INHERITANCE AND LINKAGE OF ISOZYME VARIANTS OF SILVER FIR (*ABIES ALBA* MILL.)

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ABSTRACT

Inheritance and linkage relationships of 13 enzyme systems were investigated by horizontal starch gel electrophoresis in endosperms, embryos, buds and needles of *Abies alba* Mill. A minimum of 26 loci were found to code for isozymes of the enzyme systems studied. The segregation data of allozymes in endosperms (megagametophytes) of heterozygous mother trees revealed simple Mendelian inheritance for these enzymes. No variation was found at 6 loci; at the remaining 20 variable loci 2 to 6 alleles were identified.

Two loci segregation data were available for 41 of 190 possible pairwise combinations of 20 polymorphic loci. Evidence of linkage was observed only for the pair *Idh-B* : *Ap-B*. With 37 % recombination the linkage was only moderate and homogeneous over the tested trees.

The zymogram patterns and number of controlling loci identified in *A. alba* were compared with those of other *Abies* species. Great similarity was observed.

Key words: *Abies alba*, isozymes, inheritance, linkage

INTRODUCTION

The use of isozyme-gene markers to describe and quantify genetic variation of forest trees has become an indispensable tool in forest genetic research (for review, see PAULE 1990). Before isozymes can reliably be used as gene markers, an analysis of the genetic control and their mode of inheritance must be carried out (GILLET 1993, HATTEMER *et al.* 1993, MÜLLER-STARCK 1993).

In *Abies alba* the genetic control of several enzyme systems has already been identified (STEINCHEN 1985, SCHROEDER 1989a) and these isozyme gene markers have then successfully been used for genetic studies in various populations of this species (BERGMANN *et al.* 1990, KONNERT 1992, 1993, BREITENBACH-DORFER *et al.* 1992, SCHROEDER 1989b, LONGAUER 1994).

In the course of new genetic studies on *Abies alba*, additional genetic variants (alleles, genotypes) were found at previously identified isozyme gene loci and, beyond that, additional isozyme systems not yet studied earlier were introduced to our recent studies (HUSSENDÖRFER & MÜLLER-STARCK 1994, KONNERT & BERGMANN 1995). Therefore, we want to present the results of the relevant inheritance and linkage analysis of all isozyme systems used so far. In addition, new procedures for electrophoretic separation and enzyme staining were also included. Our findings on the genetic basis of the enzyme systems studies were compared with the data published for other true fir species.

MATERIAL AND METHODS

Material

Seeds from 130 open-pollinated single trees and bulk seed lots were investigated. The seeds were collected in 1989 and 1991 from trees in fir stands of the Black Forest and in 1994 from trees in stands of Switzerland. Buds and needles were collected from numerous trees as well.

The seed was air dried and stored at temperature of –5 °C or 1 °C. The bud tissue was stored at –20 °C or –70 °C. The needles were shock frozen under the addition of insoluble PVP (Polyclar AT) in liquid nitrogen and mechanically ground (Ultra-Turrax). The grounded needles were then stored at –80 °C.

Electrophoretic methods

Extraction

The samples (endosperms, embryos, buds, needles) were homogenized in 0.08 – 0.1 M TRIS–HCl buffer,

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pH 7.0 or 7.3. To inhibit phenolics and tannins as well as to increase enzyme stability, various compounds at various concentrations were tested for their effectiveness (MÜLLER-STARCK 1993, RHODES 1977) (Tab. 1).

Table 1 Additives and their respective concentration tested for sufficiently extracting the enzymes

Additives	Concentration
2-Mercaptoetanol	0 %, 1 %, 2 %, 3 %
Na_2EDTA	0.1 %, 1 %
PVP-40 (soluble)	0 %, 2 %, 4 %, 5 %
Cystein	0 %, 0.01 %
$NaHSO_3$	0 %, 0.5 %
Na-ascorbat	0 %, 1 %

The crude extract was soaked into paper wicks (Whatman chromatography paper No. 3) and stored at –80 °C until the electrophoretic analysis was performed (non-perishable for several days).

Separation of enzymes

In order to determine the optimal resolution of the enzymes listed in Table 4 and 5, the starch gel electrophoresis was run using starch concentrations from 11 to 12 % and adding sucrose or urea (Tab. 2). The voltage range was between 15–30 $V.cm^{-1}$, the running distance approximately 10 cm.

Table 2 Composition of starch gels for isozyme analysis of *Abies alba*

Gel Nr.	Starch	Sucrose	Urea	Na_2EDTA	Buffer ml	Concentration
1	24 g	4 g	–	–	200	12 %
2	24 g	3 g	–	–	200	12 %
3	24 g	–	2 g	2 g	200	12 %
4	24 g	–	1.65 g	–	200	12 %

Various combinations of electrode and gel buffers (listed in Tab. 3) were tested for their suitability to improve the zymograms (CHELIAK & PITEL 1984, KONNERT 1992, LUNDKVIST 1979, POULIK 1957, MÜLLER-STARCK 1993).

