

Analysis of mating system in two *Pinus cembra* L. populations of the Ukrainian Carpathians

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Abstract. In natural pine populations, a mixed mating system is typical, characterized by the proportions of selfed and outcrossed seeds. Swiss stone pine (*Pinus cembra* L.) is one of the least studied European conifers in this respect. The mating system of six polymorphic allozyme loci were studied in haploid megagametophytes and diploid open-pollinated embryos in two stands located in the East Carpathians. In the 'Gorgany' population (24 trees, 198 seeds) the mean single-locus estimated outcrossing rate (t_s) was 0.731, and the multilocus estimate (t_m) was 0.773. In the 'Yayko' population the outcrossing rate was lower (27 trees, 213 seeds, $t_s=0.645$, $t_m=0.700$), suggesting 23-30% of seeds are self-pollinated. Correlation of outcrossing rate estimates among loci was less than 1, (0.300 in 'Gorgany' and 0.469 in 'Yayko') indicating biparental inbreeding occurred. Differences between t_m and t_s (0.042 in 'Gorgany' and 0.056 in 'Yayko') can also be influenced by consanguineous mating, indicated by the presence of spatial and genetic family structure. In small isolated populations of *Pinus cembra*, which are typical for the Carpathian part of the species' range, inbreeding depression may negatively affect seed quality. The high proportion of selfed seeds observed here can be expected in any seedlot of this species and should be taken into account while planning gene conservation or reforestation measures. Maternal trees in these populations showed no heterozygote deficiency at these allozyme loci, and instead showed increased proportions of heterozygotes (inbreeding coefficient $FIS = -0.200$ in 'Gorgany' and -0.142 in 'Yayko'). Balancing selection may explain heterozygosity levels up to and above equilibrium proportions.

Key words: *Pinus cembra*, Swiss stone pine, allozymes, heterozygosity, mating system, inbreeding, balancing selection

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Introduction

In forest tree stands, as well as in any higher plant population, the mating system is a crucial factor in the genetic constitution of the next

generation of plants. While assessing parameters of the mating system, the principal measure is the outcrossing rate, the proportion of outcrossed progeny out of the total analyzed F1 population. Plant mating systems have

traditionally been described by the mixed-mating model, which assumes that each mating is either a result of self-fertilization (selfing) or it originates from other individuals (outcrossing). Under model assumptions, outcrossing occurs at random with respect to the remaining population (Brown et al. 1989; Ritland 1983, 1986; Ritland & Jain 1981). In most cases, this means receiving the male gamete from any paternal plant unrelated to the maternal plant. Conifers seem to possess all the necessary adaptations for outcrossing. Usually conifers form stands with high population size and density, and the pollen of conifer trees is distributed by wind over long distances compared to the height of maternal plants. However, in some species, effective population size is low, trees are distributed sparsely, and this creates conditions limiting panmixia. Restricted seed dispersal distance also leads to formation of family structure, that in turn gives rise to inbreeding other than selfing (i.e., mating among relatives).

Swiss stone pine (*Pinus cembra* L.) occurs in the Alpine-Carpathian mountain system, occupying altitudes between 1200 and 2000 m a.s.l. (in some places up to 2600 m) (Critchfield & Little 1966; Farjon 1984; Mirov 1967). In the central part of the Alpine sector of the range it is distributed more or less continuously, while in the periphery it is highly fragmented. In the Carpathians, the species has a mosaic distribution, determined primarily by the geomorphology of this mountain system. The Carpathians lack a single central range with maximum altitudes and are characterized by chains of separate peaks, formed by river valleys that cut parallel ridges transversely (Popov 1949). To the east of the Alps, *P. cembra* occurs in the High Tatras, in the Northern Carpathians within Ukraine and Romania (these mountains are called also Eastern Carpathians; however, the easternmost range of the Carpathians lack *P. cembra*), and in the Southern Carpathians (Romania). Seeds of *P. cembra* are dispersed by birds (nutcrackers, *Nucifraga* spp.), that, along with wind-mediated pollen, dispersal create possibilities for extensive gene flow both within and among populations. Data pertaining to the level and distribution of

genetic variability on the range of Swiss stone pines are scarce. In an early allozyme study of genetic structure of isolated *P. cembra* populations, strong interpopulation genetic differentiation was reported (Szmidt 1982). Studies of *P. cembra* populations in the Italian Alps (Belletti & Gullace 1999) and in the Ukrainian Carpathians (Belokon et al. 2005) demonstrated that genetic differentiation is moderately high ($F_{ST} = 7.4\%$), in the range typical for most conifers.

