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Elevation-Dependent Genetic Variation of Plants and Seed Embryos in the Crimea Mountain Population of *Pinus pallasiana* D. Don

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Abstract—In the Crimea Mountain population of *Pinus pallasiana* D. Don, the genetic structure of adult plants and embryos was studied along an elevation gradient (150, 400, 600, and 900 m a.s.l.). Ten polymorphic loci of six enzyme systems were examined. The degree of differentiation of plants from different elevations and their progeny with respect to genetic structure proved to be low, indicating the absence of strict isolation barriers that would interfere with gene flow along the elevation gradient.

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Key words: *Pinus pallasiana*, population, elevation gradient, genetic structure and differentiation.

On mountain macroslopes, the reproductive isolation of plants growing at high and low elevations is often observed. This phenomenon is related to shifts in dates of phenophases due to differences in climatic conditions along the altitudinal gradient. Thus, according to the long-term observations of Podgornyi (1995), *Pinus pallasiana* D. Don trees growing at the timberline "bloom" every year at the time when the reproductive phase in plants growing on the same macroslope below 600 m a.s.l. has already been terminated. Such asynchronous phenology of blooming can interfere with free gene exchange within tree stands in spite of the fact that, in many species, the radius of pollen spread from an individual plant extends beyond the range of the whole population or its major part on the macroslope (Silen, 1962). Due to considerable diversity of ecological conditions along the elevation gradient and, therefore, varying pressure of natural selection and phenological isolation of plants inhabiting different elevations, continual populations may be heterogeneous in their genetic structure (Shurkhal *et al.*, 1988; Podgornyi, 1995; Petrova *et al.*, 2000). Sometimes this heterogeneity is not manifested (Knowles, Grant, 1985; Neale and Adams, 1985). It is likely that the reproductive isolation of plant subpopulations growing at different elevations would be primarily reflected in the genetic structure of seed progeny; i.e., it would already manifest itself at the embryo stage. In conifer populations, it is relatively easy to compare the genetic structure of maternal plants and embryos and to identify alleles received from paternal gametes by the isozyme pattern (Altukhov, 1989).

The objective of this work was to analyze genetic variation of plants, embryos, and their paternal gametes in *P. pallasiana* plants along the altitudinal gradient on the Mountain Crimea macroslope to elucidate the degree of differentiation within the population.

MATERIAL AND METHODS

Experiments were performed with a *Pinus pallasiana* population growing in the Mountain Crimea, at the northern boundary of the range of this species, which is characterized by narrow ecological and cenological amplitudes. The population studied is in one of the largest forests on the southern macroslope of the Main Crimea Mountain Range near the village of Nikita. The seeds of 90-year-old and older trees were collected in autumn in four places at different elevations: on Cape Mart'yan (CM, 70–150 m a.s.l.), at the lower boundary of the species range; two places in the middle zone, at elevations of 400 and 600 m a.s.l. (NIK-400 and NIK-600); and at the upper boundary of the species range at elevations of 900 m a.s.l. and higher (NIK-900).

For genetic analysis of the population, six isozyme systems were used as molecular markers: glutamate dehydrogenase (GDH), glutamate oxalacetate transaminase (GOT), superoxide dismutase (SOD), acid phosphatase (ACP), leucine aminopeptidase (LAP), and malate dehydrogenase (MDH). To compare the genotypes of the maternal plant and embryo and thereby identify the paternal alleles involved in embryo development, the isozymes from seed endosperm and embryos were separated in parallel by electrophoresis in vertical blocks of 7.5% polyacrylamide gel. In each of 92 plants tested, we analyzed eight seed endosperms and eight embryos. The procedure of isozyme electrophoresis was described in detail previously (Korshikov,

Table 1. Main indices of genetic polymorphism in samples of adult *P. pallasiana* trees and embryos along the elevation gradient in the Mountain Crimea (calculated on the basis of ten isozyme loci)