In addition, isoelectric focusing was used for the enzyme systems DIA, MNR and NDH, in order to determine their quaternary structure and also the staining specificity (for details see: WESTERMEIER 1990, MÜLLER-STARCK 1993).

Staining of enzymes

The staining of enzymes was performed according to recipes given by CHELIAK & PITEL (1984), HARRIS & HOPKINSON (1976, loc. cit. Chap.2), KONNERT (1992), RICHARDSON *et al.* (1986, loc. cit. Cap.9.2), THORMANN & STEPHAN (1993) and VALLEJOS (1983).

Verification of substrate specifity

In order to establish which isozyme zones in a zymogram are part of the enzyme system to be studied and which part are not (background staining), the staining was carried out with and without substrate for each enzyme system on replicate slabs of the same gel. The isozyme bands in the control slabs (without substrate) must be disregarded when evaluating the respective isozyme patterns.

Genetic analysis

Independence of genotype expression of sampled tissue and ontogenetic (or developmental) stage

By comparing the patterns of seed (embryo, endosperm), bud and needle tissue from the same tree we wanted to test if genotype expression depends on the type of tissue. In addition, the comparison of the isozyme phenotypes from haploid and diploid tissues allows for proving the quaternary structure of the investigated enzymes.

A verification of the complete ontogenetic stability implies that individuals of the same clone from all age classes are available (MÜLLER-STARCK 1993). Since this was not the case, a comparison between parents and progenies from two provenances (Beggingen, Switzerland and Traunstein, Bavaria) was carried out for three developmental stages – seed, 2–4 year old seedlings and 10–15 year old trees.

Verification of the genetic control and the inheritance of the respective isozyme variants through endosperm segregation analyses

Since material from controlled crosses was not available, the genetic analysis was performed using the meiotic segregation ratios observable in haploid seed endosperm (= macrogametophyte) samples from single, putatively heterozygous trees (BARTELS 1971, BERGMANN 1973, HATTEMER *et al.* 1993). With this method it is possible to assign single isozyme zones in zymograms to individual gene loci, if there is no deviation from a 1 : 1 segregation ratio. The statistical significance of the deviations observed was tested using a χ^2 test (HATTEMER *et al.* 1993). When several trees

Table 3 Tested buffer systems for starch gel electrophoresis of enzymes in Abies alba

Nr.	Electrode buffer / pH	Gel buffer / pH
	TRIS-Citrate systems *)	
1.	0.15 M TRIS-Citrate / 8.0	0.02 M TRIS-Citrate / 8.0
2.	0.15 M TRIS-Citrate / 7.5	0.02 M TRIS-Citrate / 7.5
3.	0.14 M TRIS-Citrate / 7.5	0.04 M TRIS-Citrate / 7.5
4.	0.14 M TRIS-Citrate / 8.5	0.04 M TRIS-Citrate / 8.5
5.	0.15 M TRIS-Citrate / 6.5	0.02 M TRIS-Citrate / 7.0
	ASHTON-system **)	
6.	0.03 M LiOH-0.2 M H_3BO_3 / 8.1	0.05 M TRIS-Citrate / 8.1
	POULIK-system	
7.a	0.3 M H_3BO_3-0.06 M NaOH / 8.2	0.075 M TRIS-Citrate / 8.7
7.b	0.3 M H_3BO_3-0.06 M NaOH / 8.2	0.07 M TRIS-HCl / 8.7
	TRIS-Histidine-system	
8.	0.14 M TRIS-Citrate / 7.5	0.017 M TRIS-1.4 mM Na_2EDTA-0.05 M Histidine.HCl / 6.2

were studied for a specific allelic combination, a χ^2 test was performed to test the heterogeneity of segregation. If deviation was not significant, the data were pooled over trees.

For enzyme systems in which more than one zone of activity appeared in zymograms or in zones of activity with more than one allozyme, the zones were designated with capital letters (beginning with A) and the allozymes with numbers (beginning with 1), both in decreasing order of relative mobility.

Verification of linkage between gene loci

Linkage was examined for 41 possible two-locus combinations (most of the combinations for several trees) testing the hypothesis of independence between two loci (expected segregation ratio 1 : 1 : 1 : 1). This test involved the calculation of three χ^2 values, one each for segregation at the two loci and one for linkage (HATTEMER *et al.* 1993). The recombination rate was estimated by the relative frequency of recombinant gametes. Since we had no knowledge of the parental types of the chromosomes, the less frequent gametes were assumed to be recombinants. For detailed methods, see RUDIN & EKBERG (1978) and YING & MORGENSTERN (1990).

RESULTS AND DISCUSSION

Electrophoretic methods

The best resolved zymograms for all enzyme systems from bud and seed tissue were obtained when the homogenization buffer contained 1 % mercaptoethanol, 0,1 % Na_2EDTA und 3–4 % soluble PVP–40. The addition of a small spatula tip of insoluble PVP (Polyclar AT) during the homogenization is advantageous. The resolution is becoming worse if mercaptoethanol is lacking or if its concentration is higher than 1 %.

