Linkage analysis of isozyme gene loci in *Picea abies* (L.) Karst.

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In total 14 isozyme gene loci were tested for segregation in *Picea abies*. Single tree segregation was not significantly heterogeneous. Pooled over all trees, segregation at the *Idh-1* and *Pgi-2* gene loci deviated significantly from the Mendelian expectation. Out of 91 two-locus combinations 57 could be analysed for linkage. The recombination frequencies were estimated employing the maximum likelihood and the Bayesian likelihood methods. Estimations of the recombination rates confirmed the close linkage group of *Got-1*:*Pgi-2* in Pinaceae. Also *Dia-2* appears to be linked to *Pgi-2* and *Got-1*. Moderate linkage groups of *Got-2*:*Skdh-1* and *Lap-2*:*Pgi-2* were found but not in all of the investigated trees.

INTRODUCTION

Segregation distortion and more effective linkage as cytophysiological devices can contribute to storage and protection of genetic variation. Knowledge about these components of the genetic system are important prerequisites for population studies. Segregation distortion for instance can thwart evolutionary processes and can bias the estimation of certain genetic parameters. Without linkage the formation of supergenes as adapted gene complexes is impossible. Linkage influences gametic phase disequilibrium and can play an important role when marker genes are used to predict genetic gains (Frei *et al.*, 1986).

In conifers several linkage studies have been performed and some more or less closely linked loci have been established (Guries *et al.*, 1978; Rudin and Ekberg, 1978; Adams and Joly, 1980; Conkle, 1981; Eckert *et al.*, 1981; Cheliak *et al.*, 1984; Cheliak and Pitel, 1985; Furnier *et al.*, 1986; O'Malley *et al.*, 1986; Strauss and Conkle, 1986; Muona *et al.*, 1987; Shiraishi, 1988; Müller-Starck and Liu, in press; Geburek and Wang, submitted).

MATERIALS AND METHODS

Out of 120 genotyped mature trees of three different Norway spruce stands open-pollinated

seeds of 18 multiple heterozygous trees were selected for this investigation (crop 1983/84). The forest stands are located in Schleswig-Holstein, Germany. For some trees open-pollinated seedlots of crop 1984/85 were additionally available and could be employed. The isozymes assayed, their acronyms, scored gene loci and reference of the respective genetic control are listed in table 1.

The macrogametophyte tissue was homogenized in 0.005 M tris/0.04 M HCl buffer, pH 7.5 without any addition of enzyme-protecting agents. The raw extracts were separated by means of horizontal starch gel electrophoresis. Details of the electrophoretic conditions, gel and electrode buffers as well as staining recipes for the isozymes are given in Geburek *et al.* (1987). PGI was separated and stained according to Conkle *et al.* (1982).

For the linkage analysis 57 out of 91 possible two-locus combinations could be investigated. Table 2 shows the combinations of loci tested and the number of trees studied. On the average about 280 gametes were analysed per combination.

The statistical procedures used to test for linkage and to estimate the recombination frequency are described below: a doubly heterozygous tree produces four kinds of gametes A_1B_1 , A_1B_2 , A_2B_1 and A_2B_2 . Parental and recombinant types of gametes are associated with the numbers of gametes in coupling (those in categories A_1B_1 and A_2B_2) and repulsion (those in A_1B_2 and A_2B_1).

Table 1 Isozymes, acronyms, E. C. Number, number of loci scored, and references of the respective genetic control

Isozymes	Acronyms	EC No.	No. of gene loci scored	Reference(s) of the genetic control
Acid phosphatase	APH	3.1.3.2	1	Tigerstedt (1973), Bergmann (1974), Lundkvist (1975)
Diaphorase	DIA	1.6.4.3	1	Muona <i>et al.</i> (1987)
Glutamate dehydrogenase	GDH	1.4.1.2	1	Lundkvist (1979), Cheliak <i>et al.</i> (1987)
Glutamate oxaloacetic transaminase	GOT	2.6.1.1	2	Lundkvist (1979), Poulsen <i>et al.</i> (1983)
Isocitrate dehydrogenase	IDH	1.1.1.42	1	Muona <i>et al.</i> (1987)
Leucine aminopeptidase	LAP	3.4.11.1	2	Bergmann (1973), Lundkvist (1974)
Malate dehydrogenase	MDH	1.1.1.37	1	Lundkvist (1979), Muona <i>et al.</i> (1987).
Phosphoglucosmutase	PGM	2.7.5.1	1	Poulsen <i>et al.</i> (1983).
6-Phosphogluconate dehydrogenase	6-PGDH	1.1.44	2	Poulsen <i>et al.</i> (1983)
Phosphoglucose isomerase	PGI	5.3.1.9	1	Poulsen <i>et al.</i> (1983).
Shikimate dehydrogenase	SKDH	1.1.1.25	1	Muona <i>et al.</i> (1987)

