# Studies of Allele Frequencies and Inbreeding in Scots Pine Populations by the Aid of the Isozyme Technique

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## Introduction

Our knowledge of the variability within populations of *Pinus sylvestris* is mainly limited to hardiness and growth (cf. EICHE 1966, HAGNER 1970, REMRÖD 1972, EICHE and ANDERSSON 1973). An increased knowledge of the variability of other genetically determined characteristics in Scots pine populations should be valuable especially since the total amount of seed needed for almost all reforestation in Sweden will be produced in the seed orchards established within a few years. A continuous cutting of the autochtonous stands and a reforestation with seeds from the seed orchards will in the near future narrow the gene pool of Scots pine unless measures are taken to preserve genetic variability. In this connection it is worth mentioning that STERN (1972) strongly warned against a low number of trees in the breeding population.

The number of plus trees selected all over Sweden amounts to more than 5,000 trees. This seems sufficient to guarantee a satisfactory variability. However, first of all it must be remembered that these 5,000 trees have to be divided into different breeding populations according to their origin. Today Sweden is divided into 16 different seed orchard zones which constitute the main units of reforestation. This means that each breeding population contains about 300 plus trees. However, these trees are not evenly distributed within the zones. Furthermore, the variability of the various commercially important characteristics differs between the zones. A study of the zymograms of individual trees belonging to different populations would reveal variability within as well as between the populations examined. Studies of zymograms in Scots pine (RUDIN and RASMUSON 1973) have revealed that isozyme alleles can act as codominant hereditary factors. The same mechanism works in other conifers as well (BARTELS 1971, CONCLE 1971). Based on a study of vegetatively propagated material RASMUSON and RUDIN (1971) showed that the influence of root stock and location was insignificant.

Investigations of the variability of different forest tree populations by the aid of the isozyme technique have been undertaken by several investigators during the last few years (MIYAZAKI and SAKAI 1969, SAKAI and PARK 1971, SAKAI *et al.* 1970, 1971, 1972, SAKAI and MIYAZAKI 1972, MATSURA and SAKAI 1972, TIGERSTEDT 1972). Another practical application of the isozyme technique has been suggested by BERGMANN (1971 and 1972). In this case isozyme examination should be used for seed certification.

During the previous century reforestation following forest fire seems to have been of common occurrence. In these cases the reforestation may have originated to a great extent from seeds formed on a few surviving trees widely separated from each other. The probability for selfing may thus have been relatively high (cf. JOHNSON 1945). If selfing has resulted in seed production it is expected that the new regenerated population suffered from inbreeding to a certain extent. If this process has taken place several times the degree of homozygosity may have been increased considerably. By the aid of zymogram analysis the allele frequencies in different isozyme loci can be revealed. Based on the allele frequencies the expected frequencies of the genotypes, following random mating, can be calculated. A deficit of heterozygous genotypes may indicate inbreeding, and the coefficient of inbreeding (F) may be estimated from the equation

$$F = \frac{H_0 - H_F}{H_0}$$

$H_0$ = proportion of heterozygotes expected according to random mating;

$H_F$ = proportion of heterozygotes observed (cf. CROW and KIMURA 1970).

However, there are other possible causes for deviations from expected genotype frequencies, for instance selection in favour of or against homozygotes, heterogeneity of the population and the existence of more or less isolated subpopulations, the presence of silent alleles which makes it impossible to discriminate between heterozygotes and homozygotes. Isozyme analysis is a valuable tool for revealing inbreeding, but when interpreting the obtained data due consideration must be given to other possible factors which might account for the observed deviations.

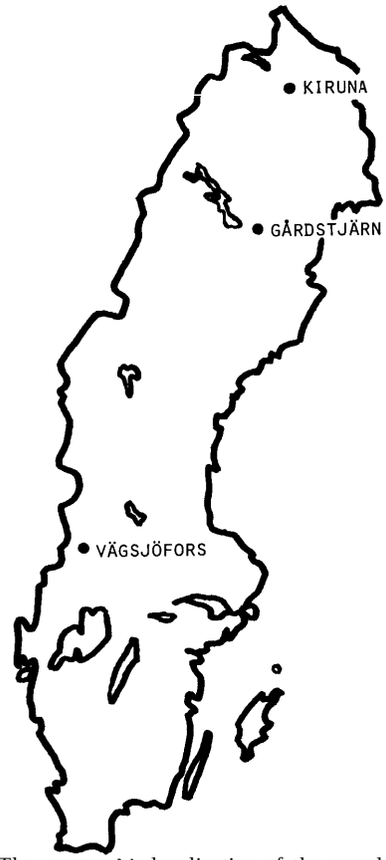


*Figure* 1. — The geographic localization of the populations included in the study.

