, but trees from other parts of Sweden were also included. The following enzyme systems and numbers of loci are represented: Acid phosphatases (PHOS) — twu loci, alcohol dehydrogenases (ADH) — two loci, esterases (EST) — one locus, glutamate oxalate transaminases (GOT) — three loci, leucine aminopeptidases (LAP) — two loci and malate dehydrogenases (MDH) — two loci. Four of these ADH-MA and -MB — LAP-B — GOT-B are associated in a well established linkage group I. Three single trees show linkage between ADH-MB in group I and GOT-A, LAP-A and MDH-MB. Possible linkage may also exist between MDH-MB — PHOS-MA — GOT-MC. This linkage group is indicated by data from five trees. Estimations of map distances have been made according to KOSAMBI (1944)and JENSEN and HELMS JÖRGENSEN (1975). Map distances between ADH-MA and -MB were found to be zero but the distance between these and LAP-B were estimated to be 32.8 ± 3.1 cM (centi Morgan) and between LAP-B — GOT-B 15.5 ± 1.4 cM. A positive interference within group I was found in two triple heterozygotes.

Key words: Pinus sylvestris, isozyme loci linkage, macrogametophytes, *interference.*

Zusammenfassung

Es wird über Untersuchungen zur Kopplung von 12 Allozym-Loci (allelische Isoenzyme) bei Makrogametophyten von Pinus sylvestris berichtet. Samen für die Isoenzymanalysen wurde im wesentlichen von Einzelbäumen eines Saatguterntebestandes in Nordschweden eingesammelt. Zusätzlich wurden einzelne Bäume aus anderen Teilen Schwedens einbezogen. Die folgenden Enzymsysteme mit den jeweiligen Anzahlen der Loci werden dargestellt: Saure Phosphatasen (PHOS) — zwei Loci, Alkohol Dehydrogenasen (ADH) — zwei Loci, Esterasen (EST) — ein Locus, Glutamat Oxalat Transarninasen (GOT) — drei Loci, Leucin Aminopeptidase (LAP) — zwei Loci und Malat Dehydrogenase (MDH) — zwei Luci. Vier von diesen, ADH-MA und -MB — LAP-B — GOT-B, sind in einer Kopplungsgruppe I vereinigt. Drei einzelne Bäume zeigen Kopplung zwischen ADH-MB in Gruppe I und GOT-A sowie MDH-MB. Kopplung könnte auch zwischen MDH-MB — PHOS-MA — GOT-Mc bestehen. Der Nachweis dieser Kopplungsgruppe basiert auf Daten von fünf Bäumen. Berechnungen für Genkarten wurden gemäß KOSAMBI (1944) und JENSEN und HELMS JÖRGENSEN (1975) durchgeführt. Der Genabstand zwischen ADH-MA und -MB scheint null zu sein, der Genabstand zwischen diesen beiden und LAP-B ist nach unseren Berechnungen 32,8 I 3,1 cM (centi Morgan), und zwischen LAP-B — GOT-B 15,5 ± 1,4 cM. Eine positive Interferenz innerhalb der Kopplungsgruppe I wurde mit Hilfe von zwei Tripelheterozygoten festgestellt.

1. Introduction

In plant breeding, gene maps facilitate research work considerably. In order to be able to draw the right conclusions from studies of population structure and dynamics it is important to know whether available loci are representatively distributed in the linkage groups of the genome. It is also desirable to have some reference loci on the chromusomes in relation to which new genes can be localized.

If economically important genes are inherited in blocks, gene markers closely linked to them are of great value. There are several examples of block inheritance reported in plant species. Gossypium fur instance has an array of closely linked modifier genes for petal plot (STEPHENS 1949, 1950). In Zea *mays* a number of genes giving rise to poor endosperm are inherited in one block (MANGELSDORF 1958). Another well-known example is that of powdery mildew resistance genes in Hordeum located close together in chromosome five (cf. NILAN 1964).

Population and genetic analyses should include the recombination frequency between interacting loci in order to facilitate a relevant interpretation of the results. Examples of this were demonstrated by GRIFFING (1960) and LEWONTIN (1964). They pointed out that an expected response to selection includes functions of the linkage between pairs of involved loci.

For most diploid species linkage studies and subsequent gene mapping is laborious and complicated. WRICHT (1976) stated that the difficulties in gene mapping are even greater in most forest trees on account of the long generation time. He pointed out the possibility of tracing linkage groups by observing to which extent traits were associated in very different populations. If the loci for the traits studied are closely linked, it might be expected that, for instance, two traits which are associated in one way in one population are associated in exactly the opposite way in another population.

For allozyme (allelic isozymes) loci in conifers, linkage studies are in some respects simplified by the use of haploid macrogametophyte analysis.

Two short papers reporting linkage studies between allezyme loci in macrogametophytes from *Picea abies* have been published by BERGMANN (1974) and LUNDKVIST (1974). BERGMANN studied linkage between two loci for each of LAP, PHOS and EST. He found an indication of linkage between the two EST-loci. LUNDKVIST did not find any link-

Table 1. — The alleles of heterozygous loci in the trees included in the present investigation.

Latitude of origin	Site	Tree nr	LOCUS											
			ADH-MA	ADH-MB	EST-MA	GOT-A	GOT-B	GOT-MC	LAP_A	LAP-B	MDH-MA	MDH-MB	PHOS-MA	PHOS-MB
65° 29'	Gårdtjärn													
		112				A1/A2	B2/B3							
		115			MA1/MA0		B2/B3							
		116		MB2/MB3			B18/B3							
		128			MA1/MA0	A1/A2	B2/B3							
		133					B18/B3	MC1/MC2			MA2/MA3	MB1/MB2	MA1/MA2	MB1/MB0
		134					B2/B3	MC1/MC2						
		135					B2/B3	MC1/MC2		B2/B3				
		136				A1/A2		MC1/MC2	A1/A2			MB1/MB2		
		138			MA1/MA0		B2/B3					MB1/MB2		
		139	MA1/MA3		MA1/MA0		B22/B3	MC1/MC2						
		140			MA1/MA0					B2/B3	MA2/MA3	MB1/MB2		
		141	MA1/MA2				B18/B3			B2/B3				
		142		MB1/MB3			B18/B3				MA1/MA2	MB1/MB2		
		145					B22/B3			B2/B3		MB1/MB4		
		148	MA1/MA2	MB2/MB3					A2/A3				MA1/MA2	
		152					B2/B22	MC1/MC2	A2/A3					
		153	MA3/MA2	MB1/MB2			B22/B3	MC1/MC2		B2/B3			MA1/MA2	
		205		MB1/MB2		A2/A3			A2/A3					
		225				A1/A2	B2/B3							
		230					B2/B3	MC1/MC2		B01/B2				
▽	▽	232		MB1/MB2					A1/A2					
56° 51'	Kosta	4		MB2/MB3			B0/B2		A0/A2					
		69					B2/B3		A2/A3					
		93		MB2/MB3	MA1/MA0			MC1/MC2				MB1/MB2	MA1/MA2	
▽	▽	97						MC1/MC2					MA1/MA2	
59° 58'	Långtora	W1038	MA1/MA2				B2/B3	MC1/MC2		B1/B2		MB1/MB2		
61° 12'	Sollerön	W2053		MB2/MB3			B2/B3		A2/A3			MB1/MB3	MA2/MA3	
61° 46'	▽	X4210						MC1/MC2		B2/B3				
63° 48'	Domsjö-	Y3001		MB2/MB3					A2/A3	B1/B2				
63° 59'	änget	Z3003								B2/B3		MB1/MB2		

age among two LAP-loci and one GOT-locus. SIMONSEN and WELLENDORF (1975) investigated linkage relationships among two LAP and two phosphoglucomutase loci in macrogametophytes of *Picea sitchensis* but the search gave negative results.