For the extraction of enzymes from needle-tissue the addition of cystein (0,01 %), $NaHSO_3$ (0,5 %) and 1 % sodium ascorbate is necessary. Without these additives no demonstrable enzyme activity from needles was observed in zymograms.

The optimal separation conditions for each enzyme system are listed in Table 4. Most of these enzymes can be satisfactorily separated be means of several buffer systems.

Table 5 lists enzyme systems for which the resolution was not sufficient. In our surveys the enzymes ACO, G3PDH, G6PDH and GRD were found to be not substrate-specific. They show the same patterns with and without substrate components in the staining solutions. It should be mentioned that LONGAUER (1994) found one gene locus with 5 alleles for the ACO system when analyzing bud tissues of silver fir from eastern Europe.

Remark: If replicate gel slabs were stained for a NADP enzyme (incl. ACO) without the specific substrate (control staining), isozyme patterns similar to the IDH patterns of the same gel could be observed in many cases. Since these background band patterns disappeared, when the separation buffer did not contain citric acid (see also BREITENBACH-DORFER *et al.* 1992), we advance the following explanation: the enzyme ACO can also use citric acid as substrate and produces isocitric acid (isocitrate), which is just the substrate for IDH. If the two enzyme zones (or their allozymes)

Table 4 Summary of investigated systems for inheritance and linkage analyses in *Abies alba*

Enzyme system and E.C. No.	Structure	Locus	No. of alleles	Gel system [1]	Buffer system [2]
Alanine aminopeptidase (AAP) E.C.3.4.11.1	monomeric	*Ap–B* *Ap–D*	2	1	6
Aspartate aminotransferase (AAT, GOT) E.C.2.6.1.1	dimeric	*Aat–A* *Aat–B* *Aat–C*	2 3 3	2, 4	4, 7a 7b
Diaphorase (DIA) E.C.1.6.4.3	tetrameric	*Dia–A*	3	1, 4	1, 3
Glutamate dehydrogenase (GDH) E.C.1.4.1.2	multimeric	*Gdh–A*	2[*]	1, 2	1, 3, 6, 7a, 7b
Isocitrate dehydrogenase (IDH) E.C.1.1.1.42	dimeric	*Idh–A* *Idh–B*	4 5	1, 3	1, 2, 3, 7
Leucine aminopeptidase (LAP) E.C.3.4.11.1	monomeric	*Ap–A* *Ap–C*	6 4	1	6
Malate dehydrogenase (MDH) E.C.1.1.1.37	dimeric	*Mdh–A* *Mdh–B*[**] *Mdh–C*	4 3	3, 4	1, 3, 5
Menadione reductase (MNR) E.C.1.6.99.2	tetrameric	*Mnr–A* *Mnr–B*	1 3	1, 4	1, 3
NADH-dehydrogenase (NDH) E.C.1.6.99.2	dimeric	*Ndh–A*	2	1, 4	1, 3
6-Phosphogluconate dehydrogenase (6-PGDH) E.C.1.1.1.43	dimeric	*6-Pgdh–A* *6-Pgdh–B*	4 3	1, 2	2, 3
Phosphoenolpyruvate carboxylase (PEPCA) E.C.4.1.1.31	–	*Pepca–A*	1	1, 2, 3	5
Phosphoglucoisomerase (PGI) E.C.5.3.1.9	dimeric	*Pgi–A* *Pgi–B*	2 3	1, 2	6, 7a, 7b
Phosphoglucomutase (PGM) E.C.5.4.2.2	monomeric	*Pgm–A* *Pgm–B*	2 2	1, 2	1, 2, 7a, 7b
Shikimate dehydrogenase (SKDH) E.C.1.1.1.25	monomeric	*Skdh–A*	2	2	1, 3

[1] gel No. from tab. 2
[2] buffer system No. from tab. 3
[*] for details see LONGAUER (1994)
[**] gene locus was not investigated

migrate in close proximity in the starch gel, the isocitrate molecules diffuse from the ACO zone to the IDH zone and the very strong IDH activity becomes visible even in control gel slabs. If allozymes of these two enzyme systems migrate in greater distance in the gel, IDH patterns could not be detected or, rarely, a part of the IDH isozymes (e.g. one band of a triple-banded heterozygous type) appeared after a long staining period. Thus, background staining in all NADP-enzyme gels and control gels, as, for instance, additional faint bands in the 6–PGDH–B zone can bee explained by IDH staining in most cases. The hypotheses that one allozyme of IDH can also use citric acid besides isocitrate as a substrate appears to be not very likely, since biochemical rules deny the capability of IDH enzymes to react with citrate molecules (CLELAND *et al.* 1969).

