Isozyme studies in seed orchards

Isozymundersökningar i fröplantager

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Abstract

Some applications of the isozyme technique for studies in seed orchards are discussed. The basis for these studies is the opportunity to use clones in the seed orchard which are isozymetically distinct from the others. If a clone in one locus carries one or two alleles unique or rare to the orchard, it may then be possible to trace the contribution of this particular clone to the seed formation. The use of this method in studies on the net effect of open pollination in a seed orchard is pointed out.

In the absence of such unique alleles for the estimation of the pollination situation, another method is suggested. This method can be made use of by collecting open-pollinated seeds from a clone and counting plants from these which, by means of the isozyme pattern, show a documented cross-pollination. In this way it is possible to obtain information on the occurrence of selfing.

Checking of the isolation technique for controlled crosses is also possible with the isozyme technique.

The theoretical opportunities for future studies of correlations between economically important traits and allozymes (allelic isozymes) are discussed briefly.

The theoretical possibilities are partly illustrated by results obtained from Swedish Scots pine seed orchards. No indication of deviation from random mating were found. The frequency of plants originating from selfing following open pollination was indicated to be in the range of 2—5 per cent. Such plants seemed to be more common in the lower than in the upper part of the crowns of the ramets. The relevance of checking controlled crosses is demonstrated by means of an applied example.

Contents

1 Introduction

The use of the isozyme technique for forest genetical purposes was first employed to any marked extent by some research groups in 1968—1969.

The advantage of this technique is due to the fact that the enzymes are the executive tools of the genes. Thus, this technique offers an opportunity to acquire informa-tion from the genome very close to the source of information. According to this the allele expression is mostly codominant at this level.

Isozymes are proteins consisting of amino acids. The determination of amino acids is controlled by the genetic code. Proteins which are very similar to each other (e.g.

Table 1. Studies of inheritance of isozymes in forest trees

Species	Enzymes studied/ number of loci	Tissue	Reference	
Picea abies	EST/1	dipl hapl	Bartels	1971
Picea abies	EST/2 LAP/2	hapl	Bergmann	1973
Picea abies	EST/2 LAP/2 PHOS/2	hapl	Bergmann	1974
Picea sitchensis	LAP/2 MDH/1 PGI/1 PGM/2	hapl	Simonsen and Wellendorf	1975
Picea glauca	PEROX/1	dipl	Feret	1971
Pinus attenuata	ADH/1 LAP/2	dipl	Conkle	1971
Pinus nigra	LAP/2	hapl	Nicolic and Bergman	1974
Pinus sylvestris	EST/3	dipl	Rudin and Rasmuson	1973
Pinus sylvestris	GOT/2	dipl hapl	Rudin	1975
Pinus sylvestris	LAP/2	dipl hapl	Rudin	1977
Pinus taeda	LAP/1 ADH/1 PEROX/1	dipl hapl	Long	1972
Ulmus pumila	PEROX/1	dipl	Feret and Stairs	1971

Abbreviations:

ADH	= Alcohol DeHydrogenases		LAP	= Leucine Amino Peptidades
dipl	= diploid tissue		MDH	= Malate DeHydrogenases
EST	= ESTerases		PEROX	= PEROXidases
GOT	= Glutamate-Oxalate Transaminases		PGI	= PhosphoGlucose Isomerases
			PGM	= PhosphoGluocoMutases
hapl	= haploid macrogametophyte (endosperm) from seeds		PHOS	= acid PHOSphatases

differing in a single amino acid) may be separated by gel electrophoresis. In this way, the presence of different genes (alleles) can be studied.

Several investigations on the inheritance of isozyme alleles are presented. At least 15 loci are now available for study (Table 1) and the number is increasing rapidly.

With these investigations as a basis, the opportunities afforded by using the isozyme technique in forest research may be summarized in the following way:

1.1 It is now possible to characterize individual trees, stands and provenances (Bergmann 1975, Lundkvist and Rudin 1976).
1.2 Several basic forest genetical problems are available for study.
    1.2.1 Linkage studies (Bergmann 1974, Simonsen and Wellendorf 1975).
    1.2.2 Evolutionary studies (Clarkson et al. 1974).
    1.2.3 Introgression (Hare and Switzer 1969).
    1.2.4 Studies of gene flow (Sakai and Park 1971, Park 1972).
    1.2.5 Effects of natural selection (Kavacs and Rone 1975).

A collection of papers concerning seed orchard management was recently compiled by Faulkner (1975). The use of isozyme gene markers for the estimation of the degree of selfing is discussed briefly by Hadders and Koski in Chapter 11. Although the isozyme technique as a tool for forest genetics is less than 10 years old, some additional applications in seed orchard studies are already emerging. This paper is mainly concerned with the possibilities of studying different components of the *net effect* of natural pollination in orchards measured by means of viable plants which are derived from open pollinated seeds, and with the checking of controlled crosses.

2 General methodological comments

There are three main ways of handling isozymes for forest genetical purposes.

The first way is to use isozyme variation in diploid tissue as it is. The primary criterion for use is repeatability of the isozyme pattern. Statistical methods are used to test the significance of different isozyme patterns.

The second way involves analysis of isozymes in diploid tissue where genetic relevance can be checked by controlled crosses. The advantages of this method are obvious. It is possible to perform many experimental studies on young plants from different crosses and other seed sources. For example, studies on the father population in natural stands and seed orchards, and on response to different kinds of selection have been carried out. The drawbacks largely concern recessive and "null" alleles which do not produce any detectable isozymes. These may lead to incorrect gene- and genotype frequencies in the diploid tissue.

The third way is to use haploid tissue, for example, the macrogametophytes of coniferous seeds. Genetical relevance can be checked by 1:1 segregation in seed lots from heterozygous trees. This method has some important advantages. Seeds are simple to transport and store, the isozyme patterns are often very distinct, recessive alleles are clearly manifested, and heterozygotes with one allele producing no detectable isozymes ("null" alleles) can be distinguished from real homozygotes. Furthermore, the interpretation of banding patterns is simpler.