Few data are available on outcrossing rates in Swiss stone pine stands and on temporal dynamics of genetic variation at different life stages. An allozyme study of the altitudinal distribution of genetic variation at juvenile and adult stages in the Austrian Alps (Klumpp & Stefsky 2004) showed that genetic diversity typical for adult populations is relatively well preserved in juveniles. Genetic structure is better reproduced in the middle montane zone, while in low and high mountain belts genetic variability of juveniles was somewhat lower than in mature trees. Only two studies focused on the mating system. A relatively low outcrossing rate was found in a sample of Swiss stone pine from Ukraine (Politov & Krutovskii 1994), and a similar but lower value was reported for a stand in the Italian Alps (Lewandowski & Burczyk 2000).

This paper presents estimates of the mating system parameters in two stands of *P. cembra* in the Ukrainian Carpathians determined using allozyme loci.

Material and methods

Plant material

Plant material comprised seeds from individual trees collected in two natural populations of Swiss stone pine in the Gorgany range (Ukrainian Carpathians). Twenty-four maternal trees were sampled on the slopes of Mt. Berezovachka, tract Dzhrurzy, Natural Reserve 'Gorgany' (sample GOR). The stand is located at altitude 1300-1500 m a.s.l and composed of 90% Norway spruce (*Picea abies* (L.) Karst.) and 10% *Pinus cembra* (southwestern slope), 70% Norway spruce and 30% Swiss

stone pine (northeastern slope). Another sample of 27 trees was collected on Mt. Yayko Ilemske on the territory of Ivano-Frankovsk oblast' of Ukraine (sample 'Yayko', YAY). Sampling was conducted at 1375-1400 m a.s.l. The stand consists of 80% Norway spruce and 20% Swiss stone pine. Mature seeds were extracted from cones and stored at +4°C until electrophoretic analysis could be performed.

Electrophoresis

Six to 11 (for most trees eight) endosperms and embryos per tree were analyzed by starch gel electrophoresis, allowing reliable discrimination of all homozygotes and heterozygotes (Morris & Spieth 1978). Endosperm and embryo from the same seed were run on adjacent lanes of the electrophoretic gel. Specimen preparation, buffer systems used, genetic interpretation of zymograms, designations of allozymes, alleles, and loci have been described elsewhere (Belokon et al. 2005; Krutovskii et al. 1987; Politov 1989). Electrophoresis of *P. cembra* embryo tissue allowed identification of diploid genotypes at the following polymorphic loci: *Adh-1*, *Fdh*, *Gdh*, *Idh*, *Lap-3*, *Mdh-2*, *Mdh-4*, and *Pgm-1*. However, poor activity and/or resolution or low variability at some isozyme zones led to exclusion of some loci from the following analyses. As a result, six loci were used to estimate outcrossing rates in each sample (*Adh-1*, *Fdh*, *Gdh*, *Mdh-2*, *Mdh-4*, *Pgm-1*). Genotypes of individual trees were inferred from segregation of alleles among endosperms.

Statistical analysis

Matrices of embryos grouped by their corresponding maternal parents were utilized to estimate single-locus (t_s) and multi-locus (t_m) outcrossing rates by the computer program MLTR 3.0 (Ritland 1984, 1986, 1990; Ritland & EL-Kassaby 1985; Ritland & Jain 1981). We used the following options: estimation by the Newton-Raphson method with allele frequencies in maternal and paternal gene pools assumed to be non-equal; information on the maternal allele in each embryo (from the megagametophyte) was supplied; resampling of entire families was used for bootstrap esti-

mation of standard errors. Since t_m is less sensitive to the violation of assumptions of the mixed mating model, the difference between t_m and t_s , and correlation of t among loci (also computed using MLTR) were calculated to reveal biparental inbreeding levels. The expected inbreeding coefficient from mating system was calculated as $F_{IS(exp)} = (1-t)/(1+t)$ (Brown et al. 1989).

