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Genetic Structure of a Pinus sylvestris *L. Seed-Tree Stand and Naturally Regenerated Understory*

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ABSTRACT. The dynamic changes in the genetic structure within a population of *Pinus sylvestris* have been studied by the isozyme technique. The stand investigated consisted of 122 seed trees on one hectare and was situated close to Vindeln, Sweden (lat. 64°). The study covered the genetic composition of adult trees, embryos of seeds of those trees, and the young trees below the stand.

The comparison of allozyme frequence showed significant differences between different stages of the life cycle at the loci LAP-A, F-EST, and ADH-B.

Genotypic frequencies in adult and young trees were close to Hardy-Weinberg expectations, but deviations were found in the embryos.

An excess of homozygotes was found in the embryos at most loci compared to the young and adult populations of the seed-tree stand. The excess of homozygosity may have been due to partial self-fertilization, which gives rise to these inbred embryos. The embryos that arose through self-fertilization seem to be eliminated from the population. By the age of 10–20 the evidence of inbreeding, excess homozygosity, disappeared. The nature of the selection against selfs and its timing can not be evaluated in this study. FOREST SCI. 31:430–436.

KEY WORDS. *Pinus sylvestris,* seed-tree stand, isozyme marker, population structure, natural regeneration, self-fertilization.

SEED-TREE STANDS are used as a means of reforestation to a considerable extent in northern Sweden. In principle, a positive selection of trees for storm resistance and good quality characters is made within the stand, and these trees are then used as a seed source for natural regeneration.

According to the Swedish Forestry Act, Swedish National Board of Forestry 1983, § 7 and the accompanying explanatory instructions, at least 75 seed trees per hectare should be used for pine regenerations in southern Sweden, and at least 50 trees per hectare in other parts of the country. In practice, the tree densities per hectare vary widely from place to place.

Plant regeneration under seed trees is affected among other things by the vari-

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ation in cone production, seed-germination capacity, competition, soil conditions, and climatic factors such as temperature and humidity.

Plants under seed trees are also influenced by strong selective factors which cause a large decrease in plant number. Most mortality appears in the early stages of development. There are some indications that there are genetic differences in this component of fitness (Shaw and Allard 1982, Tigerstedt and others 1982, Rudin and others 1977). The primary aim of this study was to get a better understanding of the genetic changes occurring in a seed-tree stand during different regeneration phases. We have investigated the changes in the genetic composition of a seed-tree stand by following allele and genotype frequencies in three different life stages, adults, embryos, and young trees. Some evidence is presented for the hypothesis that the action of natural selection causes changes in the genetic structure as the population ages.

Material and Methods

The material for this study has been collected in an approximately 100-year-old seed-tree stand at Svartberget, close to Vindeln (lat. 64°16′N, long. 19°47′E, and alt. 200 m). This stand consisted of 122 seed trees per hectare. The number of young trees after natural regeneration amounted to approximately 5,200 per hectare. Young trees were 10–20 years old at the time the adult trees were sampled. This seed-tree stand was closely surrounded by other stands of *Pinus sylvestris* and *Picea abies*.

Cones were collected separately from all 122 adult trees after felling. Buds from naturally regenerated young trees were collected in October 1981 after cessation of growing period and stored at −20°C until analysis. A total of 785 young trees were sampled following the procedure described by Yazdani and others (1984). For embryo analysis a random seed sample was not available. Thus the embryo stage is represented by one seed per adult tree.

Electrophoresis.—Allozyme patterns were assessed at nine loci for adult trees and at eight loci for embryos and young trees.

The stability of isozymes in bud tissues was checked after different periods of time. The results showed that the intact bud tissues have very stable isozyme patterns, while the homogenized tissue showed decreased enzyme activity after freezing and thawing.

Before analysis single macrogametophytes, embryos, or buds were homogenized separately in 0.1 M tris-versene-borate buffer pH 7.4 containing 1 percent (w/v) soluble polyvinylpyrolidone (M.W. 10000). The obtained crude extracts were then subjected to horizontal 12 percent starch electrophoresis. Running conditions and staining methods for enzyme markers used in this study were as described by: Rudin (1977) for leucine aminopeptidase, Rudin and Ekberg (1978) for malate- (MDH) and alcohol- (ADH) dehydrogenases, Rudin (1975) for glutamate oxalate transaminase (GOT), and Yazdani and Rudin (1982) for fluorescent esterase (F-EST). Allozymes of glutamate dehydrogenase (GDH) were separated according to Rudin (unpublished data).

