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Genetic change between life stages in Pinus sylvestris: allozyme variation in seeds and planted seedlings

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Summary

Genetic changes during artificial regeneration of pine were studied in northern Sweden. Open pollinated seeds were collected from ten maternal trees. Samples of these seeds were germinated and grown in a nursery from January 1980 to April or September 1981. Thereafter the seedlings were planted at their original site in a 2 × 2 m planting design. The genetic structure of the original seed population and that of the surviving population in the field at age three were compared. The genotypic frequencies at four polymorphic enzyme loci were obtained by electrophoresis of the embryos in seeds and buds from the surviving plants (*Got*-B, F-*Est*, *Gdh*, and *Mdh*-B). There had been no statistically significant changes in allelic frequencies. The original seed population had positive fixation indices at all loci (average 0.12), presumably due to some inbred progeny among the seeds. In the surviving population, the fixation index was 0.006, which indicates that the more inbred individuals had been virtually eliminated between the seed stage and the three year old plant stage. There was no detectable relationship between the change in heterozygosity for individual families and survival in the field.

Key words: artificial regeneration, Scots pine, allozyme variation, inbreeding, selection.

Zusammenfassung

In Nord-Schweden wurden Kiefernkulturen aus künstlicher Verjüngung auf genetische Veränderungen hin untersucht. Hierzu wurden 1980 Samen von 10 Mutterbäumen in einer autochthonen Population gesammelt, ausgesät, in einer Baumschule angezogen sowie dann wieder auf dem Ursprungsstandort in 2 × 2 m Abstand ausgepflanzt. Nach drei Jahren wurde die genetische Struktur der künstlich eingebrachten überlebenden Kiefern mit der ursprünglichen Samenpopulation verglichen. Hierzu wurden die Embryos in Samen und die Knospen der überlebenden Sämlingspflanzen auf 4 polymorphe Enzym-Loci (Got-B, F-Est, Gdh und Mdh-B) elektrophoretisch untersucht. Es gab keine signifikanten Veränderungen in den Allelfrequenzen. Die urspüngliche Samenpopulation hatte einen positiven Inzuchtkoeffizienten für alle Loci (Mittelwert 0,12), wahrscheinlich wegen teilweiser Inzucht. Bei den Pflanzen, die überlebt haben, war der Mittelwert des Inzuchtkoeffizienten 0,006. Der Anteil an Homozygoten hatte also abgenommen, weil die durch Selbstbestäubung entstandenen Pflanzen eliminiert worden waren.

Es wurde versucht, auch den Umfang der genetischen Veränderung in einzelnen Familien und die Mortalität nach der Pflanzung zu schätzen. In fast allen Familien wurde der Heterozygotiegrad höher. Zwischen der genetischen Veränderung und der Mortalität gab es keine Beziehung. Für solche Zwecke sind genauere Untersuchungen nötig.

Introduction

The reproductive system of Scots pine (*Pinus sylvestris* L.) is characterized by a very large number of seeds. Estimates of early seed production in southern Finland range from 100 and 200 seeds per m² (KOSKI and TALLQVIST, 1978). Among these seeds, severe mortality takes place. Much of this mortality is probably random, but some could be selective resulting in genetic change between different life stages of the population. Unfit plants, e.g. those suffering from inbreeding depression, may be removed from the population at an early stage.

It is well known that some Scots pine zygotes result from self-fertilization (SARVAS, 1962). Some of these selfed embryos are eliminated at a very early stage due to embryonic lethals (see KOSKI, 1973), so that the proportion of selfed embryos is lower in mature seed than in early embryogeny. Estimates of the proportion of selfed seed vary. SARVAS (1962) gave an estimate of 7%, KOSKI has suggested that only 1% of mature seed are due to selfing. Work with marker alleles has resulted in higher estimates, e.g. about 10% by MÜLLER-STARCK (1977). RUDIN *et al.* (1977) found even higher values in a seed tree stand, up to 24%. Mating between relatives may also occur, but this has not been studied in Scots pine. Inbreeding has been found to be reflected in a higher proportion of homozygotes among the seed than expected on the basis of the HARDY-WEINBERG theorem (SHAW and ALLARD, 1982, in Douglas-fir; YAZDANI *et al.*, 1985, in Scots pine). At the adult stage such excess homozygosity has disappeared, and in fact, in some cases excess heterozygosity has been found (SHAW and ALLARD, 1982; YAZDANI, *et al.* 1985). We (YAZDANI *et al.*, 1985) found earlier that in natural regeneration, excess homozygosity was eliminated in a stand aged 10—20 years. Thus adult stands of Scots pine do not seem to suffer from the presence of inbred individuals.