Results

Single-locus estimates of outcrossing rates in the Gorgany and Yayko samples were 0.706 and 0.645, respectively (table 1). Multilocus estimates were somewhat higher, 0.748 and 0.700, respectively. Using these values, the total effective selfing in Swiss stone pine populations in the Ukrainian Carpathians was estimated at 30-35%, of which 25-30% is due to selfing and 5% caused by biparental inbreeding, i.e. consanguineous mating as a result of crossing between adjacent trees more closely related than the population average.

Inbreeding was documented in embryo samples by calculating the inbreeding coefficient F_{IS} , which varied in GOR among loci from -0.025 to 0.272 (table 2). On average, F_{IS} value was positive (0.113), and at two loci (*Adh-1* and *Mdh-2*) the test for correspondence to Hardy-Weinberg equilibrium (HWE) showed highly significant deviation towards heterozygote deficiency. Even more substantial deviation from Hardy-Weinberg proportions were revealed in the YAY population. All six loci had positive values of F_{IS} , ranging from 0.114 to 0.419, indicating heterozygote deficiency. On average, F_{IS} was as low as 0.229. At four loci (*Adh-1*, *Mdh-2*, *Mdh-4*, and *Pgm-1*), genotype distributions showed significant deviation from Hardy-Weinberg proportions. In general, observed values of F_{IS} corresponded to those expected values calculated via t_s (table 1).

Adults in the studied populations (maternal trees) showed no consistent or strong heterozygote deficiency under Hardy-Weinberg expectations at these allozyme loci (table 3). Instead, we detected an increased proportion of heterozygotes. The inbreeding coefficient, averaged over all polymorphic loci,

Table 1 Estimation of outcrossing rate in two East Carpathians populations of *Pinus cembra*

Sample	Outcrossing rate		Coefficient of inbreeding	
	single-locus, t_s	multilocus, t_m	expected, $F_{IS(exp)}$	observed, F_{IS}
Gorgany	0.706±0.070	0.748±0.064	0.172	0.113
Yayko	0.645±0.058	0.700±0.054	0.215	0.229

Table 2 Levels of genetic variability at allozyme loci and inbreeding coefficient among *P. cembra* embryos

Sample	Gorgany					Yayko				
	Locus	N	H _O	H _E	F_{IS}	P	N	H _O	H _E	F_{IS}
<i>Adh-1</i>	189	0.280	0.374	0.250	0.000***	211	0.346	0.429	0.193	0.000***
<i>Fdh</i>	198	0.081	0.087	0.076	ns	212	0.047	0.064	0.261	ns
<i>Gdh</i>	198	0.000	0.000	-	ns	213	0.056	0.064	0.114	ns
<i>Mdh-2</i>	192	0.026	0.036	0.272	0.000***	212	0.061	0.077	0.203	0.003**
<i>Mdh-4</i>	198	0.409	0.399	-0.025	ns	213	0.390	0.479	0.186	0.007**
<i>Pgm-1</i>	198	0.154	0.152	-0.009	ns	212	0.175	0.300	0.419	0.000**
Mean	195.5	0.158	0.175	0.113		212.2	0.179	0.235	0.229	

N – sample size; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient; P – probability of null-hypothesis rejection by χ^2 -test for Hardy–Weinberg equilibrium (HWE): ** P<0.01 *** P<0.001, ns - not significant; F_{IS} values at loci with HWE violation are boldfaced.

was $F_{IS}=-0.150$ in 'Gorgany' and -0.093 in 'Yayko'. If only loci that were studied in embryos were included, the corresponding values were slightly less negative (e.g., for GOR $F_{IS} = -0.112$) and, for YAY close to the equilibrium point (-0.025).

