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Mating System and Genetic Diversity in Natural Populations of European Larch (*Larix decidua*) and Stone Pine (*Pinus cembra*) Located at Higher Elevations

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Summary

Mating system and genetic diversity were investigated in natural populations of European larch (*Larix decidua* MILL.) and stone pine (*Pinus cembra* L.) located at the upper limits of the species ranges in the Italian Alps. Multilocus estimates of outcrossed progeny (t_m) were relatively low in both populations ($t_m = 0.675$ for larch and 0.808 for stone pine), indicating that self-fertilization is an important component of their mating system. Mean single-locus estimates of t differed only slightly from t_m suggesting that there was a little inbreeding other than selfing, despite expected clustering of family members in those stands. The genetic structure of adult trees was consistent with the high levels of genetic diversity typically observed within populations of conifer species.

Key words: *Larix decidua*, *Pinus cembra*, allozymes, outcrossing, mixed-mating model, genetic diversity.

Introduction

Coniferous forest tree species are wind-pollinated and typically have high proportions of outcrossed progeny ($t > 0.80$) (MUONA, 1990; ADAMS and BIRKES, 1991) and high levels of genetic diversity within populations (HAMRICK et al., 1992). However, population outcrossing rates lower than 0.80 are occasionally reported (PERRY and KNOWLES, 1990; XIE et al., 1991; EL-KASSABY et al., 1994), and the levels of outcrossing vary widely both among and within species (MITTON, 1992; BURCZYK, 1998).

Density of forest tree populations decreases at limits of distribution, i.e. at higher elevations or in the far north. This

may reduce the density and movement of the pollen cloud and cause a relatively large proportion of offspring resulting from self-fertilization. The trees are usually growing in pioneer conditions. The result is that trees often exist in clusters, probably of close relatives, since seed dispersal and pollen movement may be limited. If mating occurs primarily between near neighbors, the clustering of relatives is expected to result in high levels of inbreeding and reduced effective population size.

In this paper we investigated and compared levels of allozyme variation and mating systems parameters of a forest stand composed of two coniferous species: European larch (*Larix decidua* MILL.) and Swiss stone pine (*Pinus cembra* L.). These species differ in the extent and geographic localization of their natural ranges. Natural range of European larch is restricted to four distinct areas of Central Europe (Alps, Sudety, Tatras and central part of Poland) (RUBNER, 1953). It is especially widespread in the Alps, from about 800 m elevation up to the subalpine vegetation zone. Swiss stone pine is restricted to few high elevation areas in Europe and consists of relatively small and scattered populations (HOLZER, 1975).

Materials and Methods

The two natural populations investigated in this study are located in the Alps of northern Italy, at the Stelvio National Park. The sampled populations lie at about 2000 m elevation. The main plant community of the stand was *Larici-Cembra-tum*, and the trees were loosely distributed at a density of 100 to 150 trees/ha. The trees were over 100 years old. Seeds were collected from 35 trees of European larch and 28 trees of stone pine.

Analyses of the population genetic structure were performed using macrogametophyte tissue from seeds. The following 10 enzyme systems encoded by 16 loci were used to assess genetic

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diversity in European larch: (Enzyme Commission number and locus abbreviations in parentheses: formate dehydrogenase (EC1.2.1.2; *Fdh*), glutamate dehydrogenase (EC 1.4.1.2; *Gdh*), isocitrate dehydrogenase (EC 1.1.1.4; *Idh*), leucine aminopeptidase (EC 3.4.11.1; *Lap1*, *Lap2*), malate dehydrogenase (EC 1.1.1.37; *Mdh1*, *Mdh2*, *Mdh3*, *Mdh4*), menadione reductase (*Mnr-4*), phosphoglucomutase (EC 2.7.5.1; *Pgm1*, *Pgm2*), shikimate dehydrogenase (EC 1.1.1.25, *Shdh*), sorbitol dehydrogenase (EC 1.1.1.14; *Srdh*), superoxide dismutase (EC 1.15.1.1; *Sod1*, *Sod2*). For stone pine we used 11 enzyme systems encoded by 20 loci [*Gdh*, *Got1*, *Got2*, *Got3* (glutamate oxaloacetate transaminase, EC 2.6.1.1), *Idh*, *Lap1*, *Lap2*, *Mdh1*, *Mdh2*, *Mdh3*, *Mdh4*, *Pgi1*, *Pgi2*, *Pgm1*, *Pgm2*, *Shdh1*, *Shdh2*, *Sod1*, *Sod2*, *Srdh*]. Information concerning inheritance of individual allozymes, descriptions of enzyme systems analyzed and electrophoretic conditions were described elsewhere (LEWANDOWSKI and MEJNARTOWICZ, 1990; GONCHARENKO et al., 1992).