Inheritance patterns of GOT, LAP, and F-EST in both haploid and diploid Scots pine tissues have been reported previously (Rudin 1975, 1977; Yazdani and Rudin 1982). Inheritance of MDH and ADH allozymes in Scots pine macrogametophytes has been described by Rudin and Ekberg (1978). The inheritance of MDH, ADH, and GDH allozymes in Scots pine embryos and buds has also been studied (Rudin, unpublished data). For all enzyme markers used in our study, the same gene loci have been suggested to be responsible for the observed allozyme patterns in both the haploid and diploid tissues studied. However, due

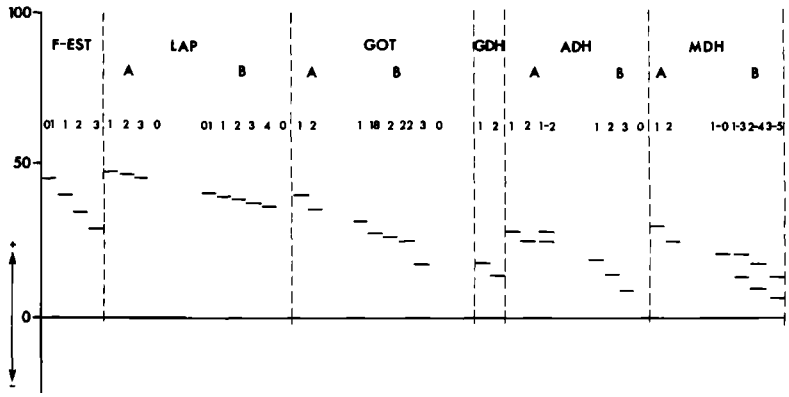

FIGURE 1. Relative mobilities of different isozymes in macrogametophyte of *Pinus sylvestris*. When more than one locus was present, loci were numbered in A, B, and C regions. Mobility of bromophenol blue is designated as 100.

to poor resolution of MDH-A and ADH-A patterns in embryos and buds studied here, these loci could have been assessed only in macrogametophytes from adult trees. The reverse was true for the GDH locus, which was studied in embryos and buds but not in macrogametophytes. The relative mobilities of the isozymes and allelic designations are given above the bands in Figure 1.

Statistical Methods.—The genotypes of individual adult trees were concluded based on seven macrogametophytes of seeds collected from each tree. We compared allelic frequencies in different life stages with χ^2-tests (Snedecor and Cochran 1967, p. 250). If numbers of alleles were too small for tests, classes were combined. If combining classes still yielded expected values too small for χ^2-tests, Fisher's exact test was used (Sokal and Rohlf 1981, p. 740). These tests were performed for all loci and between all life stages.

At three loci (LAP-A, GOT-B, and ADH-B) null alleles were detected. Both homozygotes and heterozygotes for the null allele (designated as 0 in Table 1) can be detected in adult trees, whose genotypes are scored based on haploid tissue. However, in diploid tissue, heterozygotes will go undetected and will be scored as homozygotes for the other allele. When allelic frequencies in different life stages were compared, adult trees treated in the same way, i.e., null allele heterozygotes were treated as homozygotes for the other allele to make the samples comparable. This method was only used in χ^2-tests, not when reporting the allelic frequencies.

A similar problem of different resolution in different tissues was found at the GOT-B locus, where alleles 18, 2, and 22 could not be distinguished in the embryos and buds. These three distinct alleles in adult trees were pooled into one synthetic allele.

Further analysis was conducted only on polymorphic loci, i.e., those where the most common allele had a frequency of 0.95 or less. Expected heterozygosities and their variances were estimated as described by Nei and Roychoudhury (1974). Population structure was also characterized by estimating the fixation index, F = 1 − (observed heterozygosity/expected heterozygosity). Curie-Cohen (1982) has studied different estimators of F. We used an estimator based on total observed and total expected heterozygosity, for which Curie-Cohen (1982) also gives a variance estimator. When computing average fixation indices, individual estimates were weighted with the inverses of their variances, because the accuracy of the estimates depends on the true values of the index and allelic frequencies.

TABLE 1. Allelic frequencies and sample sizes (N) in the embryo, young and adult populations of plants in the seed tree stand of Svartberget. Only statistically significant χ^2-values are shown in the table.