Table 5 Summary of further enzyme systems for which no genetic analyses were carried out

Enzyme system	E.C. No.	Abbre-viation	Observations
Acid phosphatase	3.1.3.2	ACP	provable only in seed (embryo, endosperm)
Aconitase	4.2.1.3	ACO	not substrate specific
Alcohol dehydrogenase	1.1.1.1	ADH	no coloration (lacking activity)
Esterases	3.1.1.1	EST	provable only in seed (embryo, endosperm)
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	G3PDH	not substrate specific
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH	not substrate specific
Glutathione reductase	1.6.4.2.	GRD	not substrate specific
Peroxidase	1.11.1.7	PER	no coloration (lacking activity)

For the enzymes ACP and EST only extracts from seed tissue (embryo, endosperm) showed clear zymograms. Therefore, the expression of these systems seems to be tissue-specific and no further investigation was performed. Using the described conditions the systems ADH and PER showed no activity (lack of staining) in any tissue. On the other hand LONGAUER (1994) analyzed the peroxidase system (PER) from bud tissue of silver fir. He assumed a genetic control by two gene loci, whereby *Per–A* has three alleles and *Per–B* has two. Details on methods and genetic analysis are not given.

Description of isozyme patterns and inheritance

Single-locus endosperm (megagametophyte) segregation data are summarized in Table 6. Endosperm banding patterns and their allelic designations for 24 putative loci are shown in Fig. 1.

Enzyme systems without variation

A single invariant zone of activity was observed in our studies for GDH and PEPCA.

For GDH, LONGAUER (1994) found two variants in some east European fir populations, so that one can assume that this system is probably controlled by one gene locus with two alleles. A genetic control by one gene locus with two alleles is also postulated by SCHROEDER (1989a), but segregation data are not presented. One zone of activity without variation was also observed for GDH in *A. balsamea* (NEALE & ADAMS 1981), *A. amabilis* (DAVIDSON 1990), *A. pinsapo* (PASCUAL *et al.* 1993), *A. fraseri* (JACOBS *et al.* 1984), *A. lasiocarpa* (SHEA 1988) and *A. borisii regis* (FADY & CONKLE 1992).

The system PEPCA was not studied in other *Abies* species.

Variable enzyme systems

Aminopeptidase (AP)

Remark: The comparison of the staining for AAP and LAP on two replicate slabs of the same gel revealed that several zones of activity appeared to be identical. According to this result, it was suggested to consider both enzymes as one enzyme group. The zones established to be controlled by separate gene loci with similar substrate specificity were designated with the abbreviation "AP".

Gels stained for LAP showed two variable zones of activity as it was already postulated by MEJNARTOWICZ (1979). The faster migrating zone is clearly distinguishable from other zones by migrating and staining intensity. Single bands occurred in all tissues and in addition double-banded variants were observed in embryo, bud and needle tissues in both zones of heterozygous trees, indicating a monomeric structure of the LAP system. Putatively heterozygous individuals (double-banded) produced seed endosperms with two alternative single bands exhibiting a 1 : 1 segregation ratio. Thus each zone is encoded by a separate gene locus. Comparable zones and variants occurred after staining for AAP, thus these zones and gene loci were named *Ap–A* and *Ap–C*.

Gels stained for AAP showed three clearly separated variable zones of activity in several tissues (buds, needles, seeds). The fastest migrating one was found to be identical with the fastest migrating zone of LAP, named *Ap–A*. The second zone is located between *Ap–A*