Genetic structure and diversity were assessed for the adult trees in each population by estimating the proportion of polymorphic loci, number of alleles per locus (A), and expected (H_e) and observed (H_o) heterozygosities. Estimates of H_e were corrected for small sample sizes (NEI, 1978). Observed genotype frequencies were compared to those expected under HARDY-WEINBERG equilibrium using a goodness-of-fit test (G-statistics, SOKAL and ROHLF, 1981). All tests of significance were conducted at the 0.05 probability level.

Mating system was investigated based on 384 offsprings (embryos) collected from 32 mother trees of European larch and 329 offsprings from 23 mother trees of stone pine. Only three loci (*Pgm-1*, *Pgm-2*, *Shdh* for larch; *Lap-2*, *Mdh-4*, *Shdh* for pine) were used to determine the mating system parameters in each population.

Single-locus (t_s) and multilocus (t_m) estimates of outcrossing rate were calculated for each population based on the mixed-mating model and maximum-likelihood procedures developed for conifers (RITLAND and EL-KASSABY, 1985), using the MLTF

computer program. Heterogeneity of outcrossing estimates among loci was evaluated using FISHER'S test for heterogeneity (RAO, 1973).

Results and Discussion

Genetic diversity

Of the 16 investigated loci in European larch population 9 (56%) were polymorphic (0.99 criterion) (Table 1). Most polymorphic loci were *Mnr-4* and *Shdh*. These two loci appeared to have an expected heterozygosity greater than 0.40. Individual tree heterozygosities ranged from 0 to 0.313, with a mean of 0.111. All together, 29 alleles were observed and the average number of alleles per locus was 1.81. When the observed proportions of heterozygotes were compared with those expected for a population in a HARDY-WEINBERG equilibrium, a slight but nonsignificant excess of homozygotes was detected ($F = 0.021$). The estimates of mean expected heterozygosity, mean number of alleles per locus and the percentage of polymorphic loci were lower than those already reported for European larch populations (LEWANDOWSKI and MEJNARTOWICZ, 1991; MAIER, 1992; BELLETTI et al., 1997; LEWANDOWSKI, 1997).

The population of Swiss stone pine was less variable. Only 9 (45%) of the 20 investigated loci were polymorphic. The most polymorphic loci were *Lap-2*, *Mdh-4* and *Shdh-1* (Table 2). The average number of alleles per locus was 1.57. Individual tree heterozygosities ranged from 0 to 0.200, with a mean of 0.100. Similarly as in the European larch population, a nonsignificant excess of homozygotes was detected and the mean value of WRIGHT'S fixation index was $F=0.014$. The observed level of allozyme variation was similar to that previously reported for *Pinus cembra* by POLITOV and KRUTOWSKII (1994), but lower than in other studies (SZMIDT, 1982; BELLETTI and GULLACE, 1999).

Comparison between our results and those presented in literature are hampered by differences in the number and the sets of loci studied, but also in the geographical scale considered.

Table 1. – The parameters of genetic structure of the European larch population.

	Loci									Mean
	<i>Lap-1</i>	<i>Lap-2</i>	<i>Mdh-2</i>	<i>Mdh-3</i>	<i>Mdh-4</i>	<i>Mnr-4</i>	<i>Pgm-1</i>	<i>Pgm-2</i>	<i>Shdh</i>	
Allele										
1	0,986	0,129	0,986	0,914	0,014	0,386	0,900	0,029	0,243	
2	0,014	0,857	0,014	0,086	0,986	0,614	0,100	0,886	0,014	
3	–	0,014	–	–	–	–	–	0,087	0,714	
4	–	–	–	–	–	–	–	–	0,029	
H_o	0,029	0,229	0,029	0,171	0,029	0,314	0,143	0,229	0,514	0,187
H_e	0,028	0,249	0,028	0,157	0,028	0,474	0,180	0,207	0,430	0,198
F	-0,014	+0,080	-0,014	-0,094	-0,014	+0,337	+0,206	-0,102	-0,197	0,021
G-test	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	0,046	<i>ns</i>	<i>ns</i>	<i>ns</i>	
p -value										

Table 2. – The parameters of genetic structure of the stone pine population.