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

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. <i>Aph-2</i>		—	3/-	—	4/1	—	1/-	—	1/-	—	—	2/1	—	2/1
2. <i>Dia-2</i>	—		—	1/1	—	1/-	—	—	—	—	—	1/1	1/1	1/2
3. <i>Gdh-1</i>	ns	—	—	—	4/-	1/1	1/-	1/-	—	—	3/-	3/-	4/-	—
4. <i>Got-1</i>	—	*	—	—	—	1/-	—	-/1	-/1	—	—	1/1	1/1	1/1
5. <i>Got-2</i>	ns	—	ns	—	—	2/-	1/-	2/-	4/-	2/-	5/-	8/1	5/-	3/2
6. <i>Idh-1</i>	—	ns	ns	ns	ns	—	—	1/-	—	—	—	—	3/-	1/-
7. <i>Lap-1</i>	ns	—	ns	—	ns	—	—	—	—	1/-	1/-	—	1/-	—
8. <i>Lap-2</i>	—	—	ns	ns	ns	ns	—	—	2/1	—	2/-	2/-	3/2	—
9. <i>Mdh-3</i>	ns	—	—	ns	ns	—	—	ns	—	1/-	4/-	5/-	2/-	1/-
10. <i>Pgm-1</i>	—	—	—	—	ns	—	ns	—	ns	—	2/-	1/-	1/-	—
11. <i>6-Pgdh-2</i>	—	—	ns	—	ns	—	ns	ns	ns	ns	—	5/-	5/-	—
12. <i>6-Pgdh-3</i>	ns	ns	ns	ns	ns	ns	—	ns	ns	ns	ns	—	5/1	3/1
13. <i>Pgi-2</i>	—	*	ns	*	ns	ns	ns	*	ns	ns	ns	ns	—	1/1
14. <i>Skdh-1</i>	ns	ns	—	ns	*	ns	—	—	ns	—	—	*	ns	—

Number before the slash = number of analysed single-tree seedlots, crop 1983/84.

Number after the slash = number of analysed single-tree seedlots, crop 1984/85.

— = Two-locus combination not tested.

ns = not significant.

* = significant linkage at least at 5 per cent level in at least one seed sample

However, because the breeding history of a certain tree is unknown it is not clear whether the parental category is associated with the coupling or the repulsion phase. This complicates the estimation of the recombination fraction and is disregarded in a maximum likelihood estimation of the recombination frequencies (θ), if θ is estimated as quotient of the number of the putative recombinants related to the number of all analysed gametes.

The estimation of this θ is especially biased when a small sample size is analysed and/or weak linkage is present at the respective loci combination.

$$\text{bias} = E\hat{\theta} - \theta = f(\theta, n).$$

This was the reason why in this investigation a likelihood procedure was used, which considers the fact that the putative recombinants are not necessarily the *de facto* recombinants.

When n_i is the number of analysed gametes of the i th tree and k_i is the number of the smaller class in repulsion or coupling phase the likelihood is denoted by

$$L(\theta | n_i, k_i, i = 1, \dots, N)$$

which is expressed for a single tree as

$$L(\theta_{1i} | n_i, k_i) = \binom{n_i}{k_i} (\theta_{1i}^{k_i} (1 - \theta_{1i})^{(n_i - k_i)} + \theta_{1i}^{(n_i - k_i)} (1 - \theta_{1i})^{k_i}) \quad \text{for } k_i = n_i/2.$$

The maximum likelihood estimator is that value of θ_{1i} which maximizes $L_i(\theta_{1i}|n_i, k_i)$ and was designated $\hat{\theta}_1$. The maximum likelihood estimator was found by grid search. This was done by increasing θ_i in 5×10^{-4} steps starting from zero and computing the respective likelihoods.

The likelihood of θ pooled over N trees is

$$L(\theta_1) = \prod_{i=1}^N L(\theta_{1i}|n_i, k_i).$$

The 95 per cent maximum likelihood confidence interval (CI) was determined as

$$CI(\theta_1|95\%) =$$

$$\{\theta_1 | -2 \log L(\theta_1) < -2 \log L(\hat{\theta}_1) + x_{1,0.5}^2\}$$

and the upper and lower borders were found by grid search.