The purpose of the present study was to investigate the occurrence of linkage among 12 allozyme loci identified in the macrogametophytes of *Pinus sylvestris*.

2. Material and Methods

2.1 Material

The criterion for the choice of the trees mentioned below was that each of them was heterozygous for at least two allozyme loci. All seeds were collected from single *Pinus sylvestris* trees and kept separate. The 30 heterozygous trees investigated for allozyme loci are presented in *Table 1*. Most of these trees (21 trees) originated from a 120—130 year old seed tree stand at Gårdstjärn (lat. 65° 29', long. 19° 17' E Gr, alt 400 m) with 10—18 trees/ha. Cones were harvested in late October 1974 and 1975.

Furthermore, four trees from a 70—80 year old, full density stand close to Kosta in southern Sweden, at latitude 56° 51', longitude 15° 14' E Gr and altitude 280 m were included. Cones were harvested in March 1974.

Seeds were also collected from three clones from two seed orchards in central Sweden — Långtora and Sollerön — and two clones from one seed orchard in northern Sweden, Domsjöänget. All these clones originated from an area between latitude 60°—64° in Sweden. The harvest in the seed orchards was undertaken in September and October 1975. Additional seeds came from two clones (W 1038 and Y 3001) during the same period in 1976.

In order to intensify the isozyme staining, seeds were germinated on a surface of distilled water during one to four days before analysis. This treatment did not change the isozyme pattern for any isozyme locus within a period of one to seven days.

2.2 Biochemical methods

The macrogametophyte was separated from the embryo in each seed and the former was homogenized in 0.2 ml Tris-borate-EDTA buffer at pH 7.4 in single plastic mortars 8 mm in diameter (cf. RUDIN 1975 and 1977). The homogenates were filtered through Kleenex tissue into pieces of cellulose filter paper and inserted into a 12% starch gel for electrophoretic separation. The separation and isozyme staining methods used were as follows:

Acid PHOSphatases (PHOS) were separated according to JONSSON ad BLOMQUIST (1976) and stained according to SHAW and PRASAD (1970); however 1 mM Mg^{2+} was added to the stain buffer.

Malate DeHydrogenases (MDH) were separated according to SHAW and PRASAD (1970), but 120 g urea was added to the gel buffer. Glass fiber filter paper was used instead of cellulose. Staining was done according to SHAW and PRASAD (1970), modified in the following way. In 0.18 M glycerine buffer at pH 8.7 the following chemical concentrations were added: 1.3 mM neutralized DL malic acid, 2 mM NBT, 1.2 mM PMS and 7 mM NAD.

Esterases (EST) were separated according to ASHTON and

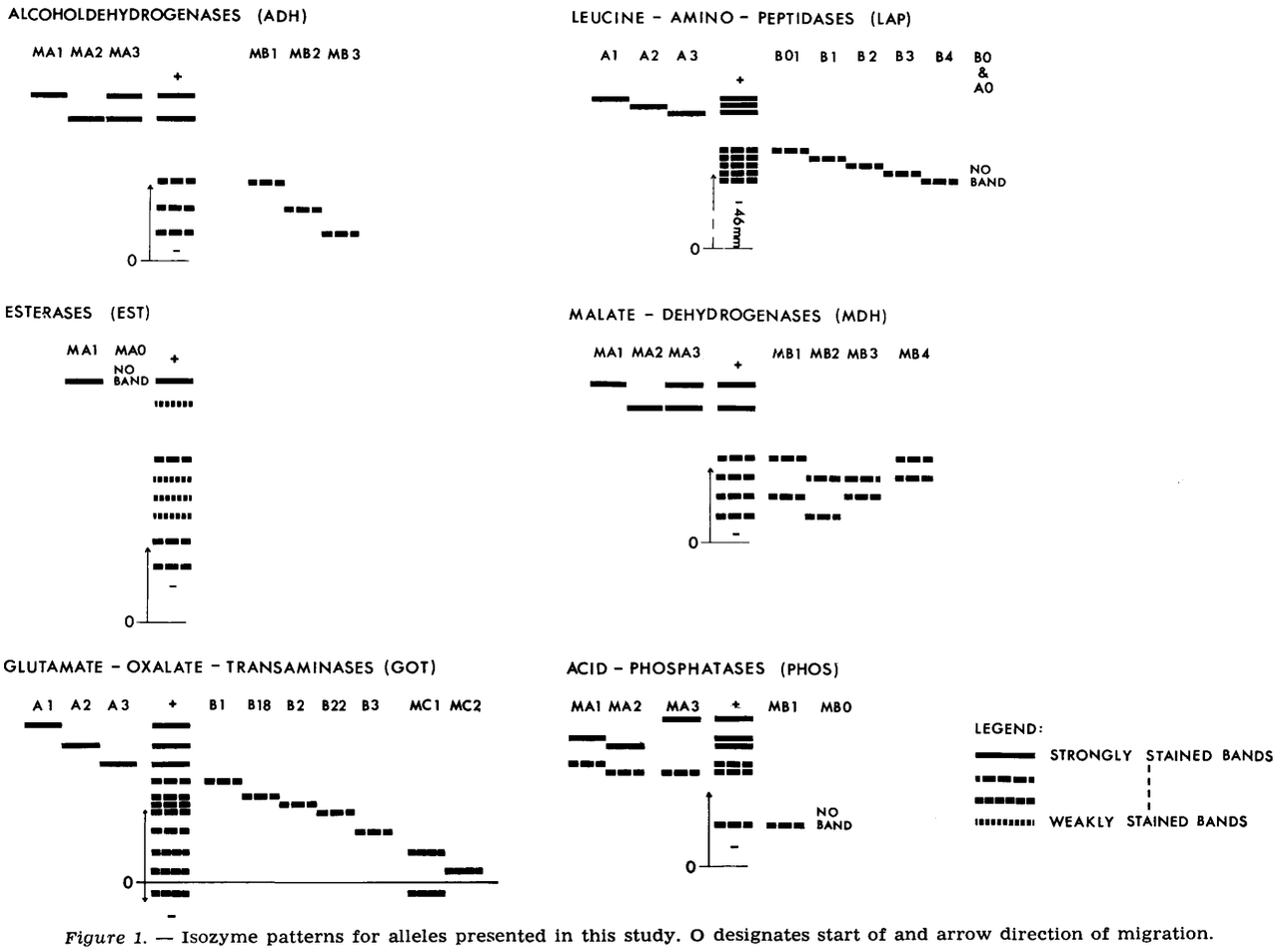

Figure 1. — Isozyme patterns for alleles presented in this study. O designates start of and arrow direction of migration.

