Theor Appl Genet (1995) 90:811–818

K. V. Krutovskii · D. V. Politov

Allozyme evidence for polyzygotic polyembryony in Siberian stone pine (*Pinus sibirica* Du Tour)

Received: 2 July 1994 / Accepted: 9 August 1994

Abstract Approximately 4000 mature seeds from 350 trees in nine populations (12–75 trees per population) of Siberian stone pine were investigated for multiple embryos (polyembryony). Haploid megagametophytes and embryos were genotyped for eight allozyme loci. Eighty-one seeds (2.11%) had more than 1 embryo. Of these, 71 seeds had 2 embryos (1.85%), 6 seeds had 3 embryos (0.16%), 3 seeds had 4 embryos (0.08%) and 1 seed had 6 embryos (0.026%). Allozyme comparison of megagametophytes and embryos could distinquish two types of polyembryony in 56 of the 81 seeds. In 28 seeds (50%) the polyembryony was polyzygotic (independent fertilizations of more than one egg cell in the ovule); 25 seeds (45%) had most likely monozygotic polyembryony (genetically identical embryos resulting from the cleavage of a single proembryo) and 3 seeds had both genetically different and genetically identical embryos. To the best of our knowledge, this is the first genetic evidence for the form of polyembryony in conifer seeds.

Key words Polyembryony · *Pinus sibirica* Du Tour · Siberian stone pine · Allozyme loci

Introduction

Polyembryony (PE), i.e. the occurrence of more than one embryo in a single seed, is a rare derivative phenomenon in angiosperms but very common in gymnosperms, including all conifers (Chamberlain 1966). This phenomenon has been quite thoroughly studied both embryologically and cytologically in different pine species, including the Siberian stone pine (e.g. Kozubov et al. 1982; Tret'yakova 1982, 1990).

Micro- and megagametophyte development, fertilization and embryogenesis are rather complicated in conifers. They consist of many stages, and in some genera there is an overwintering dormancy between pollination and fertilization. However, these processes have many common features, even in different genera or families of conifers (Chamberlain 1966; Kozubov et al. 1982). In Siberian stone pine three or four archegonia (rarer – two or five), each containing one egg cell, usually develop in a single ovule (Tret'yakova 1982, 1990). All egg cells and other megagametophyte cells are genetically identical, since they originate mitotically from one of four megaspores, which are products of meiosis. All of them, except for the basal megaspore, supposedly, degenerate and do not participate in the formation of a mature megagametophyte with an egg cell.

Cytological examinations made after normal wind pollination indicate that usually two, and on rare occasions, one or three, pollen grains penetrate the ovule nucellus and germinate. As a result, more than one egg cell (but usually not more than two) can be independently fertilized (Tret'yakova 1982, 1990). If after such a fertilization both or more zygotes develop and give embryos, this PE is called simple, archegonial, polyzygotic or non-cleavage (Dogra 1967). The embryos are heterogenic: they have identical maternal but different paternal haplotypes. Another type of PE is called monozygotic, or cleavage. It results from the cleavage of a developing zygote or proembryo into several, usually, four, embryos during early embryogenesis. These embryos have identical maternal and paternal haplotypes. Later, during embryogenesis, due to different development conditions and, probably, also for genetic reasons in the case of polyzygotic PE, one of the embryos as a rule dominates in the embryo cavity, the other embryo(s) either degenerating and disappearing or remaining very small and underdeveloped. We shall

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Communicated by P. M A. Tigerstedt

K. V. Krutovskii[1] (✉) · D. V. Politov
Laboratory of Population Genetics, N. I. Vavilov Institute of General Genetics, Russian Academy of Sciences, GSP-1 Moscow 117809 B-333, Russia

Present address:
[1] Department of Forest Science, Oregon State University, FSL 020, Corvallis, OR 97331-7501, USA

use polyzygotic PE (PZPE) and monozygotic PE (MZPE) as the terminology most appropriately characterizing the genetic differences between the two types of PE.

Tret'yakova (1982) observed more than four embryos in the embryo cavity of Siberian stone pine seeds and considered this to be evidence for the presence of both PE types in a single seed. However, despite numerous cytological and embryological investigations of conifers, there is still no quantitative data on PZPE:MZPE ratios in conifer seeds.