The obstacles may be: lack of seeds (especially for *Picea* in the northern part of Scandinavia) and difficulties in obtaining a representative seed sample if no single tree collections are available. If female gametophyte segregation deviates markedly from 1:1 in a locus, estimations of gene frequencies for the stand itself will be incorrect if no single tree collection is made.

To become an efficient tool for breeding purposes, the available isozyme loci should be satisfactorily polymorfic and together represent a major part of the linkage groups of the genome. Therefore, linkage studies on available loci are important.

The most useful allelic constitution of an isozyme locus probably comprises at least two alleles with frequencies just below 0.50, together with some rare alleles.

It is also of great advantage to have a sufficient number of loci available to facilitate allozyme profilation of several clones.

In addition, some experiences of the development of the isozyme technique and sampling are reported by Rudin (1976). These studies concerned:

— biochemical methods of electrophoresis and isozyme staining
— the season for collection of needles in relation to stability of isozyme patterns
— where in a tree to collect needles
— isozyme patterns in relation to environment.

3 Theoretical aspects

3.1 Studies of the relative contribution to the pollination by different clones and random mating

It is obvious that there are good possibilities to identify clones by the isozyme technique. These possibilities are increasing rapidly, since the number of available isozyme loci are also increasing.

For studies on the relative contribution by different clones, it is suitable to find clones in the seed orchard, which have rare or, to the orchard, unique isozyme alleles. If there is a clone homozygous for, e.g. LAP—A2, and no other clone in the orchard carries this allele, that particular clone may be traced as the father whenever it appears among the open-pollination progeny (Fig. 1, clone 5). If there is a heterozygote LAP—A1/A3, and A1 is the unique allele, only half the progeny from this clone can be traced (Fig. 1, clone 9). It is of value to be able to analyse many isozyme loci in order to distinguish as many clones as possible from the others. Obviously it is very difficult to obtain a complete picture of the contributions by all clones, but the method affords a means of calculating the magnitude of the differences in pollination capacity between clones. Therefore, it is always possible to test the deviation from a random mating case. An applied example is shown in 4.2.

3.2 Studies of the proportion of plants originating from selfing following open pollination

3.2.1 Using rare alleles

To study the proportion of selfing, at least three methods are available, one of them referring to the situation mentioned above. If there are clones, each of which is homozygous and unique to the orchard, it is easy to count the homozygotes among the open-pollinated progeny (Fig. 1, clone 5). Such homozygous mother clones will produce only homozygous individuals after selfing.

Also clones heterozygous for a unique allele may be used. Only one-quarter of the individuals after spontaneous selfing of a heterozygous mother clone (Fig. 1, clone 9) will be homozygous for the unique allele. The possibilities of estimating the propor-

Figure 1. An example of a zymogram (the result of an isozyme separation) from mother clones. One band indicates a homozygote, two bands a heterozygote.

Designation of the allele A1								—		
A2					—					
A3	— — — —				— — — — — —					
A4										

| Mother clone No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |

Types of progeny after selfing of mother
Clone Nos. 5 and 9
Segregations

A2 — (clone 5)
A1 — — (clones 7, 8)
A3 — — (clones 10, 11)

all   1 : 2 : 1

Only this type is useful   These types may also be created by cross pollination

Figure 2. Photo illustrating detectable Individuals After Cross-Pollination (IACPs). It shows a gel stained for the GOT—A and -B regions. The mother clone, Y 4501 (M), has the genotype GOT—B2/B2. Heterozygotes in the B-region have three bands. All heterozygotes are IACPs. 0 designates start of, and arrow direction of, migration of isozyme bands in the gel.

tion of selfing of a heterozygous clone are thus considerably lower than is the case for an individual carrying a unique allele in a homozygous condition. An applied example is presented in 4.3.1.

3.2.2 *Using detectable cross-pollination*

It is possible to assess relative selfing rates at different levels in the crown of clones with no unique alleles by a count of the progenies resulting from detectable cross-pollination. If it is assumed that a mother clone has the isozyme genotype B2/B2, the number of seedlings with an isozyme genotype comprising other alleles than B2 can be used to estimate the frequency of cross-pollination (Figure 2). Different isozyme genotypes offer different opportunities to estimate the true number of these individuals. To evaluate the ability of such clones to distinguish the cross-pollinated progenies, a diagnostic value (DV) may be calculated according to the following example:

Alleles at locus A	A1	A2	A3	A4
Gene frequencies among the cross-pollinating clones	0.1	0.3	0.5	0.1

Alleles at locus B	B1	B2	B3
Gene frequencies among the cross-pollinating clones	0.1	0.6	0.3

Consider a clone of the genotype A1/A3 and B3/B3. To identify an Individual After Cross-Pollination (IACP) in the offspring, it is sufficient to find one of the alleles A2, A4, B1 or B2. The probability of finding an IACP in locus A is 0.3; (for A2), +0.1; (for A4) = 0.4; and in locus B, 0.1; (for B1), +0.6; (for B2) = 0.7. The probability of not finding an IACP then becomes 0.6 for locus A and 0.3 for B. The probability of identification of an IACP in neither locus A nor locus B is $0.6 \times 0.3 = 0.18$. Finally, the probability of identifying an IACP in either locus A or B or both is $1.00—(0.6 \times 0.3) = 0.82$ (DV). Any number of loci can be included in a DV only by multiplication of additional probabilities of not finding an IACP. To get a high DV it is favourable to have clones with rare alleles. The DV $(0 \le DV \le 1)$ should be used as a measure of the usefulness for clones in a related type of study. They may be used as weights for results, or progenies from clones with a low value may be omitted from further calculations and discussion of results. Based on the number of documented outcrossed plants (b) among all analysed plants (c), and the probability that a case of outcrossing becomes documented (DV), a selfing index (I_s) may be calculated. The expected frequency of cases with documented outcrossing will be $(1—I_s) \cdot DV$. Putting this equal to b/c and evaluating I_s the following formula is attained:

$$I_s = 1 - b/cDV$$

9

To improve the approximations, the male flowering of the different clones may be estimated or the genotypic composition of the fertilizing pollen cloud may be estimated by direct isozyme analysis. An applied example is demonstrated in 4.3.2.