BRADEN (1961) but gel buffer was diluted with 50% more distilled water according to BECKMANN *et al.* (1967). Staining was according to LAWRENCE *et al.* (1960).

Glutamate Oxalate Transaminases (GOT) were separated according to the same method used for EST. Staining was done according to SCHWARTZ *et al.* (1963).

Leucine Amino Peptidases (LAP) were separated according to the same method as for EST. Staining was done according to BECKMANN *et al.* (1964).

Alcohol DeHydrogenases (ADH) were separated and stained according to the same method as for MDH. DL malic acid, however, was replaced by 1.2 ml 95% ethanol in 40 ml of the staining solution.

2.3 *Loci and alleles*

Twelve loci were available for this study. Their zymogram phenotypes are illustrated in *Figure 1*. Four of them, GOT-A and -B, LAP-A and -B, have been utilized in our laboratory for some years (RUDIN 1975 and 1977) and manifest themselves in needles as well as in macrogametophytes. The locus EST-A, identified in the macrogametophytes, seems to correspond to the locus EST-A observed in needles and is described by RUDIN (1973). The remaining loci are presented for the first time in this publication. The inheritance for each of them was checked by a χ^2-analysis of 1:1 segregation. Loci represented by the fastest migrating region in the gels are designated by -A, the second fastest by -B etc. M designates that the loci have so far been identified only in macrogametophytes.

Zymograms of PHOS had two stained regions each varying independently. However, they need a somewhat trained eye for interpretation. Locus PHOS-MA had three alleles, each represented by two bands in different positions. Locus PHOS-MB had two alleles. One was manifested by one band and the other by no band ("null") allele.

In spite of broad, somewhat blurred bands, the MA-region in ADH-gels was easy to interpret. However, the isozyme bands of this region were not always visible. In spite of that, this locus was included in the analysis, since the alleles of this locus were segregating as expected when the banding pattern was visible. Furthermore, no discrepancies of the estimated recombination frequencies were observed. Three possible combinations of the two bands in this region represent the three alleles. The B-region in ADH-gels showed three bands, each representing one allele. This region was stable and easy to interpret.

In the EST-zymograms at least 11 bands could be identified, three of which appeared and disappeared out of our control. In addition there were also stable bands, which were inherited. In this study, however, only one locus with two alleles was included because of incomplete information of the inheritance of the other loci. One allele was manifested by one band and the other by no band.

The enzyme system GOT offered three polymorphic regions with corresponding loci. All of them were easy to interpret if the seed material was fully developed. Undeveloped seeds might result in faint and blurred bands, especially in regions B and C. In region A three bands, each identifying one allele, were found. The B-region showed bands in five positions, each representing one allele. The C-band might migrate towards the anode (+) or the catode (—) in the gels. One allele in this locus was represented by two bands, one migrating in each direction. The second allele was represented by one band which migrated very slowly towards the anode.

All the LAP-bands were easy to interpret. Three bands in region -A and five bands in region -B represented one allele each. Both loci LAP-A and -B had one additional — no band — ("null") allele.

Zymograms stained for MDH showed a lot of polymorphism in the heavily stained banding pattern. At least two independently varying regions could be identified. Two of the alleles in the MDH-MA locus were represented by single bands, the third allele by double bands. All four alleles in the MDH-MB locus were easily identified by double bands. One additional, extremely slowly migrating region was independently varying too. Until now, however, we have incomplete information about the inheritance of isozymes in this region.

2.4 *Statistical methods*

Recombination frequencies were estimated as the proportion of macrogametophytes showing recombination genotypes to the total number of macrogametophytes analysed. It was always assumed that the smallest class contained recombinants. This method cannot present higher values than 0.50 in spite of the fact that the frequency should be normally distributed about 0.50 in the case of independent segregation between two loci.

In order to determine a desirable number of macrogametophyte analyses from a single tree and for different recombination values and levels of significance, *Figure 2* was

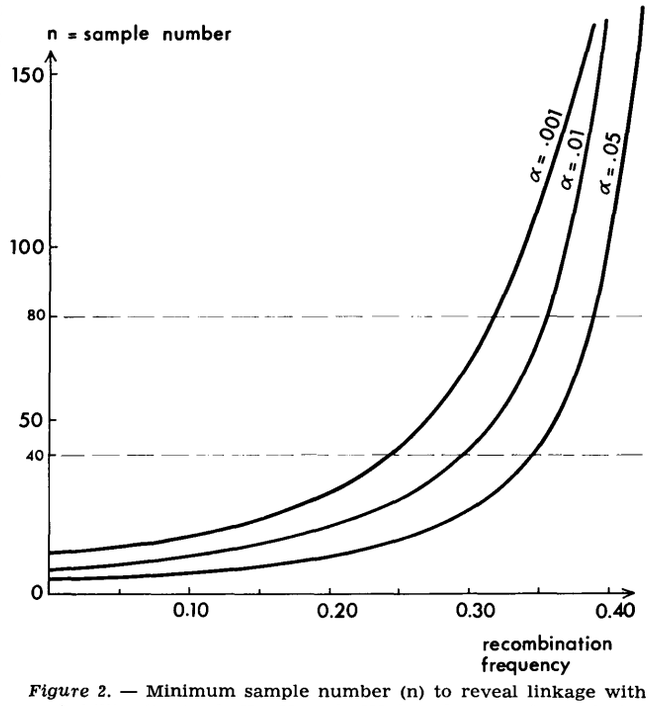

Figure 2. — Minimum sample number (n) to reveal linkage with a probability (1—α) of .95, .99 and .999 based on a χ^2_L analysis of deviation from 1 : 1.

drawn. This is based on a χ^2-analysis of linkage derived from deviations from a 1 : 1 segregation of the parental in relation to recombination gametes (cf. MATHER 1951).

Unbalanced segregation of one pair of alleles does not affect these χ^2_L-analyses (BAILEY 1961). If both loci, however,

are unbalanced this must be taken into account. Therefore separate χ^2-tests of all segregating pairs of alleles in each combination of loci must be performed. For significantly unbalanced segregation in both loci a special test to determine linkage was suggested by MATHER (1951) and BAILEY (1961).