Since most of the excess embryos degenerate early in embryogenesis, usually only a small proportion of the mature seeds are polyembryonic. However, climatic disturbances may greatly increase their number (Doyle 1957; Dogra 1967; Simak 1973). Zemlyanoi (1973) noted that under more or less optimal conditions of the Northeastern Altai, Siberian stone pine PE seeds constituted about 1% of the filled seeds; however, this could reach 39–45% in the highlands or under the influence of low temperature. Iroshnikov (1974) also found that Siberian stone pine PE seeds constituted about 0.2–3.0% of the filled seeds under optimal conditions of the Western Sayan foothill populations (also used in our study), but could reach 3–10% (sometimes 15–25%) in subalpine populations of this region. Simak (1973) proposed that embryo elimination or suppression occurs as a result of some inhibiting activity of the dominant embryo, but if this activity is decreased under some circumstances, additional embryos have a greater chance to undergo development and differentiation. Possibly light frost and a sharp decrease in temperature weaken competition between embryos, thereby allowing the development of several embryos in one ovule.

As well as being influenced by environmental effects, PE frequency also can be under genetic control. Observing reproduction over many years of the same Siberian stone pine trees Iroshnikov (1974) found that in every population that he studied there are trees with a stable year-to-year high proportion of PE seeds (up to 38–44%).

The most precise data on PZPE and MZPE can be obtained only by comparing embryo genotypes characterized by a set of polymorphic genetic markers. Codominantly inherited isozymes controlled by polymorphic allozyme loci are quite effective genetic markers for such analysis and remain the most available. Unfortunately, conifer seeds are generally very small. This makes isozyme analysis of endosperms and embryos, especially poorly developed rudimentary ones in PE seeds, difficult. In this respect, Siberian stone pine seeds are a nice exception. This species is characterized by relatively large (10- to 14-mm) seeds with usually well-developed embryos. This fact, plus its wide distribution in Eurasia and the considerable information available from previous work on its genetic variability, made us choose this species as an object for our study.

Materials and methods

We used seeds sampled separately from 350 trees from nine natural populations located over a range of Siberian stone pine sites. Five populations, Filin Klyuch, Mutnaya Rechka, Malyi Kebezh, Sobach'ya Rechka and Listvyanka, are located in the Ermakovskii District of the Western Sayan Mountains (Krasnoyarsk Territory, East Siberia); the Yailyu population is found in the Altai Mountains (Gorno-Altaiskaya Autonomous Region, East Siberia); and Smokotino, Zorkal'tsevo-1 and Zorkal'tsevo-3 are in the Tomsk Region (West Siberia). A more detailed description of the populations can be found elsewhere (Iroshnikov 1974; Krutovskii et al. 1988, 1989, 1990, 1994; Politov 1989; Politov et al. 1992; Politov and Krutovskii 1994).

Endosperms (haploid megagametophytes) and embryos of 3845 seeds from 350 trees (12–75 trees per population) were genotyped for eight allozyme loci (*Adh-1, Dia-2, Fe-2, Lap-3, Mdh-2, Pgi-2, Pgm-1* and *Skdh-1*; see footnote to Table 1 for definitions). Tissue preparation, electrophoretic conditions, buffer systems, genetic interpretation of isozyme patterns, designation of allozymes, alleles and loci have been described elsewhere (Krutovskii et al. 1987; Politov 1989). Maternal haplotype was defined by endosperm tissue analysis and corresponded to the haplotype of the megagametophyte and the egg cell. Paternal haplotype was inferred by comparison of the embryo genotype with the endosperm haplotype. Often only one embryo among PE seeds was well-developed, and others were tiny and underdeveloped. We could not determine the genotypes of all of the allozyme loci in some embryos because of weak enzyme activity (especially in the undeveloped embryos). This decreased the discriminating power of our analysis for some seeds.