3.2.3 *Comparing the observed and the expected numbers of progeny*

A third possibility to discover tendencies of selfing is to compare the established and the expected numbers of progeny belonging to different genotypes. One or three genotypes may occur following selfing. If the proportion of these genotypes is higher than expected, this will indicate selfing. There are also opportunities to apply advanced statistical analysis based on this idea using the method of "maximum likelihood". Brown et al. (1975) have used this method for analysis of isozyme data of *Eucalyptus obliqua* in relation to questions of selfing.

3.3 Studies of the contamination with pollen from surrounding stands

Opportunities for a closer study depend on a situation in which the surrounding stands have alleles which deviate considerably from those found in the orchard. It is suitable to estimate the allele composition in the pollen cloud from the surrounding stands during the years when no male flowers are formed. It is then possible to estimate the gradual decrease of the impact from the surrounding stand as the orchard develops. The estimations are based on gene frequencies in the pollen cloud manifested in open-pollinated progeny. It is of course important that no considerable alternation of genotype composition of surrounding stands occur during the period of study.

If the orchard already produces male flowers, a more elaborate method would be to sample surrounding stands, in order to calculate the proportion of unique alleles in the contaminating pollen cloud in relation to the orchard. In order to elucidate the total influence from these stands, the proportion of the open-pollinated progeny

which carries a certain unique allele must be divided by the gene frequency for this allele in the surrounding stands.

3.4 Formation of provenance hybrids in a seed orchard

Several seed orchards are composed of clones of different origin. A reason for this is that hybrids may show hybrid vigour. If there are no hybrids formed, no hybrid vigour can be exploited. The formation of hybrids may be checked by isozymes. There should be good opportunity to characterize at least some of the clones from different provenances in a provenance-crossing seed orchard. In one provenance alleles are sought which are not represented in the other, so that attention is limited to the isozyme loci which best distinguish the provenances. It is then easy to recognize the alleles unique to one provenance in the open-pollinated progenies of the other. Theoretically, the proportion of the progenies which results from the successful inter-provenance crosses may be calculated in the following manner, but with additional profiling alleles and clones.

A hypothetical seed orchard is composed of ten clones from each of provenances I. and II. . Clone nos. 6 and 9 from provenance I. are of the genotype EST—B2/B4 and EST—B4/B4, respectively. None of the clones from provenance II., nor from the rest of provenance I., carries the B4 allel. If the pollen contribution is equal for all clones, the pollen cloud originating from provenance I. will carry the EST—B4 allel in the frequency $\frac{3}{20} = 0.15$.

It is assumed that pollen distribution, fertilization and other factors up to provenance-cross progeny formation are not distorted in any way. In random mating there will be a gene frequency of EST—B4 $= \frac{3}{20 + 20} = 0.075$ among the progenies derived from provenance II. If the frequency becomes markedly higher than expected for alleles unique to provenance I. in progenies

10

from provenance II, this will indicate a high proportion of successful interprovenance crosses. A frequency significantly lower than expected of some alleles unique to provenance I. among the progeny derived from its own provenance, points in the same direction.

3.5 Checking of the crossing technique for controlled crosses

For work in forest genetics it is of importance to make controlled crosses. Unfortunately, it may happen that the actual pedigree is not the same as the expected one. Through investigation of the isozyme type of mother, father and progeny, illegitimate matings may be discovered. For such investigations the prerequisites mentioned in 3.1 and 3.2 must be fulfilled.

The probability of detecting illegitimate progenies depends on the genotype of the parents and the contaminating pollen. When these facts are known, the percentage of pollen contamination may be estimated.

The occurrence of pollen contamination may be especially severe following controlled selfing. A reason for this is that a selfpollinated embryo frequently aborts. This might be explained either by the homozygosity for one lethal or by the accumulation of several semilethals (Andersson et al. 1974). Nonetheless, such embryos will often be inferior in comparison with embryos produced by cross-pollination. Therefore, illegitimate pollens have a greater chance of forming viable seeds in selfed families. An applied example is demonstrated in 4.4.

3.6 Studies of correlations between economically important traits and the isozyme genotype

Studies of such correlations are interesting and important, but are also complicated and laborious. For instance, correlations between isozyme genotype and wood production, resistance to fungi and hardiness may be studied.

Geldermann (1975) is optimistic as regards the use of monohybrid inherited characters (such as isozymes) as markers for sections of chromosomes. He maintains that this method allows a measure of the substitution effects on quantitative characters in natural and breeding populations of animals. Von Weissenberg (1976) has discussed the effectiveness of indirect selection using gene markers and finds the possibilities not too promising. His considerations are made on the basis of studies of fungus disease and insect resistance. The problems he discusses include:

— the causality between marker and desired trait
— heritability of the marker and the desired trait
— genetic control of correlation between the marker and the desired trait
— pleiotropic effects on marker and desired trait.

The advice he gives in cases of noncausal relationships between marker and desired trait is to look for markers as a by-product of general studies in breeding populations.

Weir et al. (1972) measured fitness values for locus pairs in ten separate generations of barley. Four loci were available for this study. The results indicated that single-locus selection estimates bear little relationship to two-locus estimates. Their conclusion is that complex epistatic selective forces operate in that population. This and other studies of *Avena barbata* strongly indicate that natural selection acts to structure the genome (Clegg et al. 1972, Hamrick and Allard 1972) into coadapted gene complexes. This could also apply to forest trees. If so, it is an encouraging fact for studies of natural populations employing allozymes (allelic isozymes). This presents a much greater probability of obtaining a linkage between a locus of practical interest and isozyme gene markers in a coadapted gene group, than when single gene selection is pronounced.

Tigerstedt (1973), supported by Levins (1963), emphasizes an opposite opinion on the basis of his results from allozyme studies

of marginal and central populations in the following way: "The restriction of genetic variability in *Picea abies* is not due to co-adapted gene complexes." If *Pinus* and *Picea* have coadapted gene complexes or not must be judged in the future. But it must be pointed out that if the gene complexes are there, it will be somewhat more difficult to study associations between characters of interest and isozyme loci by progeny from orchards, because these co-adapted gene complexes might be broken up in the artificial offspring population from orchards.

