GENETIC MAPPING OF ALLOZYME LOCI IN FOUR TWO-NEEDLE PINE SPECIES OF EUROPE

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ABSTRACT

In the analysis of 24 allozyme loci in four European two-needle pine species 14 genes in 4 linkage groups in *Pinus sylvestris* L., 14 genes in 4 groups in *P. mugo* Turra , 12 genes in 3 groups in *P. nigra* Arn. and 4 genes in one linkage group in *P. brutia* Ten. were mapped. The order and the locations of homologous genes in the linkage groups in the four species studied are similar. The data obtained suggest that in the course of separate development of the species of the *Pinus* genus that has lasted for millions of years, there was not any large inversion, translocation, or other significant chromosomal change, at least in the gene blocks analyzed. Potentialities of further mapping of allozyme loci in pines are discussed.

Key words: isozymes, linkage, *Pinus sylvestris*, *Pinus mugo*, *Pinus nigra*, *Pinus brutia*.

INTRODUCTION

Regardless of great economic importance of pines their genomes have scarcely been investigated for a long period of time in contrast to genomes of other commercial plants. The main reason is that conifers have a prolonged life cycle and there were no accessible gene markers. The situation has changed with the advent of molecular-genetic methods, including isosyme electrophoresis which permitted to initiate research on genetic mapping of pines (RUDIN & EKBERG 1978, GURIES *et al.* 1978, O'MALLEY *et al.* 1979, ADAMS & JOLY 1980, CONKLE 1981, ECKERT *et al.* 1981, MORAN *et al.* 1983, STRAUSS & CONKLE 1986, O'MALLEY *et al.* 1986, FURNIER *et al.* 1986, NIEBLING *et al.* 1987, SHIRAISHI 1988, SZMIDT & MUONA 1989, GEBUREK *et al.* 1990, MORGANTE *et al.* 1993, GONCHARENKO *et al.* 1994a). It should be noted that American pines appeared to be better investigated as far as linkages among allozyme genes have been analysed and reported for ten of them. At the same time allozyme genes have been mapped in only three pine species occurring in the European continent, *Pinus sylvestris* L., *P. leucodermis* Ant., *P. pallasiana* Asch. (RUDIN & EKBERG 1978, NIEBLING *et al.* 1987, SZMIDT & MUONA 1989, MORGANTE *et al.* 1993, GONCHARENKO *et al.* 1994a).

The purpose of our study was to construct genetic maps for four European two-needle pines, *Pinus sylvestris, P. mugo* Turra, *P. nigra* Arn.. and *P. brutia* Ten., using allozyme genes.

MATERIALS AND METHODS

This study is based on seeds from 920 trees in twenty-eight *P. sylvestris* natural populations, 118 trees in five *P. mugo* populations, 215 trees in nine *P. nigra* populations and 176 trees in seven *P. brutia* populations occurring in Latvia, Poland, Belarus, the Ukraine and Russia.

The enzymes were electrophoresed on 13–14% starch gel. For electrophoresis three buffer systems were used: (a) tris-EDTA-borate, pH 8.6, (b) tris-citrate, pH 6.2, (c) tris-citrate, pH 6.2 (electrode buffer) / tris-HCl, pH 8.0 (gel buffer) (GONCHARENKO *et al.* 1992). Recipes for enzyme extraction and histochemical enzyme staining followed the standard methods (CONKLE *et al.* 1982, CHELIAK & PITEL 1984, GONCHARENKO *et al.* 1989) with insignificant modifications. Seed material from each tree was analyzed using 14 gene-enzyme systems. The data on genetic control of allelic variants of 23 loci in the pine species assayed were presented in previous studies (GONCHARENKO *et al.* 1994b, 1995, 1998; SILIN &

Table 1 Enzymes, their abbrevations , enzyme commission numbers, number of loci scored and buffer systems used for electrophoresis

Enzyme	Abbreviation	E.C. No.	Buffer	Loci scored
Aspartate aminotransferase	AAT	2.6.1.1	A, C	3
Alcohol dehydrogenase	ADH	1.1.1.1	A	2
Glucose phosphate isomerase	GPI	5.3.1.9	B	1
Diaphorase	DIA	1.6.4.3	C	2
Leucine aminopeptidase	LAP	3.4.11.1	A, C	2
Fluorescent esterase	FL-EST	3.1.1.2	A, C	1
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	B	2
Glutamate dehydrogenase	GDH	1.4.1.2	A	1
Malate dehydrogenase	MDH	1.1.1.37	B	4
Phosphoglucomutase	PGM	2.7.5.1	A	2
Acid phosphatase	ACP	3.1.3.2	B	1
Isocitrate dehydrogenase	IDH	1.1.1.42	C	1
Aconitase	ACO	4.2.1.3	B	1
Peptidase	PEP	3.4.13.1	A	1

GONCHARENKO 1996). The results of the genetic analysis of the *Pep–3* locus presented here for the fist time also support genetic control of the electromorphs revealed. Individual trees were genotyped using 8 to 10 megagametophytes. The enzymes assayed, their abbreviations, the buffer systems used and the number of loci consistently scorable are given in Table 1.

Genetic mapping was based on the study of haploid megagametophytes that are immediate product of meiosis in conifers, which with the availability of trees heterozygous for two and more loci permits to analyze linkages without making special crossings. Segregation of two-locus combinations divided megagametophytes into two classes, "normal" and "recombinant". Since it was not known beforehand which was "normal", the least in number was taken as "recombinant" (RUDIN & EKBERG 1978). The frequency of recombination (R) between loci was calculated by the formula: $R = r/N$, where r is the number of recombinants and N is the total number of the megagametophytes analyzed. The χ^2-test was used to detect deviation of gamete classes from 1:1 segregation ratio. If, for a pair of loci there were trees with more than 40% of recombinants, these loci were not regarded as linked. In the analysis of several trees that exhibited reliable linkages for a particular pair of loci pooled recombination frequencies were calculated. The genetic distances between loci were calculated in centiMorgans (cM) by the formula Kosambi $D = 25 \ln [(1 + 2R) / (1–2R)]$, where R is the recombination frequency (KOSAMBI 1944). The linear order of genes was determined from analysis of trees heterozygous for three and more loci. The number of megagametophytes used to study linkages in each pair of loci ranged from 10 to 728 and averaged about 80 per pair of loci analyzed.

RESULTS AND DISCUSSION

Linkage analysis of allozyme loci in the four pine species

Pinus sylvestris

A total of 24 loci in the *P. sylvestris* natural populations studied were polymorphic. Of the 276 possible two-locus combination which can be formed from 24 polymorphic loci, 232 pairs of allozyme loci were compared in at least one tree (Table 2 in Appendix). In the investigated material linkage was recognised in only 18 pairwise combinations among 14 loci. Data from individual trees are presented for these linked loci in Table 3. From this table it is obvious that fourteen of twenty-four polymorphic loci of Scots pine fell into four linkage groups.

