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Genetic Interpretation of Malate Dehydrogenase (MDH) Isozyme Gene Loci Using a New Staining Approach and the Genetic Control of Ten Other Isozymes in *Pinus roxburghii* SARG.

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Abstract

Genetic control of malate dehydrogenase and ten other enzymes have been investigated in *Pinus roxburghii*, the most extensively distributed pine of India. Seeds were collected from eight natural populations covering about one third of the species' distribution range. In total, 2560 samples consisting of equal number of embryos and endosperms were analysed for ACO, AAT, GDH, IDH, LAP, MDH, MNR, PGI, PGM, 6PGDH and SKDH using starch gel electrophoresis. Eighteen polymorphic loci were found to code for these enzymes. Two to three alleles per locus were identified. Mostly the isozyme loci followed the expected 1:1 segregation ratio. MDH was stained

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with two staining methods to distinguish between different isozyme gene loci. Three loci proved to be variable. Intra-locus as well as inter-locus heterodimeric hybrid bands were identified, the latter giving evidence for the existence of a gene duplication.

Key words: Pinus roxburghii, malate dehydrogenase, isozymes, genetic control.

FDC: 165.3; 174.7 *Pinus roxburghii*; (540).

Introduction

Pinus roxburghii SARG. commonly known as Indian Long Leaved Pine, is the most extensively distributed pine in India. Confined to the subtropical and warm temperate monsoon belt, the species grows between 450 m to 2300 m altitude, 26°N to 36°N latitude and 71°E to 93°E longitude in the Shiwaliks and the main Himalayan river valleys from Kashmir to Bhutan (DOGRA, 1985). Being pioneer in nature, it is an active coloniser of the degraded sites particularly the drier south facing slopes. Much of the degraded area in its natural habitat is deficient of organic matter, water holding capacity, nutrient availability and thus lacking in all the desirable physical, chemical and biological characteristics necessary to support valuable species other than *P. roxburghii*. Hence, the species has great potential in afforestation programmes. It provides not only timber, fuel, and pulpwood but also meets the demand for packing cases and stakes for vegetable cultivation.

With the development of isozyme analysis and its application to the field of forest genetics, there has been an increasing interest to study the variation present in different tree species and to exploit it for development of strategies for tree improvement programmes. The malate dehydrogenases (MDH) in higher plants represent a complex system composed of various isozymes functioning in different cell compartments (YANG and SCANDALIOS, 1974). The assessment of genetic control of MDH is a complex problem due to duplication of a certain gene locus and formation of inter- and intralocus heterodimeric hybrid bands which make interpretation of the isozyme bands somewhat difficult (HARRY, 1983; ADAMS et al., 1990; THORMANN and STEPHAN, 1993). Many scientists have interpreted the same pattern of MDH in different ways which has led to controversy in the literature (RUDIN and EKBERG, 1978; SZMIDT and MUONA, 1989). Thus, there are problems in the interpretation of MDH patterns. The present study was undertaken to define the genetic control of MDH using two staining methods and of other isozyme gene loci in *P. roxburghii*.

Material and Methods

The study is based on the seed material collected from open pollinated trees from eight natural *P. roxburghii* populations covering Shiwalik and Himalayan ranges of the species' distribution in Himachal Pradesh in India (*Figure 1* and *Table 1*). From each population twenty trees of different ages were selected keeping minimum distance of 50 m between tree to tree. Two to three fully developed and ripened cones were harvested from each tree during March and April depending upon the cone ripening period under different climatic conditions of the stands. The cones were dried in open sun and the seeds were extracted manually. After cleaning, the seeds from each tree were put in separate polythene bags with their individual identity and stored at 4 °C.

Isozyme analysis was carried out with the help of widely used horizontal starch gel electrophoresis based on the methods given by SHAW and PRASAD (1970), CONKLE et al. (1982) and CHELIAK and PITEL (1984) with slight modifications. From each tree eight seeds were excised and endosperm and embryo were homogenised separately in 80 micro litres of extraction buffer which contained the following chemicals per 100 ml of Tris-HCl, pH 7.5: ascorbic acid (0.106 g), saccharose (17.165 g), lystein-HCl (0.105 g), tween-80 (1.0 ml), PVP (8.0 g), NAD (0.029 g), bovine albumin (0.1 ml), dithiothreitol (0.015 g), EDTA (0.015 g), tergitol (1 ml), mercaptoethanol (0.7 g). Other more simple extraction buffers may give satisfactory resolution, however, for the set of enzymes analysed, the given buffer proved to be the best.