	Loci									Mean
	<i>Lap-2</i>	<i>Mdh-2</i>	<i>Mdh-4</i>	<i>Pgi-1</i>	<i>Pgi-2</i>	<i>Pgm-1</i>	<i>Shdh-1</i>	<i>Sod-2</i>	<i>Srdh</i>	
Allele										
1	0,054	0,018	0,375	0,929	0,964	0,875	0,589	0,018	0,036	
2	0,696	0,982	0,625	0,071	0,036	0,125	0,375	0,982	0,964	
3	0,250	–	–	–	–	–	0,036	–	–	
H_o	0,393	0,036	0,393	0,000	0,071	0,250	0,464	0,036	0,071	0,190
H_e	0,450	0,035	0,469	0,133	0,069	0,219	0,511	0,035	0,069	0,221
F	0,126	-0,018	0,162	–	-0,037	-0,143	0,091	-0,018	-0,037	0,014
G-test	<i>ns</i>	<i>ns</i>	<i>ns</i>	0,0001	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	
p -value										

However, the low level of allozyme variation in the investigated populations of European larch and Swiss stone pine may be the result of a reduction of the gene pool of populations occupying sites near the timber line. BERGMANN and HATTEMER (1995) maintained that genetic drift and isolation cannot be the only relevant factors influencing genetic variation patterns in stone pine populations. They postulated that *Pinus cembra* has evolved by duplications of invariant isozyme loci in response to constant or predictable environmental conditions. The observed excess of homozygotes could be due to self-fertilization and mating between closely related trees. Neither can we exclude the hypothetical action of natural selection acting against heterozygotes at certain developmental stages (KARKKAINEN et al., 1996). Similarly, a slight excess of homozygotes has been observed in the most elevated European larch populations from the Alps (BELLETTI et al., 1997) and in five populations of Swiss stone pine in alpine areas of Piedmont and the Valle d'Aosta in NW Italy (BELLETTI and GULLACE, 1999). In an old stand of low elevation populations of Polish larch LEWANDOWSKI et al., (1991) observed constant excess of heterozygotes at all variable loci.

Mating system analysis

Single- and multilocus estimates of outcrossing in *Larix decidua* and *Pinus cembra* are presented in table 3. In *Larix decidua* the single-locus rates (t_s) ranged from 0.627 to 0.663. All of the estimates significantly differed from 1.0, and they were very consistent over the loci studied. Generally, both methods of estimation (t_m and t_s) gave very similar results (Table 3). Estimates of outcrossing rates of *Pinus cembra* were slightly greater. The single-locus rates (t_s) ranged from 0.701 to 0.869, and were significantly heterogeneous over the loci studied (at $p=0.03$ level). Observed heterogeneity among single-locus outcrossing rates is probably due to a restricted number of maternal trees sampled. In such a case, each marker locus may test overlapping sets of mating events with unequal precision (BROWN et al., 1985). The multilocus estimate (t_m) was calculated to be 0.808 and it was higher than the mean of single-locus estimates ($t_s=0.751$).

Table 3. – Estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates of *Larix decidua* and *Pinus cembra* populations (standard deviations in parentheses).

<i>Larix decidua</i>		<i>Pinus cembra</i>	
Locus	Outcrossing rate	Locus	Outcrossing Rate
<i>Pgm-1</i>	0.663 (0.077)	<i>Lap-2</i>	0.869 (0.048)
<i>Pgm-2</i>	0.663 (0.080)	<i>Mdh-4</i>	0.701 (0.067)
<i>Shdh</i>	0.627 (0.064)	<i>Shdh</i>	0.684 (0.065)
mean t_s	0.651	Mean t_s	0.751 ^a
t_m	0.675 (0.045)	T_m	0.808 (0.036)

^a) parameter t_s significantly heterogeneous across loci at $p=0.0302$ level

Coniferous species are known to be predominantly outbreeding (STERN and ROCHE, 1974), however a variable and sometimes significant amount of selfing has been observed in natural populations of different species (KNOWLES et al., 1987; PERRY and KNOWLES, 1990; MORGANTE et al., 1991; XIE et al., 1991; EL-KASSABY et al., 1994; MEJNARTOWICZ et al., 1994). The outcrossing rate observed so far for European larch appeared to be higher than presented in this paper, both in natural stands (LEWANDOWSKI et al., 1991), and in seed orchards (BURCZYK et al., 1991; PAULE and GOMORY, 1992; BURCZYK et al., 1997).