The first linkage group included eight loci, *Aat–1, Gpi, Dia–2, Pep–3, Adh–1, Adh–2, Lap–2,* and *Aat–2.* Close linkage was observed between the *Adh–1* and *Adh–2* loci. Of the 728 megagametophytes analyzed only 8 appeared to be recombinants, *i.e.* the genetic distans between them was 1.1 cM after Kosambi (Table 3). A close linkage relationship between two alcohol dehydrogenase loci was reported for *P. sylvestris, P. contorta* Dougl. ex Loud., and *P. jeffreyi* Grev. et Balf. (RUDIN & EKBERG 1978, CONKLE 1981, SZMIDT & MUONA 1989).

Two trees were heterozygous for *Aat–1* and *Gpi* (Table 3). Interestingly, that one of them is a 200-year-old tree growing in the national park "Belovezh-

Table 3. Pairs of loci for which significant levels of linkage were detected in *Pinus sylvestris*

Combination Locus A : locus B / Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (*R*)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Aat–1:Gpi									
BP–126	25	1	2	18	0.783	1.391	34.783***	0.065	6.6
BP–45/5	0	35	31	0	0.242	0.242	66.000***	0	0
						Pooled	100.321***	0.027	2.7
Aat–1:Adh–1									
PD–38	4	21	24	3	0.077	0.308	27.769***	0.135	13.8
Aat–1:Adh–2									
BP–126	3	23	16	4	0.783	1.391	22.261***	0.152	15.7
PD–38	21	4	3	.24	0.077	0.308	27.769***	0.135	13.8
						Pooled	50.000***	0.143	14.7
Gpi:Dia–2									
TB–69	1	23	21	1	0.087	0.087	38.348***	0.043	4.4
Gpi:Adh–1									
NB–1	3	8	7	2	0.200	0	5.000*	0.250	27.5
KUR–101	8	22	13	3	4.261*	0.348	12.252***	0.239	26.0
TB–69	19	5	4	18	0.087	0	17.044***	0.196	20.7
						Pooled	34.321***	0.223	24.0
Gpi:Adh–2									
NB–18	11	2	1	10	0.167	0	13.500***	0.125	12.8
KUR–101	22	8	3	13	4.261*	0.348	12.252***	0.239	26.0
BP–126	2	25	17	2	1.391	1.391	31.391***	0.087	8.8
						Pooled	55.172***	0.155	16.0
Dia–2:Adh1									
TB–69	3	19	20	4	0.087	0	22.261***	0.152	15.7
TB–45	19	2	2	24	0.532	0.532	32.262***	0.085	8.6
TB–23	5	8	11	2	0	1.385	5.538*	0.269	30.1
						Pooled	57.891***	0.151	15.6
Dia–2:Adh–2									
TB–45	2	19	24	2	0.532	0.532	32.362***	0.085	8.6
TB–22	3	17	26	2	1.333	2.083	30.083***	0.104	10.6
						Pooled	62.411***	0.095	9.6
Pep–3:Adh–1									
MG–20	9	0	0	11	0.200	0.200	20.000***	0	0
US–24	1	22	25	0	0.083	0.333	44.083***	0.021	2.1
						Pooled	64.059***	0.015	1.5
Pep–3:Lap–2									
US–24	6	17	19	6	0.083	0.083	12.000***	0.250	27.5
Adh–1:Adh–2									
KUR–101	0	21	25	0	0.348	0.348	46.000***	0	0.
BP–294/19	0	12	10	2	0	0.667	16.667***	0.083	8.4
CK–321/4	0	19	21	0	0.100	0.100	40.000***	0	0
B–28	3	83	87	1	0.023	0.207	158.368***	0.023	2.3
B–15	47	1	1	41	0.400	0.400	82.178***	0.022	2.2
S–6	0	19	17	0	0.111	0.111	36.000***	0	0

Table 3 (continued)

Combination Locus A : locus B Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (*R*)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Adh–1:Adh–2									
R-9	0	33	27	0	0.600	0.600	60.000***	0	0
R-4	12	0	0	16	0.571	0.571	28.000***	0	0
K-9	0	29	26	0	0.164	0.164	55.000***	0	0
TB-45	0	21	26	0	0.532	0.532	47.000***	0	0
BP-111	21	0	0	15	1.000	1.000	36.000***	0	0
PD-38	0	28	24	0	0.308	0.308	52.000***	0	0
TK-28	0	21	19	0	0.100	0.100	40.000***	0	0
						Pooled	696.352***	0.011	1.1
Adh–1:Lap–2									
CK–312/4	15	4	2	19	0.100	0.900	19.600***	0.150	15.5
KUR-101	6	15	20	5	0.348	0.783	12.522***	0.239	26.0
CK-312/12	3	8	8	2	0.047	0.047	5.762*	0.238	25.9
I-6	7	17	19	5	0	0.333	12.000***	0.250	27.5
BP-111	16	5	5	10	1.000	1.000	7.111**	0.278	31.3
TB-45	7	14	17	9	0.532	0.081	4.787*	0.340	41.5
US-24	20	6	5	17	0.333	0.083	14.083***	0.229	24.8
						Pooled	72.504***	0.248	27.2
Adh–2:Lap–2									
CK-312/4	2	19	15	4	0.100	0.900	19.600***	0.150	15.5
KUR-101	20	5	6	15	0.348	0.783	12.522***	0.239	26.0
I-4	7	15	13	5	0.400	0	6.400*	0.300	34.7
TB-45	17	9	7	14	0.532	0.021	4.787*	0.340	41.5
BP-111	16	5	5	10	1.000	1.000	7.111**	0.278	31.3
						Pooled	46.895***	0.263	29.3
Adh-1:Aat–2									
KUR–101	4	12	7	6	0.310	1.690	2.793	0.345	42.4
TK-28	7	14	10	9	0.100	0.533	1.600	0.400	54.9
PD-53	3	9	7	1	0.800	0	7.200***	0.200	21.2
TK-119	4	7	8	4	0.043	0.043	2.130	0.348	42.9
BP-111	8	13	11	4	1.000	0.111	4.000*	0.333	40.2
TB-23	4	12	8	2	1.335	0.154	7.538**	0.231	25.0
						Pooled	22.092***	0.322	38.2
Lap-2:Aat–2									
KUR-101	10	7	1	11	0.862	1.690	5.828*	0.276	31.0
NB–6	25	1	0	22	0.333	0.833	44.083***	0.021	2.1
PD-28	0	13	17	2	1.125	0.125	24.500***	0.063	6.3
I-8	20	5	7	16	0.083	0.750	12.000***	0.250	27.5
BP-111	4	17	15	0	1.000	0.111	21.778***	0.111	11.3
						Pooled	100.109***	0.140	14.4
Lap-1:Fl-Est									
TB-7	12	5	4	10	0.290	0.032	5.452*	0.290	33.2
NM-4a	3	13	9	3	0.571	0.571	9.143**	0.214	22.9
NB–6	15	5	8	20	1.333	0.083	10.083**	0.271	30.3
CK-348/4	4	16	19	1	0	0.900	22.500***	0.125	12.8
MK-30	3	7	7	5	0.182	0.182	1.636	0.364	46.1
BP-45/5	6	27	24	7	0.063	0.250	22.563***	0.203	21.6
BP-45/29	5	21	16	3	1.089	0.200	18.689***	0.178	18.6
						Pooled	85.309***	0.223	24.0