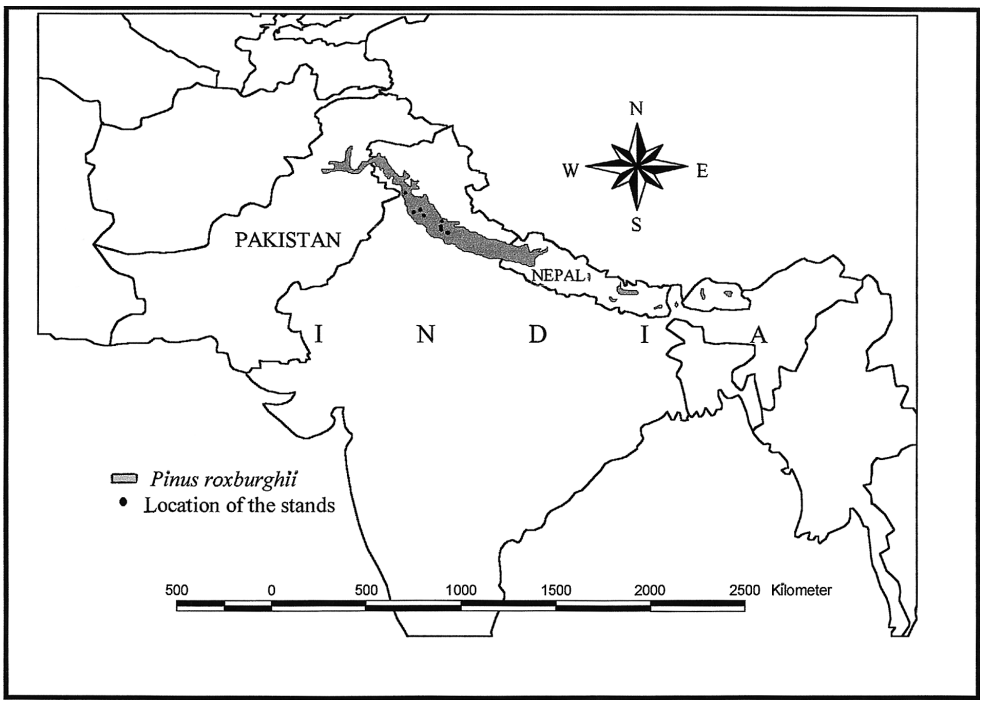

Figure 1. – Map showing distribution of *Pinus roxburghii* and locations of the stands.

Table 1. – Geographic location of *Pinus roxburghii* stands selected for isozyme studies.

Population	Number of plants	Altitude (m)	Latitude (° N)	Longitude (° E)
Sulyali	20	490	32.3	75.9
Aghar	20	800	31.7	76.5
Banethi	20	1370	30.6	77.3
Gagret	20	535	31.5	76.3
Nauni	20	1450	31.3	76.4
Nihari	20	1200	30.8	77.1
Shilly	20	1550	30.9	77.1
Shalaghat	20	2000	31.1	77.2

Table 2. – Composition of Tris-citrate and Ashton gels.

Gel	Composition	Quantity	Concentration
Tris-Citrate gel	gel buffer(Tris-cit.) starch saccharose	250ml 30g 6g	12%
Ashton gel	gel buffer electrode buffer starch saccharose	228ml 22ml 30g 6g	12%

Table 3. – Electrode and gel buffers.

Name	Gel buffer	Electrode buffer	Enzyme system
Tris-citrate system	electrode buffer in the ratio of 1:2 with distilled water	18.0g / l tris 10.0g / l citric acid pH 7.2	ACO, IDH, MDH MNR, PGI, PGM 6PGDH, SKDH
Ashton system	6.2g / l tris 2.0g / l citric acid pH 8.3	11.8g / l boric acid 1.0g / l lithium hydroxide pH 8.1	AAT GDH LAP

Toronto starch, the molecular sieve used for the study was the product of Biomol (Hamburg). The gel composition and concentration are given in *table 2*.