Nordheim *et al.* (1983) showed that a Bayesian approach, using a prior distribution that places equal probabilities on all values of θ is superior to the maximum likelihood procedure when weak linkage is observed. Therefore an additional estimator was calculated (Jeffreys, 1961).

$$\hat{\theta}_2 = \int_0^{0.5} \theta L(\theta|n, k) d\theta / \int_0^{0.5} L(\theta|n, k) d\theta.$$

The integrals were calculated by approximating the likelihood function by a step function.

Contingency-tables were employed for the linkage analysis and to test if the segregation at the different gene loci followed the Mendelian 1:1 expectation. Unbalanced segregation of one pair of alleles does not affect linkage analyses (Mather,

1951). Estimation of homogeneity of $\hat{\theta}$ over all trees was performed following Rao (1973, p. 389):

$$x_{n-1}^2 = \sum_{i=1}^n \frac{N_i(\theta_i - \hat{\theta})^2}{\hat{\theta} - \hat{\theta}^2}$$

n = number of trees, N_i = number of analysed gametes for the i th tree, θ_i = estimated recombination frequency for the i th tree, $\hat{\theta}$ = overall estimated recombination frequency.

The map distance in centimorgans (cM) was calculated after Kosambi (1944) employing $\hat{\theta}_2$. Before estimating this distance pooled over several trees, each single tree map distance was weighted with G according to Fisher (1937):

$$G_i = (N_i(1 - 4\hat{\theta}_i^2)^2) / (\hat{\theta}_i(1 - \hat{\theta}_i)).$$

RESULTS

Segregation

In total 14 isozyme gene loci were tested for segregation (table 3). There was no significant heterogeneity among samples for the same loci. Only at *Idh-1* and *Pgi-2* segregation distortion was significant ($P < 0.05$) resulting in allelic proportions of 324:272 and 567:642 respectively.

Linkage

Table 4 shows that the maximum likelihood and Bayes estimates of θ were very similar. Compared to the maximum likelihood estimation the Bayes estimates result in lower values when the recombi-

Table 3 Segregation at single isozyme gene loci in *Picea abies*

Isozyme gene locus	No. of trees	Alleles	Numbers	Heterogeneity χ^2 (df)	Deviation χ^2 (1 df)
<i>Aph-2</i>	6	2-3	300:263	5.66 5 ns	2.43 ns
<i>Gdh-1</i>	5	1-2	474:482	5.89 4 ns	0.07 ns
<i>Got-1</i>	3	1-2	152:128	3.22 2 ns	2.06 ns
<i>Got-2</i>	14	1-2	824:820	11.34 13 ns	0.01 ns
<i>Dia-2</i>	2	1-2	85:87	0.22 1 ns	0.02 ns
<i>Idh-1</i>	4	1-2	324:272	5.44 3 ns	4.54 *
<i>Lap-1</i>	2	1-2	73:80	0.85 1 ns	0.32 ns
<i>Lap-2</i>	7	3-4	375:351	2.52 6 ns	0.80 ns
<i>Mdh-3</i>	6	3-4	388:393	5.19 5 ns	0.03 ns
<i>Pgm-1</i>	2	1-2	78:79	0.07 1 ns	0.01 ns
<i>6-Pgdh-2</i>	7	1-2	473:499	2.47 6 ns	0.70 ns
<i>6-Pgdh-3</i>	13	1-2	941:937	18.45 12 ns	0.01 ns
<i>Pgi-2</i>	11	1-2	567:642	13.26 10 ns	4.65 *
<i>Skdh-1</i>	7	1-2	429:391	9.15 6 ns	1.76 ns

ns = Not significant.

* = $P < 0.05$.

Table 4 Estimates of the recombination frequencies (θ) and measures for significance