Sample	Sample size, ind.	Proportion of polymorphic loci (P_{99})	Average number per locus			Average heterozygosity		Wright's fixation index (F)
			alleles	genotypes	alleles in the pool of paternal gametes	expected (H_E)	observed (H_O)	
MM								
Adult trees	18	0.700	2.000	2.300	–	0.204 ± 0.026	0.194 ± 0.024	0.049
Embryos	144	0.700	2.100	2.700	1.900	0.192 ± 0.009	0.133 ± 0.008	0.307
NIK-400								
Adult trees	29	0.800	2.300	2.600	–	0.176 ± 0.020	0.186 ± 0.019	–0.057
Embryos	232	0.700	2.500	3.100	2.200	0.151 ± 0.007	0.113 ± 0.006	0.251
NIK-600								
Adult trees	20	0.800	2.400	2.600	–	0.180 ± 0.024	0.190 ± 0.025	–0.055
Embryos	160	0.700	2.400	3.200	2.200	0.177 ± 0.009	0.140 ± 0.008	0.209
NIK-900								
Adult trees	25	0.900	2.200	2.500	–	0.205 ± 0.023	0.240 ± 0.023	–0.17
Embryos	200	0.800	2.400	3.200	2.100	0.192 ± 0.008	0.120 ± 0.007	0.375
Entire population								
Adult trees	92	0.800	2.200	3.400	–	0.193 ± 0.012	0.203 ± 0.012	–0.051
Embryos	736	0.700	2.300	4.100	2.300	0.179 ± 0.004	0.125 ± 0.004	0.302

Pirko, 2002). For four plant samples, the frequencies of alleles and genotypes, main indices of genetic polymorphism, and the coefficients of Wright's F-statistics and Nei's G-statistics were estimated (Altukhov, 1989; Goncharenko *et al.*, 1992; Korshikov *et al.*, 2002). For calculations, the BIOSYS-1 program was used (Swofford and Selander, 1981). Multilocus evaluation of the system was performed with the MLTR program (Ritland, 2002) based on three polymorphic loci (*Gdh, Got-2*, and *Got-3*) by calculating a standard coefficient of the proportion of cross-pollination (t_m).

RESULTS AND DISCUSSION

In 92 maternal *P. pallasiana* plants and 736 embryos from their seeds, 29 alleles of 10 polymorphic loci were identified. In the population pool of pollen that fertilized ovules with subsequent development of high-quality seeds, only 26 alleles were found. In the total samples of plants and embryos, 33 and 41 genotypes were detected, respectively. The highest and lowest levels of genetic diversity in the plant, embryo, and male gamete samples were observed in plants from the middle zone and from Cape Mart'yan, respectively (Table 1). These differences may be related to a greater plant density in the middle zone, where favorable ecological conditions permit the development of continuous forest cover extending for tens of kilometers along the Main Mountain Range 400–800 m above sea level. On Cape Mart'yan, only single plants occur.

In adult trees, the proportion of polymorphic loci increased at higher elevations. The highest values of expected (H_E) and observed (H_O) heterozygosity were characteristic of the plants sampled at the upper boundary of *P. pallasiana* range on the macroslope (NIK-900), although they did not differ significantly from other samples according to Student's *t*-test. In another study with 20 allozyme loci (Korshikov *et al.*, 2002), we observed a similar tendency; the average observed heterozygosity of the population did not change considerably: $H_O = 0.192 \pm 0.008$. In the embryos, the proportion of polymorphic loci was, as a rule, lower, whereas the values of observed heterozygosity were much lower (by 26.3–50%) than in the maternal plants. No significant correlation was revealed between heterozygosity at any locus and the elevation at which the plants and their progeny were sampled.