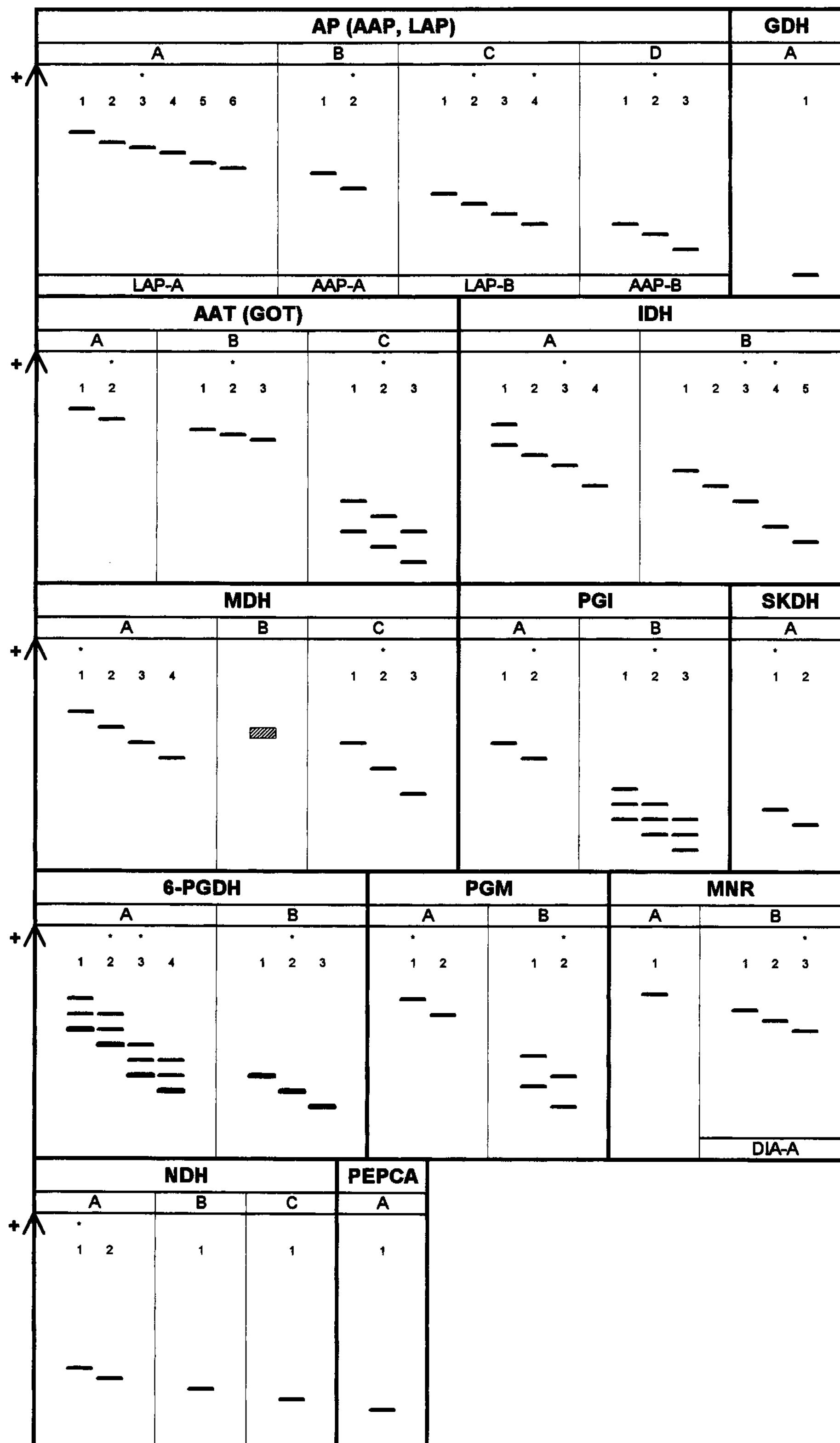

Figure 1 Schematic illustration of isozyme phenotypes in megagametophytes of *Abies alba*. The symbol * above the allele designations indicates the most frequent types for each locus

Table 6 Segregation at single loci *Abies alba*

Locus	Alleles	No. of trees	Ratio	Total	χ^2 segregation if significant	χ^2 heterogenity if significant
Aat–A (*Got–A*)	1–2	1	36 : 41	77	n. s.	–
Aat–B (*Got–B*)	1–2	4	124 : 118	242	n. s.	n. s.
Aat–C (*Got–C*)	1–2	1	18 : 22	40	n. s.	–
	2–3	5	142 : 132	274	n. s.	n. s.
Ap–A (*Lap–A*)	3–4	3	44 : 46	90	n. s.	n. s.
	3–5	9	304 : 300	604	n. s.	10.14*
	4–5	1	14 : 16	30	n. s.	–
Ap–B (*Aap–A*)	1–2	5	183 : 183	386	n. s.	n. s.
Ap–C (*Lap–B*)	1–2	2	28 : 32	60	n. s.	n. s.
	1–4	1	16 : 14	30	n. s.	–
	2–3	2	33 : 27	60	n. s.	n. s.
	2–4	5	80 : 70	150	n. s.	n. s.
	3–4	2	31 : 29	60	n. s.	n. s.
Ap–D (*Aap–B*)	1–2	2	29 : 31	60	n. s.	n. s.
Dia–A (*Mnr–B*)	1–3	7	451 : 399	850	n. s.	n. s.
Idh–A	1–3	6	214 : 196	408	n. s.	n. s.
	1–4	3	73 : 72	145	n. s.	n. s.
	2–3	1	16 : 14	30	n. s.	–
	3–4	6	186 : 168	354	n. s.	n. s.
Idh–B	3–4	9	290 : 308	548	n. s.	n. s.
Mdh–A	1–2	1	14 : 11	25	n. s.	–
	1–3	8	241 : 220	461	n. s.	n. s.
Mdh–C	0–3	1	8 : 10	18	n. s.	–
	1–3	1	9 : 15	24	n. s.	–
	2–3	1	12 : 8	20	n. s.	–
6–Pgdh–A	1–3	1	13 : 17	30	n. s.	–
	2–3	9	303 : 286	589	n. s.	n. s.
6–Pgdh–B	1–2	1	75 : 65	140	n. s.	–
	2–3	2	95 : 102	197	n. s.	n. s.
Pgi–A	1–2	1	13 : 18	31	n. s.	–
Pgi–B	1–2	1	25 : 16	41	n. s.	–

and *Ap–C* (*Ap–C* attributed to LAP) and named *Ap–B*. The *Ap–D* zone is the slowest and overlapps partially with the *Ap–C* zone. Hence, the common staining for LAP and AAP and the comparison of resulted zymograms is neccessary for a sure interpretation. Segregation studies of endosperms from maternal trees exhibiting double–banded *Ap–B* and *Ap–D* zones showed

a 1 : 1 segregation ratio with two single bands and exhibited Mendelian inheritance (Tab. 6).