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When artificial regeneration is used, both seeds and young plants probably experience less severe conditions than those in natural stands. Favorable conditions are provided in nurseries, and it is possible that inbred individuals may survive the initial stages, get planted in the field, yet grow poorly due to inbreeding depression. In the present paper, we report genetic changes that took place in the early phases of artificial regeneration. We compared the genetic structure of an original seed population with there year old plants that were first grown in the nursery and then planted in the field, and found that excess homozygosity was eliminated by age three, but that the genetic changes within families were unrelated to mortality in the field.

Materials and Methods

We studied as stand of Scots pine in northern Sweden, Svartberget, close to Vindeln (64°16'N, 19°47'E, altitude 200 m). The natural regeneration of this stand had been studied earlier (YAZDANI *et al.*, 1985). The stand was cut in 1979, and at that time cones were collected from 10 trees to compare artificial and natural regeneration. Part of the seed of each family was used for electrophoretic analysis, part of it was used for planting at the original site.

For planting at the original site, 300 seeds of each family were sown in peat pots in January 1980 and were initially grown in a greenhouse. Germination was even. The plants were inventoried in April 1980, at which time it was found that because of uneven watering and fertilization, only 73% of the plants were alive. The seedlings overwintered outside before planting in 1981. No conscious selection was applied during this stage.

The experimental area in Svartberget consisted of two blocks, 26 × 40 m² each, which were used for inventorying natural regeneration. One of the blocks was prepared by hand for planting. Spacing between rows and between planting sites in a row was 2 m. There were thus 20 × 13 = 260 plantings spots, 26 for each family. At each planting spot, one seedling was planted in the spring of 1981 (June 4th) and one in the fall of 1981 (September 29th). From the original 300 seeds, 52 seedlings were chosen from each family. Healthy vigorous seedlings were planted. On August 13, 1983, the plants were measured and their condition recorded.

Electrophoretic analyses were made on the embryo stage and the three year old plants. Of the remaining seed, about 40—100 embryos per family were analyzed. The genotypes of three year old plants were determined based on bud samples collected from all families in the fall of 1983. Only lateral buds were collected because the seedlings are to be used for further comparisons between artificial and natural regeneration, and destructive sampling could not be used. Both embryos and buds were analysed with respect to five enzyme systems. These systems were leucine aminopeptidase *(Lap)* (see RUDIN, 1977 for description in Scots pine), glutamate-oxaloacetate-transaminase *(Got)* RUDIN 1975), fluorescent esterase *(F-Est)* (YAZDANI and RUDIN, 1982), glutamic dehydrogenase *(Gdh)* (staining modified from e.g. VALLEJOS, 1983, but with 0.1 M phosphate buffer pH 6.5), malate dehydrogenase *(Mdh)* (RUDIN and EKBERG, 1978), and aconitase *(Aco)* (SZMIDT and YAZDANI, 1986). These systems allow genotypic determination at nine loci. However, only four sufficiently polymorphic loci are used in this study, because relatively monomorphic loci are not informative for comparisons between life stages. These four loci were *Got-B*, *F-Est*, *Gdh*, and *Mdh-B*. For one family we obtained so few electrophoretic data that it was excluded, leaving 9 families for statistical analysis.

Statistics. Aside from expected and observed heterozygosities and allelic frequencies we also computed fixation indices and their variances ($F = 1 - H_{obs}/H_{exp}$, where H is heterozygosity) based on total expected and observed heterozygosity, as described by CURIE-COHEN (1982). Allelic distributions and fixation indices between life stages were compared with X^2-tests. Estimates of outcrossing for the whole population were obtained using the single locus method described by BROWN *et al.* (1975), which is based on progeny arrays from single maternal parents. Outcrossing estimates for homozygous individual maternal trees were obtained. The method for estimating outcrossing rates and their variances has been described by BROWN and CLEGG (1984). Our data consisted of embryo genotypes. Megagametophyte data were not available, hence, statistical methods designed especially for conifers could not be used.

Minimum variance means of parameters over families or loci were computed by weighting individual estimates whith the inverses of their variances. Mortalities between families were compared with χ^2-tests.

Results and Discussion

The basic data for the total population are given in *Table 1* for four loci. There were no statistically significant changes in allelic frequencies between the two life stages. At individual loci, the genotypic distributions did not differ either. Observed heterozygosities were consistently higher in the population of young plants than in the embryos. However, it is better to compare fixation indices, which do not depend on allelic frequencies. Positive fixation indices are due to excess homozygosity compared to HARDY-WEINBERG expectation, negative ones to homozygote deficiency. The locus *Got-B* had quite large positive fixation indices in both life stages At the other loci, embryos had positive fixation indices, but young plants had negative indices. All loci had positive fixation indices in the embryos. This excess of homozygotes is most probably due to inbreeding, including selfing. Allelic frequency differences between subpopulations are also a possible cause of excess homozygosity (WAHLUND effect).