The adult plant samples manifested a uniform genetic structure, as H_O and H_E values did not differ significantly. Embryo genetic structure was shifted due to a deficiency in heterozygotes, which is confirmed by high positive values of Wright's fixation index (see Table 1). Wright's F-statistics coefficients (F_{IS}, individual inbreeding relative to population, and F_{IT}, individual inbreeding relative to species) indicate a 2.9% excess and 1.1% deficiency in adult plants and a considerable deficiency in heterozygotes in embryos (14.1 and 15%, respectively).

The observed distribution of *P. pallasiana* genotypes sampled at different elevations did not differ sig-

Table 2. Significant deviations of observed *P. pallasiana* genotype distribution from the Hardy–Weinberg law in embryo samples from different elevations in Mountain Crimea

Locus	Sample				In the entire population
	CM	NIK-400	NIK-600	NIK-900	
Gdh	n.s.	72.71** (0.18)	n.s.	n.s.	59.13** (0.67)
Got-2	4.59* (1.00)	12.73** (1.00)	18.68** (1.00)	26.96** (1.00)	65.53** (1.00)
Got-3	n.s.	n.s.	24.89** (0.71)	83.46** (0.88)	44.79** (0.90)
Lap-1	5.75* (0.83)	n.s.	n.s.	26.86** (0.87)	161.13** (2.31)
Lap-2	59.28** (0.93)	n.s.	4.28* (1.00)	25.82** (1.42)	11.06** (0.58)
Acp	107.34** (2.74)	19.89** (1.10)	51.31** (2.40)	13.93** (0.84)	404.80** (3.68)
Mdh-2	–	n.s.	–	n.s.	n.s.
Mdh-3	n.s.	4.99* (0.64)	n.s.	16.58** (1.00)	n.s.

Note: The number of degrees of freedom is indicated in parentheses; * difference is significant at $P < 0.05$, ** difference is significant at $P < 0.001$; n.s. – not significant.

nificantly from equilibrium according to the Hardy–Weinberg equation (the only exceptions were three different loci in different samples). In embryo samples, significant shifts in genotype distribution (for 7 loci) were recorded in 18 cases (Table 2). These deviations in genotype distribution in all embryo samples were characteristic of two highly variable loci, *Got-2* and *Acp.* In the total embryo sample from the entire population, significant deviations of observed genotype distribution from the expected one were observed in six cases, whereas none was found for plants. In embryos, all significant deviations from equilibrium distribution were related to heterozygote deficiency. An increased number of deviations from the genotype equilibrium in the embryos was noted in many conifers and is explained by a high proportion of self-pollinated plants in natural populations (Politov *et al.*, 1992).

The multilocus analysis of the plant breeding system in the *P pallasiana* samples showed that the highest proportion of cross-pollination (t_m) was characteristic of plants growing on Cape Mart'yan (0.894 ± 0.099) and the middle-altitude zone NIK-600 (0.820 ± 0.118), and the lowest proportion, in the upper zone of the continuum (0.418 ± 0.056). At the same time, thinned tree stands on Cape Mart'yan produced the smallest proportion of high-quality seeds and the largest proportion of hollow seeds (Korshikov *et al.,* 2002). The production of high-quality seeds in this tree stand is primarily associated with the high frequency of cross-pollination by pollen from the middle-altitude zone. In the year of our observations, the average t_m value for the population studied was 0.657 ± 0.056, which is the lowest limit for natural conifer stands (Politov *et al.*, 1992).

A comparison of heterogeneity of allele frequencies in plant and embryo samples using a χ^2 test confirmed that, in the seed progeny from different elevations and in the entire population, the allele diversity was maintained in all ten loci studied. In the embryos, frequencies for the *Acp* locus differed markedly in three samples, and in the sample from the upper elevation zone, in four loci. This is in agreement with the excessive homozygosity of the plant progeny in the upper zone of the continuum.