Tree	Crop	Observed no.				Chi-square (1 df)			Maximum likelihood estimated θ_1	Bayes estimates θ_2	Maximum likelihoods confidence (CI 95 per cent)	Kosambi map distance (cM)
		A ₁ B ₁	A ₁ B ₂	A ₂ B ₁	A ₂ B ₂	Segregation						
		Locus 1	Locus 2	Linkage								
<i>Dia-2: Got-1</i>												
G8	83/84	6	22	23	9	0.27	0.07	15.13**	0.250	0.258	0.152–0.369	28.5
	84/85	17	20	27	10	0.00	2.65	5.41*	0.365	0.368	0.261–0.486	47.1
Heterogeneity ¹ (1 df): $\chi^2 =$						2.04 ns	1.86 ns	0.313	0.316	0.238–0.395	33.8	
<i>Dia-2: Pgi-2</i>												
G8	83/84	33	5	8	32	0.05	0.21	34.80***	0.167	0.175	0.095–0.259	18.3
	84/85	17	11	13	23	1.00	0.25	3.75	0.375	0.378	0.263–0.500	49.3
Heterogeneity ¹ (1 df): $\chi^2 =$						7.89**	7.46**	(0.261	0.264	0.193–0.337	21.6)	
<i>Got-1: Pgi-2</i>												
G8	83/84	7	37	32	7	0.30	0.30	36.05***	0.169	0.176	0.098–0.259	19.0
	84/85	8	68	56	12	0.44	1.78	73.84***	0.139	0.144	0.089–0.202	14.8
Heterogeneity ¹ (1 df): $\chi^2 =$						0.37 ns	0.43 ns	0.150	0.153	0.107–0.200	16.1	
<i>Got-2: Skdh-1</i>												
G1	83/84	34	9	7	36	0.00	0.19	33.91***	0.186	0.193	0.113–0.277	20.4
	84/85	36	6	15	34	0.54	1.33	27.31***	0.231	0.237	0.152–0.324	25.8
G2	83/84	20	18	23	20	0.31	0.31	0.01	0.500	0.456	0.391–0.500	77.0
G7	83/84	25	27	23	25	0.16	0.16	0.00	0.500	0.461	0.403–0.500	80.1
	84/85	24	6	9	18	0.16	1.42	12.35***	0.263	0.271	0.161–0.387	30.4
Heterogeneity ¹ (4 df): $\chi^2 =$						35.89***	25.22***	(0.343	0.344	0.280–0.410	25.1)	
<i>Lap-2: Pgi-2</i>												
TH97	83/84	17	36	35	23	0.22	0.44	8.84**	0.361	0.363	0.275–0.452	46.0
	84/85	7	14	11	5	0.67	0.03	4.47*	0.324	0.332	0.189–0.500	40.0
G2	83/84	18	24	34	34	6.14*	0.33	0.50	0.500	0.457	0.326–0.500	77.6
G1	83/84	14	13	14	18	0.42	0.15	0.39	0.500	0.439	0.349–0.500	68.4
	84/85	33	17	17	23	1.11	1.11	4.85*	0.378	0.380	0.280–0.488	49.8
Heterogeneity ¹ (4 df): $\chi^2 =$						8.31 ns	3.49 ns	0.409	0.410	0.360–0.461	48.4	
<i>6-Pgdh-3: Skdh-1</i>												
G1	83/84	13	15	17	13	0.07	0.07	0.61	0.500	0.434	0.336–0.500	66.3
G2	83/84	23	27	19	24	0.53	0.87	0.03	0.500	0.459	0.389–0.500	78.8
G8	83/84	18	27	34	21	1.00	0.16	4.67*	0.390	0.392	0.298–0.500	52.8
	84/85	41	45	31	36	2.36	0.53	0.03	0.500	0.468	0.421–0.500	85.2
Heterogeneity ¹ (3 df): $\chi^2 =$						3.68 ns	25.4 ns	0.477	0.467	0.421–0.500	60.5	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant; ¹, first χ^2 value refers to $\hat{\theta}_1$, second to $\hat{\theta}_2$.

nation rate is high and *vice versa*. Tight linkage blocks were indicated for certain two-locus combinations. *Got-1* was linked tightly to *Pgi-2* and to *Dia-2*. For these two-locus combinations one tree

with seed samples of different crops was available. Pooled over the two seed samples recombination frequencies (Bayes estimates) for *Dia-2: Got-1*, *Dia-2: Pgi-2*, and *Got-1: Pgi-2* were 0.316, 0.264,

and 0.153, respectively. *Got-2:Skdh-1* could be analysed employing the seeds of three trees. For two of them seed samples of different years were available. Whereas for tree G1 very similar results were obtained when calculating $\hat{\theta}$ based on the two seed samples, $\hat{\theta}$ for G7 differed strongly between the two years. Pooled over all seed samples and trees $\hat{\theta}_2$ of 0.344 was calculated. *Lap-2:Pgi-2* showed moderate linkage resulting in $\hat{\theta}_2$ of 0.410, when the data of five seed samples of three trees were considered. The gamete frequencies of tree G1 resulted in contradictory $\hat{\theta}$ estimates with one sample showing linkage, the other not. *6-Pgdh-3* and *Skdh-1* seem to be also moderately linked in one of the three investigated trees. This combination showed moderate linkage in one tree with a $\hat{\theta}_2$ of 0.392, whereas the other seed sample of the same tree and the two other trees showed no linkage.