The electrode and gel buffers used for different enzymes are given in *table 3*.

After loading of paper wicks soaked in crude homogenate onto the gels, currents of 260 mA and 60 mA with the voltage at maximum possible (500 V) were applied to each Tris and Ashton gel, respectively. The gels were kept cool at 4 °C during this process. The optimum running time for Tris gel was 5.30 h whereas for Ashton it was 4.30 h.

The gels were cut into four slices of about 2 mm thickness. The top one was discarded. The gel slices were then transferred into the enzyme specific chemical solutions and kept at 37 °C to undergo the reaction. The time taken for staining varied from 30 minutes to about three hours. MDH being a difficult system to interpret was stained with two staining methods (THORMANN and STEPHAN, 1993) given below;

Method 1: Staining of gel with the staining solution containing 100 ml Tris-HCl (pH 9.0), 33 mg NAD, 150 mg L-malic acid and 366 mg fast blue BB.

Method 2: Staining solution contained 100ml Tris-HCl (pH 8.0), 33 mg NAD, 33 mg MTT, 150 mg L-malic acid, 3 ml PMS and 3 ml $MgCl_2$ (10%).

Description and Genetic Evaluation of the Zymograms

The enzymes which showed more than one zone of activity were designated with capital letters A, B and so on starting from the most anodal end. Similarly in each zone, the bands resp. the allozymes to which these bands stand for were assigned numbers 1, 2 and 3 in the order of their decreasing migration distances on the gels i.e. the fastest band was designated by 1. Whereas the undetectable allozymes encoded by null alleles were designated by 0. The interpretation of the genetic control of the putative enzyme loci was proved using the meiotic segregation ratios observed. (BARTELS, 1971; BERGMANN, 1973; HATTEMER et al., 1993). The statistical significance of the deviations (1:1) observed was tested using χ^2-test (HATTEMER et al., 1993).

Results and Discussion

Enzymes studied

In total, 2560 samples were assayed for 11 enzymes. Whereas in the beginning 20 enzymes were stained, a clear resolution was observed only in 11 (*Table 4*).

The enzymes which showed very weak or no resolution were acid phosphatase, alcohol dehydrogenase, coniferyl alcohol dehydrogenase, esterase, formate dehydrogenase, phosphoenolpyruvate carboxylase, peroxidase, nicotinamide adenine dinucleotide dehydrogenase, sorbitol dehydrogenase. These were excluded from the further analyses.

For the 11 enzymes studied, 24 presumable loci were identified out of which 18 were found to be polymorphic. Number of alleles as observed for different putative enzyme gene loci are shown in *table 4*. The detailed description of each enzyme is as follows.

Malate dehydrogenase (MDH)

A differing number of bands ranging from 4 to 7 were visible on the gels stained for MDH. Only 4 to 6 bands are shown in

Table 4. – Enzymes, enzyme gene loci, their allelic variants and the structure of proteins encoded by the gene loci in *P. roxburghii*.

Enzymes	Gene loci	No. of alleles	Protein structure
Aconitase (ACO) E.C. 1.1.1.42	ACO-A	2	monomeric
Aspartate aminotransferase (AAT) E.C. 2.6.1.1	AAT-A AAT-B AAT-C	1 3 3	dimeric
Glutamate dehydrogenase (GDH) E.C. 1.4.1.2	GDH-A	2	hexameric
Isocitrate dehydrogenase (IDH) E.C. 1.1.1.42	IDH-A IDH-B	1 2	dimeric
Leucine-amino peptidase (LAP) E.C. 3.4.11.1	LAP-A LAP-B	2 2	monomeric
Malate dehydrogenase (MDH) E.C.1.1.1.37	MDH-A MDH-B MDH-C MDH-D	2 3 3 1	dimeric
Menadione reductase (MNR) E.C. 1.6.99.2	MNR-A MNR-B MNR-C	2 2 1	tetrameric
Phosphoglucose isomerase (PGI) E.C. 5.3.1.9	PGI-A PGI-B	2 2	dimeric
Phosphoglucomutase (PGM) E.C. 2.7.5.1	PGM-A PGM-B	3 2	monomeric
6-Phosphogluconate dehydrogenase (6PGDH) E.C. 1.1.1.44	6PGDH-A 6PGDH-B	2 1	dimeric
Shikimic acid dehydrogenase (SKDH) E.C. 1.1.1.25	SKDH-A SKDH-B	2 1	monomeric