In this situation the most efficient way to look for associations between isozyme profiles and economically important characteristics is to make selections for different traits in full sib families in order to keep the genetical background as isogenic as possible.

4 Some applied studies in Swedish Scots pine seed orchards

4.1 Material and methods

4.1.1 *Material*

Cones were collected following open pollination in the seed orchards at Nedansjö and Solleron. Seeds from these were grown to two-year-old plants. Needles from all plant material from the last growing season were harvested during the dormancy period: in October 1971 for material from Nedansjö and Solleron, and in November 1974 for Skogsnäbben.

Nedansjö. The 405 Nedansjö (Stöde) seed orchard is situated at lat. 62° 23′ N, long. 16° 48′ E, 70 m above sea level. The seed orchard comprises two separate sectors which will be designated A and B. Each sector comprises 25 clones. Three of them are common (Z 4000, Z 4005 and Z 4018). The purpose is to use seeds from the different sectors in different climatic zones. In the analyses below, it will be assumed that the two sectors may be regarded as different units. However, certainly some pollen will fly from one sector to another. This has to be kept in mind on analysis of the data. Cones were collected at two different levels in the crowns of the ramets; the top level (*H*igh) and the bottom level (*L*ow). Offsprings from 25 ramets representing 15 clones were grown to plants.

Solleron. The 442 Solleron seed orchard is situated at lat. 60° 55′ N, long. 14° 36′ E, 165 m above sea level. The orchard comprises 21 clones. Progeny from five clones were included in this study.

Skogsnäbben. The 49 Skogsnäbben seed orchard is situated at lat. 55° 57′ N, long. 13° 06′ E, 85 m above sea level. The orchard comprises 30 clones, six of which have been selfed. Needles from four-year-old plants from these selfed families were analysed.

4.1.2 *Biochemical and genetical methods*

Isozyme separations and the interpretation of the band pattern were performed according to the methods described by Rudin and Rasmuson (1973) and Rudin (1975 and 1977). The isozyme systems analysed are esterases (EST), leucine-amino peptidases (LAP) and glutamat-oxalate transaminases (GOT). Only one locus per enzyme system was utilized in this study.

For all three loci, a rough check of genotypes and segregations of clones investigated in this study was made by analysis of genotype distribution in half sib families (one example, see Table 2). In a few cases

Table 2. Genotype distribution of open pollinated progeny from the high level of clone Z 3001. Data from two ramets are summarized. Only combinations in thick type are correct if inheritance is regular. In this case no improper combinations occurred.

Clone genotype EST — **B01/B2**

B01	B1	**B2**	B3	B4	Allele comb.
—	—	11	1	—	**B01**
	—	—	—	—	B1
		19	3	1	**B2**
			—	—	B3
				—	B4

Clone genotype GOT — **B1/B2**

B1	**B2**	B3	Allele comb.
—	6	4	**B1**
	15	8	**B2**
		—	B3

Clone genotype LAP — **B1/B2**

B1	**B2**	B3	Allele comb.
1	14	—	**B1**
	19	1	**B2**
		—	B3

genotypes of plants could not be related to the mother clone. Results from these plants were omitted from further calculations.

4.1.3 Confidence interval of a low proportion

For the proportions calculated in this study, a 95 per cent confidence interval will be given. This means that in 95 per cent of all random samples the true population mean will be within the confidence interval. In 2.5 per cent of the samples the true value will be below the lower limit, and in 2.5 per cent, the true value will be above the upper limit.

The technique of calculating the intervals may be studied, e.g. in Pearson and Hartley (1956 pp. 74— and corresponding tables). If there is a low frequency of plants carrying an allele, the Poisson distribution may be used. The confidence limits are tabulated in Pearson and Hartley (1956 p. 203). The statistical technique is illustrated by an example (cf. 4.2.1).

Among 444 analysed plants eight were found to be heterozygous for GOT—B1. The tables mentioned by Pearson and Hartley give us a probability of less than 0.025 of obtaining 8 or more heterozygotes if the expected number is below 3.45. The probability of obtaining 8 or less is less than 0.025, if the expected number is above 15.76. The confidence interval of the proportion of heterozygous plants (X) is obtained by dividing these expected numbers by 444.

$$0.0078 < X < 0.0355$$

The table mentioned stops at 50. If the number of "deviating" elements is higher, the binomial distribution may be used and the following expression will be sufficiently accurate for applications to confidence intervals:

$$X \pm z \sqrt{X(1-X)/N}$$

The area above $+z$ or below $-z$ of the standardized normal distribution corresponds to the confidence level. If $z = 1.96$, 0.025 of the distribution will be above $+z$, and 0.025 below $-z$, thereby corresponding to a 95 per cent confidence interval.

N = size of the random sample
X = proportion of "deviating" elements, e.g. originating from a heterozygous clone in the orchard with a rare allele.

4.2 Studies concerning the effective father population

4.2.1 The impact from single clones studied by use of rare or unique alleles

Clone Z 3001 from Nedansjö, section A, carries two unique alleles in a heterozygous condition. GOT—B1 is unique to the whole orchard, while LAP—B1 is unique to section A. In section B another clone, Z 4019, is also a heterozygous carrier of this allele.

Among the 444 analyzed offsprings (excluding offsprings from Z 3001 itself), eight heterozygous GOT—B1 plants were found. If the two homologous alleles contribute equally, this indicates the following contribution of this clone as a father to the other clones in the orchard (p):

$$2 \times 8/444 = 0.036$$

A 95 per cent confidence interval is calculated as outlined in 4.1.3. As it is only possible to trace half of the offspring, since only one allele is known, the confidence limits given for the proportion of heterozygous plants are doubled; thus:

$$0.016 < p < 0.071$$

There were 8 LAP—B1 plants among 633 investigated. These reveal a contribution of $2X = 0.025$ from Z 3001 and

$$0.011 < p < 0.050$$

derived in the same way as above.