Table 3 (continued)

Combination Locus A : locus B / Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (*R*)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Idh:Gdh									
TB-23	0	31	27	0	0.276	0.276	58.000***	0	0
TB-13	0	18	18	0	0	0	36.000***	0	0
						Pooled	94.000***	0	0
Pgm-2:Mdh-3									
KUR-1	0	14	10	0	0.667	0.667	24.000***	0	0
TB-23	27	0	1	30	0.276	0.069	54.069***	0.017	1.7
TB-20	0	14	18	0	0.500	0.500	32.000***	0	0
BP-45/5	0	30	29	1	0	0.067	56.067***	0.017	1.7
						Pooled	166.092***	0.011	1.1

*** Significant linkage: p<0.05 (*); p<0.01 (**); p<0.001 (***)

skaya Pushcha" (Belarus) was also heterozygous for *Adh–2*. These enabled us to establish the order *Aat–1, Gpi* and *Adh–2* on the genetic map constructed for *P. sylvestris* (Figure 1). Linkage between the loci presumably corresponding to our *Aat–1* and *Gpi* has been reported for *P. rigida* Mill., *P. taeda* L., *P. contorta, P. jeffreyi, P. strobus* L. and *P. radiata* D.Don. and *P. attenuata* Lemm. (GURIES *et al.* 1978, ADAMS & JOLY 1980, CONKLE 1981, ECKERT *et al.* 1981, MORAN *et al.* 1983, O'MALLEY *et al.* 1986, STRAUSS & CONKLE 1986).

Of more than 900 individual trees of *P. sylvestris* assayed, only one (TB-69) appeared to be heterozygous for the *Gpi* and *Dia–2* loci, and the genetic distance between them was 4.4 cM (Table 3). The genetic distance between these loci in the four pine species ranges from 14.1 to 23.9 cM (CONKLE 1981, STRAUSS & CONKLE 1986, NIEBLING *et al.* 1987). We used the symbol of *Dia–2* to indicate the slow zone which appeared on diaphorase gels. Two other zones between *Dia–1* and *Dia–2* stained inconsistently and were not scored in the current work. Analysis of 46 megagametophytes in a tree (TB–69) heterozygous for *Gpi, Dia-2* and *Adh–1* allowed us to establish the order of the three loci (Figure 1). According to our results *Adh–2* is located closer to the loci *Gpi* and *Dia–2*, than *Adh–1* (Table 3).

The close linkage of the loci *Pep–3* and *Adh–1* with a distance of 1.5 cM was revealed by using data from two diheterozygous trees (Table 3), one of which was also heterozygous for *Lap–2*. This allowed to locate Pep–3 to the left of the *Adh–1* locus. Taking into account that in our study *Dia–2* is located farther from *Adh–1* (15.6 cM) than *Pep–3* (1.5 cM) we have localized *Pep–3* in the first linkage group between *Dia–2* and *Adh–1* (Figure 1). It is necessary to note that the localization of the gene coding one of peptidases in the first linkage group was described only by American researchers (NIEBLING *et al.* 1987).

We found independent segregation between the loci *Lap–2, Aat–2* and the loci *Aat–1, Gpi, Dia–2* (Table 2 in Appendix). *Lap–2* (RUDIN & EKBERG 1978, ADAMS & JOLY 1980, CONKLE 1981, ECKERT *et al.* 1981, STRAUSS & CONKLE 1986, NIEBLING *et al.* 1987, SZMIDT & MUONA 1989) and *Aat–2* (RUDIN & EKBERG 1978, CONKLE 1981, NIEBLING *et al.* 1987, SZMIDT & MUONA 1989, GEBUREK *et al.* 1990) together with *Aat–1, Gpi, Dia–2, Pep–3, Adh–1*, and *Adh–2* have been located in one linkage group in some pine species, *Lap–2* and *Aat–2* being located to the right of the alcohol dehydrogenase loci (Figure 1).

The order of the 8 loci of the first linkage group in *P. sylvestris* established in this study is similar to that reported by other researchers (RUDIN & EKBERG 1978, NIEBLING *et al.* 1987, SZMIDT & MUONA 1989). However, it should be noted that these earlier studies did not present simultaneous analysis of *Gpi* and *Aat–1*. For example, SZMIDT & MUONA (1989), and NIEBLING *et al.* (1987) used only *Pgi–B* (that presumably corresponds to our *Gpi*), and RUDIN & EKBERG (1978) used only *Got–A* (presumably corresponding to *Aat–1* in the current study). Also in contrast to our conclusions, NIEBLING *et al.* (1987) mapped *Dia–C* (presumed to be the same as our *Dia–2*) to the left of *Pgi–B*.

The second linkage group we considered consists of *Lap–1* and *Fl–Est* (Figure 1). The distance between these loci was 24.0 cM (Table 3), somewhat