Locus	Population	Allele designations and frequencies						N	Heterogeneity
		1	2	3	0				
LAP-A	Embryo	.009	.967	.005	.019			107	$\chi^2_{(2)} = 12.79^{**}$
	Young	.006	.984	.010				778	
	Adult	.004	.934	.048	.013			114	$\chi^2_{(1)} = 12.31^{**}$
		1	2	3	01				
LAP-B	Embryo	.018	.959	.005	.018			110	
	Young	.005	.966	.024	.005			775	
	Adult	.004	.956	.035	.005			115	
		1	2						
GOT-A	Embryo		1.000					120	
	Young	.003	.997					765	
	Adult	.004	.996					115	
		1	18	2	22	3	0		
GOT-B	Embryo			.556		.444		99	
	Young	.024		.507		.469		765	
	Adult	.018		.491		.487	.004	113	
		1	2	3	01				
F-EST	Embryo	.825	.058	.117				103	$\chi^2_{(2)} = 16.8^{***}$
	Young	.689	.129	.174	.008			776	$\chi^2_{(2)} = 12.3^{**}$
	Adult	.682	.120	.190	.008			121	$\chi^2_{(4)} = 17.6^{**}$
		1	2						
MDH-A	Adult	.031	.969					114	
		1–3	2–4	3–5					
MDH-B	Embryo	.685	.315					119	
	Young	.693	.307					762	
	Adult	.691	.305	.004				110	
		1	2	1–2					
ADH-A	Adult	.288	.680	.032				111	
		1	2	3	0				
ADH-B	Embryo	.077	.908	.015				98	$\chi^2_{(2)} = 25.9^{***}$
	Young	.082	.775	.143				785	$\chi^2_{(2)} = 22.7^{***}$
	Adult	.066	.781	.145	.008			114	$\chi^2_{(4)} = 26.7^{***}$
		1	2						
GDH	Embryo	.361	.639					122	
	Young	.338	.662					772	

*P < 0.05 **P < 0.01 ***P < 0.001.

Results and Discussion

Allelic and genotypic frequencies were estimated for nine loci in the adult trees, and for eight loci in the embryos and young trees. All life stages have seven loci in common.

Table 1 gives the allelic frequencies and sample sizes for different loci at three life stages. Statistically significant χ^2-values for allele frequency comparisons are also given.

At the LAP-A locus, allele 2 was less frequent in the adults than in the other life stages. This difference is partly due to the fact that heterozygotes for the null allele could be detected in the adults, but not in the other samples, where frequencies are based on diploid tissue. However, this was taken into account in performing the χ^2-test, which still remained significant. The GOT-A locus was

TABLE 2. Observed and expected heterozygosities and fixation indices for different life stages in the seed-tree stand in Svartberget.

Locus	Population	Observed heterozyg.	Expected heterozyg. (H_e)	Var (H_e)	Fixation index F	Variance *F*
GOT-B	Embryo	0.384	0.494	0.00017	0.227	0.0097
	Young	.499	.522	.00003	.043	.0012
	Adults	.531	.522	.00021	−.018	.0073
F-EST	Embryo	.301	.302	.00293	.003	.0063
	Young	.491	.479	.00032	−.026	.0006
	Adults	.512	.485	.00196	−.057	.0038
MDH-A	Adults	.061	.060	.00091	−.032	.0002
MDH-B	Embryo	.412	.432	.00100	.046	.0086
	Young	.399	.426	.00017	.063	.0014
	Adults	.445	.430	.00121	−.036	.0085
ADH-A	Adults	.387	.453	.00143	.145	.0082
ADH-B	Embryo	.082	.169	.00237	.517	.0220
	Young	.375	.372	.00038	−.006	.0008
	Adults	.386	.365	.00264	−.057	.0038
GDH	Embryo	.344	.461	.00061	.260	.0081
	Young	.477	.448	.00012	−.065	.0012

monomorphic in the embryos, but in the samples of adults and young trees another allele was found with a low frequency. The embryo sample size may not have been large enough to detect variation. Both F-EST and ADH-B showed a similar pattern: old and young trees did not differ, but embryos had a higher frequency of the main allele of the locus. Allelic frequencies at the loci LAP-B, MDH-B, and GDH did not differ significantly between the life stages.

In general, there seemed to be more differences between embryos and young trees than between young trees and adults. This embryo sample was generated by the adults that were studied here. Each maternal tree was represented by one seed. Therefore, our method of sampling prevents us from detecting effects of different female fertilities in the trees. Any real differences in allelic frequencies are then most likely due to differences between trees in male fertility, and differences in viability from fertilization to seed stage. Pollen and seeds from surrounding stands may play a role in the genetic composition of young trees. If the allele frequencies of those stands are different, migration may contribute to the observed differences found between adults, embryos, and young trees.