Discussion

In previous studies the outcrossing rate in Swiss stone pine was estimated by allozyme loci at 0.808 in the Italian Alps (Lewandowski & Burczyk 2000) and at 0.686 (t_m) and 0.707 (t_s) in the Ukrainian Carpathians (Politov & Krutovskii 1994). Our new data for the two Ukrainian populations do not significantly differ from these values. In a closely related species, Siberian stone pine (*Pinus sibirica* Du Tour), the outcrossing rate was higher, with multilocus estimate values ranging from 0.846 to 0.980 (mean 0.894) (Politov & Krutovskii 1994), which is close to the mean for wide-spread conifers (see review in Politov et al. 1990). Relatively low values of outcrossing for the Carpathian *P. cembra* stands are com-

icina L. (Knowles et al. 1987), *L. decidua* L. (Knowles et al. 1987) and *Abies alba* L. (Pirko 2005). In *Larix*, pollen lacks air sacs which limit pollen dispersal distance and promotes selfing. In *Abies* and *Pinus* there should be other reasons for such low values. In the case of the studied *Pinus cembra* populations, inbreeding can be caused by the low effective size of populations and stand density. Similar low outcrossing rate were observed in relict low density stands of *Pinus sylvestris* and *P. sylvestris* var. *cretacea* in Ukraine (Mudrik 2006).

In small isolated populations of *Pinus cembra* which are typical for the Carpathian part of the species' range, both selfing and mating among close relatives may negatively affect seed quality due to inbreeding depression. A high proportion of selfed seeds observed in the two spatially separated parts of the range can be expected in any seed lot of this species, and that should be taken into account while planning gene conservation or reforestation measures.

The question arises of why this significant heterozygote deficiency is not observed in

Table 3 Levels of genetic variability at allozyme loci and inbreeding coefficient among *P. cembra* maternal trees

Sample Locus	Gorgany						Yayko					
	N	A	Ho	He	F_{IS}	P	N	A	Ho	He	F_{IS}	P
<i>Adh-1</i>	24	4	0.500	0.413	-0.210	ns	27	4	0.333	0.322	-0.036	0.036*
<i>Adh-2</i>	24	2	0.708	0.499	-0.419	0.040*	27	2	0.407	0.489	0.167	ns
<i>Fdh</i>	24	2	0.083	0.080	-0.043	ns	27	2	0.037	0.036	-0.019	ns
<i>Fe-2</i>	8	1	0.000	0.000	-	ns	9	1	0.000	0.000	-	ns
<i>Gdh</i>	24	1	0.000	0.000	-	ns	27	2	0.074	0.071	-0.038	ns
<i>Got-1</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Got-2</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Got-3</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Idh</i>	23	1	0.000	0.000	-	ns	27	2	0.185	0.168	-0.102	ns
<i>Lap-1</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Lap-2</i>	24	2	0.458	0.353	-0.297	ns	27	2	0.074	0.071	-0.038	ns
<i>Lap-3</i>	24	2	0.583	0.500	-0.167	ns	27	2	0.741	0.499	-0.484	0.012*
<i>Mdh-1</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Mdh-2</i>	24	2	0.042	0.041	-0.021	ns	27	2	0.148	0.137	-0.080	ns
<i>Mdh-3</i>	24	2	0.125	0.117	-0.067	ns	27	2	0.259	0.226	-0.149	ns
<i>Mdh-4</i>	24	2	0.500	0.413	-0.210	ns	27	2	0.370	0.483	0.233	ns
<i>Mnr-1</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Pepca</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Pgd-1</i>	22	2	0.182	0.165	-0.100	ns	27	2	0.333	0.278	-0.200	ns
<i>Pgd-2</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Pgd-3</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Pgi-1</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Pgi-2</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Pgm-1</i>	24	3	0.167	0.155	-0.073	ns	27	3	0.333	0.287	-0.160	ns
<i>Pgm-2</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Skdh-1</i>	16	3	0.313	0.275	-0.135	ns	18	2	0.111	0.105	-0.059	ns
<i>Skdh-2</i>	24	1	0.000	0.000	-	ns	27	1	0.000	0.000	-	ns
<i>Sod-2</i>	24	2	0.042	0.041	-0.021	ns	21	1	0.000	0.000	-	ns
<i>Sod-3</i>	24	2	0.083	0.080	-0.043	ns	15	2	0.067	0.064	-0.034	ns
<i>Sod-4</i>	11	2	0.455	0.351	-0.294	ns	14	2	0.571	0.408	-0.400	ns
All variable loci	22.7	1.60	0.141	0.116	-0.150		25.1	1.60	0.135	0.122	-0.093	
Loci analyzed in embryos					-0.112						-0.020	