In summary, one can assume that the AP–system (LAP and AAP) is controlled by four polymorphic gene loci. The absence of hybrid bands in putative heterozygous individuals suggested a monomeric structure of the aminopeptidase enzyme system.

Two zones of activity were reported for LAP also in *A. balsamea* (NEALE & ADAMS 1981), *A. fraseri* (DIEBEL & FERET 1991), *A. borisii-regis* (FADY & CONKLE 1992), and *A. pinsapo* (PASCUAL *et al.* 1993). AAP was not studied in other *Abies* species.

Aspartate aminotransferase (AAT)

AAT (also known as GOT) had three zones of activity in zymograms. *Aat–A* and *Aat–B* were variable, each with 3 single bands in endosperms. Segregation tests revealed that each zone is controlled by a separate locus with 3 alleles in tested material. A genetic control by three gene loci was also postulated for GOT by MEJNARTOWICZ (1979), STEINCHEN (1985) and MOLLER (1986).

Embryo and bud extracts showed either single bands, in the same position as in endosperms or triple-banded variants with an additional band with intermediate mobility (hybrid band). This isozyme configuration appeared in heterozygotes and suggests a dimeric structure for AAT.

The zone closest to the cathode – *Aat–C* – showed three double-banded variants, which behaved as alleles at one locus. The observed segregation at *Aat–C* is not significantly different from the expected 1 : 1 ratio (Tab. 6). Additional five-banded variants in putative heterozygous embryos or buds also confirmed the dimeric structure.

Three loci have also been reported to code for this dimeric enzyme in *A. balsamea* (NEALE & ADAMS 1981,) *A. borisii-regis* (FADY & CONKLE 1992), *A. pinsapo* (PASCUAL *et al.* 1993), *A. equi-trojani, A. bornmuelleriana* (KONNERT *et al.* 1992), *A. amabilis* (DAVIDSON 1990) and *A. lasiocarpa* (SHEA 1988).

Isocitrate dehydrogenase (IDH)

There were two zones of activity on gels stained for IDH, as described by STEINCHEN (1985), MOLLER (1986) and SCHROEDER (1989a). The slower migrating zone (*Idh–B*) was stained more intensively than the faster zone (*Idh–A*). At *Idh–A* four variants – one double–banded, three single-banded – were observed. The segregation (single trees or pooled) fits the Mendelian expectation of a 1 : 1 ratio of gametes, with no detectable heterogeneity among trees (Tab. 6).

Idh–B was variable possesing five single-banded variants in endosperms. They were segregating as alleles at a single locus (Tab. 6). Embryos showed in zone A and zone B triple-banded variants when heterozygous suggesting a dimeric structure for this enzyme system.

Two gene loci also control this enzyme system in several other *Abies* species, whereby *Idh–A* was monomorphic in *A. nordmanniana, A. bornmülleriana, A. equi-trojani*(KONNERT *ET al.* 1992), *Idh–B* in *A.fraseri* and *A. pinsapo* (DIEBEL & FERET 1991, PASCUAL *et al.* 1993). *A. pinsapo* had two alleles at *Idh–A* (PASCUAL *et al.* 1993). In *A. balsamea* (NEALE & ADAMS 1981) and *A. amabilis* (DAVIDSON 1990) the IDH system was claimed to be controlled by only one gene locus. According to the description given by the above authors this locus is equivalent to our *Idh–B* locus.

Malate dehydrogenase (MDH)

The malate dehydrogenases (MDH) in higher plants represent a complex system composed of various isozymes which function in different cell compartments (YANG & SCANDALIOS 1974). If individual MDH controlling gene loci have recently been duplicated, there frequently appear interlocus hybrid bands (heterodimers) in zymograms, which additionally impede the interpretation of MDH patterns.

In silver fir the MDH zymograms are very difficult to be interpreted due to similar migration rates of several isozyme zones and the overlapping of bands between them. A possible resolution of the zymogram patterns and their subsequent genetic interpretation is based on the fact that MDH in plants consists of two enzyme types: (1) non-decarboxylating MDH (E.C. 1.1.1.37) and (2) oxaloacetate-decarboxylating MDH (E.C. 1.1.1.38). Both MDH types using NAD as coenzyme can be stained after electrophoretic separation with the common tetrazolium method, where NADH + H^+ reduces a tetrazolium salt leading to the blue formazan. However, the non-decarboxylating MDH which produces oxaloacetate can also be stained with a diazonium salt like Fast Blue BB (THORMANN & STEPHAN 1993). Based on the latter staining method with Fast Blue BB, three isozyme zones can easily be recognized in zymograms from endosperm extracts of silver fir, of which the middle zone turned out to be an interlocus hybrid band. The fastest migrating and most intensely stained zone is called *Mdh–A* and the next and weaker stained zone is called *Mdh–C*, since another zone (*Mdh–B*) is found to be located between these two zones, if the zymograms are stained with the tetrazolium method. Hence, the MDH patterns of silver fir can be resolved through the differentiation between decarboxylating and non-decarboxylating isozymes.