This was in fact a χ^2-analysis of heterogeneity. A simplified way of carrying out such calculations would be to use the ordinary χ^2-analysis, but to take the actual gene segregation into account when calculating the expected numbers. Assume a genotype of a mother tree $\frac{A1\ \ B1}{A2\ \ B2}$ for two linked loci. Then the following isozyme types of gametes are possible:

	A1/B1 parental	A1/B2 crossing	A2/B1 over	A2/B2 parental	Total number
Extected	$n\,p_A p_B$	$np_A(1-p_B)$	$n(1-p_A)p_B$	$n(1-p_A)(1-p_B)$	n
Observed	a	b	c	d	n

$$p_A = \frac{a+b}{n} \qquad p_B = \frac{a+c}{n}$$

The corrected χ^2_L is then reached as follows:

$$\chi^2_L = \frac{(a+d-np_Ap_B-n(1-p_A)(1-p_B))^2}{np_Ap_B+n(1-p_A)(1-p_B)} + \frac{(b+c-np_A(1-p_B)-n(1-p_A)p_B)^2}{np_A(1-p_B)+n(1-p_A)p_B}$$

There is one degree of freedom, since the initial three are reduced by two causes of variation, namely the segregation of alleles from the actual two loci studied. Special cases where the above related test has to be used, however, were rare.

No recombination frequency above 0.40 for a single distance has seriously been discussed in this paper. Linkage was only accepted if χ^2_L was significant at the 5% level and no more than one of the segregating loci showed a deviation from 1 : 1 at the 5% level. The Yates correction for discontinuity was used for all χ^2-estimates of deviation from a 1 : 1 segregation. It is also important to keep in mind that it is not possible to make an identification of the coupling and the repulsion phases in a proper way using macrogametophyte analyses. Therefore, a calculation of mean values, based on recombination data from more than one tree, was made only for those loci where linkage had been established.

2.5 *Choice of function for map distances*

The recombination values obtained in this study were so high that a function for the transformation of these values into map distances was necessary. A proper mapping function should involve a distance from the centromere and from the chromosome arm terminus in order to take the effect of interference into account. Neither the information about the location of the centromeres nor the total arm lengths in mapping units for any chromosome in *Pinus sylvestris* were available. As soon as a pronounced affinity between non-homologous chromosomes is discovered, the centromere can be located (WALLACE 1957). Furthermore, it was even less possible to reach the total arm length in this study.

In the present situation it must be sufficient to choose a mapping function with a fixed level of chiasma interference. The best alternative was then the KOSAMBI (1944) formula in combination with the JENSEN and HELMS JÖRGENSEN (1975) method of maximum likelihood estimation of map distances.

KOSAMBI (1944) tried to take the problem of chiasma interferene into account. The influence of this, however, was fixed at a level mostly found in the central parts of the long arm of the chromosome. His suggested formula was:

$$x = \frac{1}{4}\ln\frac{1+2y}{1-2y} \qquad x = \frac{\text{map distance in cM}}{100}$$

y = recombination frequency

OWEN (1950) suggested a general index (K) with the purpose of testing the interference before using the KOSAMBI formula. The calculated K is not allowed to deviate significantly from unity if the map distances obtained by the KOSAMBI formula are to attain a good precision.

Based on observed data from a triple backcross, the formula for the estimation of K takes on the following form (OWEN 1953):

$$\hat{K} = \frac{n^2 a_{12}}{2(a_1+a_{12})(a_2+a_{12})(a_1+a_2)}$$

a_1 = recombination events in region I

a_2 = recombination events in region II

a_{12} = double recombination events in both I and II

n = number of gametes studied

The large sample variance was estimated by

$$\text{var } K = \hat{K}^2\left(\frac{1}{a_{12}} + \frac{5}{a_1+a_2} - \frac{1}{a_1+a_{12}} - \frac{1}{a_2+a_{12}} - \frac{4}{n} - \frac{8a_{12}\hat{K}}{n^2}\right)$$

(OWEN 1953 rearranged by BAILEY 1960)

Standard deviations of map distances (S_{md}) were calculated according to the following formula (OWEN 1950):

$$S_{md} = \pm S_y \frac{2500}{2500-y^2}$$

y = recombination frequency

S_y = st.d. of recombination frequency

The KOSAMBI formula has been utilized for chromosome mapping in a variety of organisms and was found to give quite good results. This formula was used for gene mapping of Punnett's sweet pea (BHAT 1948) and for rice (BHAT 1950). WALLACE (1957) used it for the mapping of chromosome five in the house mouse. JENSEN and HELMS JÖRGENSEN used it as a base for their maximum likelihood method applied to the mapping of chromosome five in barley. In spite of its simplicity it seems therefore to have a wide range of applications.

Before estimating an average, the map distances from single trees (X_i) were weighted with I_{X_i} as suggested by FISHER (1937) using the following formula:

$$I_{X_i} = \frac{n\,(1 - 4y^2)^2}{y\,(1 - y)}$$

I_{X_i} = weight for a map distance estimated from tree X_i
y = recombination frequency

3. Results and discussion

The linkage study was based on isozymes in macrogametophytes. Therefore it was only possible to study linkage based on the segregation in megaspore mother cells.

The number of loci studied was 12, and 48 different pairs of these were available for a test of linkage *(Table 1)*. At least four loci are linked in group I *(Figure 3)*. Three different loci observed in single trees were associated with this group. Slight evidence of linkage between three other loci was also obtained.

3.1 *Unbalanced segregation within a locus*

Different loci had a varying tendency to unbalanced segregation, that is, a significant deviation from 1 : 1 segragation of alleles in heterozygous trees. The segregation was studied in 98 cases of heterozygosity. Out of these, eleven deviated with slight significance (at 5% level) and one with high significance (at 0.1% level) from 1 : 1 segregation *(Table 2)*. Six loci showed a regular segregation. Three loci had only one incident each with a slightly significant deviation from a 1 : 1 segregation. Three loci had a more marked tendency of deviation from regular segregation. These loci, GOT-B, GOT-MC and MDH-MB showed each three incidents of deviation out of 21, 12 and 12 heterozygous trees examined, respectively. There was only one case of unbalanced segregation for two loci simultaneously in a linkage test *(Table 2)*. Such a double deviation might affect the recombination value, but in this case the value was close to the average of seven estimates between the same loci. When the suggested simplified way of testing for significant linkage (cf. 2.4) was applied, a χ^2-value of 0.53 was

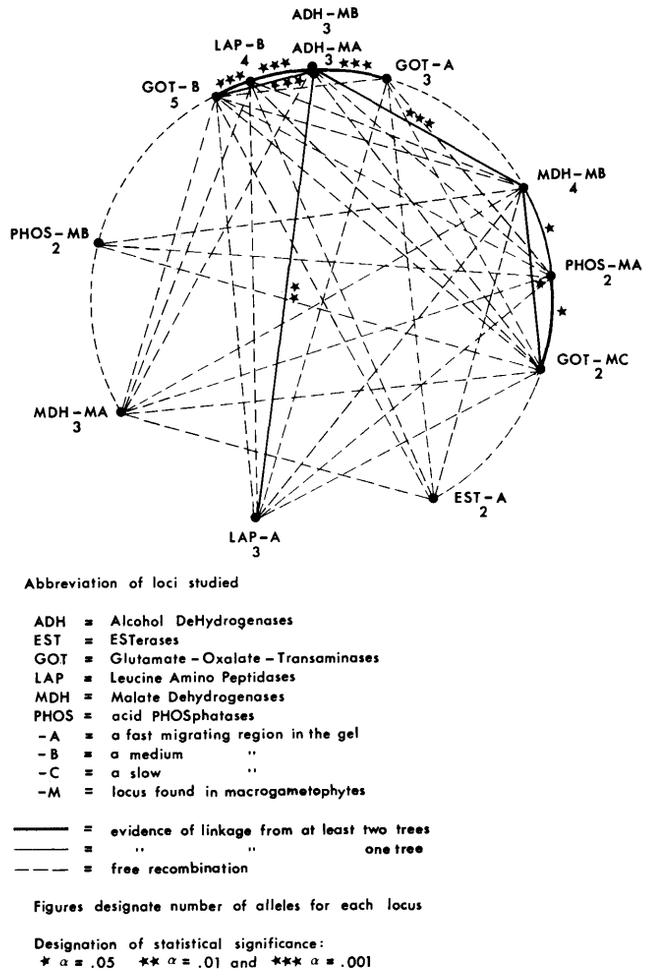

Figure 3. — Schematic illustration of linkage groups. All trees represented by a solid line in this figure have not reached designated level of significance, mostly because of lack of seeds. Distance between linked loci lying on the perifery are proportional to map units.