In this case, however, the true impact from Z 3001 may be somewhat lower, owing to pollen from Z 4019 in sector B.

The expected contribution from one clone to the other 24 in the sector (excluding selfing) is 1/24 = 0.042. Owing to pollen dispersal between sections, this proportion may be a little lower. This figure agrees well with the findings with respect to Z 3001.

Also at Sollerön, there is one clone with an allele which is unique to the seed orchard, viz. clone W 4013, heterozygous for LAP—B4. Segregation of B2 and B4 in macrogametophytes revealed a ratio of 0.43: 0.57, which is no significant deviation from 0.50: 0.50. Four plants out of 110 investigated carried this allele, thus:

$$2X = 0.072 \ (0.020 < p < 0.186)$$

The expected value following random mating, excluding selfing, is 0.050. This value is also close to the one observed. Thus it may be stated that two investigated clones in two different seed orchards transmitted their genes on the male side to about the same extent as would have been expected following random mating. Certainly there may have been other clones deviating from random mating.

To obtain the best information from this type of study it is necessary:

— to include more isozyme systems in order to identify more unique alleles;
— to investigate more plants in order to improve the statistics;
— to collect the cones in a proper way (cf. e.g. Lindgren and Lindgren 1977);
— to check allele segregation in heterozygotes;
— to count male strobili.

4.2.2 Comparison between expected and observed father population

The most frequent allele of locus LAP—B is B2. A number of offsprings from LAP—B2/B2 grafts were studied. In these cases it is known that the mother contributes a B2, so the genotypes of fertilizating pollens may be determined. The results obtained at Nedansjö, and a comparison with the results expected if all clones in the seed orchard contributed equally, are shown below:

	LAP—B1	LAP—B2	LAP—B3
Expected sector A	0.02	0.92	0.06
Expected sector B	0.02	0.94	0.04
Observed numbers	10	595	31
Observed proportions	0.016	0.936	0.049

Expected and observed values agree remarkably well. This indicates no selection against nor in favour of B1 and B3.

4.2.3 Effective father population for different ramets

It is obvious that close neighbours may contribute to a considerable extent to the pollen cloud in special situations. (A special study of this phenomenon is under way in which the impact of an allele unique to the seed orchard is studied in different neighbours of a ramet carrying the unique allele.) It may be possible to trace such a difference as a heterogenous distribution of rare alleles among the progenies. This was tested with LAP—B. From each seed sample (High and Low kept separate), 15—20 plants were analyzed. Because the results from High and Low did not differ, the results were pooled. The distribution of the events when B1 or B3 were found among progenies from different levels of the crown in ramets not carrying the allele was as follows:

	Number of B1 or B3 in sample from each ramet and level				
	0	1	2	3	4
B1 observed	29	10	2	0	0
B1 expected	29.1	9.9	1.7	0.2	0
B3 observed	16	14	3	3	1
B3 expected	15.2	13.5	6.0	1.8	0.5

The expected values were calculated according to a Poisson distribution. As can be seen, the agreement is good, showing that no indications of local differences in the pollen cloud could be found. However,

15

it has to be emphasized that important effects may pass unnoticed owing to the small number of investigated ramets and the few loci and alleles studied.

4.3 Studies of the proportion of open pollinated plants originating from spontaneous selfing

4.3.1 *Using rare alleles*

One means of obtaining information about the frequency of selfing in seed orchards is to study the occurrence of a unique allele in the homozygous condition in the progeny following open pollination of a heterozygous mother. In the material mentioned earlier, homozygotes of the following types were found in the progenies from two heterozygous mothers:

Z 3001	LAP—B1/B1	1/70
Z 3001	GOT—B1/B1	0/76
W 4013	LAP—B4/B4	0/25
Pooled		1/171

If there is Mendelian segregation, four times as many self-fertilizations will be expected thus the proportion of selfing (s) will be estimated to 0.023.

The confidence interval will be:

$0.0006 < s < 0.13$

The interval will be somewhat larger as the analysis of LAP—B1 and GOT—B1 was made on the same plants.

However, this calculation may be misleading owing to the following considerations:

— The calculation is based on a single homozygous plant
— The plant may originate from a contamination from the other sector of Nedansjö; thus, the incidence of selfing may be overestimated
— there might be gametic selection against the rare alleles; thus the amount of selfing may be underestimated in specific cases.

LAP—B3 is a rare, but not unique, allele. There were 5 homozygotes found in 193 offsprings of heterozygotes. Assuming random mating without selfing, 3.2 would have been expected. The excess of 1.8 might be interpreted as the result of selfing. It does not seem probable that the cause is intensive pollen production of B3 carriers, as the incidence of LAP—B3 in the pollen cloud agreed well with the expected incidence following random mating (4.2.2). A very rough estimate of the proportion of selfing is: $4 \times 1.8/193 = 0.037$. As the lower confidence limit is 0, and s cannot be below that, it seemed justified to allow a 5 per cent confidence on the high confidence level in this case.

$0 \leqq s < 0.15$

4.3.2 *Using detectable cross-pollination*

Selfing may be analysed based on the frequency of plants with a documented cross-pollinated origin compared with expected frequency (Diagnostic value, DV, see 3.2.2). In this example needles were collected from plants originating from a high (H) and a low (L) level in the crowns of 25 ramets of 15 clones in the seed orchard at Nedansjö. The needles were collected in 1969 and 1970. Diagnostic values were calculated based on a cross-pollinating pollen-cloud, with each of the 24 other clones in that sector of the orchard providing an equal share. The gene frequency of the three isozyme loci used for the calculations is presented in Table 3. The genotype of each clone is given in Table 4. The calculated DV are found in Table 5. The DV's of most genotypes utilized in this investigation are somewhat too low to give reliable results.

I_s (see 3.2.2) was estimated for each sample. The statistical accuracy of each sample was low. Therefore, a statistical analysis including I_s for all samples was carried out.

Average values and 95 per cent confidence intervals are shown in Table 6.

It seems as if the incidence of selfing is low in the "High" cones but high in the

"Low" cones. The difference is significant if based on a comparison between the two categories within each ramet.