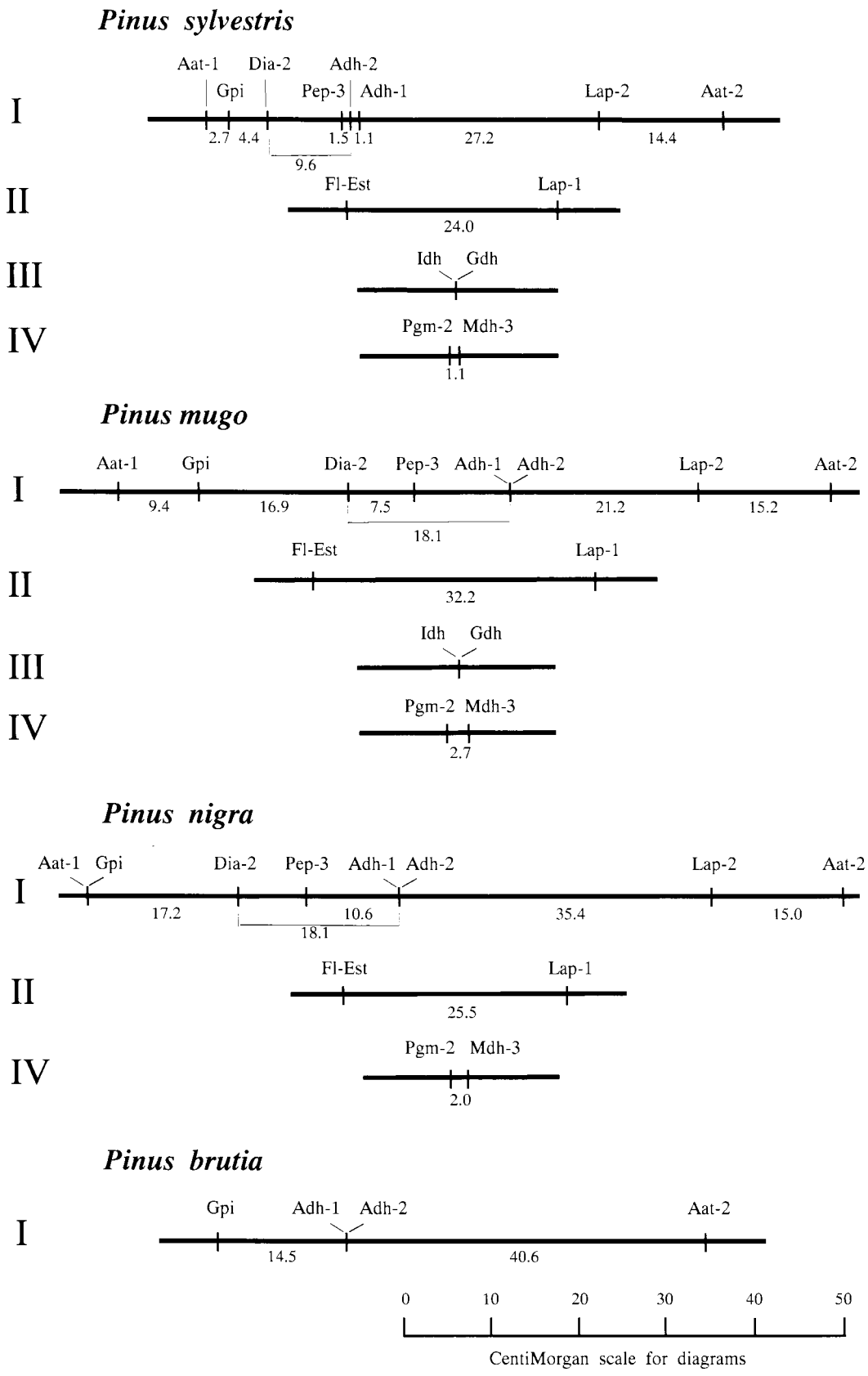

Figure 1. Genetic maps of four Eastern European pine species constructed on the basis of linkage of allozyme genes. Distance in centiMorgans (cM).

Table 5. Pairs of loci for which significant levels of linkage were detected in *Pinus mugo*

Combination Locus A : locus B / Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (R)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Aat-1:Gpi									
U-28	16	2	1	14	0.273	0.030	22.091***	0.091	9.2
U-1	4	1	0	5	0	0.400	6.400*	0.100	10.1
						Pooled	28.488***	0.093	9.4
Gpi:Dia-2									
P-1	0	4	6	1	0.818	0.091	7.364**	0.091	9.2
R-16	4	14	12	2	0.500	0	12.500***	0.188	19.7
						Pooled	19.558***	0.163	16.9
Gpi:Adh-1									
U-9	4	11	9	2	0.615	0	7.538**	0.231	25.0
U-21	7	4	4	10	0.360	0.360	3.240	0.320	37.9
						Pooled	10.373**	0.275	30.8
Gpi:Adh-2									
U-28	12	5	4	12	0.030	0.030	6.818**	0.273	30.6
U-13	5	10	12	7	0.471	0	2.941	0.353	43.9
						Pooled	9.328**	0.313	36.8
Dia-2:Pep-3									
DV-19/3	2	12	13	0	0.037	0.333	19.593***	0.074	7.5
Dia-2:Adh-1									
D-1/2	2	18	18	6	0.364	0.364	17.818***	0.182	19.1
U-15	11	1	4	15	1.581	0.032	14.226***	0.161	16.7
						Pooled	32.013***	0.173	18.1
Dia-2:Adh-2									
D-1/2	2	18	18	6	0.364	0.364	17.818***	0.182	19.1
U-15	11	1	4	15	1.581	0.032	14.226***	0.161	16.7
						Pooled	32.013***	0.173	18.1
Adh-1:Adh-2									
U-3	5	0	0	7	0.333	0.333	12.000***	0	0
U-15	18	0	0	20	0.105	0.105	38.000***	0	0
D-1/2	0	20	24	0	0.364	0.364	44.000***	0	0
D-1/12	0	15	19	0	0.471	0.471	34.000***	0	0
						Pooled	128.000***	0	0
Adh-1:Lap-2									
U-15	14	3	5	15	0.243	0.027	11.919***	0.216	23.1
U-21	2	8	11	2	0.391	0.391	9.783***	0.174	18.1
						Pooled	21.600***	0.200	21.2
Adh-2:Lap-2									
U-15	14	3	5	15	0.243	0.027	11.919***	0.216	23.1
U-28	9	7	6	11	0.030	0.273	1.485	0.394	53.3
						Pooled	11.200***	0.300	34.7
Lap-2:Aat2									
U-15	18	3	3	19	0.023	0.023	22.349***	0.140	14.3
P-27	1	11	10	3	0.040	0.360	11.560***	0.160	16.6
						Pooled	33.882***	0.147	15.2

Table 5 (continued)

Combination Locus A : locus B / Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (*R*)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Lap-1:Fl-Est									
D-1/6	4	10	16	2	0.500	2.000	12.500***	0.188	19.7
U-28	4	7	11	5	0.926	0.333	3.000	0.333	40.2
U-21	7	3	6	9	1.000	0.040	1.960	0.360	45.4
U-15	12	5	6	14	0.243	0.027	6.081*	0.297	34.2
DV-19/3	12	3	4	8	0.333	0.926	6.259*	0.259	28.7
						Pooled	27.676***	0.284	32.2
Idh:Gdh									
D-2/13	0	6	4	0	0.400	0.400	10.000***	0	0
Pgm-2:Mdh-3									
D-1/2	9	0	0	11	0.200	0.200	20.000***	0	0
P-15	7	0	1	9	0.529	0.059	13.235***	0.059	5.9
						Pooled	33.108***	0.027	2.7

*** significant linkage $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)

less than in SZMIDT & MUONA's (1989) study on Scandinavian populations of *P. sylvestris*. Linkage between *Lap–1* and *Fl–Est* has been reported for 4 other pine species, the genetic distances between them being 25.6 cM in *P. jeffreyi*, 35.6 cM in *P. contorta*, 45.6 cM in *P. taeda* (CONKLE 1981), and 35.0 cM in *P. attenuata* (STRAUSS & CONKLE 1986).

The third linkage group is represented by a block of two tightly linked loci, *Idh* and *Gdh* (Table 3). These loci have been reported to be tightly linked in two North American pine species, the map distance between them in *P. contorta* being 2.7 cM and no recombinants were found in *P. taeda* (CONKLE 1981).