N, number of embryos analyzed; A, number of alleles per locus; Ho, observed heterozygosity; He, expected heterozygosity; F_{IS} , coefficient of inbreeding; P, probability of rejecting the null hypothesis of no deviation from Hardy-Weinberg equilibrium; * $P < 0.05$; other designations as in table 2.

adult adult samples. Selection against inbred progeny is considered the main factor responsible for the rise of heterozygosity up to and (in the case of overdominance) above equilibrium proportions (Altukhov 1991; Bush & Smouse 1992; Politov & Krutovskii 2004). Selection eliminates inbred progeny and even can lead to heterozygote excess. In the case of *P. cembra*, taking into account the difference in

inbreeding level of embryo samples between the two samples, we can retrospectively trace dynamics in the level of inbreeding. If the initial level of inbreeding among embryos which founded the GOR stand was lower, this might explain the higher heterozygote excess in the corresponding adult sample. The initial degree of inbreeding of the YAY embryo sample was higher, and the adult population

had observed heterozygosity at, but not above, the HWE point. We suggest balancing selection may be one possible explanation for this. The difference between corresponding *FIS* estimates in embryos and adult trees remains the same in both samples. We note that these data are indirect estimates, since direct estimation is extremely challenging due to the late maturation of conifers.

The lower heterozygosity observed in the Alps at low and high altitudes in the juvenile part of the population is evidence that inbreeding effects can last for a long time, and similar research is required in the Carpathians. Due to its more fragmented range, Carpathian *P. cembra* stands are more vulnerable to disturbance and population impacts, and special programs for conservation of genetic resources of this ecologically important species should be developed where data on the mating system should be incorporated.

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- gur locus (t_s) a fost de 0,731 iar aceeași rată de încrucișare estimată pentru mai muți loci (t_m) a fost de 0,773. În populația Yavko (27 arbori, 213 semințe) rata de încrucișare a fost mai mică ($t_s=0.645$, $t_m=0.700$) sugerând că 23-30% din semințe au rezultat din auto polenizare. Corelația valorilor ratei de încrucișare dintre loci a fost sub valoarea 1 (0.300 în Gorgany și 0.469 în Yayko) indicând că depresiunea consangvină biparentală a avut loc. Diferențele dintre t_m și t_s (0.042 în Gorgany și 0.056 în Yayko) pot de asemenea fi influențate de încrucișarea consangvină fapt indicat de prezența structurii spațiale și genetice a familiei. În populații mici și izolate de *P. cembra*, populații care sunt tipice pentru o parte a arealului speciei în Carpați, depresiunea consangvină poate afecta negativ calitatea seminței. Proporția ridicată a semințelor din consangvinizare observată aici poate fi prezentă în oricare lot de semințe aparținând acestei specii fapt care trebuie luat în considerare atunci când se planifică acțiunea de conservare a genelor sau măsurile de împădurire. Arborii materni din aceste populații nu au arătat existența deficienței de heterozigoție la acești loci izoenzimatici, dar în schimb au arătat proporții crescute ale heterozigoților (coeficientul de heterozigoție $F_{IS} = -0.200$ în Gorgany and -0.142 în Yayko). Selecția balansată poate explica nivelele de heterozigoție până la și deasupra proporțiilor de echilibru.
- Cuvinte cheie:** *Pinus cembra*, zâmbru, izoenzime, heterozigoție, sistem de încrucișare, consangvinizare, selecție balansată.
- (Tradus de I. Blada)

Rezumat. Politov D.V., Pirkov Y.V., Pirkov N.N., Mudrik E.A., Korshikov I.I., 2008. Analiza sistemului de încrucișare în două populații de *Pinus cembra* din Carpații Ucrainieni. *Ann. For. Res.* 51:11-18.

În populațiile naturale de pin, sistemul mixt de încrucișare este tipic și este caracterizat prin proporțiile de semințe rezultate din consangvinizare și din încrucișare îndepărtată. Zâmburul (*Pinus cembra* L.) este unul din cele mai puțin studiate conifere din Europa. Sistemul de încrucișare a celor șase loci izoenzimatici polimorfici au fost studiați la megagametofitele haploide și la embrionii diploizi rezultați din polenizare liberă în două arborete din Carpații de Est (Ucraina). În populația Gorgany (24 arbori, 198 semințe) rata medie de încrucișare estimată pentru un sin-