Table 2. — Trees with significant deviation from 1 : 1 segregation of alleles.

Mother tree no	Locus GOT-B					Locus GOT-MC				Locus PHOS-MB			
	Endosperm segregation, number of alleles				Test 1 : 1 χ^2-value	Endosperm segregation, number of alleles			Test 1 : 1 χ^2-value	Endosperm segregation, number of alleles			Test 1 : 1 χ^2-value
	B18	B2	B3	B4		MC1	MC2	MC0		MB1	MB2	MB0	
1112		52	33		3.81*								
225		21	9		4.03*								
230		50	30		4.51*	28		52	6.61*				
133						29	50		5.06*	[1])28		50	5.65*
Kosta 97						43	25		4.25*				

Mother tree no	Locus LAP-A				Locus LAP-B				Locus MDH-MB			
	Endosperm segregation, number of alleles			Test 1 : 1 χ^2-value	Endosperm segregation, number of alleles			Test 1 : 1 χ^2-value	Endosperm segregation, number of alleles			Test 1 : 1 χ^2-value
	A1	A2	A3		B1	B2	B3		MB1	MB2	MB0	
135						106	60	12.2***				
136	104	76		4.05*								
140									71	46		4.92*
Z3003									26	12		4.45*
Kosta 93										71	47	4.48*

[1]) Not identical macrogametophytes.
* designates significance at 5% level, *** at 0.1% level.

Table 3. — χ^2-analysis of segregation, recombination data and map distance of linkage group I. All means are weighted according to FISHER (1937).

Loci I/II	Tree no	No of endosperm anal.	χ^2_I 1 : 1 df = 1	χ^2_{II} 1 : 1 df = 1	χ^2_L 1 : 1 df = I	recomb. % (y) $\pm S_y$	Map distance according to KOSAMBI (1944) in cM $\pm S_{md}$ (OWEN 1950)	JENSEN and JÖRGENSEN (1975) in cM
ADH-MA/ADH-MB								
	148	40	2.03	2.03	38.13***	00	.00	
	153	80	2.11	2.11	78.01***	00	.00	0.0
	Σ	120			118.01***	00		
ADH-MA/LAP-B								
	141	39	.00	.41	.92	41.0 ± 7.9	57.8 ± 24.11	
	W1038	98	.09	.26	15.52***	29.6 ± 4.6	34.0 ± 7.1	
	Σ	137			7.47**	$\bar{y}$ = 32.8 ± 4.0	$\bar{x}$ = 35.8 ± 8.6	32.8 ± 3.1
ADH-MB/LAP-B								
	153	120	.03	.13	22.72***	27.5 ± 4.1	30.9 ± 5.9	
	Y3001	76	1.07	.65	8.22**	32.9 ± 5.4	39.9 ± 9.5	
	Σ	196			31.20***	$\bar{y}$ = 29.6 ± 3.3	$\bar{x}$ = 33.3 ± 6.9	
LAP-B/GOT-B								
	135	126	4.96*	.39	44.64***	19.8 ± 3.5	20.9 ± 4.2	
	141	78	1.04	1.04	21.55***	23.1 ± 4.8	25.0 ± 6.1	
	145	125	1.79	.01	65.72***	13.5 ± 3.0	13.8 ± 3.2	
	153	204	.04	.12	103.06***	14.2 ± 2.4	14.6 ± 2.6	15.5 ± 1.4
	230	80	.11	4.51*	32.51***	17.5 ± 4.2	17.5 ± 4.8	
	W1038	100	.09	.00	56.25***	12.0 ± 3.2	12.2 ± 3.4	
	Σ	713			326.73***	$\bar{y}$ = 16.1 ± 1.4	$\bar{x}$ = 15.7 ± 3.5	
ADH-MA/GOT-B								
	139	36	.03	1.36	2.25	36.1 ± 8.0	45.6 ± 16.7	
	141	39	.00	.00	2.56	35.9 ± 7.7	45.2 ± 15.9	
	W1038	98	.09	0.1	3.68	39.8 ± 4.9	54.9 ± 13.4	
		173			9.95**	$\bar{y}$ = 38.2 ± 3.7	$\bar{x}$ = 49.4 ± 8.6	
ADH-MB/GOT-B								48.3 ± 3.1
	116	37	2.70	.43	1.73	37.8 ± 8.0	49.3 ± 18.7	
	142	39	.41	.00	2.56	35.9 ± 7.7	45.2 ± 15.9	
	153	159	.23	.05	13.31***	35.2 ± 3.8	43.8 ± 7.5	
	W2053	100	.01	3.61	6.25*	37.0 ± 4.8	47.5 ± 10.6	
	Σ	335			25.27***	$\bar{y}$ = 36.1 ± 2.6	$\bar{x}$ = 45.4 ± 10.3	
GOT-A/ADH-MB								
	205	113	1.27	.89	12.78***	32.7 ± 4.4	39.1 ± 7.7	
LAP-A/ADH-MA								
	148	73	.49	1.97	.22	46.6 ± 5.8		
LAP-A/ADH-MB								
	205	117	.77	.55	.19	42.7 ± 4.6		
	232	40	1.23	.03	.63	42.5 ± 7.8		
	Kosta 4	87	.74	2.25	9.01**	33.5 ± 5.1	40.2 ± 9.2	
	Σ	244						
MDH-MB/ADH-MA								
	W1038	105	.00	.15	.61	45.7 ± 4.9		
MDH-MB/ADH-MB								
	142	40	.06	1.27	.20	46.8 ± 5.6		
	W2053	61	.07	.00	11.08***	27.9 ± 5.7	31.5 ± 8.3	
	Kosta 93	86	2.62	2.62	.29	45.5 ± 5.4		
	Σ	187						
MDH-MB/PHOS-MA								
	133	104	.47	.24	6.01*	37.5 ± 4.7		
	W2053	70	3.21	2.41	.00	50.0 ± 6.1		
	Kosta 93	36	1.36	2.25	.03	47.2 ± 8.3		
	Σ	210						
PHOS-MA/GOT-MC								
	133	79	.81	5.06*	5.06*	36.7 ± 5.4		
	152	52	.02	.17	1.56	40.4 ± 6.8		
	Kosta 93	35	1.83	1.03	4.11*	31.4 ± 7.8		
	Kosta 97	68	.13	4.25*	.37	45.6 ± 6.0		
	Σ	234						
MDH-MB/GOT-MC								
	133	102	.48	.79	2.21	42.2 ± 4.9		
	136	100	.21	.09	3.61	40.0 ± 4.8		
	W1038	100	.01	2.25	4.41*	39.0 ± 4.9		
	Kosta 93	118	4.48*	.21	.08	48.3 ± 4.6		
	Σ	420						