The high degree of similarity in genetic structure was characteristic of *P. pallasiana* samples. In pairwise comparisons (six pairs), allele heterogeneity was detected in only two cases (different loci in two pairs: CM–NIK-400 and CM–NIK-900), genotypic heterogeneity was detected in three cases (different loci in three pairs: CM–NIK-400, CM–NIK-900, and NIK-400–NIK-900). In embryo samples, significant heterogeneity was determined for eight out of ten loci (Table 3). It was observed in 3–6 loci in comparisons of alleles (31 out of 60 cases) and in 4–7 loci in comparisons of genotypes (32 cases). No evident quantitative dependence of these indices on elevation was noted. Within the limits of the continuum, a significant inequality in the spatial distribution of alleles and genotypes was characteristic of only one locus in the plants and of seven loci in the embryos. Among the latter, three loci most significantly contributed to the genetic heterogeneity of embryos along the elevation gradient: *Lap-1, Acp*, and *Lap-2*.

Paternal gametes contribute to the formation of genetic heterogeneity of *P. pallasiana* embryos sampled at different elevations. Considerable differences in allele frequencies in the pool of paternal gametes of these samples were observed for four to six loci. In loci *Acp* and *Lap-1*, significant heterogeneity was noted in all six pairs of samples. Negative correlations were revealed between the frequency of predominant allele in the paternal gamete pool and sample elevation above sea level for loci *Got-3* ($r = –0.996$) and *Mdh-3* ($r = –0.874$). Similar interrelations were found for predominant allele in the locus *Got-3* of embryos ($r = –0.929$).

Calculation of the coefficients F_{ST} and G_{ST} that characterize the population heterogeneity showed that ~98.3% of variation in adult plants and 98.7% in

Table 3. Heterogeneity of (A) alleles and (G) genotypes in embryos and in the pool of (P) paternal gametes in the samples of *P. pallasiana* collected at different elevations on the macroslope of the Crimea Mountain (standard χ^2 test)

Locus	Heterogeneity between all samples					Embryos																	
	plants		embryos			CM – NIK-400			CM – NIK-600			CM – NIK-900			NIK-400 – NIK-600			NIK-600 – NIK-900			NIK-400 – NIK-900		
	A	G	A	G	P	A	G	P	A	G	P	A	G	P	A	G	P	A	G	P	A	G	P
Gdh				40.4	16.0		15.0		6.1	6.4	6.5					22.9	7.6	7.7	7.0			10.3	
				(9)	(6)		(3)		(2)	(2)	(2)					(3)	(2)	(2)	(2)			(3)	
	n.s.	n.s.	n.s.	***	*	n.s.	**	n.s.	*	*	*	n.s.	n.s.	n.s.	n.s.	***	*	*	*	n.s.	n.s.	*	n.s.
Got-1			17.7	10.7																	4.6	4.7	
			(3)	(3)	–	–	–	–	–	–	–	n.s.	n.s.	–	–	–	–	n.s.	n.s.	–	(1)	(1)	–
	n.s.	n.s.	*	*																	*	*	
Got-2			13.3	15.3	14.5			7.0	13.1	7.9	13.6				4.4			5.1		4.6			
			(2)	(6)	(3)			(1)	(1)	(2)	(1)				(1)			(1)		(1)			
	n.s.	n.s.	**	*	**	n.s.	n.s.	**	***	*	***	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.	*	n.s.	n.s.	n.s.
Got-3			25.1	29.9	13.51			4.1	9.3	10.1	6.6	18.9	17.3	11.8							10.8	14.8	11.83
			(6)	(9)	(3)			(1)	(2)	(3)	(1)	(2)	(3)	(1)							(2)	(3)	(1)
	n.s.	n.s.	***	***	**	n.s.	n.s.	*	**	*	**	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	**	***
Lap-1	16.0		112.7	109.6	76.1	78.8	50.8	46.6	20.1	26.7	13.3	54.6	34.4	31.5	35.4	39.7	38.6	13.1	21.9	12.3	12.3	10.3	12.2
	(6)		(6)	(12)	(6)	(3)	(3)	(2)	(2)	(3)	(2)	(2)	(4)	(2)	(3)	(4)	(2)	(2)	(4)	(2)	(2)	(4)	(2)
	*	n.s.	***	***	***	***	***	***	***	***	**	***	***	***	***	***	***	***	***	**	**	*	**
Lap-2		21.6	89.7	76.8	70.8		10.3		14.5	12.9	10.6	9.8		13.5	40.5	32.7	30.5	32.5	24.5	22.4	26.8	26.3	22.2
		(9)	(9)	(15)	(6)		(4)		(2)	(4)	(2)	(3)		(2)	(3)	(4)	(2)	(2)	(4)	(2)	(3)	(3)	(2)
	n.s.	*	***	***	***	n.s.	*	n.s.	***	*	**	**	n.s.	***	***	***	***	***	***	***	***	***	***
Acp			122.8		77.7	29.9	23.5	17.9	68.5	66.6	45.1	19.8	17.6	14.1	30.7	21.8	21.2	37.5	33.9	27.4	35.3	30.6	25.4
			(9)		(9)	(3)	(6)	(3)	(3)	(6)	(3)	(3)	(5)	(2)	(3)	(5)	(3)	(2)	(6)	(3)	(3)	(6)	(3)
	n.s.	n.s.	***	n.s.	***	***	***	***	***	***	***	***	**	***	***	***	***	***	***	***	***	***	***
Mdh-3			37.3	53.1		13.5	19.6					7.2	9.9		15.8	48.9		8.5	10.9		15.9	12.5	
			(9)	(15)		(3)	(4)					(2)	(3)		(3)	(5)		(2)	(4)		(2)	(3)	
	n.s.	n.s.	***	***	n.s.	**	***	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	**	***	n.s.	*	*	n.s.	***	**	n.s.