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In the present paper data from three populations of *Pinus sylvestris* will be presented. Two of them were selected for isozyme analysis of special reasons. They will be presented in another context. Their geographic localization is shown in *Fig. 1.*

## Material and Methods

Needles for this investigation were collected on the south-west side of the crowns of the trees. The geographic data of the three localities are summarized below.

| Name of locality | Latitude | Longitude | Altitude | Date of collection | Individuals tested |
|---|---|---|---|---|---|
| Vägsjöfors | | | | | |
| Ladtjärnstorp | 60° 18' | 13° 06' | 180 | January 1973 | 99 |
| Bomtorpet | 60° 17' | 13° 07' | 200 | January 1973 | 42 |
| Gårdstjärn | 65° 29' | 19° 17' | 400 | December 1972 | 132 |
| Kiruna | | | | | |
| Kauppinen | 67° 51' | 20° 27' | 355 | March 1973 | 19 |
| Nokutosvara | 67° 51' | 20° 16' | 480 | March 1973 | 20 |

The trees belonging to the Kiruna population constitute mother trees of the populations included in the country-wide provenance experimental series established during the early fifties by Eiche (cf. Eiche 1966). The collection of material was done from grafts growing in a clone archive at Röskär, Bogesund, nine kilometers north-east of Stockholm.

The stand Ladtjärnstorp at Vägsjöfors was an ordinary full density stand about 100 years old. It is worth mentioning that more than 10 per cent of the trees suffered from *Peridermium pini.* At Bomtorpet the stand had a density of about 0.6 and was 110 years old.

The age of the trees at Gårdstjärn amounts to about 120 years. The average number of trees per hectar within this seed-tree stand varies between 10—18 trees.

The twigs with needles were stored at +6° C for less than two months before investigation. The needles were ground in an automatic mortar grinder (own construction). Buffer, staining methods for esterases and other details were made according to the simplified method earlier described by Rudin and Rasmuson (1973).

For leucine-amino-peptidases (LAP) and glutamate-oxalate-transaminases (GOT) the electrophoretic separation was carried out in a 12% starch-gel pH 8.2 (Ashton and Braden 1961) for 4.5 hours in room temperature and at 20 volts per cm. However, the gel buffer was diluted with 50% more dest. water (Beckman *et al.* 1967) than the original Ashton and Braden buffer.

In order to develop the LAP-bands the gel was preincubated in 0.2 M Tris-maleat buffer pH 5.4 for 15 minutes. The buffer was removed and the staining solution was added. This consists of 55 ml of the above mentioned Tris-maleate buffer, 5 mg L-leucyl-$\beta$-naphtylamide HCl and 7.5 mg Black K salt (Beckman *et al.* 1964). After two hours at 37° C the LAP-pattern was recorded. Fixation of the gels was done in a solution consisting of water, metanol and acetic acid in the ratio of 5 : 5 : 1. The GOT-bands were stained with the following staining solution: 75 ml 0.1 M phosphate buffer pH 7.4, 0.375 mg pyridoxal-5-phosphate, 172 mg L-asparic acid and 150 mg Fast Blue BB-salt (Schwartz *et al.* 1963, slightly modified). The pattern was recorded after 1½ hours in 37° C and in darkness. Fixation of the bands was done in glycerol + dest. water (1 : 1).

*Table 1.* — The allele frequencies in three isozyme loci from three populations of *Pinus sylvestris*