obtained. With one degree of freedom, the probability of getting this deviation, or greater, from expected values, when assuming independent segregation, was P = 0.40 — 0.50. This should be compared with an uncorrected χ^2_L = 1.51 with one degree of freedom which revealed a P = 0.20 — 0.25. A χ^2-test of heterogeneity according to MATHER (1951) resulted in a χ^2-value of 0.53 which, with one degree of freedom, revealed a P = 0.40 — 0.50.

3.2 *Linkage groups and gene maps*

3.2.1 Linkage group I

The seven loci which could be involved in this group were GOT-A and/or LAP-A and/or MDH-MB — ADH-MA and -MB — LAP-B — GOT-B in the order mentioned. Basic data for the following results are listed in *Tables 3—6.*

ADH-MA — MB

The two loci ADH-MA and -MB were very closely linked. Out of 120 analyses no recombination was found. Evidence for the loci to be different is presented in *Table 1.* This shows that the alleles in the two loci vary independently, because the allele composition is independent for the two loci.

ADH-MA, -MB — LAP-B

Locus LAP-B was linked to ADH-MA. Three double heterozygous trees showed a high significance for linkage. One tree from Gårdstjärn did not. A test of inconsistency according to JENSEN and HELMS JÖRGENSEN (1975) did not point out any single data for omission. The average map distance according to KOSAMBI (1944) was found to be 34.2 ± 7.4 cM and the maximum likelihood estimated map distance was found to be 32.8 ± 3.1 cM.

LAP-B — GOT-B

The linkage between LAP-B and GOT-B was determined by analyses of macrogametophytes from six trees, five from Gårdstjärn and one plus tree. The recombination per cent varied between 12.0 ± 3.2 and 23.1 ± 4.8 but supported linkage in a proper way. In spite of the great variation no recombination values could be omitted based on tests of inconsistency. The average map distance was found to be 15.7 ± 3.5 cM and the maximum likelihood estimated map distance was found to be 15.5 ± 1.4 cM.

ADH-MA, -MB — GOT-B

According to the results presented above, the four loci ADH-MA, -MB, LAP-B and GOT-B seemed to belong to the same linkage group. It should be possible to establish the sequence of the four loci on the chromosome, if the recombination frequency between ADH-MA, -MB and GOT-B was known. At Gårdstjärn there was one double heterozygous tree showing highly significant linkage. At the southern stand (Kosta) there was one double heterozygote which, however, showed independent segregation and was therefore omitted from further calculations. Two plus trees showed significance or almost significance for linkage. Four other trees at Gårdstjärn showed a clear tendency to linkage. On account of the lack of seeds the analysed numbers of the four latter were too low to permit significance for such a long average map distance as 46.5 ± 10.3 cM.

The maximum likelihood estimated summed map distance was found to be 48.3 ± 3.1 cM. The sequence, average recombination per cent and map distances in cM for these four loci will be as follows:

Map distances	ADH-MA, -MB	32.8 ± 3.1	LAP-B	15.5 ± 1.4	GOT-B
Average recombination %		30.9 ± 2.5		16.1 ± 1.4	

It was also clearly demonstrated that this part of the linkage group I was confirmed by trees from northern and central Sweden *(Table 3).* However, it has not yet been possible to establish this linkage group in southern Sweden.

GOT-A — ADH-MB

Linkage between GOT-A and ADH-MB could only be tested in one tree from Gårdstjärn, but this was highly significant. The map distance was estimated to 39.1 ± 7.7 cM. There was no double heterozygote for GOT-A and LAP-B but for GOT-A and GOT-B there were two trees which showed independent segregation. This indicated that GOT-A should be located on the opposite side of ADH-MB relative to LAP-B.

LAP-A — ADH-MB

One tree from southern Sweden (Kosta) showed signficant linkage between LAP-A and ADH-MB *(Table 3 and 4.)* The map distance was estimated to 40.2 ± 9.2 cM. Three trees from Gårdstjärn did not show any linkage for this combination. Furthermore, no linkage was found between LAP-A and GOT-B, which indicated that LAP-A should be located on the opposite side of ADH-MA, -MB relative to LAP-B and GOT-B. It should be recalled, however, that the linkage group I has not yet been established in the southern stand at Kosta.

The question as to whether or not the linkage group LAP-A — ADH-MB belongs to linkage group I could therefore not be answered by this study.

MDH-MB — ADH-MA, -MB

In one of the plus trees from central Sweden, MDH-MB and ADH-MB were highly significantly linked *(Table 5).* The map distance was estimated to 31.5 ± 8.3 cM. The segregation of the alleles in these two loci was also analysed in another plus tree as well as in one tree at Gårdstjärn and one tree at Kosta. All these trees showed independent segregation. Furthermore, free recombination had also been found between MDH-MB and LAP-A (two trees), LAP-B (four trees), GOT-A (one tree) and GOT-B (five trees). This means that, so far, MDH-MB only showed linkage to one locus (ADH-MB) in linkage group I. If MDH-MB and ADH-MB were linked, then the linkage group I and the possible linkage group described below might be associated. In such a situation seven out of the 12 loci studied might be linked which is highly unlikely.

3.2.2 *Possible linkage*

MDH-MB — PHOS-MA

Linkage was obtained between MDH-MB and PHOS-MA in one tree from Gårdstjärn which indicated a slight significance. No linkage was observed for the double heterozygote at Kosta or for one of the plus trees tested *(Table 4 and 5).*

MDH-MB — GOT-MC

Linkage was observed in one plus tree which was indicated by a slight significance, whereas two trees at Gårdstjärn and one tree at Kosta showed free recombination.

PHOS-MA — GOT-MC

Indications of linkage were found in one tree at Gårdstjärn and one from Kosta. A second tree at Gårdstjärn as

Table 4. — Recombination between allozyme loci in macrogametophytes from trees at Kosta.

	PHOS-MA	MDH-MB	LAP-A	GOT-MC	GOT-B
		1	1	1	1
ADH_MB		86	** 87	77	87
		free	33.3 ± 5.1	free	free
		1		1	
EST-A		84		77	
		free		free	
			2		
GOT-B			128		
			free		
	2	1			
GOT-MC	103	118			
	free	free			
	1				
MDH-MB	36				
	free				
				2	Number of trees
			*** 100		Number of analyses
	Weighted recombination %(r) ± S_y		32.7 ± 4.4		

Levels of significance: * = 5%; ** = 1%; *** = 0.1%.