One can thus assume that for *A. alba* the system MDH is controlled by at least 3 gene loci. Both *Mdh–A* and *Mdh–C* are variable with 3 and 4 alleles, respectively. *Mdh–B* is monomorph. The dimeric structure of the enzyme is verified by the appearance of the inter– and intralocus-hybrid bands.

A control by three gene loci has also been assumed for MDH in *A. amabilis* (DAVIDSON 1990) and *A. pinsapo* (PASCUAL *et al.* 1993), whereby NEALE & ADAMS (1981) could only verify two gene loci for *A. balsamea.* A direct comparison of the banding patterns, of loci and alleles is difficult, because the results of different authors are contradictory. This is probably due to the complexity of the MDH–system. Thus it is especially important to use the recommended staining method (with Fast Blue BB) to assure a reliable interpretation.

Menadione reductase (MNR)

Gels stained for MNR showed five zones of activity. A comparative study revealed that only the two fastest zones belong to the MNR system, while the three slower zones occur also after staining for NDH (s.b.). The two zones of activity of the MNR system were clearly distinguishable by migrating and staining intensity. The faster zone was less intensely stained, whereas the slower zone stained very intensively. In the faster zone only a single band appeared in several tissues. In the slower migrating zone three single-banded variants occurred in all tissues. In embryo tissues, bud tissues und needle tissues additional five-banded variants were found using starch gel electrophoresis and also isoelectric focusing. Studies of seed endosperms from those phenotypes showed a 1 : 1 segregation ratio with two single bands and exhibited Mendelian inheritance (Tab. 6). Hence it is postulated that each zone is controlled by a separate gene locus (*Mnr–A* and *Mnr–B*). The *Mnr–A* locus seems to be invariant. The *Mnr–B* locus is variable with three single–banded variants representing three alleles. Embryos, buds and needle tissues from heterozygous trees also showed five–banded variants, suggesting a tetrameric structure for this enzyme.

Similar results were found for *A. pinsapo* (PASCUAL *et al.* 1993) and *A. borisii-regis* (FADY & CONKLE 1992).

Remark: Staining for MNR compared with staining for DIA revealed that the fastest migrating zone of DIA is identical to the *Mnr–B* zone. A similar result was also found for two pine species (YI 1992, YI & KIM 1994). According to YI and KIM (1994) the results of DIA and MNR comparisons were interpreted that *Dia–A* and *Mnr–B* are produced by the same isozyme.

Diaphorase (DIA) (see Menadione reductase)

NADH–dehydrogenase (NDH)

There were three zones of activity on gels stained for NDH. A comparison revealed that the three zones also appeared with staining for MNR and DIA. In each zone a single band was found in all tissues. Occasionally bud and needle tissues exhibited a three-banded variant in the fastest migrating zone. Therefore it is assumed that this enzyme is encoded by three gene loci. The *Ndh–A* locus seems to be polymorphic with two bands corresponding to two alleles. On the basis of the three--banded phenotypes observed in zymograms from putative heterozygous individuals, a dimeric structure of the enzyme is suggested.

Because seed from single trees heterozygous for NDH was not available, the genetic analysis could not be carried out. Therefore, the hypothesis on the genetic control must be considered preliminary.

6–Phosphogluconate dehydrogenase (6–PGDH)

Two zones were found in gels stained for 6–PGDH. Since the migration rate of both zones is not very different, they showed partially overlapping banding patterns in zymograms.

In the faster migrating zone (*6-Pgdh–A*), four triple-banded variants were observed, which showed a 1 : 1 segregation ratio. In the slower migrating zone (*6–Pgdh–B*) three single-banded variants were identified. Segregation analysis revealed no significant deviation from a 1 : 1 ratio (Tab. 6). The heterozygous embryos showed phenotypes with five bands in zone A and three bands in zone B indicating that the functional form of 6–PGDH is a dimer.

Thus 6–PGDH appears to be controlled in *A. alba* by two gene loci, as it was described by SCHROEDER (1989a). Because of partial overlapping of the isozyme variants in zymograms, the analysis of diploid material can be quite difficult in some cases.

Two gene loci and dimeric enzyme structure have also been found in *A. fraseri* (JACOBS *et al.* 1984), *A. lasiocarpa* (SHEA 1988), *A. pinsapo* (PASCUAL *et al.* 1993). Other authors report of only one controlling locus for *A. balsamea* (NEALE & ADAMS 1981), *A. borisii-regis* (FADY & CONKLE 1992) and *A. amabilis* (DAVIDSON 1990).