Taking into account the absence of or weak linkage between the allozyme loci used (Politov et al. 1989; Politov and Krutovskii 1994), we estimated the probability of independent fertilization of m egg cells in an single ovule in the case of a PE seed with embryos of the same allozyme genotype by $(\Pi_{i=1}^{n} p_i)^m$, where m is the number of compared embryos in the mature seed and p_i is the frequency of the corresponding allele in the outcrossing pollen pool for the i-th locus ($i = 1, 2 \ldots n$), studied in all of the embryos compared. Allozyme allele frequencies in the outcrossing pollen pool were estimated using the computer program MLT (Ritland 1990) and were based on the genotypes of all the embryos studied earlier in our research on stone pine mating system (Politov and Krutovskii 1994).

Results

Of the 81 seeds (2.11%) that were PE, the majority, 71 seeds (1.85%) had 2 embryos; 6 seeds (0.16%) had 3 embroys; 3 seeds (0.08%) had 4 embryos; and 1 seed (0.026%) had 6 embryos. Of these 81 seeds, 56 could be used to determine PE type. Twenty-eight (50%) of them could be considered to be unequivocally the result of PZPE because paternal haplotypes differed at one (e.g. seed nos. 27 and 47 of tree no. 10 from Filin Klyuch, Table 1) or more (e.g. seed no. 9 of tree no. 38 from the same population) loci. The maximum difference was in four loci and was found between the embryos in seed no. 12 of tree no. 213 from Mutnaya Rechka (Table 1).

Estimated frequency of PZPE has to be considered to be the minimum possible because there is a probability of embryos from independent fertilizations having the same allozyme genotype. Thus, in all cases of embryos of the same genotype, the probability of independent simultaneous fertilization of egg cells in an ovule by different pollen grains of the same allozyme haplotype has been estimated. If, because of a high occurrence of some

Table 1 Multilocus allozyme genotypes of embryos of Siberian stone pine polyembryonal seeds sampled in nine populations and supposed types of polyembryony (PE)

Number of		Embryo genotype[a]								*P*[b]	Type of PE[c]
Trees	Seeds	*Adh-1*	*Dia-2*	*Fe-2*	*Lap-3*	*Mdh-2*	*Pgi-2*	*Pgm-1*	*Skdh-1*		
Filin Klyuch (1287[d])											
10	27[e]	–[f]	**32**[g]	–	–	–	–	–	–		