It is not quite certain that a high I_s-value may be interpreted as a high incidence of selfing. Other types of deviation from random mating may also be responsible; e.g., if the proportion of the most common allele in the pollen-cloud is under-estimated by the equal-share assumption, a false positive I_s may easily occur. However, a difference between Low- and High-cones from the same ramets is very hard to explain other than being a real occurrence of self-fertilization.

The I_s estimates are more certain the higher the diagnostic value is. Thus, it may be justified to weight the I_s values with the DV values, in calculation of the average, in order to obtain a higher statistical accuracy. This was done in the last column of Table 6. The weighted values indicate a somewhat higher level of self-fertilization than the unweighted values.

In conclusion, it may be said that a certain frequency of selfing is expected in a seed orchard. In this special case there are tendencies for the frequency of selfing at the lower levels of the ramets to be higher than that expected. At the higher levels, however, the selfing frequency is significantly lower than at the lower levels. The same tendency was found by Hadders (1971) for the same material by means of empty seed frequencies, but not for the frequencies of abnormal one-year-old plants. Based on an investigation of filled seeds in a stand of Loblolly Pine, Franklin (1971) estimated the natural self-fertilization in upper crowns to be 7 per cent and in lower crowns 34 per cent.

Table 3. Gene frequencies estimated from isozyme analyses in the seed orchard at Nedansjö.

Enzyme system	Allele	Gene frequencies	
		Sector A	Sector B
Esterases (EST)	B01	0.10	0.02
	B1	0.02	0.14
	B2	0.74	0.66
	B3	0.10	0.14
	B4	0.04	0.04
Glutamate-oxalate transaminases (GOT)	B1	0.02	—
	B2	0.70	0.50
	B3	0.28	0.50
Leucine-amino peptidases (LAP)	B1	0.02	0.02
	B2	0.92	0.94
	B3	0.06	0.04

Table 4. Clones from which seeds were collected at two levels in the crown and their genotypes.

	EST—B	GOT—B	LAP—B
Sector A			
Y 4500	B2/B2	B2/B2	B2/B2
Y 4501	B2/B2	B2/B2	B2/B2
Y 4506	B2/B2	B2/B2	B2/B3
Y 4507	B2/B2	B2/B3	B2/B2
Y 4508	B2/B2	B2/B2	B2/B2
Y 4510	B2/B2	B2/B2	B2/B2
Z 3001	B01/B2	B1/B2	B1/B2
Z 4000	B2/B2	B2/B2	B2/B2
Z 4002	B2/B2	B2/B3	B2/B2
AC 4103	B2/B3	B2/B2	B2/B2
AC 4106	B01/B2	B2/B3	B2/B3
AC 4109	B2/B2	B2/B3	B2/B2
Sector B			
Z 4006	B2/B2	B2/B2	B2/B3
Z 4012	B01/B2	B2/B2	B2/B2
Z 4013	B2/B4	B2/B2	B2/B2

4.4 Checking of the crossing technique for controlled selfing

Selfing pollen usually have a considerably lower probability of producing plants than have outcrossing pollen. (The quantitative consequences were discussed by Lindgren 1976.) Therefore, a rather low percentage of contaminating pollen in artificial selfing may cause a high fraction of plants originating from outcrossing. The occurrence of such contaminations has been tested in progenies from clones at the Skogsnäbben seed orchard. A considerable incidence of contamination was suspected.

Table 5. Number of plants after discovered cross pollination fram the high (H) and low (L) levels in the crown of investigated ramets.

IACP = Individuals After Cross Pollination

Year of collection	Clone		Seed lot No.	Diagnostic value (DV)	IACP plants b	Analysed plants c	Average frequency of IACP-plants b/c	Selfing index $1 - \dfrac{b}{c \cdot DV}$
70	Y 4500—2	H	109	0.540	9	20	0.450	+ 0.167
		L	110		11	20	0.550	− 0.018
70	Y 4501—4	H	113	0.540	15	20	0.750	− 0.388
		L	114		14	19	0.737	− 0.364
69	Y 4501—8	H	39	0.540	9	19	0.474	+ 0.124
		L	40		5	20	0.250	+ 0.537
70	Y 4506—1	H	115	0.509	12	16	0.750	− 0.473
		L	116		14	18	0.778	− 0.528
70	Y 4508—2	H	129	0.540	15	19	0.790	− 0.461
		L	130		10	18	0.556	− 0.280
69	Y 4508—4	H	55	0.540	5	18	0.278	+ 0.486
		L	56		2	16	0.125	+ 0.769
70	Y 4510—2	H	137	0.540	11	17	0.647	− 0.197
		L	138		7	20	0.350	+ 0.352
69	Z 3001—3	H	1	0.447	11	20	0.550	− 0.231
		L	2		10	18	0.556	− 0.244
69	Z 3001—9	H	5	0.447	5	15	0.333	+ 0.254
		L	6		9	17	0.529	− 0.185
70	Z 4000—1	H	79	0.540	10	20	0.500	+ 0.075
		L	80		10	20	0.500	+ 0.075
69	Z 4000—9	H	9	0.540	11	20	0.550	− 0.018
		L	10		7	20	0.350	+ 0.352
69	Z 4001—2	H	15	0.345	8	20	0.400	− 0.158
		L	16		4	20	0.200	+ 0.421
70	Z 4002—1	H	91	0.345	6	18	0.333	+ 0.035
		L	92		6	20	0.300	+ 0.131
69	Z 4002—4	H	19	0.345	9	19	0.474	− 0.371
		L	20		8	19	0.421	− 0.219
69	Z 4002—5	H	23	0.345	7	20	0.350	− 0.013
		L	24		7	20	0.350	− 0.013
70	Z 4006—8	H	99	0.697	17	20	0.850	− 0.220
		L	100		13	20	0.650	+ 0.067
69	Z 4012—1	H	101	0.701	7	18	0.389	+ 0.445
		L	102		5	20	0.250	+ 0.643
70	Z 4012—10	H	103	0.701	7	20	0.350	+ 0.500
		L	104		10	20	0.500	+ 0.286
70	Z 4013—1	H	105	0.691	12	20	0.600	+ 0.132
		L	106		9	19	0.474	+ 0.315
69	Z 4013—7	H	33	0.691	13	20	0.650	+ 0.060
		L	34		8	20	0.400	+ 0.421
69	AC 4103—3	H	61	0.475	5	20	0.250	+ 0.474
		L	62		6	18	0.333	+ 0.298
69	AC 4103—6	H	63	0.475	12	20	0.600	− 0.264
		L	64		8	19	0.421	+ 0.113
70	AC 4106—1	H	147	0.293	6	20	0.300	− 0.068
		L	148		7	20	0.350	− 0.246
69	AC 4106—6	H	65	0.293	8	20	0.400	− 0.424
		L	6		9	20	0.450	− 0.602
69	AC 4109—2	H	69	0.345	5	19	0.263	+ 0.238
		L	70		2	20	0.100	+ 0.711