The fourth linkage group was also characterized by two tightly linked loci, *Pgm–2* and *Mdh–3* (Figure 1). Of the 174 megagametophytes analyzed only 2 appeared to be recombinants (Table 3). This has also been reported for *P. rigida* in which the frequency of recombination was 0.013 (O'MALLEY *et al.* 1986).

Pinus mugo

In natural populations of *P. mugo* 23 loci were found to be polymorphic. There was a total of 253 possible locus pairs with 23 polymorphic loci. Of these combinations, 169 pairs of allozyme loci were compared in at least one tree (Table 4 in Appendix). We revealed reliable linkages at 14 pairs of loci. Table 5 gives detailed results of linkage analysis of these pairs of loci. As the result of the study on *P. mugo* we have mapped 14 loci that fell into four linkage groups (Fig. 1).

The linear order of loci in the linkage groups in *P. mugo* as a whole is identical to that in *P. sylvestris*. The first linkage group was characterized by a block of eight loci, *Aat–1, Gpi, Dia–2, Pep–3, Adh–1, Adh–2, Lap–2* and *Aat–2*. Contrary to Scots pine, no recombinants were found between *Adh–1* and *Adh–2* in mountain pine (Table 5). Therefore relative positions of these loci are arbitrary. We had only one tree diheterozygous for *Pep–3* and one locus of the first linkage group. This individual (DV-19/3) exhibited reliable linkage between *Pep–3* and *Dia–2* at a distance of 7.5 cM (Table 5). By analogy with *P. sylvestris*, the *Pep–3* locus was located between *Dia–2* and *Adh–1* on the map constructed for *P. mugo*. It should be noted that the genetic maps for the first, second and fourth linkage groups in *P. mugo* appeared to be somewhat longer than those constructed for *P. sylvestris* (Figure 1).

Pinus nigra

The results of our study on black pine showed that two loci, *Idh* and *Acp*, were monomorphic and some loci were slightly polymorphic. Therefore of the 231 possible two-locus combinations which can be formed from 22 polymorphic genes in *P. nigra* we analyzed 125 ones (Table 6 in Appendix). Reliable linkages were established at 14 pairs of loci. Table 7 lists the results of linkage analysis in some detail. On the basis of the findings of the study of *P. nigra*, we revealed three linkage groups that included 12 loci (Figure 1).

The first linkage group consisted of 8 loci. The order of loci in this linkage group in *P. nigra* was identical to that in *P. sylvestris* and *P. mugo*. But no

Table 7. Pairs of loci for which significant levels of linkage were detected in *Pinus nigra*

Combination Locus A : locus B Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (R)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Aat-1:Gpi									
DM-1/3	9	0	0	11	0.200	0.200	20.000***	0	0
Aat-1:Adh-1									
DM-1/3	10	3	3	12	0.143	0.143	9.143***	0.214	22.9
SD-6	10	4	2	8	0.667	0	6.000*	0.250	27.5
						Pooled	15.077***	0.231	25.0
Gpi:Dia-2									
DM-1/5	1	14	16	3	0.471	0	19.882***	0.118	12.0
AO-9	12	4	4	20	1.600	1.600	14.400***	0.200	21.2
AL-1/a-6	12	2	3	15	0.500	0.125	15.125***	0.156	16.2
AL-1/b-9n	13	3	2	9	0.926	0.333	10.704***	0.185	19.4
						Pooled	59.556***	0.165	17.2
Gpi:Pep-3									
NK-1/3	8	37	18	4	7.786**	3.358	27.597***	0.179	18.7
Gpi:Adh-1									
NK-1/3	35	10	5	17	7.896**	2.522	20.433***	0.224	24.1
NK-2/7	3	5	5	2	0.067	0.067	1.667	0.333	40.2
DM-1/3	7	2	2	9	0.200	0.200	7.200**	0.200	21.2
DM-1/24	3	15	19	5	0.857	0.095	16.095***	0.190	20.1
DM-1/21	5	3	3	9	0.800	0.800	3.200	0.300	34.7
IG-3/7	9	13	11	6	0.641	0.026	2.077	0.385	50.9
AL-1/a-6	10	4	6	12	0.500	0	4.500*	0.313	36.7
						Pooled	50.557***	0.268	29.9
Gpi:Adh-2									
DM-1/45n	8	3	1	8	0.200	0.200	7.200**	0.200	21.2
IG-3/7	9	13	11	6	0.641	0.026	2.077	0.385	50.9
SD-16m	13	4	3	10	0.533	0.133	8.533**	0.233	25.3
AL -1/b-6	6	2	0	4	1.333	0	5.333*	0.167	17.3
						Pooled	20.050***	0.277	31.2
Dia-2:Adh-1									
NK-1/2	11	3	2	11	0.037	0.037	10.704**	0.185	19.4
NK-2/23	3	9	12	4	0.571	0.143	7.000**	0.250	27.5
AL-1/a-6	12	3	4	13	0.125	0	10.125**	0.219	23.5
AL-1/a-3	5	25	20	2	1.231	0.077	27.769***	0.135	13.8
DM-1/36a	11	3	4	14	0.500	0.125	10.125**	0.219	23.5
DM-1/19n	22	3	1	16	1.524	0.381	27.524***	0.095	9.6
						Pooled	90.709**	0.174	18.1
Dia-2:Adh-2									
AL-1/a-3	5	25	20	2	1.231	0.077	27.769***	0.135	13.8
NK-2/23	3	9	12	4	0.571	0.143	7.000**	0.250	27.5
						Pooled	33.800***	0.175	18.3
Pep-3:Adh-1									
NK-1/3	3	23	37	4	3.358	2.522	41.925***	0.104	10.6

Table 7 (continued)

Combination Locus A : locus B / Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (*R*)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Adh-1:Adh-2									
AL-1/a-3	25	0	0	27	0.077	0.077	52.000***	0	0
IG-3/6m	0	35	41	0	0.474	0.474	76.000***	0	0
IG-3/7	20	0	0	19	0.026	0.026	39.000***	0	0
IG-3/8n	43	0	0	35	0.821	0.821	78.000***	0	0
NK-2/23	15	0	0	13	0.143	0.143	28.000***	0	0
						Pooled	273.000***	0	0
Adh-1:Lap-2									
AL-1/a-6	13	3	5	11	0	0.500	8.000**	0.250	27.5
DM-1/24	6	16	16	4	0.095	0.095	11.524***	0.238	25.9
NK-1/3	14	26	16	11	2.522	0.731	4.313*	0.373	48.2
						Pooled	21.454***	0.305	35.4
Lap-2:Aat-2									
DM-1/24	3	16	15	2	0.111	0	18.778***	0.139	14.3
AL-1/b-8	1	4	6	1	0.333	0.333	5.333*	0.167	17.3
						Pooled	24.083***	0.146	15.0
Lap-1:Fl-Est									
AL-1/a-5	5	15	11	3	1.059	0.118	9.529**	0.235	25.5
Pgm-2:Mdh-3									
IG-3/7	22	0	0	17	1.059	1.059	39.000***	0	0
IG-1/10	0	11	15	1	0.926	0.333	23.148***	0.037	3.7
AL-1/b-6	7	1	0	4	1.333	0.333	8.333**	0.083	8.4
AO-9	0	9	11	0	0.200	0.200	20.000***	0	0
						Pooled	90.163***	0.020	2.0

*** significant linkage $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)

recombinants were found between the *Aat–1* and *Gpi* loci (Table 7). Therefore on the map they were jointly located. We revealed no recombinants between the loci encoding *Adh–1* and *Adh–2* either (Table 7). In order to map *Pep–3* in *P. nigra* we had a single tree (NK-1/3) that appeared to be heterozygous for three loci, *Gpi, Pep–3* and *Adh–1*. The results of the linkage analysis enabled us to map *Pep–3* between *Gpi* and *Adh–1* at a distance of 10.6 cM from *Adh–1*.