Phosphoglucoisomerase (PGI)

Gels stained for PGI had two variable zones of activity with single-banded variants in the faster migrating and triple-banded variants in the slower migrating zone.

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Table 7 Numbers of trees tested for each pair of loci in *Abies alba*

Locus	*Ap–B*	*Aat–B*	*Aat–C*	*Dia–A*	*Idh–A*	*Idh–B*	*Mdh–A*	*6–Pgdh–A*	*6–Pgdh–B*
Ap–A	5	3	1	1	4	6	1	5	–
Ap–B	–	2	1	–	4	4	2	3	–
Aat–A		–	1	–	–	1	1	–	–
Aat–B		–	2	1	2	3	2	1	–
Aat–C			–	2	2	3	2	3	1
Dia–A				–	2	2	3	2	–
Idh–A					–	8	4	9	1
Idh–B						–	–	10	1
Mdh–A								4	–
6–Pgdh–A								–	1

Segregation data support the hypothesis of one di-allelic locus for *Pgi–A* and one tri-allelic locus for *Pgi–B*. Since the system rarely showed variation, the segregation could only be tested for one tree. Although heterozygous embryos were rare, two different five-banded patterns could be founded at *Pgi–B*, suggesting that PGI is a dimeric enzyme.

PGI was found to be encoded by two gene loci also for other Abies species, e.g. *A. balsamea, A. fraseri, A. lasiocarpa, A. borisii–regis* and *A. pinsapo* (NEALE & Adams 1981, JACOBS *et al.* 1984, SHEA 1988, PASCUAL *et al.* 1993, FADY & CONKLE 1992,). *Pgi–A* was monomorphic in almost all cases, *Pgi–B* showed little variation with two to three alleles.

Phosphoglucomutase (PGM)

Two zones of activity were observed for PGM. The faster migrating zone (*Pgm–A*) revealed two variants. In endosperms only one single band was detected. In bud tissue we also found an additional double–banded variant. Based on our findings and on the fact that PGM is monomeric in most conifers, we assumed that this zone is controlled by one gene locus with two alleles.

The slower migrating zone (*Pgm–B*) showed two double–banded variants in endosperms. The variation was observed in endosperms of bulked seed. Unfortunately no seed from single trees, which are heterozygous at this locus, was available, so that segregation could not be verified. Since the variation was independent of zone A, we assumed that *Pgm–B* is controlled by another gene locus with two alleles.

Almost identical patterns, i.e. single–banded variants in *Pgm–A* and double–banded variants in *Pgm–B* were observed by NEALE & ADAMS (1981) in *A. balsamea*. In *A. pinsapo* two gene loci also exists for PGM but the *Pgm–B* zone always stains faintly and is invariant (PASCUAL *et al.* 1993). Similary, in *A. lasiocarpa Pgm–B* was too faint to be scored (SHEA 1988). In *A. balsamea* only one locus with four alleles was identified (DAVIDSON 1990).

Shikimate dehydrogenase (SKDH)

Only one zone could be detected in zymograms of SKDH (see also SCHROEDER 1989a). In most of the *Abies alba* populations *Skdh–A* consisted of a single band without any variation. Another single-banded variant was only found in a bulk seed sample from Calabria, Italy. Because seeds from individual trees were not available from this population, it was not possible to verify the segregation at *Skdh–A*. The assumption that SKDH in *A. alba* is controlled by one gene locus must be considered as preliminary.

For *Abies* species, few investigations have chosen SKDH. A single invariant zone of activity has been reported for *A. balsamea* (JACOBS *et al.* 1984). For *A. pinsapo*, on the other hand, the SKDH system appeared to be controlled by two gene loci, which however show little variation (PASCUAL *et al.* 1984).

Linkage

Twenty polymorphic loci were available to study linkage relationships. Of the 190 possible two–locus combinations 41 pairs were investigated in double–-heterozygous trees. The number of trees used for linkage analysis and the tested pairs are given in Table 7.

Evidence of linkage was observed only for the pair *Idh–B : Ap–B*. With 37 % recombination the linkage was only moderate and homogeneous over the three trees tested. In one tree *Idh–B* was found to be slightly linked with *Got–C* (36 % recombination). Analysis of two additional trees, however, refute the assumption of linkage between these two loci.

The linkage of several enzyme gene loci was also studied in *A. borisii-regis* (FADY & CONKLE 1993) and *A. balsamea* (NEALE & ADAMS 1981). Comparable linkage groups with these two *Abies* species were not

found for *A. alba*. The reason for this lack can be due to the methods used since different systems were included in the studies. From our available material we could not test the pairs *Lap–B : Got–A*, *Got–B : Pgi–B* and *Got–A : Got–C*, which are strongly linked in *A. balsamea*. On the other, hand we could not find any references to the linkage of *Idh–B : Ap–B* (syn. *Aap–A*) in conifers.

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