		–	**33**[h]	–	–	–	–	–	–	1.000	PZPE
	47	–	22	–	**33**	–	–	–	–		
		–	22	–	**31**	–	–	–	–	1.000	PZPE
	69	23	22	22	11	11	21	22	23		
		23	22	22	11	11	21	22	–	0.0003	MZPE
	71	23	22	22	11	**12**	21	22	23		
		23	–	–	–	**11**	21	–	–	1.000	PZPE
11	24	–	–	**22**	–	11	22	–	–		
		–	–	**21**	–	11	22	–	–	1.000	PZPE
	38	–	22	12	31	11	22	22	32		
		–	22	–	31	11	22	22	–	0.0074	MZPE
37	12	–	22	22	33	11	22	22	33		
		–	22	22	33	11	22	22	33	0.0020	MZPE
38	9	–	22	**11**	**33**	11	22	22	22		
		–	22	**12**	**31**	11	22	22	23	1.000	PZPE
	15	33	22	12	31	11	22	22	22		
		33	22	12	–	11	22	22	22	0.0041	MZPE
41	1	22	–	22	11	11	22	22	33		
		22	–	22	11	11	22	22	33	0.0544	MZPE
44	5	22	22	22	13	11	21	22	33		
		22	22	22	13	11	21	22	–	$5.7\cdot10^{-4}$	MZPE
Mutnaya Rechka (885)											
51/2	6	22	22	12	11	11	22	22	22		
		22	–	12	–	–	22	–	–	0.3197	?[k]
69	8	22	22	22	11	11	22	22	23		
		–	–	22	–	–	22	–	–	0.4996	?
213	1	22	–	22	–	11	22	22	–		
		–	–	22	–	11	–	22	–	0.5060	?
	7	32	32	22	11	11	22	22	22		
		–	–	22	–	11	22	22	–	0.4377	?
	12	**33**	–	**22**	**11**	11	22	22	**32**		
		32	–	**21**	**13**	11	22	22	**33**	1.000	PZPE
		33	–	**22**	**11**	11	22	22	**33**	1.000	PZPE
289	10	22	–	21	11	11	22	–	33		
		22	–	21	11	11	22	–	33	0.0076	MZPE
299	2	22	–	–	–	11	–	22	–		
		22	–	–	–	11	–	22	–	0.5607 (2)[i]	?
		22	–	–	–	11	–	22	–	0.4199 (3)	?
		22	–	–	–	11	–	22	–	0.3144 (4)	?
		22	–	–	–	11	–	22	–	0.2354 (5)	?
		22	–	–	–	11	–	22	–	0.1763 (6)	?
812	3	22	–	21	11	11	22	22	33		
		–	–	–	11	11	22	22	33	0.1907	?
Malyi Kebezh (118)											
178	6	22	22	22	21	11	22	22	22		
		22	22	22	21	11	22	22	22	0.0506	MZPE
	8	22	22	22	11	11	22	22	22		
		22	22	22	11	11	22	22	22	0.0506	MZPE
Sobach'ya Rechka (97)											
159	1	**23**	32	12	11	11	22	22	33		
		22	32	12	11	11	22	22	33	1.000	PZPE
Listvyanka (377)											
90	1	32	22	22	11	11	22	**22**	**22**		
		32	22	22	11	11	22	**21**	**21**	1.000	PZPE
217	3	22	22	22	11	11	22	22	22		
		22	22	22	11	11	22	22	22	0.0276	MZPE
624	4	22	22	22	11	11	22	22	22		
		22	22	22	11	11	22	22	22	0.0276 (2)	MZPE
		22	22	22	11	11	22	22	22	0.0046 (3)	MZPE
808	7	22	22	22	31	11	22	22	**22**		
		22	22	22	31	11	22	22	**22**	0.0276 (2)	MZPE
		22	22	22	31	11	22	22	**22**	0.0046 (3)	MZPE
		22	22	22	31	11	22	22	**21**	1.000	PZPE
	10	–	22	–	31	11	22	22	13		
		–	22	–	31	11	–	22	–	0.5440	?