Note: The number of degrees of freedom is indicated in parentheses. * Difference is significant at $P < 0.05$; ** difference is significant at $P < 0.01$; *** difference is significant at $P < 0.001$; n.s.—not significant.

Table 4. Nei's genetic distances (Nei, 1972) between the regions of *P. pallasiana* continuum differing in elevation (above the diagonal, values for maternal plants; below the diagonal, values for embryos)

Population	CM	NIK-400	NIK-600	NIK-900
CM	–	0.004	0.004	0.004
NIK-400	0.005	–	0.003	0.005
NIK-600	0.009	0.004	–	0.003
NIK-900	0.005	0.003	0.004	–

embryos manifested itself within the samples. This is indicative of similarity in the genetic structure of plants and embryos taken along the elevation gradient. However, the greatest contribution to heterogeneity within the population was made by two loci in plants—Sod-4 (4%) and Lap-1 (4%)—and by three loci in embryos—*Lap-1* (4.1%), *Acp* (3.1%), and *Lap*-2 (2.1%). Low calculated values of Nei's genetic distance (D_N) (Table 4), which includes all differences in the allele frequencies for ten studied loci, confirm insignificant genetic differentiation of plant and embryo samples from different elevations. Thus, the average values of D_N were 0.0037 for plant samples and 0.005 for embryo samples. A higher D_N value for embryos was explained by greater differentiation of embryo samples than of plant samples from Cape Mart'yan. Based on D_N values, dendrograms were constructed by means of UPGMA. The dendrograms were similar for plant and embryo samples, thus confirming that the reproduction of the genetic structure in the progeny within mountain *P. pallasiana* population is not accidental.

Thus, in the typical *P. pallasiana* population at different elevations on the macroslope of Crimea Mountain, the level of heterozygosity of embryos was substantially lower than that of plants. Differences in ecological factors along the elevation gradient and related incomplete reproductive isolation of plants at different elevations (Podgornyi, 1995) did not lead to distinct segregation of their seed progeny into subpopulations. It is likely that more strict isolation barriers than incomplete coincidence of plant reproductive phases are required for producing such a result.

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