The second and fourth linkage groups in *P. nigra* were also identical to those in *P. sylvestris* and *P. mugo* (Figure 1). Unfortunately, owing to the fact that all the *P. nigra* tree individuals appeared to be monomorphic for *Idh* we did not succeed in mapping the *Gdh* and *Idh* loci that, by analogy with the above two species, could have comprised the third linkage group.

Pinus brutia

The results of our investigation of *P. brutia* showed that half of the 24 loci were monomorphic. Trees were available to test for linkage among 49 of the 66 pairwise possible combinations for 12 polymorphic genes in the *P. brutia* populations studied (Table 8 in Appendix). Among them reliable linkages were revealed at five pairs of loci (Table 9). This permitted to map four loci, *Gpi, Adh–1, Adh–2* and *Aat–2*, that were linked to each other in the first linkage group, as with the other species studied by us (Figure 1).

Potentialities of further mapping of allozyme loci in pines

In their earlier studies on pines, a number of research ers have reported linkages among some other loci

Table 9. Pairs of loci for which significant levels of linkage were detected in *Pinus brutia*.

Combination Locus A : locus B / Tree	Observed numbers by allelic combinations				χ^2 (1 d.f.)			Recombination fraction (*R*)	Kosambi distance (cM)
	A_1B_1	A_1B_2	A_2B_1	A_2B_2	locus A	locus B	linkage		
Gpi:Adh–1									
KK–36	24	3	1	14	3.429	1.524	27.524***	0.095	9.6
KK–4	9	3	4	10	0.154	0	5.538*	0.269	30.1
KK–21c	17	4	2	13	1.000	0.111	16.000***	0.167	17.3
KK–7	2	10	15	3	1.200	0.533	13.333***	0.167	17.3
KK–38	12	1	4	11	1.429	0.571	11.571***	0.179	18.7
						Pooled	72.000***	0.167	17.3
Gpi:Adh–2									
KK–36	3	24	14	1	3.429	1.524	27.524***	0.095	9.6
KK–21c	4	17	13	2	1.000	0.111	16.000***	0.167	17.3
MN–1a	1	7	10	3	1.190	0.048	8.048**	0.190	20.1
						Pooled	50.919***	0.141	14.5
Adh–1:Adh–2									
KK–23	0	10	18	0	2.286	2.286	28.000***	0	0
KK–36	0	25	17	0	1.524	1.524	42.000***	0	0
KK–21c	0	19	17	0	0.111	0.111	36.000***	0	0
DJ–1	0	5	7	0	0.333	0.333	12.000***	0	0
DJ–12c	17	0	0	10	1.815	1.815	27.000***	0	0
DJ–16b	0	14	22	0	1.778	1.778	36.000***	0	0
						Pooled	181.000***	0	0
Adh–1:Aat–2									
KA–18a	7	12	15	10	0.818	0	2.273	0.386	51.4
KK–23	4	6	11	7	2.286	0.143	1.286	0.393	53.0
KK–36	9	16	10	7	1.524	0.381	2.381	0.381	50.0
KK–21c	13	6	4	13	0.111	0.111	7.111**	0.278	31.3
KK–38	6	10	8	4	0.574	0	2.286	0.357	44.8
						Pooled	14.045***	0.360	45.3
Adh–2:Aat–2									
KA–22	6	12	16	2	0	1.778	11.111***	0.222	23.9
KK–23	11	7	4	6	2.286	0.143	1.286	0.393	53.0
KK–36	10	7	9	16	1.524	0.381	2.381	0.381	50.0
KK–21c	4	13	13	6	0.111	0.111	7.111***	0.278	31.3
KK–26a	16	11	8	14	0.510	0.020	2.469	0.388	51.7
						Pooled	20.780***	0.335	40.6

*** significant linkage $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)

used in the current study. For instance, NIEBLING *et al.* (1987) succeeded in mapping two genes in *P. sylvestris* encoding malate dehydrogenases, *Mdh–A* and *Mdh–C* (presumably corresponding to our *Mdh–1* and *Mdh–3*). *Mdh–C* was linked to *Lap–A* (presumed to be homologous to our *Lap–1*), with a distance of 51.5 cM (NIEBLING *et al.* 1987). As indicated above, in our study *Lap–1* was linked to *Fl–Est* in the second group and *Mdh–3* was linked to *Pgm–2* in the fourth group (Figure 1). Analysis of several trees heterozygous for *Mdh–3, Lap–1* and *Fl–Est* revealed no linkage between *Mdh–3* and *Lap–1* or between *Mdh–3* and *Fl–Est* (68.2 cM and 74.6 cM, respectively). It should be noted that NIEBLING *et al.* (1987) mapped *Mdh–A* together with *6–Pgd–A* and *Got–C* (presumed to be the same as our *6–Pgd–1* and *Aat–3)* into one linkage group. We were not able to map the above three genes because we revealed reliable linkage for none pair of loci regardless of our having analyzed material from six *P. sylvestris* trees heterozygous for

Aat–3 and *6–Pgd–1* (67.9 cM), from three individuals heterozygous for *Mdh–1* and *6–Pgd–1* (60.7 cM) and from two trees heterozygous for *Aat–3* and *Mdh–1* (90.3 cM).

The tight linkage relationships between *6–Pgd–B* and *Pgi–B* have been reported for *P. leucodermis* by MORGANTE *et al.* (1993). On the basis of the results of the special–purpose study of the four pine species, we revealed linkage between the above loci in none of the species (64.8 to 81.5 cM).

In his study on *P. jeffrey* CONKLE (1981) has reported linkage between Idh and Aco with a distance of 2.8 cM. We had trees heterozygous for *Idh* and *Aco* in none of the species surveyed. But we could carry out linkage analysis of *Aco* and *Gdh* which was tightly linked to Idh. Data from *P. sylvestris, P. mugo* and *P. nigra* trees heterozygous for *Aco* and *Gdh* did not provide evidence for linkage between these loci (72.3 to 101.1 cM).