Table 1 (Continued)

Number of		Embryo genotype[a]								*P*[b]	Type of PE[c]
Trees	Seeds	*Adh-1*	*Dia-2*	*Fe-2*	*Lap-3*	*Mdh-2*	*Pgi-2*	*Pgm-1*	*Skdh-1*		
	11	–	22	–	31	11	22	22	**12**		
		–	22	–	31	11	–	22	**13**	1.000	PZPE
	14	–	–	–	–	–	–	22	–		
		–	–	–	–	–	–	22	–	0.9274 (2)	?
		–	–	–	–	–	–	22	–	0.8931 (3)	?
		–	–	–	–	–	–	22	–	0.8600 (4)	?
X2	17	–	22	22	33	11	22	–	22		
		22	22	22	33	11	22	–	22	0.0009	MZPE
Yailyu (204)											
13c	3	22	22	22	11	11	22	22	**21**		
		22	22	22	11	11	22	22	**22**	1.000	PZPE
	4	22	22	22	**11**	11	22	22	**13**		
		22	22	22	**13**	11	22	22	**11**	1.000	PZPE
14c	3	22	22	12	13	11	22	23	22		
		22	22	12	13	11	22	23	22	$4.8\cdot10^{-7}$	MZPE
15	4	22	22	22	11	11	22	12	33		
		22	22	22	11	11	–	12	–	0.2964	?
27	7	22	32	22	11	11	22	22	–		
		22	32	22	11	–	–	22	–	0.3112	?
46	1	23	22	**12**	11	11	22	**22**	23		
		23	22	**11**	11	11	22	**23**	–	1.000	PZPE
	3	22	22	11	11	11	22	33	33		
		22	22	11	11	11	22	33	33	$1.6\cdot10^{-6}$	MZPE
	4	22	22	11	11	11	22	22	**22**		
		22	22	11	11	11	22	22	**23**	1.000	PZPE
51c	1	22	22	22	11	11	22	22	32		
		22	22	22	11	11	22	22	32	0.0394	?
137	4	32	23	22	11	21	**21**	22	12		
		32	–	22	11	21	**22**	22	–	1.000	PZPE
	5	32	32	22	11	11	22	22	12		
		–	–	–	11	11	22	22	12	0.0944	?
	8	32	–	22	11	11	22	22	22		
		–	–	22	11	11	–	–	–	0.5376	?
138	6	22	22	22	11	11	22	22	23		
		22	–	22	11	–	22	22	–	0.2676	?
139	1	**22**	–	**21**	11	11	22	22	33		
		22	–	**22**	–	11	22	–	–	1.000	PZPE
		23	–	**22**	–	11	22	–	–	1.000	PZPE
		23	–	**22**	–	11	22	–	–	0.0201	MZPE
	6	**22**	22	12	**11**	11	22	22	**21**		
		22	22	12	**13**	11	22	22	**23**	1.000	PZPE
		23	–	12	**11**	11	–	22	–	1.000	PZPE
145	1	23	22	21	11	11	11	22	33		
		23	22	21	11	11	11	22	33	$2.2\cdot10^{-6}$	MZPE
	2	32	22	**11**	11	11	**22**	22	**11**		
		32	22	**12**	11	11	**21**	22	**13**	1.000	PZPE
147	1	22	32	21	11	11	22	22	32		
		22	32	21	11	11	22	22	32	0.0013	MZPE
	8	33	32	21	11	11	21	22	31		
		33	32	21	11	11	21	22	31	$4\cdot10^{-8}$	MZPE
156	2	32	22	12	11	11	22	22	**13**		
		–	–	–	11	11	–	22	**12**	1.000	PZPE
	3	22	22	11	31	11	–	22	32		
		22	22	11	31	11	–	22	–	0.0100	MZPE
190	2	22	22	**12**	11	11	22	22	33		
		22	22	**11**	11	11	22	22	33	1.000	PZPE
		22	22	**11**	11	11	22	22	33	0.0021	MZPE
	5	**22**	22	12	31	11	22	22	33		
		23	22	12	31	11	22	22	33	1.000	PZPE
		22	22	12	31	11	22	22	33	0.0615	?
Mm	4 (1)	–	22	22	–	11	22	22	–	0.3550 (1-2)[j]	
	4 (2)	–	22	22	11	11	22	22	23	0.0949 (2-3)	?
	4 (3)	33	22	22	11	11	22	22	23	0.4492 (1-3)	?
										0.1094 (all 3)	
	5	32	22	22	11	11	22	22	23		
		32	22	22	11	11	22	22	23	0.0615	?
	6	32	22	22	11	11	22	22	22		

Table 1 (Continued)