A tentative map of the gene loci is given in fig. 1. *Lap-2* was found to be linked to *Pgi-2*. However no tree was available to study linkage between *Lap-2* and either *Got-1* or *Dia-2*. Therefore *Lap-2* could be located on the other side of this linkage group.

DISCUSSION

Segregation distortion

Some enzymes seem to exhibit more frequent segregation distortion than others. There are several studies in conifers, which show that GOT, LAP, 6-PGDH, and APH often do not segregate in the expected 1:1 ratio (cf. Strauss and Conkle, 1986). At the *Gdh* locus Cheliak *et al.* (1987) found segregation distortion in Norway spruce. In the present study segregation distortions were observed at *Idh-1* and *Pgi-2* pooled over all trees. Several factors could contribute to such meiotic drive processes and/or gametic selection. Distortion genes linked to isozyme gene loci or linkage of embryonic lethal genes and the respective

isozyme gene locus can cause segregation distortion. Viability selection after fertilization can also contribute to segregation distortion because only those maternal gametes could be scored that were associated with viable embryos.

Linkage

The presented data indicate that *Mdh-3*, *Idh-1*, *6-Pgdh-2*, *6-Pgdh-3*, *Gdh-1*, *Got-2*, *Pgm-1*, *Aph-2*, and *Lap-2* are not linked in *P. abies*. This is in good agreement with the results elaborated in earlier studies in this tree species. Our data confirm six pair-wise combinations, which were also analysed by Lundkvist (1979) and resulted in free recombination in *P. abies*.

Out of 29 two-locus combinations also investigated by Muona *et al.* (1987) the data of the present study confirm their findings of 27 combinations. One of their pair-wise comparisons showed weak linkage (*Aph-2:Gdh-1*; $\hat{\theta}_1 = 0.417$) in one of three trees analysed. However, linkage between these loci could not be confirmed in any of the three trees of the present study analysing in total 287 gametes. The moderate linkage of *Lap-2:Pgi-2* found in our material was not observed by Muona *et al.* (1987). However, in two of our five samples linkage was not found to be significant and the estimated distance between these loci was about 48 cM. Estimated recombination frequencies of *Got-1:Pgi-2*, *Dia-2:Pgi-2*, and *Dia-2:Got-1* of our material were in the same frame and increased in identical order as found by Muona *et al.* (1987). An earlier linkage study in Norway spruce indicated the following linkage groups (Poulson *et al.* 1983):

- (1) *Lap-1:Got-3:6-Pgdh-3*, (*Got-3* is considered to be identical with *Got-2* of this study)
- (2) *Mdh-2:Mdh-3*,
- (3) *Got-1:Lap-2*.

Concerning linkage in many loci analysed in their study there is agreement with our results. However linkage of *Got-2:6-Pgdh-3* could not be confirmed in any of the eight trees regarding 832 gametes. The one tree tested for of *Lap-1:Got-2*, also showed no linkage. The linkage blocks of *Mdh-2:Mdh-3* and *Got-1:Lap-2* could not be studied in the present investigation. Linkage of *Pgi-2* and *6-Pgdh-2*, 3 in *P. glauca* (King and Dancik, 1983) is not supported by our data.

Significant linkage was found in tree G8 between *6-Pgdh-3* and *Skdh-1* analysing seed of crop 83/84. But the other three seed samples included indicate no linkage. The different θ esti-

Figure 1 Schematic linkage map in *Picea abies*.

mates among two seed samples of G8, as well as no linkage in the other trees leads us to expect free combination of these loci. Muona *et al.* (1987) investigated this combination employing one Norway spruce clone and also found no linkage.

The recombination rates varied among the tested trees but environmental as well as genetic factors could contribute to this phenomenon (Allard, 1963; Grell, 1966; Moran *et al.*, 1983). Thus mapping of isozyme gene loci can only be tentative in this study. The numbers of trees investigated were fairly small. Nevertheless our mapping is in agreement with the findings in other Pinaceae (see gene mapping in five *Pinus* species reviewed in Conkle, 1981). *Lap-2* may be located either on the right or left of *Pgi-2*. Since Poulsen *et al.* (1983) have calculated a map distance between *Got-1* and *Lap-2* of about 20 cM, it is more probable that *Lap-2* is located on the left of *Pgi-2* when regarding our estimated distances. Also *Aco* could be part of this linkage block (Cheliak *et al.*, 1987). Especially striking is the highly conservative linkage group of *Got-1* and *Pgi-2* resulting in a distance estimate of 16 cM which agrees well with other studies in the *Picea* genus (King and Dancik, 1983; Boyle and Morgenstern, 1985; Barret *et al.*, 1987; Muona *et al.* 1987).

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