Table 2 gives some estimated parameters describing population structure for the polymorphic loci. At those loci where different life stages can be compared, allelic diversity (=expected heterozygosity) in adult trees was at least as great as in the younger stages. The fixation index compares the observed proportion of heterozygotes to that expected from Hardy Weinberg equilibrium. A lack of heterozygotes results in a positive fixation index, an excess in a negative fixation index. The fixation index for embryos was positive at all loci, indicating an excess of homozygotes. At five of the six loci the adult trees had a negative fixation index, which indicates an excess of heterozygotes. These trends are also reflected in the averages of the estimates, computed for loci polymorphic in all life stages (Table 3). The average fixation index was 0.124 in embryos, 0.007 in the young trees and a negative −0.046 in the adults.

It is well known that there is some self-fertilization in Scots pine (e.g., Rudin and others 1977). How is this reflected in the genetic structure of the population?

TABLE 3. Averages for observed and expected heterozygosities and fixation indices, based on those 4 loci that are polymorphic in all life stages.

Population	Average observed heterozygosity	Average expected heterozygosity	F*
Embryo	0.295	0.349	0.124+
Young	.441	.450	.007+
Adults	.469	.451	−.046+

* The average fixation is a weighted mean. The weights are inverses of variances at each locus.

When there is partial self-fertilization, it is desirable to obtain an estimate of the frequency of self-fertilization in order to adequately interpret the genotypic data. Such an estimate was found in another parallel study with the aid of rare or unique alleles. The method is inefficient, because only trees and loci with rare alleles can be used. However, it may still give an approximate estimate of the magnitude of selfing. The estimated mean selfing proportion in this study was 11.8 percent (Yazdani and others 1984).

The selfing rate of 0.118 should give rise to an equilibrium fixation index of $F = s/(2 - s) = 0.063$ in the zygotes, where s is the proportion of selfing. In fact, we found somewhat higher values in embryos. There was much between-locus variation in the estimates, but the average fixation index was 0.124. Thus, our estimate of selfing does not quite account for the degree of homozygosity found in embryos. The adult trees had a slightly negative average fixation index (−0.046). There were also some differences in allelic frequency between adult trees and embryos. Differences in fertility of male parents may have caused allelic frequency differences. The decrease in heterozygosity is most likely due to the effects of self-fertilization, which occurs in this seed-tree stand at the rate of about 12 percent (Yazdani and others 1984). In fact, the embryos were even a little more inbred than predicted by this amount of selfing. The cause of the extra homozygosity is not known.

The same adult trees gave rise to the sample of 10–20 year old trees. There are two obvious reasons for genetic differences between embryos and young trees. First, our sample of embryos was not random, but all seed trees were equally represented. This may not have been so in the embryo sample that gave rise to our young trees. Variation in seed production may have contributed to these genetic differences. At F-EST and ADH-B there were differences in allelic frequency between embryos and young trees. Secondly, a more important difference is that the average inbreeding coefficient had decreased and was very small in the young trees. There was no more excess homozygosity in the young trees. This is probably due to the elimination of inbred individuals from the population through viability selection.

The young trees and adult trees in our study cannot be directly compared, because the zygote population that gave rise to these adults was generated more than 100 years ago in a natural stand, whereas the young trees are from a seed-tree stand. The initial composition of that population may have been different. However, we may suspect that due to self-fertilization, there was excess homozygosity compared to Hardy-Weinberg expectation. At present, there is no evidence of inbreeding. In fact the fixation index was even slightly negative. There may be several reasons for such an excess, e.g., heterozygote advantage or associative overdominance (see Shaw and Allard 1982 for a discussion). Results on young trees indicate that the elimination of inbreds mostly occurs rather early, but some further genetic change may still occur as the population grows older.

The results of our study can be compared to those obtained by Shaw and Allard (1982). They studied natural stands of Douglas-fir, and found an excess of homozygotes at the embryo stage corresponding to the degree of selfing. In the adult trees, fixation indices were slightly negative for most loci. Tigerstedt and others (1982) studied a very old stand of *Pinus sylvestris*. The 300–400-year-old stand had Hardy-Weinberg genotypic frequencies, but the younger generation (100 years old) still contained an excess of homozygotes.

Our results agree with both of these studies in that adults were found to be close to Hardy-Weinberg equilibrium. As Shaw and Allard (1982), we also found some inbreeding in seeds due to partial self-fertilization. Our results differ from those of Tigerstedt and others (1982) in the stage of elimination of inbreds from the population. They found evidence of inbreeding even in 100-year-old trees, whereas in our material most inbreds had been eliminated before the trees were 10–20 years old.

It is well known that conifers display a high degree of inbreeding depression. The avoidance of inbreeding is based on elimination of inbred individuals after fertilization. In the early development, allelic lethals cause the death of many such individuals (e.g., see Koski 1982). More detailed studies on the timing of mortality are underway.

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