The multilocus estimate of outcrossing rate ($t_m = 0.686$) calculated by POLITOV and KRUTOWSKII (1994) in a population of *P. cembra* with a low stand density from the Eastern Carpathians was lower than our estimate. However, t_m in a related species - *P. sibirica*, growing in populations with high density was higher and ranged from 0.846 to 0.980, with a mean of 0.894 (POLITOV and KRUTOWSKII, 1994).

Comparison of single-locus and multilocus estimates of outcrossing rate allows for the inference on the amount of inbreeding other than selfing (RITLAND and JAIN, 1981). Single-locus estimates are known to be lowered by any form of inbreeding in addition to selfing, such as mating among relatives, that may result from family spatial structure of populations (SHAW and ALLARD, 1982). In our study t_m was greater than the mean of the single-locus estimates (t_s) for both species. However, those differences appeared to be negligible (0.02 in larch and 0.06 in pine). This implies a rather weak effect of mating between relatives and suggests that self-fertilization may be the main factor influencing inbreeding in the studied populations.

Lower pollen production in highly elevated populations of *L. decidua* and *P. cembra* investigated in this paper, can be a reasonable explanation for the increased rate of self-fertilization. Density of trees in the highly elevated populations is generally decreased, therefore, one may expect that the average pollen production per square meter is also lowered. KOSKI and TALLQVIST (1978) estimated that in northern forests, where the density of trees is smaller than that in the southern ones, the average pollen production per square meter is three times lower. In wind-pollinated plants, the rate self-fertilization is assumed to be higher when the relative concentration of self pollen is higher around crowns of individuals (ZIEHE and GREGARIUS, 1988). KARKKAINEN et al. (1996) and HEDRICK et al. (1999) found that lower pollen production in northern forests in Finland may increase levels of self-fertilization.

The high level of self-fertilization found in high elevated populations of *L. decidua* and *P. cembra* could also be explained by reduction of the number of recessive embryonic lethals. The ability to produce viable seeds after self-fertilization due to reduction of the number of recessive embryonic lethals may represent a selective advantage in harsh climatic conditions near or above timber line. This could assure that at least some viable seeds of individuals would be produced and contributed to a new generation. It is particularly important when pollen is in short supply and the population is small or low in density. KARKKAINEN et al. (1996) showed that populations of Scots pine from northern Finland have a lower number of embryonic lethal equivalents than those from southern Finland. They proposed that this difference in inbreeding depression could be caused by either more self-fertilizations occurring in northern than in southern populations, or by a stronger selection against detrimental alleles in the northern than in the southern populations.

Differences in self-fertility due to different numbers of lethal equivalents could explain the variability in outcrossing rate among populations from low and high elevations. This hypothesis, of course, needs to be tested through comparative analysis of progenies from controlled self- and cross-pollinations.

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Classifying *Abies* Species (*Pinaceae*) Based on the Sequence Variation of a Tandemly Repeated Array Found in the Chloroplast DNA *trnL* and *trnF* Intergenic Spacer

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Abstract

DNA sequences of the chloroplast spacer region between the *trnL* and *trnF* genes were determined in 18 species of *Abies* MILL. and another five species of *Pinaceae* (*Keteleeria davidiana*,

na, *Tsuga sieboldii*, *Larix kaempferi*, *Pseudotsuga menziesii*, and *Picea bicolor*). A tandem repeat sequence composed of a 14-bp core sequence was found in all *Abies* species analyzed. This tandem repeat array was specific for genus *Abies*, since this array was not detected in the other five species of *Pinaceae*. Comparison of the tandem repeat region of *Abies* species revealed variation in the number of repeats and in the nucleotide sequences of the units among species. The number of repeats varied from two to five, and there were eight different unit types in nucleotide sequences. Eighteen *Abies*

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