| Locus | EST-B | | | | | | GOT-B | | | | | | LAP-B | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Allele | B01 | B 1 | B 2 | B 3 | B 31 | B 4 | B 1 | B 13 | B 2 | B 22 | B 3 | B 4 | B01 | B 1 | B 2 | B 3 | n |
| Vägsjöfors | 0.04 | 0.07 | 0.69 | 0.17 | — | 0.03 | 0.02 | — | 0.38 | 0.01 | 0.59 | — | 0.004 | 0.02 | 0.94 | 0.04 | 282 |
| Gårdstjärn | 0.05 | 0.11 | 0.62 | 0.18 | 0.03 | 0.01 | 0.04 | — | 0.43 | 0.04 | 0.48 | 0.004 | — | 0.003 | 0.94 | 0.06 | 264 |
| Kiruna | 0.03 | — | 0.86 | 0.09 | 0.01 | 0.01 | — | 0.01 | 0.55 | 0.08 | 0.36 | — | — | 0.03 | 0.95 | 0.03 | 78 |
| Test of homogeneity | $\chi^2 = 18.804$<br>df = 8<br>$0.025 < p < 0.05$<br>B 31 and B 4 pooled<br>B 2 versus other alleles:<br>$\chi^2 = 15.897$<br>df = 2<br>$p < 0.001$<br>B 3 versus other alleles:<br>$\chi^2 = 4.025$<br>df = 2<br>$0.10 < p < 0.20$ | | | | | | $\chi^2 = 26.439$<br>df = 6<br>$p < 0.001$<br>B 1 + B 13 + B 4 pooled<br>$\chi^2 = 19.93$<br>df = 4<br>$p < 0.001$<br>B 1 + B 13 + B 4 pooled<br>B 2 + B 22 pooled<br>B 2 versus other alleles:<br>$\chi^2 = 7.481$ df = 2 $p < 0.05$<br>B 2 + B 22 versus other alleles:<br>$\chi^2 = 14.595$ df = 2 $p < 0.001$<br>B 3 versus other alleles:<br>$\chi^2 = 15.549$ df = 2 $p < 0.001$ | | | | | | $\chi^2 = 0.101$<br>df = 2<br>$0.95 < p < 0.99$<br>B01 + B 1 + B 3 pooled | | | | |

## Results and Discussion

The zymogram analysis of the two populations at Vägsjöfors showed a close relationship between the two populations. Therefore, the trees of these two populations can be regarded as components of just one population.

Owing to the low number of trees in the two populations at Kiruna they were regarded as one population to make comparisons with the other two populations meaningful.

The allele frequencies in the three investigated isozyme loci are compiled in *Table 1*. To perform tests of homogeneity among populations the alleles with the lowest frequencies had to be pooled, as indicated in the table. The reason for pooling the GOT-B alleles B2 and B22 will be given below. The tests revealed heterogeneity of allele frequencies for the EST-B locus (5% significance) and for the GOT-B locus (0.1% significance) whereas an extremely high p value for homogeneity was obtained for the LAP-B locus.

The most common alleles were tested separately against all other alleles. In the EST-B locus a significant heterogeneity was found for B2 but not for B3. In the GOT-B locus B2, B2 + B22, as well as B3 showed heterogeneity among populations. The resulting $\chi^2$ values are also given in *Table 1*.

A separate test of homogeneity was also carried out for the Vägsjöfors and Gårdstjärn populations. In that case the test of homogeneity revealed that the differences in allele frequencies were significant at the one per cent level both as regards the EST-B and GOT-B loci.

The genotype frequencies of the two populations were tested with respect to the agreement with the HARDY-WEINBERG law. The data from this analysis are compiled in *Table 2*.

*Table 2.* — Summary of the test of agreement with random mating genotype frequencies within three isozyme loci from three different populations of *Pinus sylvestris*.

| | EST-B | GOT-B | LAP-B |
|---|---|---|---|
| Vägsjöfors | $0.3 < p < 0.5$ | $0.5 < p < 0.7$ | $0.8 < p < 0.9$ |
| Gårdstjärn | $p < 0.001$ | $p < 0.1$ [1)] | $0.8 < p < 0.9$ |
| Kiruna | $0.10 < p < 0.20$ | $0.8 < p < 0.9$ | $0.90 < p < 0.95$ |

1) If the figures for the genotypes B 2/B 2, B 2/B 22, B 22/B 22 are summed the p value will fall between 0.5 and 0.7

The GOT-B locus needs a special comment. In the population from Gårdstjärn four homozygous B 22/B 22 trees were found whereas the theoretically expected number based on the frequency of this allele was calculated to be 0.24. This deviation from the expectation explains the low p value obtained in this case. However, it is a delicate task to separate the genotypes B 2/B 22 and B 2/B 2 from each other. It was therefore preferable to test the agreement with the HARDY-WEINBERG law also following pooling of the genotypes B 2/B 2, B 2/B 22, and B 22/B 22. In doing so the p value falls between 0.5 and 0.7. With this in mind it may be concluded that only the EST-B locus in the Gårdstjärn population showed a significant deviation from the expectation of random mating.