Table 5. — Recombination between allozyme loci in macrogametophytes from plus trees in seed orchards.

	PHOS-MA	MDH-MB	LAP-B	LAP-A	GOT-MC	GOT-B
		1	1		1	1
ADH-MA		105	*** 98		98	98
		free	29.6 ± 4.6		free	free
		1	1	2		2
ADH-MB		*** 61	** 76	177		* 100
		27.9 ± 5.7	32.9 ± 5.4	free		37.0 ± 4.8
		2	1	1	1	
GOT-B		161	*** 100	100	100	
		free	12.0 ± 3.2	free	free	
		1	2			
GOT-MC		* 100	140			
		39.0 ± 4.9	free			
		1	1			
LAP-A		61	113			
		free	free			
		2				
LAP_B		138				
		free				
	1					
MDH-MB	70					
	free					
					2	Number of trees
				*** 100		Number of analyses
		Weighted recombination %(r) ± S_y		32.7 ± 4.4		

Levels of significance: * = 5%; ** = 1%; *** = 0.1%.

well as one tree at Kosta showed free recombination.

One tree from Gårdstjärn (133) was heterozygous for all three loci. The recombination values from this tree indicated the following sequence of loci in a possible additional linkage group MDH-MB — PHOS-MA — GOT-MC. As mentioned above, one plus tree (W 2053) showed a highly significant linkage between MDH-MB and ADH-MB. This fact might indicate a connection of the possible additional linkage group to linkage group I, but this is for the present unsure.

3.3 *Interference studies*

In the vicinity of a point of recombination additional recombination is commonly markedly diminished by chiasma interference. This will gradually decrease as the distance between the markers increases. Further, there is evidence that the interference increases within a constant map distance if this slides from the centromere to the chromosome terminus (Bailey 1961). This phenomenon must, of course, be taken into account when the map distances based on recombination frequencies are calculated. The impact of chiasma interference is estimated by the Coefficient Of Coincidence (COC) which is defined as the quotient between the observed and the expected frequency of double recombinations. If $0 < COC < 1$ interference is positive and above unity, interference is negative between the studied loci. If $COC = 1$ no interference exists and if $COC = 0$ the interference is total. To be able to estimate a COC three heterozygous loci must be available within the same individual.

Fortunately, three trees had the loci ADH-MA or -MB — LAP-B — GOT-B in a heterozygous condition. A sufficient number of analyses (98 and 120) are available for two of them. The calculated COC for these are 0.28 and 0.61, respectively. These indicate positive interference which emphases the utilization of the Kosambi formula for calculations of map distances.

In two single trees the discrepancies are 8.5 and 8.8 cM between the summed distances and that calculated between ADH-MA, -MB and GOT-B directly (*Figure 4*). The positive discrepancy would indicate that the Kosambi level of interference is too low for the loci studied. On the other hand,

Table 6. — Recombination between allozyme loci in macrogametophytes from trees at Gårdstjärn.

	PHOS-MB	PHOS-MA	MDH-MB	MDH-MA	LAP-B	LAP-A	GOT-MC	GOT-B	GOT-A	ADH-MB
					1	1	2	2		2
ADH-MA					39	73	76	* 75		*** 120
					free	free	free	36.0 ± 5.5		.00
		1	1	1	1	2	1	3	1	
ADH-MB		63	40	79	*** 120	157	80	*** 235	*** 113	
		free	free	free	27.5 ± 4.1	free	free	35.7 ± 3.1	32.7 ± 4.4	
			2	1	1		1	4	1	
EST-A			157	79	79		80	200	40	
			free	free	free		free	free	free	
		1	1			2	1	2		
GOT-A		91	100			335	140	115		
		free	free			free	free	free		
	1	2	3	2	5	1	7			
GOT-B	77	164	191	110	*** 613	119	762			
	free	free	free	free	16.8 ± 1.5	free	free			
	1	2	2	1	3	2				
GOT-MC	60	* 131	202	56	244	249				
	free	38.2 ± 4.2	free	free	free	free				
		2	1							
LAP-A		197	140							
		free	free							
			2	1						
LAP-B			71	77						
			free	free						
	1	1	3							
MDH-MA	78	106	322							
	free	free	free							
	1	1								
MDH-MB	74	* 104								
	free	37.5 ± 4.7								
	1									
PHOS-MA	128									
	free									

2 Number of trees

*** 100 Number of analyses

Weighted recombination %(r) ± S_y 32.7 ± 4.4

Levels of significance: * = 5%; ** = 1%; *** = 0.1%.

those with I_x, according to FISHER (1937) weighted and pooled data *(Table 3)*, showed something of the opposite situation with a discrepancy of —3.4, which in fact was a very good precision. The even better method according to JENSEN and HELMS JÖRGENSEN (1975), however, presented a discrepancy of ±0 and markedly lower standard deviations *(Figure 4)*.

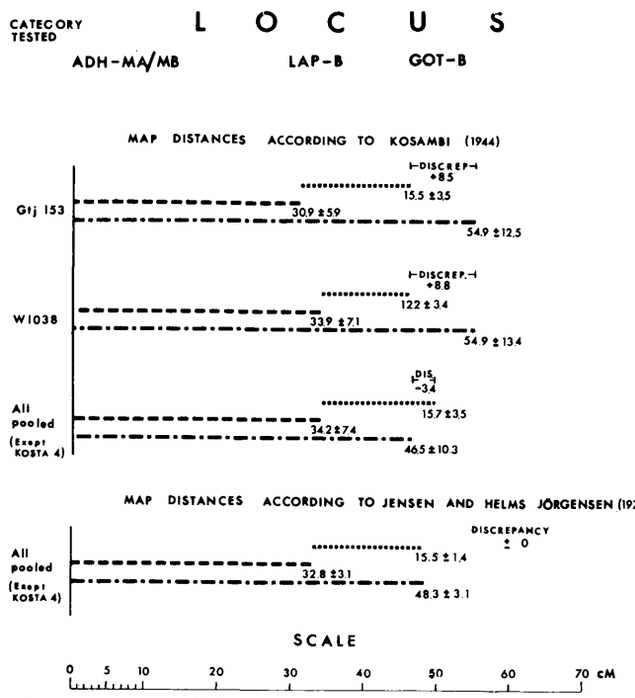

Figure 4. — Map distances for linkage group I according to different mapping principles.

As pointed out above in section 2.5 the Kosambi coefficient, quantifying the interference, should not deviate significantly from unity for a proper utilization of the Kosambi mapping function. For the two trees in which such calculations were possible, these coefficients were estimated to $K_{Gtj\ 153} = 0.81 \pm 0.46$ and $K_{W\ 1038} = 0.35 \pm 0.35$, which in both cases did not deviate significantly from unity, which once more supported the utilization of the Kosambi formula.