Number of		Embryo genotype[a]								P^b	Type of PE[c]
Trees	Seeds	*Adh-1*	*Dia-2*	*Fe-2*	*Lap-3*	*Mdh-2*	*Pgi-2*	*Pgm-1*	*Skdh-1*		
		32	22	22	11	11	–	22	22	0.0508	?
Ff	5	22	22	22	11	11	22	22	21		
		22	22	22	11	11	22	22	21	0.0011	MZPE
Smokotino (338)											
1	6	22	22	**12**	11	–	22	22	–		
		22	22	**11**	11	–	22	22	–	1.000	PZPE
11	7	**22**	22	22	11	11	21	22	32		
		23	22	22	11	11	21	22	32	1.000	PZPE
18	3	23	32	12	11	11	21	32	**23**		
		23	32	12	11	11	21	32	**22**	1.000	PZPE
	8	22	22	22	11	11	22	32	32		
		22	22	22	11	11	22	32	32	0.0139	MZPE
20	1	**23**	22	**21**	11	11	22	22	**31**		
		22	22	**22**	11	11	22	22	**33**	1.000	PZPE
40	7	22	22	22	11	11	–	22	23		
		22	22	22	11	11	–	22	23	0.0517	?
43	4	32	22	21	11	11	22	32	33		
		32	22	–	11	11	–	32	33	0.1092	?
	5	33	22	11	11	11	21	33	32		
		–	22	–	11	11	–	–	32	0.0922	?
46	2	33	22	22	11	11	22	22	33		
		33	22	22	11	11	22	22	33	0.0063	MZPE
	3	**32**	22	**22**	**13**	11	22	22	33		
		33	22	**21**	**11**	11	22	22	33	1.000	PZPE
48	2	22	22	12	11	11	–	22	33		
		–	22	12	11	11	–	22	–	0.2822	?
57	4	22	22	22	11	11	11	22	31		
		22	22	22	11	11	11	22	31	$3.9 \cdot 10^{-6}$	MZPE
	6	22	22	21	11	11	**11**	22	33		
		22	22	21	11	11	**12**	22	33	1.000	PZPE
65	7	22	22	22	–	11	–	–	33		
		22	22	22	–	11	21	–	33	0.0016	MZPE
68	1	23	22	22	33	11	22	22	33		
		23	22	22	33	11	22	22	33	0.0002	MZPE
72	2	32	22	22	**13**	11	**21**	22	**33**		
		32	22	22	**11**	11	**22**	22	**31**	1.000	PZPE
88	6	23	22	22	11	11	22	22	22		
		–	22	22	11	11	22	–	–	0.2494	?
89	4	22	32	12	11	11	22	22	33		
		22	–	12	–	11	22	22	–	0.1503	?
Zorkal'tsevo-1 (276)											
52	13	22	22	21	**13**	11	**21**	22	33		
		22	22	21	**11**	11	**22**	22	33	1.000	PZPE
55	3	22	23	22	11	11	21	22	23		
		–	–	22	11	11	–	–	–	0.2163	?
423	7	22	22	22	13	11	**22**	22	23		
		22	22	22	13	11	**21**	22	23	1.000	PZPE
Zorkal'tsevo-3 (262)											
13	8	23	22	22	11	11	22	22	22		
		23	22	22	11	11	22	22	22	0.0029	MZPE
99	3	22	22	21	11	**11**	22	22	**32**		
		22	22	21	11	**12**	22	22	**33**	1.000	PZPE
	8	22	22	21	**11**	11	22	22	22		
		22	22	21	**13**	11	22	22	–	1.000	PZPE

[a] *Adh-1*, alcohol dehydrogenase; *Dia-2*, diaphorase; *Fe-2*, fluorescent esterase; *Lap-3*, leucine aminopeptidase; *Mdh-2*, malate dehydrogenase; *Pgi-2*, phosphoglucose isomerase; *Pgm-1*, phosphoglucomutase; *Skdh-1*, shikimate dehydrogenase

[b] *P*-probability of egg cells in one ovule to have been fertilized by sperms of different pollen grains, i.e., probability of an additional embryo to have resulted from PZPE

[c] PZPE and MZPE, polyzygotic and monozygotic types of polyembryony (PE), respectively

[d] Total number of seeds studied

[e] Genotype of a larger dominant embryo is listed as the first for each seed; the following genotypes are those of additional smaller embryos

[f] Genotype was not defined because of small embryo size or weak enzyme activity

[g] Maternal allele (allele of macrogametophyte) is listed as the first allele in embryo genotypes; paternal allele (allele of sperm), as the second one

[h] Genotypes of embryos orginating from the same seed but differing in paternal alleles are marked by bold font

[i] Probability that two (2), three (3), four (4), five (5) or six (6) embryos have simultaneously resulted from PZPE

[j] Serial numbers of embryos used for pairwise evaluation of probability

[k] High level of probability of PZPE does not allow reliable discrimination of two different types of PE