During the analysis of the compiled data it was found that the deviations were all caused by an excess of homozygous individuals. Inbreeding coefficients were therefore calculated according to the equation given in the introduction *(Table 3)*. This calculation is just another way of expressing the deviations from the HARDY-WEINBERG law, and in agreement with the previous test the only significant coefficient of inbreeding was found in the Gårdstjärn population for the EST-B locus. This inbreeding coefficient was remarkably high, amounting to 0.33 which is even higher than that expected following half sib mating.

As pointed out earlier the significance of this observation should not be overestimated. No significant excess of homozygotes was found in the other two loci, but selection might act differently on the various genotypes of the tested loci, or on loci linked to them, which may obscure the inbreeding effect. A close study of our data shows that the differences between the gene frequencies in the GOT-B locus of the three populations are greater than for the corresponding ones in the EST-B locus. — Furthermore preliminary date from a new investigation of stands distributed all over Sweden (RUDIN unpubl.) suggest that the most common EST-B alleles — B2 and B3 — occur in the same frequency in the very north and in the very south of Sweden. — This probably means that selection on a geographical basis is less pronounced for the EST-B locus. Moreover, the population at Gårdstjärn is an artificial one. The trees have been selected according to their good characteristics with respect to growth, trunk straightness etc. The occurrence of an excess of homozygotes might be connected with these characteristics of practical value. Since the trees which are rejected on the occasion of selection cannot be analysed, it is impossible to come to any conclusions concerning this question. It suffices to add that the excess of B 1/B 1 trees was most pronounced but B 3/B 3 trees were also in great excess.


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## Abstract

The allele frequencies in three isozyme loci of three Swedish pine populations were estimated (cf. *Table 1*). The populations were tested with respect to homogeneity. The tests revealed heterogeneity of allele frequencies for the EST-B locus (5 per cent significance) and for the GOT-B locus (0.1 per cent significance) whereas high p value for homogeneity was obtained for the LAP-B locus. The populations were tested for agreement with expected genotype frequencies following random mating. A general excess of homozygotes was found, but the deviations were significant only for the EST-B locus of the Gårdstjärn population (lat. 65° 29' alt. 400 m). The calculated inbreeding coefficient for this population for the EST-B locus was 0.33.



*Key words:* *Pinus sylvestris*, population genetics, isoenzyme studies, esterases, leucine aminopeptidases, glutamate-oxalat-transaminases, allele frequencies, inbreeding, genetic variability.


## Literature Cited


ASHTON, G. C., and BRADEN, A. W. H.: Serum-globulin polymorfism in mice. Aust. J. Biol. Sci. **14**: 248—253 (1961). — BARTELS, H.: Isoenzyme und ihre Bedeutung für Forstpflanzenzüchtung und -gene-

*Table 3.* — Expected and observed frequency of heterozygotes in three isozyme loci from three populations of *Pinus sylvestris* as well as the inbreeding coefficients (F).

| | EST-B | | | GOT-B | | | LAP-B | | |
|---|---|---|---|---|---|---|---|---|---|
| | expected | observed | F | expected | observed | F | expected | observed | F |
| Vägsjöfors | 0.4863 | 0.4184 | 0.14 | 0.5054 | 0.4610 | 0.09 | 0.1145 | 0.1064 | 0.07 |
| Gårdstjärn | 0.5650 | 0.3788 | 0.33$^{xxx}$ | 0.5816 | 0.5909 | - | 0.1150 | 0.1136 | 0.01 |
| Kiruna | 0.2526 | 0.1538 | 0.39 | 0.5613 | 0.5385 | 0.04 | 0.0979 | 0.1026 | - |