Table 6. Selfing index (I_s) for High- and Low-cones.

Selfing index (I_s)

Category	Arithmetic average	95 per cent confidence interval	DV-weighted average
H-cones	− 0.020	− 0.138 < I_s < 0.114	0.013
L-cones	0.122	− 0.032 < I_s < 0.275	0.158
Difference L—H	0.134	0.020 < diff < 0.248	0.145

Table 7. Alleles and gene frequencies of fathers for crosses in the Skogsnäbben seed orchard.

Locus	Alleles and gene frequencies		
LAP—A	A2 0.98	A3 0.02	
LAP—B	B1 0.02	B2 0.94	B3 0.04
GOT—A	A2 1.00		
GOT—B	B1 0.02	B2 0.45	B3 0.53

In order to screen the possibilities to detect illegitimate pollination, all the mother clones and all the fathers which took part in the crossing programme of that particular year were isozyme analysed. The gene frequencies for these fathers were compared with the genotypes of the six mother clones that had been selfed (Table 7 and 8). One family (H 55) was found to be suitable for further tests. As can be seen in Table 8, the mother of this family is homozygous GOT—B2/B2.

This allele is present at a frequency of 0.45. The probability of discovering an illegitimate pollination is 1.00—0.45 ≈ 0.55. The corresponding probability for the other selfed families varied between 0.00 and 0.06. A total probability of discovery of an illegitimate pollination may be calculated in the same way as the diagnostic value (DV), (see 3.2.2).

Five out of the six mother clones had a DV = 0.10, and one mother clone (H 55) had a DV = 0.59. From this latter mother clone 32 plants from selfing were isozyme analysed. Seven of these plants were classified as a result of illegitimate pollination. They had one band of the mother type and one band in a position which did not correspond to the type of the mother. The average height of these plants was 95.7 cm, ranging between 77 and 122 cm. For the 25 other plants the average height was 58.2 cm, ranging between 26 and 98 cm. A t-test between the average values resulted in t = 4.363***. This result supports our classification of the seven plants as being a result of illegitimate pollination. With further polymorphic loci in needle analyses the possibility to distinguish contamination increases rapidly.

Table 8. Allele composition of selfed mother clones at Skogsnäbben.

Mother clone	Locus and alleles				
	LAP—A	LAP—B	GOT—A	GOT—B	DV
G 51	A2/A2	B2/B2	A2/A2	B2/B3	0.10
H 16	A2/A2	B2/B2	A2/A2	B2/B3	0.10
H 55	A2/A2	B2/B2	A2/A2	B2/B2	0.59
H 76	A2/A2	B2/B2	A2/A2	B2/B3	0.10
H 98	A2/A2	B2/B2	A2/A2	B2/B3	0.10
H 125	A2/A2	B2/B2	A2/A2	B2/B3	0.10

5 Sammanfattning

Användning av isozymmetodiken vid skogs-genetiska undersökningar i fröplantager diskuteras och belyses med några exempel.

Isozymerna är proteiner som direkt avspeglar genotypen. Mycket obetydliga genetiska skillnader (utbyte av enstaka aminosyror) kan upptäckas genom att proteinmolekylerna rör sig olika snabbt genom ett elektriskt fält.

Om flera polymorfa isozym-loci är tillgängliga utgör den totala isozymprofilen underlag för identifikation av kloner använda i ett förädlingsprogram.

Om en klon är ensam om en viss isozymtyp (allel) i en plantage, kan denna klons faktiska bidrag till pollineringen i plantagen studeras.

Genom jämförelser mellan moderns och avkommans genotyper kan slutsatser dras rörande den effektiva faderpopulationens sammansättning. Bl.a. diskuteras följande typer av frågeställningar:

— eventuella avvikelser från slumpmässighet beträffande de olika klonernas genbidrag till avkomman

— graden av självbefruktning
— förekomsten av pollen från källor utanför plantagen
— frekvensen av hybridfrö i en proveniens-korsningsplantage
— förekomsten av pollenkontaminering vid kontrollerade korsningar, särskilt självpollinering
— eventuella kopplingar mellan isozymtyp och praktiskt värdefulla karaktärer.

Praktiska applikationer av isozymtekniken har utförts vid tallfröplantagerna Nedansjö, Sollerön och Skogsnäbben. Resultaten kan sammanfattas enligt följande:

— Ingen indikation på avvikelser från slumpmässig parning föreligger.
— Självbefruktningsfrekvensen verkar röra sig om 2—5 procent.
— Kottar från nedre delen av kronan förefaller innehålla mer självpollineringsfrö.
— En stor andel av avkommor från kontrollerad självbefruktning kan vara ett resultat av pollenkontaminering.

6 Acknowledgements

References

Andersson, E., Jansson, R. and Lindgren, D. 1974. Some results from second generation crossings involving inbreeding in Norway spruce (*Picea abies*). — Silvae Genet. 23: 34—43.

Bartels, H. 1971. Genetic Control of Multiple Esterases from Needles and Macrogametophytes of *Picea abies*. — Planta (Berl.) 99: 283—289.