haplotypes in the population pollen pool, this probability (P) turned out to be high enough to expect PE seeds with such PZPE embryos (N_e) in the sample of N PE seed studied (i.e. if $N_e = P \cdot N > 1$), these PE seeds were considered to be an indefinite case of PE and were excluded from both PZPE and MZPE estimation. If the probability of finding genetically identical embryos that resulted from PZPE was low (i.e. if $N_e \ll 1$), these PE seeds, having embryos of the same genotype, were considered to be the result of MZPE. For example, the second embryo in seed no. 6 of tree no. 51 from Mutnaya Rechka was genotyped for only three loci: *Adh-1*, *Fe-2*, and *Pgi-2* (Table 1). The paternal haplotype of this embryo was $Adh\text{-}1^2/Fe\text{-}2^2/Pgi\text{-}2^2$, or simply *222*. Frequency of allele $Adh\text{-}1^2$ in the outcrossing pollen pool of this population equaled 0.80, the frequency of allele $Fe\text{-}2^2 = 0.76$ and that of allele $Pgi\text{-}2^2 = 0.93$. Thus, haplotype *222* theoretically can be found in this population at the frequency of $0.80 \cdot 0.76 \cdot 0.93 = 0.5654$. Probability of fertilization of one ovule by two different pollen grains of the same haplotype *222* was $P = 0.5654^2 = 0.3197$. The estimated probability was too high to consider this seed to be MZPE, since even among the 8 PE seeds studied in this population more than 2 PZPE seeds with such a haplotype can be found by chance (i.e. $N_E = P \cdot N = 0.3197 \cdot 8 = 2.6 > 1$). Another example is the second embryo in seed no. 10 of tree no. 299 from the same population. Its paternal haplotype *211123* has been defined for 6 loci, *Adh-1*, *Fe-2*, *Lap-3*, *Mdh-2*, *Pgi-2*, and *Skdh-1*. The expected frequency of this multilocus haplotype was estimated as 0.0873 by multiplication of frequencies of the corresponding alleles of these loci in the outcrossing pollen pool of the Mutnaya Rechka population. The probability of two different pollen grains of this haplotype fertilizing one ovule was very low and equaled $0.0873^2 = 0.0076$. We could not expect to find by chance such a case of PZPE among the 8 seeds studied because the expected number of such seeds was only $0.0076 \cdot 8 = 0.06 \ll 1$. Hence, this case of PE can be reliably considered to be MZPE. Similar analysis of all additional isogenic embryos in PE seeds from the nine populations identified 25 seeds (44.6% of all classified PE seeds) that could be considered to have resulted from MZPE (Table 2).

We also observed three cases (5.4%) of composite or "mixed" PE, when 1 (seed no. 7 of tree no. 808 from Listvyanka) or 2 (seed no. 1 of tree no. 139 and seed no. 2 of tree no. 190 from Yailyu) additional embryos resulted from PZPE and the other one(s) of the same seed from MZPE. For example, 1 out of 4 embryos of seed no. 7 of tree no. 808, differing in the paternal allele of locus *Skdh-1*, resulted from PZPE, but the other 3 were isogenic and could be considered to have resulted from MZPE because the probability of finding PZPE embryos isogenic by chance equaled only 0.0276 for 2 isogenic embryos and 0.0046 for 3 isogenic embryos. In this case, the expected number of such seeds in our sample of 7 seeds was only $7 \cdot 0.0276 = 0.19$ ($m = 2$) or even $7 \cdot 0.0046 = 0.03$ ($m = 3$) with, respectively, 2 or 3 isogenic embryos having resulted from PZPE.

Foothill populations of the Western Sayan and plain populations of Tomsk Region (except Smokotino) do not differ substantially in PE, nor in PZPE and MZPE frequency (Table 2). However, the Yailyu subalpine population (~ 1600 m above the see level) had a substantially high percentage (13.24%) of PE seeds. All populations, including Yailyu, with more than 2 classified PE seeds had approximately equal numbers of PZPE and MZPE types.

Discussion

PE frequency

The PE frequency of 0.85–1.86% (Table 2) that we found in foothill populations of the Western Sayan (Filin Klyuch, Mutnaya Rechka, Malyi Kebezh, Sobach'ya Rechka, and Listvyanka) was in good agreement with the data obtained by Iroshnikov (1974) for the same populations (0.2–3.0%). The PE frequency that we observed in Yailyu population (13.24%) confirmed the higher frequency of PE in Siberian stone pine subalpine populations observed by Zemlyanoi (1973) and Iroshnikov (1974).