$^{xxx}$ significantly different from 0 at the 0.1 per cent level

tik. Allgem. Forstzschr. **26**: 50—52 (1971). — Beckman, L., Scandalious, J. G., and Brewbaker, J. L.: Genetics of leucine aminopeptidase isozymes in maize. Genetics **50**: 899—904 (1964). — Beckman, L., Beckman, G., Christodoulou, C., and Ifekwunigwe, A.: Variations in human placental alkaline phosphatase. Acta Genet. (Basel) **17**: 406—412 (1967). — Bergmann, F.: Genetische Untersuchungen bei *Picea abies* mit Hilfe der Isoenzym-Identifizierung. I. Möglichkeiten für genetische Zertifizierung von Forstsaatgut. Allgem. Forst- u. Jagdz. **142**: 278—280 (1971). — Bergmann, F.: Experiments on possibilities for genetic certification of forest seeds. IUFRO — SABRAO Joint Symposia, Tokyo, A-6 (V): 1—5, 1972. — Conkle, Th.: Inheritance of alcohol dehydrogenase and leucine aminopeptidase isozymes in knobcone pine. For. Sci. **17**: 190—194 (1971). — Crow, J. F., and Kimura, M.: An introduction to population genetics theory. Harper & Row, Publishers, New York, Evanston, and London, 1970. — Eiche, V.: Cold damage and plant mortality in experimental provenance plantations with Scots pine in Northern Sweden. (Köldskador och plantdöd i proveniensförsök med tall i Norrland.) Stud. For. Suec. **36**: 1—218 (1966). — Eiche, V., and Andersson, E.: Survival and growth in Scots pine *(Pinus silvestris* L.). Provenance experiments in Northern Sweden. Theoret. Appl. Genet. (In press 1973). — Hagner, M.: A genecological investigation of the annual rhythm of *Pinus silvestris* L. (En genekologisk undersökning av årsrytmen hos *Pinus silvestris* L.). Stud. For. Suec. **80**: 1—40 (1970). — Johnson, L. P. V.: Reduced vigour, chlorophyll deficiency, and other effects of self-fertilization in *Pinus*. Can. J. Res. **23**: 145—149 (1945). — Matsumura, T., and Sakai, K.-I.: Geographical variation on an isozyme level in *Abies sachalinensis*. IUFRO — SABRAO Joint Symposia, Tokyo, A-9 (V): 1—12, 1972. — Miyasaki, Y., and Sakai, K.-I.: Use of zymography for identification of a clone in *Cryptomeria japonica* D. Don. J. Japan. Forestry Soc. **51**: 235—239 (1969). — Rasmuson, B., and Rudin, D.: Variations in esterase zymogram patterns in needles of *Pinus silvestris* from provenances in Northern Sweden. Silvae Genet. **20**: 39—41 (1971). — Remröd, J.: Resultat från tallproveniensförsök i Norrlands inland och höglägen. En analys av skogsodlingsresultatet efter 21 år samt dettas samband med proveniensernas förflyttning. Thesis. Royal College of Forestry, Stockholm 1972 (Mimeographed 41 pp.). — Rudin, D., and Rasmuson, B.: Genetic variation in esterases from needles of *Pinus silvestris* L. Hereditas **73**: 89—98 (1973). — Sakai, K-I., Hayashi, S., and Tomita, K.: Clone analysis and genetical study of quantitative characters in a natural forest of *Cryptomeria japonica*. Silvae Genet. **19**: 124—128 (1970). — Sakai, K.-I., Iyama, S., Miyazaki, Y., and Iwagami, S.: Genetic studies in natural populations of forest trees. IUFRO — SABRAO Joint Symposia, Tokyo, A-4 (I): 1—13, 1972. — Sakai, K.-I., and Miyazaki, Y.: Genetic studies in natural populations of forest trees. II. Family analysis: A new method for quantitative genetic studies. Silvae Genet. **21**: 149—154 (1972). — Sakai, K-I., Miyasaki, Y., and Matsumura, T.: Genetic studies in natural populations of forest trees. I. Genetic variability on the enzymatic level in natural forests of *Thujopsis dolabrata*. Silvae Genet. **20**: 168—173 (1971). — Sakai, K-I., and Park, Y-G.: Genetic studies in natural populations of forest trees: III. Genetic differentiation within a forest of *Cryptomeria japonica*. Theoret. Appl. Genet. **41**: 13—17 (1971). — Schwartz, M. K., Nisselbaum, J. S., and Bodansky, O.: Procedure for staining zones of activity of glutamic oxalo-acetic transaminase following electrophoresis with starch gel. Am J. Clin. Pathol. **40**: 103—106 (1963). — Stern, K.: Breeding population and productive population in forest tree breeding. IUFRO — SABRAO Joint Symposia, Tokyo, A-2 (I): 1—9, 1972. — Tigerstedt, P.: Genetical characteristics and adaptation of the tree populations in northern forests. Mimeographed 8 pp., 1972.