BAILEY (1961) pointed out the opportunity to use the estimated K as an indication of rough orientation of studied loci in relation to the midarm of a long chromosome. If $K > 1$ they are situated between the centromere and the midarm, but if $K < 1$ they are situated on the opposite side in relation to the midarm. Because both calculated K values fell within the interval $0 < K < 1$, they therefore may possibly indicate that the linkage group I is situated between the midarm and the arm terminus, if the studied *Pinus* sylvestris chromosomes behaved as postulated by BAILEY.

3.4 *General discussion*

It must be emphasized that these linkage studies were based on female gametes. In spite of the general observation that gamete selection is less pronounced on the female than on the male side in flowering plants, female gamete selection might have influenced recombination data. Until now only faint indications of selection against certain gametes were discovered. On account of fewer selection steps, the need to use corrections for different gamete and

genotype fitness is much less using macrogametophytes than diploid material.

3.4.1 Undiscovered linkage

Isozyme bands classified as representing one allele, in some cases certainly contain a number of alleles. This fact may influence the possibilities to trace linkage. If one of two loci, responsible for the bands migrating to the same positions in the gels, is linked to a third locus, linkage can only be effectively detected if the unlinked locus is homozygous for a "null" or non-interfering allele. Then it is obvious that all possible linkages between studied loci have not been discovered.

RUNQUIST (1968) observed an average chiasma frequency of 2.40 in *Pinus sylvestris* from central Sweden. This result indicated relatively long chromosomes. The same observation was reported in other cytological studies (AASS 1957, SAYLOR 1972 and KORMUTAK 1975). It must then be possible that loci on the same chromosome might be so widely separated that the recombination data cannot diagnose the loci as linked.

3.4.2 Possible influence of inversions

Single crossovers within the inverted segment in inversion heterozygotes usually give rise to lethal recombinant gametes. Therefore, loci located within such an inversion often show a strong linkage. Double crossovers within inversions, however, may in certain cases give rise to vital recombinant gametes. The probability of double crossovers within the inversion is mainly dependent on the size of the inversion and the crossing-over frequency in the actual chromosome segment. By this mechanism the recombination frequency between ADH-MA and ADH-MB might be estimated too low. Studies of meiosis should be undertaken in order to provide indications of this. On the other hand, it is unlikely that the two trees representing double ADH heterozygotes, should both be inversion heterozygotes.

3.4.3 Discrepancies between southern and northern Sweden

ANDERSSON *et al.* (1969) surveyed a great deal of meiotic irregularities in conifers. A high frequency of events leading to, for instance, inversions, translocations and univalents were observed. Furthermore, *Pinus sylvestris* colonized Sweden from the south about 7000 B.C. (VON POST 1924) and less than 1000 years later from the north (HULTÉN 1950). On account of these facts it would not be surprising to find slightly different chromosomal arrangement of *Pinus sylvestris* in southern compared to northern Sweden. The analysed trees from a stand in southern Sweden (Kosta) indicated this by showing a deviating linkage pattern. ADH-MB showed linkage to LAP-A in one Kosta tree but not in other trees. ADH-MB showed linkage to GOT-B in the others but not in the Kosta trees. At this stage of the study the validity of the results must therefore be confined to investigated stands and trees.

3.4.4. Varying recombination data for LAP-B — GOT-B

The recombination frequencies between LAP-B and GOT-B occupies two different levels, one of about 13% and the other of about 20%. The reasons for this might be firstly an inbalance in recombination gamete segregation indicated by under-representation of the gamete LAP-B3/GOT-B22. The two trees representing the lack of this gamete type show the lowest recombination value in the Gårdstjärn stand.

Gamete selection favouring certain combinations of unlinked loci have been found, among others, by CLEGG *et al.* (1972) in *Avena barbata*. Allozyme analyses presented 32 possible allele combinations from five loci in the gametes. However they pointed out that one combination dominated (91%) in a more mesic environment and gradually decreased in frequency in xeric environments where quite different combinations of alleles predominated. An evaluation of possible multilocus organization in this material will be published in a separate paper.

Secondly, high temperature is well known to cause an increase in the crossing-over frequency. By a rise in temperature from 25° to 35° C GRELL (1966) reported a threefold increase of the crossing-over frequency in *Drosophila melanogaster* females. Seeds for analyses were collected during 1974, which had a very hot early summer especially in northern Sweden and during 1975 with an ordinary early summer. The cones collected in 1975 passed meiosis during the hot period in 1974 with air temperatures at noon of 28—29° C. Therefore they might have had a higher chiasma frequency, which could have resulted in a higher recombination frequency. From the two trees with the highest recombination frequency between LAP-B and GOT-B, cones for this study were collected exclusively during the autumn of 1975. From the others, with one exception, collection was mainly made during 1974. These results might indicate a possible influence by temperature. Therefore the temperature factor should be taken into account in future linkage studies.

4. Conclusions

It can be concluded that there are data to suggest environmental or genetic mechanisms which could influence the recombination frequency. With the possible exception of gamete selection and temperature the results obtained do not appear to indicate that such mechanisms are likely to constitute a disturbing influence.

The levels of recombination frequency between the loci ADH-MA, -MB — LAP-B — GOT-B in linkage group I, indicate a certain order between them. The existence of and the order within the possible linkage group MDH-MB — PHOS-MA — GOT-MC is less certain. The reason for this was that a number of single recombination values from different trees were low. If future macrogametophyte analyses record data appropriately, the indicated fragments of gene maps will grow rapidly both in precision and extension.

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An Analysis method to improve statistical efficiency of a randomized complete block design[1])

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Summary

A procedure, called the "moving average" method is described whereby statistical efficiency can be made as high for experiments following randomized complete block designs as for those following more complex lattice or incomplete block designs. Briefly, the procedure is to consider each plot in relation to few of its neighbors and adjust its mean according to the performance of those neighbors. The method is most useful in experiments having large blocks with large amounts of within-block variation and effectively converts them into experiments consisting of numerous small, uniform blocks. As used in five plantations, the moving average method reduced error sums of squares by 30—50% and changed seedlots means by 0—16%.

Key words: Moving average method.

Zusammenfassung

Ein Verfahren, bezeichnet als „moving average"-Methode, wird beschrieben, nach dem die Auswertung von randomisierten vollständigen Blockanlagen mit gleich hoher statistischer Effizienz möglich ist wie diejenige von den komplexen lateinischen oder unvollständigen Blockanlagen. Bei diesem Verfahren wird jede Versuchsparzelle mit wenigen Nachbarzellen in Beziehung gebracht und das Parzellenmittel entsprechend korrigiert. Diese Methode eignet sich besonders für Versuchsanlagen mit großen Blocks und großem Variationsanteil für die Ursache „innerhalb Blocks" und wandelt diese in zahlreiche kleine gleichmäßige Blocks um. Durch die Anwendung des beschriebenen Verfahrens wurde bei der Auswertung von 5 Versuchsanlagen mit *Pinus resinosa*-Herkünften die Summe der Abweichungsquadrate für den Versuchsfehler um 30 bis 50% verringert. Die Herkunft-Mittelwerte änderten sich dabei um 0—16%.

Introduction

A randomized complete block design, in which each seedlot is reprsented by one plot in each block, is the simplest replicated design to install and analyze. In large experi-

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