At *Got-B*, genotypic distributions in both seeds and young plants differed significantly from the HARDY-WEINBERG proportions. At other loci, the smaller differences were not statistically significant. At both life stages, the estimates of fixation indices were significantly heterogeneous between loci ($X^2_{emb} = 27.3$, df = 3, $P < 0.001$) ($X^2_{young} = 17.8$, df = 3, $P < 0.001$). However, we are here mainly interested in the direction of change, and this was similar at al loci.

The average fixation index (weighted by the inverses of the variances of the individual estimates) in embryos was $\hat{F}_{emb} = 0.12$, in young plants, $\hat{F}_{young} = 0.006$. The fixation indices in seeds and planted seedlings were compared locus by locus with X^2-tests. All fixation indices in seeds were larger than in the young plants, but the difference was statistically significant only at Gdh ($X^2 = 3.96$, $P < 0.05$), and close to significance at *F-Est* ($X^2 = 2.94$, $P < 0.10$). Thus a change had taken place in the population during the first three years: excess homozygosity was eliminated. It is probable that this change was due to selective elimination of inbreds. When each family was considered separately, the fixation index decreased

Table 1. — Allelic frequencies, expected and observed heterozygosities (H_{exp} and H_{obs}), fixation indices (F) and their standard deviations, estimates of outcrossing rates (t) and their standard deviations, and equilibrium fixation indices, given t, (F_e), in embryos (E) and seedlings (Y) of *Pinus sylvestris* in Svartberget.

Locus	Life-stage	N	Allele					H_{exp}	H_{obs}	F	SD (F)	t	SD (t)	$F_e=(1-t)/(1+t)$
			1	2	3	4	5							
Got-B	E	419			.468		.532	.498	.334	.329	.046	0.77	0.15	0.13
	Y	183	.003		.454		.544	.499	.366	.266	.071			
F-Est	E	515	.785	.084	.130	.001		.361	.338	.063	.039	1.04	0.10	0
	Y	149	.748	.128	.107			.408	.430	-.052	.055			
Gdh	E	625	.268	.730	.002			.396	.378	.046	.040	1.08	0.09	0
	Y	199	.231	.769				.355	.392	-.103	.063			
Mdh-B	E	516	.698	.302				.422	.388	.081	.044	0.88	0.10	0.06
	Y	163	.687	.313				.430	.442	-.027	.077			

between the embryo and seedling stage in 8 of the 9 families (data not shown).

Unfortunately, we were not able to determine the stage when the genetic change took place. The 3000 seeds originally planted had undergone some mortality (or non-germination) in the nursery before planting. From the 300 seeds planted per family, only 52 were planted in the field, which caused selection for size and vigor. After planting, the overall mortality in the field in the first years was 10%. Of the 520 plants, 466 were still alive in the third summer. Further, buds were collected only from plants with lateral buds, which may have been a source of bias. If we make some highly simplifying assumptions, it is possible to estimate the minimum mortality needed to change the fixation index from X to 0. Initially, the proportion of heterozygotes is 2pq(1-X). Let us assume that selection acts only to remove excess homozygosity, without changing allelic frequencies. Then a mortality proportion of X will result in F = 0 among the survivors. Thus 12% mortality would be required to change the average fixation index from 0.12 to zero. It is evident that in the field nearly sufficient mortality has taken place to account for the genetic change. However, it is of course much more likely that at least part of this genetic change took place during the nursery phase, either through mortality or at the stage when plants were chosen for planting, and after field mortality, at the stage when buds were collected. Even though we cannot distinguish between the causes here, it is of interest that inbred progeny disappear at a relatively early stage.

Outcrossing estimates (t̂) are listed in *Table 1.* The low outcrossing values at *Got-B* are concordant with the excess homozygosity we observed. The between locus heterogeneity ($X^2_{(3)} = 4.4$ $P > 0.05$) was not statistically significant. The expected equilibrium fixation index, given t, is $F_{eq} = (1-t)/(1+t)$. This predicted F is shown for each locus separately in *Table 1.* For *Got-B* there was a deficiency of heterozygotes compared to what is expected on the basis of the mating system. *F-Est* and *Gdh* should have zero or negative F-values, but they are difficult to treat because the outcrossing estimates are large than one. For *Mdh-8* the mating system estimate predicts an F-value of 0.06 and 0.081 was actually observed. These fixation indices for individual loci can also be compared to a previous dataset from the same population in Svartberget, namely the embryo population in Yazdani *et al.* (1985). As here, *Got-B* had a large positive index in the embryos. *F-Est* and *Mdh-B* were close to Hardy-Weinberg expectation. The only difference is that in the earlier study we also found a large positive fixation index for *Gdh* in the seed population.