Thus, of the 24 allozyme loci we could map exactly only 14 ones. However, the data obtained make it possible to perform a comparative analysis of genetic maps constructed for the pines studied. As is seen from Figure 1, the order and the locations of homologous genes in the linkage groups in the four species investigated are similar. The data obtained suggest that in the course of separate evolution of the species of the *Pinus* genus that has lasted for several millions of years (BOBROV 1978, KOZUBOV & MURATOVA 1986) there was not any large inversion, translocation, or other significant chromosomal change, at least in the gene blocks analyzed.

The similarities in the linear orders of genes in the linkage groups in the pine species surveyed support once again the data of cytological and genetic studies (PEDERICK 1970, SAYLOR 1972, CONKLE 1981) showing that the genetic arrangement is highly conservative in the pines studied.

On the whole, taking into account localization of genes encoding isozymes, to date genetic maps covering 200 to 300 cM have been constructed for some species of pines (CONKLE 1981; NIEBLING *et al.*, 1987). In map units it is about 10% of the genome of pines if to assume the size of 2500 cM proposed by NEALE & WILLIAMS (1991) as a basis. It should be noted that to date only a part of the allozyme loci used has been mapped. In this respect the potentialities of genes encoding isozymes have not been exhausted as yet. Localization of allozyme genes together with DNA-markers also holds much promise. In their study DEVEY and co-workers constructed a genetic map for loblolly pine that covered more than 600 cM using a variety of RFLP-markers and several allozyme loci (DEVEY *et al.* 1994). Currently RAPD-markers have found wide use in construction of genetic maps. This has enabled researchers to extend significantly genetic maps for pines (NELSON *et al.* 1994, PLOMION *et al.* 1995, YAZDANI *et al.* 1995, KUBISIAK *et al.* 1996).

Regardless of widespread use of direct DNA-markers (RFLP and RAPD) further mapping of allozyme genes that represent the structural and functional part of genomes in pines remains an important task because there is scarcely any information about other structural genes in conifers.

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APPENDIX

Table 2. Two-locus combinations and number of trees employed for the linkage analysis (upper half) and the results of statistical testing (lower half) in *Pinus sylvestris*

Locus	*Aat -1*	*Aat -2*	*Aat -3*	*Adh -1*	*Adh -2*	*Gdh*	*Gpi*	*Dia -1*	*Dia -2*	*Lap -1*	*Lap -2*	*Mdh -1*	*Mdh -2*	*Mdh-3*	*Mdh -4*	*Fl-Est*	*Pgm -1*	*Pgm -2*	*Pgd -1*	*Pgd -2*	*Idh*	*Acp*	*Aco*	*Pep -3*
Aat-1		2	3	1	2	3	2	4	–	3	2	1	–	2	4	3	2	2	2	4	–	2	–	–
Aat-2	n		8	6	6	5	4	5	4	4	5	2	2	3	5	5	3	2	5	4	2	1	1	1
Aat-3	n	n		7	5	7	6	4	4	4	4	2	1	7	7	5	2	3	6	4	1	2	2	2
Adh-1	***	***	n		13	4	3	6	3	3	7	2	1	5	4	6	2	2	3	2	1	2	2	2
Adh-2	***	n	n	***		6	3	5	2	3	5	2	1	4	4	3	2	2	5	5	–	2	3	–
Gdh	n	n	n	n	n		2	4	3	2	1	2	1	4	6	4	3	2	4	5	2	4	2	–
Gpi	***	n	n	***	***	n		3	1	4	3	1	–	4	3	4	2	1	3	3	–	1	–	–
Dia-1	n	n	n	n	n	n	n		4	5	5	1	1	5	3	6	2	2	6	4	1	2	–	1
Dia-2	–	n	n	***	***	n	***	n		–	1	–	–	2	3	4	2	1	3	2	1	2	2	–
Lap-1	n	n	n	n	n	n	n	n	–		6	2	–	5	4	7	2	1	5	4	1	2	–	–
Lap-2	n	***	n	***	***	n	n	n	n	n		1	–	6	2	5	1	1	3	5	–	2	–	1
Mdh-1	n	n	n	n	n	n	n	n	–	n	n		–	1	2	3	1	–	3	3	–	1	–	–
Mdh-2	–	n	n	n	n	n	–	n	–	–	–	–		2	1	1	1	–	1	1	–	–	–	–
Mdh-3	n	n	n	n	n	n	n	n	n	n	n	n	n		7	6	2	4	3	3	2	1	–	1
Mdh-4	n	n	n	n	n	n	n	n	n	n	n	n	n	n		5	4	4	4	5	2	4	2	–
Fl-Est	n	n	n	n	n	n	n	n	n	***	n	n	n	n	n		3	2	6	4	2	3	1	–
Pgm-1	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n		1	2	2	–	2	1	–
Pgm-2	n	n	n	n	n	n	n	n	n	n	n	–	–	***	n	n	n		2	1	1	1	–	–
Pgd-1	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n		5	–	4	2	1
Pgd-2	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n		1	3	2	1
Idh	–	n	n	n	–	***	–	n	n	n	–	–	–	n	n	n	–	n	–	n		–	–	–
Acp	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	n	n	n	n	–		2	1
Aco	–	–	n	n	n	n	–	–	n	–	–	–	–	–	n	n	n	–	n	n	–	n		1
Pep-3	–	n	n	***	–	–	–	n	–	–	***	–	–	n	–	–	–	–	n	n	–	n	n	

– two-locus combinations not tested
n not significant
*** significnat linkage at $p < 0.05$ (*), $p < 0.01$ (**); $p < 0.001$ (***)

Table 4. Two-locus combinations and number of trees employed for the linkage analysis (upper half) and the results of statistical testing (lower half) in *Pinus mugo*