Bergmann, F. 1973. Genetische Untersuchungen bei *Picea abies* mit Hilfe der Isoenzym-Identifizierung. II Genetische Kontrolle von Esterase- und Leucinamino-peptidase Isoenzymen im haploiden Endosperm ruhenden Samen. — Theoret. Appl. Genet. 43: 222—225.

— 1974. The genetics of some isoenzyme systems in Spruce endosperm. (*Picea abies*). — Genetica (Yugoslavia) 6: 353—360.

— 1975. Herkunfts-Identifizierung von Forstsaatgut auf der Basis von Isoenzym-Genhäufigkeiten. — Allg. Forst- u. Jagdz. 146: 191—195.

Brown, A. H. D., Matheson, A. C. and Eldrige, K. G. 1975. Estimation of Mating System of *Eucalyptus obliqua*. — L'Hérit. by using Allozyme Polymorphism. — Aust. J. Bot. 23: 931—949.

Clarkson, R. B., Huang, F. H., Cech, F. C. and Gihgerich, L. A. 1974. Variability of Soluble Seed Proteins, peroxidase and Leucine aminopeptidase in populations of *Juglans nigra*. — Biochem. Syst. Ecol. 2: 59—66.

Clegg, M. T., Allard, R. W. and Kahler, A. L. 1972. Is the Gene the Unit of Selection? Evidence from Two Experimental Plant Populations. — Proc. Nat. Acad. Sci. USA 69: 2474—2478.

Conkle, M. T. 1971. Inheritance of alcohol dehydrogenase and leucine aminopeptidase isozymes in Knobcone pine. — Forest Sci. 17: 1970.

Faulkner, R. 1975. Seed orchards — Forestry Commission Bulletin 54.

Feret, P. P. 1971. Isozyme variation in *Picea glauca* (Moench). Voss seedlings — Silvae Genet. 20: 46—50.

Feret, P. P. and Stairs, G. R. 1971. Peroxidase inheritance in *Ulmus pumila*. — Forest Sci. 17: 472—475.

Franklin, E. C. 1971. Estimates of frequency of natural selfing and on inbreeding coefficients in Loblolly pine. Silvae Genet. 20: 262—265.

Geldermann, H. 1975. Investigations on Inheritance of Quantitative Characters in Animals by Gene Markers. I Methods — Theoret. Appl. Genet. 46: 319—330.

Hadders, G. 1972. Pollineringssituationen i tallplantager. — Föreningen Skogsträdförädling, Inst f skogsförbättring, Årsbok 1971: 111—139.

Hadders, G. and Koski, V. 1975. Probability of inbreeding in seed orchards. In Seed Orchards, edited by R. Faulkner. — Forestry Commission Bulletin 54: 108—116.

Hamrick, J. L. and Allard, R. W. 1972. Microgeographical variation in allozyme frequencies in *Avena barbata*. — Proc. Nat. Acad. Sci. USA, 69: 2100—2104.

Hare, R. C. and Switzer, G. L. 1969. Introgression with Shortleaf pine may explain rust resistence in western Loblolly pine. — U.S. Forest Service. Note 50—88. 2 p.

Kavacs, J. and Rone, N. 1975. Peroxidase isoenzymes in the populations of Norway spruce. — Genetitjeskie issledovanija drevcsich v latvijskoj SSR, Riga 1975. 58—62. Eng. summary.

Levins, R. 1963. Theory of fitness in a heterogenous environment. II. Developmental flexibility and niche selection. — Am. Natur. 97: 75—90.

Lindgren, K. and Lindgren, D. 1976. Blomningen i en granfröplantage. — Forskningsrapport till Statens Råd för Skogs- och Jordbruksforskning.

Long, E. M. 1972. Genetic polymorphism of isozymes in Loblolly pine seed. — Thesis for Ph. D. Texas A and M University 1972. 55 p.

Lundkvist, K. and Rudin, D. 1977. Genetic variation in 11 populations of *Picea abies* in Sweden as determined by isozyme analysis. — Hereditas 85: 67—74.

Nicolic, D. J. and Bergmann, F. 1974. Genetic variation of Leucine aminopeptidase isoenzymes in seeds of *Pinus nigra Arn.* — Genetica (Yugoslavia) 6: 361—365.

Park, Y. G. 1972. Gene flow in natural forest of *Cryptomeria japonica* by means of isozyme polymorphisms. — Research report of Inst of forest genetics, S. Korea. No. 9: 77—85.

Pearson, E. S. and Hartley, H. O. 1956. Bio-metrika tables for statisticans, Vol 1 — University press, Cambridge.

Rudin, D. 1975. Inheritance of glutamate oxalate-transaminases (GOT) from needles and endosperms of *Pinus sylvestris*. — Hereditas 80: 296—300.

— 1976. Application of isozymes in tree breeding. — Proceedings of the IUFRO Joint Meeting on Advanced Generation Breeding, Bordeaux. 20 p.

— 1977. Leucine-amino-peptidases (LAP) from needles and endosperms of *Pinus sylvestris L.* — a study of inheritance of allozymes. — Hereditas 85: 219—226.

Rudin, D. and Rasmuson, B. 1973. Genetic variation in esterases from needles of *Pinus silvestris L.* — Hereditas 73: 89—98.

Sakai, K.-I. and Park, Y.-G. 1971. Genetic studies in natural populations of forest trees. III. Genetic differentiation within a forest of *Cryptomeria japonica.* — Theoret. Appl. Genet. 41: 13—17.

Simonsen, V. and Wellendorf, H. 1975. Some polymorphic isoenzymes in the seed endosperm of sitka spruce *(Picea sitchensis (Bong.) Carr.).* — Forest Tree Improvement 9: 1—21.

Tigerstedt, P. M. A. 1973. Studies on isozyme variation in marginal and central populations of *Picea abies.* — Hereditas 75: 47—60.

Weir, B. S., Allard, R. W. and Kahler, A. L. 1972. Analysis of complex allozyme polymorphisms in a barley population. — Genetics 72: 505—523.

von Weissenberg, K. 1976. Indirect selection for improvement of desired traits. — In Modern Methods in Forest Genetics, edited by J. P. Miksche. 217—228. Springer Verlag, Berlin, Heidelberg, New York.