We can obtain a mean outcrossing rate by weighting individual estimates whith inverses of their variances. This results in a mean of 0.97. This level of outcrossing is well within those observed previously, even somewhat higher. An earlier estimate from the same stand, $\hat{t} = 0.88$, indicated more selfing or mating between relatives (see Yazdani *et al.*, 1985). Our observed outcrossing estimate, 0.97, would be consistent with a low fixation index of 0.02. We observed $\hat{F}_{emb} = 0.12$, which is more consistent with the estimate of outcrossing from Yazdani *et al.* 1985. The most likely cause of the excess homozygosity is still inbreeding, even if we did not detect it with our average selfing rates.

We also tried to look for association between rate of selfing, degree of genetic change, and mortality within families. However, we found no associations. The differences between families were small, and we had too few families to detect such associations.

The focus of this study was to obtain a general picture of the genetic changes due to artificial regeneration as compared to natural regeneration. Our earlier study demonstrated that inbred trees are eliminated during natural regeneration by the time the trees are 10—20 years old (Yazdani *et al.*, 1985). The present study showed that a similar genetic change occurred in artificial regeneration by the time the plants were three years old. However, we do not know at exactly which stage the genetic changes occurred. More detailed observations are needed to find the causes and the stages at which inbreds are selectively eliminated. We plan to collect data on the parallel genetic changes that take place in natural and artificial regeneration in the Svartberget area. Such comparisons are needed to evaluate in more detail the effects of artificial and natural regeneration.

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Investigations on Chromosomes of Siberian Spruce (Picea obovata Ledeb.)

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Summary

38 individuals of the species *Picea obovata* Ledeb. were studied with special regard to their chromosome number and chromosome morphology. The chromosome number within the diploid (somatic) set is 2n = 24. Socalled accessory or B-chromosomes were not found.

Within two specimens of the investigated plants, a pair of homologous chromosomes (no. 11), was found which differed in length. Thus there exists polymorphism in chromosome length of pair no. 11.

Key words: *Picea obovata*, karyotype, polymorphism, B-chromosomes.

Zusammenfassung

38 verschiedene Bäume der Art *Picea obovata* Ledeb. wurden in bezug auf Chromosomenzahl und -morphologie untersucht. Die Chromosomenzahl beträgt im diploiden (somatischen) Satz 2n = 24. Sog. akzessorische oder B-Chromosomen konnten nicht nachgewiesen werden.

Es wurden bei zwei Exemplaren der untersuchten Pflänzchen unterschiedlich lange Chromosomen beim Paar Nr. 11 gefunden, d. h. es liegt hier ein Polymorphismus der Chromosomenlänge vor.

1. Introduction

Since its designation as a species by Ledeb. (1833) dissension exists as to the systematical position of Siberian Spruce in the genus *Picea* (Schmidt-Vogt, 1974). Teplouchov (1898) concluded that of the characteristics described by Ledeb., i.e. the position of the cones and the form of needle-tips and cone-scales, only the form of the cone-scales allows a clear differentiation.

As to further macroscopic morphological characteristics, there are recent indications in the literature that *Picea obovata* differs from *Picea abies* in the occurrence of so-called „B- chromosomes" in addition to the regular 24 chromosomes in the somatic set (Kruklis, 1971; Pravdin *et al.*, 1976; Pravdin and Rostovtsev, 1979).

In the present paper, the karyotype of Siberian Spruce (origin Altai-mountains, 52° northern latitude, 86° eastern longitude, 1500 m above sea level) was investigated with special regard to the number and morphology of the chromosomes.

2. Material and Methods

The material for the studies were 4-year-old plants, grown from a seed lot from the above-mentioned origin[1]).

A total of 482 samples (root tips) from 47 different trees were investigated. Thereof, 109 samples from 38 different trees contained useful metaphase-stages for karyotype-analysis.

The root tips were taken between late July and late September from potted plants, rinsed with Aqua dest. and subsequently treated as follows:

In order to increase the number of metaphase-stages, the root tips were incubated in colchicine-solution (0.01 to 0.05%) at 24° C for 4 to 24 hours. After the colchicine-treatments, the samples were rinsed throughly in Aqua bidest. and subsequently fixed in ethanol-acetic acid (3:1) at 4° C for 8 to 24 hours. The samples were macerated in different ways, the most favourable method proving to be the acetic-treatment (45%) for one hour at 60° C. Other methods, such as the treatment with 45% acetic acid for only 10 minutes at 60° C (Kondo and Hizume, 1982) or incubation with 0.2 to 1 N hydrochloric acid at different temperatures (Mergen and Novotny, 1957; Greilhuber, 1973 and 1974; Wochok *et al.*, 1980; D'Amato *et al.*, 1981; Mac Pherson and Filion, 1981; Schlarbaum and Tsuchiya, 1981) showed less satisfactory results. Also, the use of cellulase

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