Locus	*Aat -1*	*Aat -2*	*Aat -3*	*Adh -1*	*Adh -2*	*Gdh*	*Gpi*	*Dia -1*	*Dia -2*	*Lap-1*	*Lap-2*	*Mdh-2*	*Mdh -3*	*Mdh -4*	*Fl-Est*	*Pgm -1*	*Pgm -2*	*Pgd -1*	*Pgd -2*	*Idh*	*Acp*	*Aco*	*Pep -3*
Aat-1		–	–	–	1	–	2	1	–	1	1	–	–	–	1	–	–	–	–	–	–	–	–
Aat-2	–		2	2	3	4	3	4	4	6	2	4	1	2	1	–	1	4	1	–	–	1	–
Aat-3	–	n		3	2	1	1	4	1	4	2	3	1	1	3	–	–	2	2	–	2	2	1
Adh-1	–	n	n		4	1	2	3	2	3	2	2	2	2	3	–	1	2	2	–	2	–	–
Adh-2	n	n	n	***		–	2	3	2	4	2	3	3	4	4	1	1	2	3	–	3	–	–
Gdh	–	n	n	n	–		2	1	2	3	–	2	1	1	–	–	1	3	1	1	–	1	–
Gpi	***	n	n	**	**	n		2	2	3	2	1	–	–	2	–	–	1	1	–	–	–	–
Dia-1	–	n	n	n	n	n	n		2	4	3	3	1	1	3	–	–	2	2	–	2	2	1
Dia-2	–	n	n	***	***	n	***	n		3	2	4	2	2	2	–	2	4	3	–	2	1	1
Lap-1	n	n	n	n	n	n	n	n	n		4	4	2	3	5	1	1	3	2	–	2	2	2
Lap-2	n	***	n	***	***	–	n	n	n	n		–	–	–	3	–	–	1	–	–	–	–	–
Mdh-2	–	n	n	n	n	n	n	n	n	n	–		2	3	2	–	1	3	3	–	3	2	2
Mdh-3	–	n	n	n	n	n	–	n	n	n	–	n		4	2	1	2	2	3	–	3	–	–
Mdh-4	–	n	n	n	n	n	–	n	n	n	–	n	n		2	1	2	2	3	–	3	–	–
Fl-Est	n	n	n	n	n	–	n	n	n	***	n	n	n	n		1	–	1	3	–	3	1	1
Pgm-1	–	–	–	–	n	–	–	–	–	n	–	–	n	n	n		–	–	1	–	1	–	–
Pgm-2	–	n	–	n	n	n	–	–	n	n	–	n	***	n	–	–		2	1	–	1	–	–
Pgd-1	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n		2	–	1	1	–
Pgd-2	–	n	n	n	n	n	n	n	n	n	–	n	n	n	n	n	n	n		–	3	1	1
Idh	–	–	–	–	–	***	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–
Acp	–	–	n	n	n	–	–	n	n	n	–	n	n	n	n	n	n	n	n	–		1	1
Aco	–	n	n	–	–	n	–	n	–	n	–	n	–	–	–	–	–	n	–	–	–		1
Pep-3	–	–	n	–	–	–	–	n	***	n	–	n	–	–	n	–	–	–	n	–	n	n	

– two-locus combinations not tested
n not significant
*** significnat linkage at $p < 0.05$ (*), $p < 0.01$ (**); $p < 0.001$ (***)

Table 6. Two-locus combinations and number of trees employed for the linkage analysis (upper half) and the results of statistical testing (lower half) in *Pinus nigra*

Locus	*Aat -1*	*Aat -2*	*Aat -3*	*Adh -1*	*Adh -2*	*Gdh*	*Gpi*	*Dia -1*	*Dia -2*	*Lap -1*	*Lap -2*	*Mdh -1*	*Mdh -2*	*Mdh -3*	*Mdh -4*	*Fl-Est*	*Pgm -1*	*Pgm -2*	*Pgd -1*	*Pgd -2*	*Aco*	*Pep -3*
Aat-1		–	1	2	–	–	1	–	–	–	–	–	–	1	–	–	–	1	1	–	–	–
Aat-2	–		2	7	3	3	4	2	4	1	2	–	1	4	2	1	–	3	2	2	1	–
Aat-3	n	n		3	1	–	2	2	1	1	–	–	–	2	–	1	–	2	1	1	–	–
Adh-1	***	n	n		5	2	7	5	6	1	3	1	1	6	1	2	1	4	2	2	1	1
Adh-2	–	n	n	***		–	4	2	2	–	–	–	1	4	–	1	–	2	1	2	–	–
Gdh	–	n	–	n	–		3	1	2	–	–	–	–	2	–	–	1	2	2	1	2	–
Gpi	***	n	n	***	***	n		2	4	–	5	1	–	3	–	1	2	3	1	2	1	1
Dia-1	–	n	n	n	n	n	n		6	–	1	–	1	2	–	–	–	2	2	1	1	–
Dia-2	–	n	n	***	***	n	***	n		–	–	–	1	2	1	–	1	1	–	2	2	–
Lap-1	–	n	n	n	–	–	–	–	–		–	–	–	1	–	1	–	–	–	–	–	–
Lap-2	–	***	–	***	–	–	n	n	–	–		1	–	2	–	1	–	3	–	–	–	1
Mdh-1	–	–	–	n	–	–	n	–	–	–	n		–	–	–	1	–	1	–	–	–	1
Mdh-2	–	n	–	n	n	–	–	n	n	–	–	–		1	–	–	–	–	–	–	–	–
Mdh-3	n	n	n	n	n	n	n	n	n	n	n	–	n		–	2	2	4	2	2	1	–
Mdh-4	–	n	–	n	–	–	–	–	n	–	–	–	–	–		–	–	–	–	–	–	–
Fl-Est	–	n	n	n	n	–	n	–	–	**	n	n	–	n	–		–	2	1	–	–	1
Pgm-1	–	–	–	n	–	n	n	–	n	–	–	–	–	n	–	–		2	–	1	1	–
Pgm-2	n	n	n	n	n	n	n	n	n	–	n	n	–	***	–	n	n		2	2	1	1
Pgd-1	n	n	n	n	n	n	n	n	–	–	–	–	–	n	–	n	–	n		1	–	–
Pgd-2	–	n	n	n	n	n	n	n	n	–	–	–	–	n	–	–	n	n	n		–	–
Aco	–	n	–	n	–	n	n	n	n	–	–	–	–	n	–	–	n	n	–	–		–
Pep-3	–	–	–	***	–	–	***	–	–	–	n	n	–	–	–	n	–	n	–	–	–	

– two-locus combinations not tested
n not significant
*** significnat linkage at $p < 0.05$ (*), $p < 0.01$ (**); $p < 0.001$ (***)

Table 8. Two-locus combinations and number of trees employed for the linkage analysis (upper half) and the results of statistical testing (lower half) in *Pinus brutia*

Locus	*Aat–2*	*Adh–1*	*Adh–2*	*Gdh*	*Gpi*	*Lap–1*	*Mdh–1*	*Mdh–3*	*Pgd–1*	*Pgd–2*	*Acp*	*Aco*
Aat–2		5	5	2	5	–	3	2	1	3	2	1
Adh–1	***		6	1	5	–	3	3	1	5	2	1
Adh–2	***	***		1	3	–	4	2	2	3	–	1
Gdh	n	n	n		1	2	2	3	–	2	–	–
Gpi	n	***	***	n		–	3	3	1	3	1	1
Lap–2	–	–	–	n	–		–	2	–	1	–	–
Mdh–1	n	n	n	n	n	–		3	1	4	1	1
Mdh–3	n	n	n	n	n	n	n		–	2	–	1
Pgd–1	n	n	n	–	n	–	n	–		1	–	–
Pgd–2	n	n	n	n	n	n	n	n	n		1	1
Acp	n	n	–	–	n	–	n	–	–	n		–
Aco	n	n	n	–	n	–	n	n	–	n	–	

– two-locus combinations not tested
n not significant
*** significnat linkage at $p < 0.05$ (*), $p < 0.01$ (**); $p < 0.001$ (***)