The anomalously high level of PE in tree no. 808 found by Iroshnikov (1974) was also observed in our study. The peculiarity is, obviously, characteristic of this

Table 2 Number and percentage of polyembryonal seeds and polyembryony types in Siberian stone pine populations (*PZPE* polyzygotic polyembryony, *MZPE* monozygotic polyembryony, *ES* East Siberia, *WS* West Siberia)

Population (region)	All seeds studied	Polyembryonal seeds	PZPE	MZPE	Mixed type
Filin Klyuch (ES)	1287	11 (0.85%)	5 (45.5%)	6 (54.5%)	–
Mutnaya Rechka (ES)	885	8 (0.90%)	1 (50%)	1 (50%)	–
Malyi Kebezh (ES)	118	2 (1.70%)	–	2 (100%)	–
Sobach'ya Rechka (ES)	97	1 (1.03%)	1 (100%)	–	–
Listvyanka (ES)	377	7 (1.86%)	2 (33.3%)	3 (50%)	1 (16.7%)
Yailyu (ES)	204	27 (13.24%)	8 (47.1%)	7 (41.1%)	2 (11.8%)
Smokotino (WS)	338	18 (5.33%)	7 (58.3%)	5 (41.7%)	–
Zorkal'tsevo-1 (WS)	276	3 (1.09%)	2 (100%)	–	–
Zorkal'tsevo-3 (WS)	262	3 (1.15%)	2 (66.7%)	1 (33.3%)	–
Total	3845	81 (2.11%)	28 (50%)	25 (44.6%)	3 (5.4%)

tree and was stable for several years of observation: 40.5% PE seeds were observed in 1966; 44.2%, in 1967; 38.4%, in 1969; and 41.6%, in 1970 (Iroshnikov 1974). We have obtained a similar value of 37.5% for the crop of 1987. These data suggest that PE can be under strong genetic control. All types of PE seeds (PZPE, MZPE and "mixed") have been found in this tree.

However, initial PE frequencies are much higher, and early embryonal selection and elimination usually lead to the survival of only 1 embryo in mature seeds. Sarvas (1962) observed a large loss of second and third embryos during embryogenesis in *Pinus sylvestris.*

PE types

From our study we are able to conclude that both PZPE and MZPE types of PE occur at nearly equal frequencies (50% and 45%, respectively; Table 2) among Siberian stone pine PE seeds. The existence of both types of PE may increase this species adaptation potential allowing embryonal selection without a significant decrease of seed productivity.

Such data are of interest in connection with a supposedly important role of PE in the evolution and adaptation of conifers (Sorensen 1982). By providing the opportunity for early embryo selection, PZPE promotes the survival of viable seeds, probably with the most adapted embryo, and thus maintains the seed productivity of a tree and decreases the "cost" of selection. If one takes into account the relatively high frequency of self-pollination among conifers, including Siberian stone pine (Politov and Krutovskii 1990, 1993), the successful replacement of a dead inbred embryo by an outcrossed one owing to PZPE allows the most optimal, at the moment, level of polymorphism in a population without a considerable reduction of seed productivity. Both PZPE and MZPE can also reduce detrimental environmental effects, because an additional functional embryo could replace the degenerating one. The ratio between the two types of PE can be regulated by genetic-environment interactions.

There are polycross tests that indicate that the paternity ratio of embryos is not the same as the male parent ratio in the pollen mix (e.g. Moran and Griffin 1985; Cheliak et al. 1987). A number of factors could be involved, and selection among PZPE embryos is one of them.

It is noteworthy, that 2 out of 10 PE seeds with more than 2 embryos had 3 embryos of three different paternal haplotypes (see no. 12 of tree no. 213 from Mutnaya Rechka, and seed no. 1 of tree no. 139 from Yailyu; Table 1). These data seem to be the first direct genetic confirmation of cytological observations of fertilization of one ovule by more than two different pollen grains. Moreover, we have obtained genetic evidence of PE seeds being able to have a composite, "mixed" origin – 3 seeds were found that contained embryos that resulted from both types of PE.

Acknowledgement We are very thankful to Frank Sorensen for his invaluable comments on the manuscript that helped us improve our paper.

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