figure 2b. However, a distinct zonation of the bands was not apparent. In endosperm extracts 4 to 5 bands were observed, whereas in embryo extracts 4 to 7 bands were detected with both the staining methods. Though there was no difference in number of bands observed with the two staining methods yet striking differences were observed with respect to staining intensities of certain corresponding bands. With staining Method 1, bands with slower migration rate showed heavy staining in comparison to the bands with faster migration on the gels. One comparatively faint band with slower migration rate was, however, observed (lane 2). In contrast, an intensely stained band with highest migration rate was unique as compared to other bands of same migration rate which were relatively faint. With Method 2, some faintly stained bands with faster migration rate were observed as compared to other bands which were darker. Separation of the bands of the embryo extracts was better observable with this method as compared to the other method (lane 1, *Figure 2b*).

THORMANN and STEPHAN (1993) have observed 2 to 5 bands in endosperms and 4 to 9 bands in corresponding embryos in *P. sylvestris* with the two staining methods. The staining intensities of the bands were also different. The authors have suggested the two methods of staining for easier recognition of the MDH isozyme loci which is based on the fact that MDH in

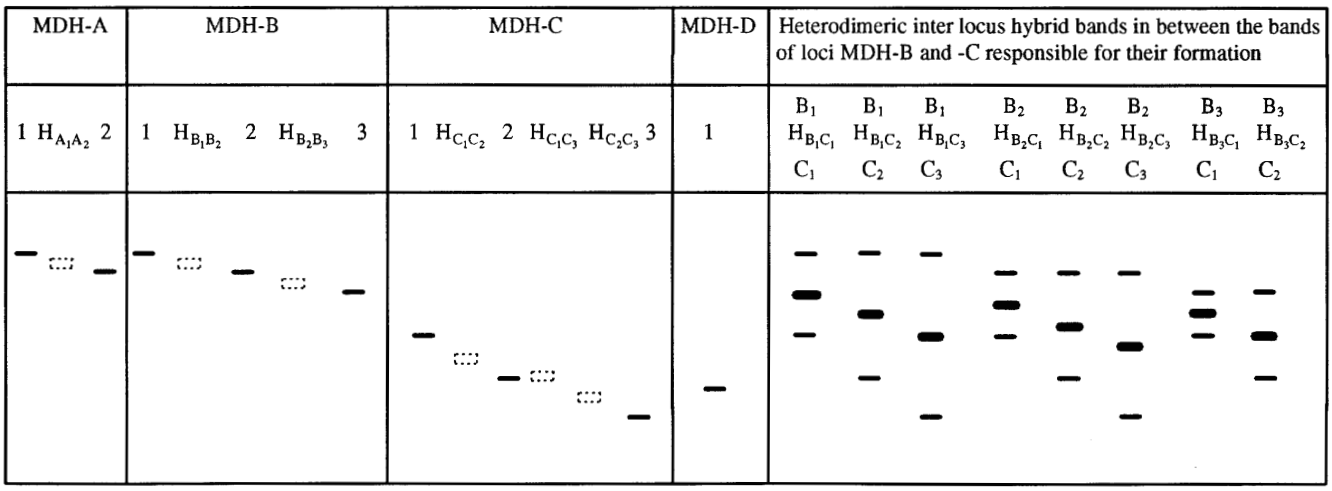

Figure 2a. – Schematic illustration of the banding patterns found for MDH in *Pinus roxburghii*. The diagramme shows the position of all the bands observed and the putative genetic interpretation of the alleles of respective loci. Bands marked by H show the inter (fat, solid) and intra (dashed) locus hybrid bands. B_3 allele was found only in a few embryos as a contribution from the male parents in the putative combination B_2B_3.

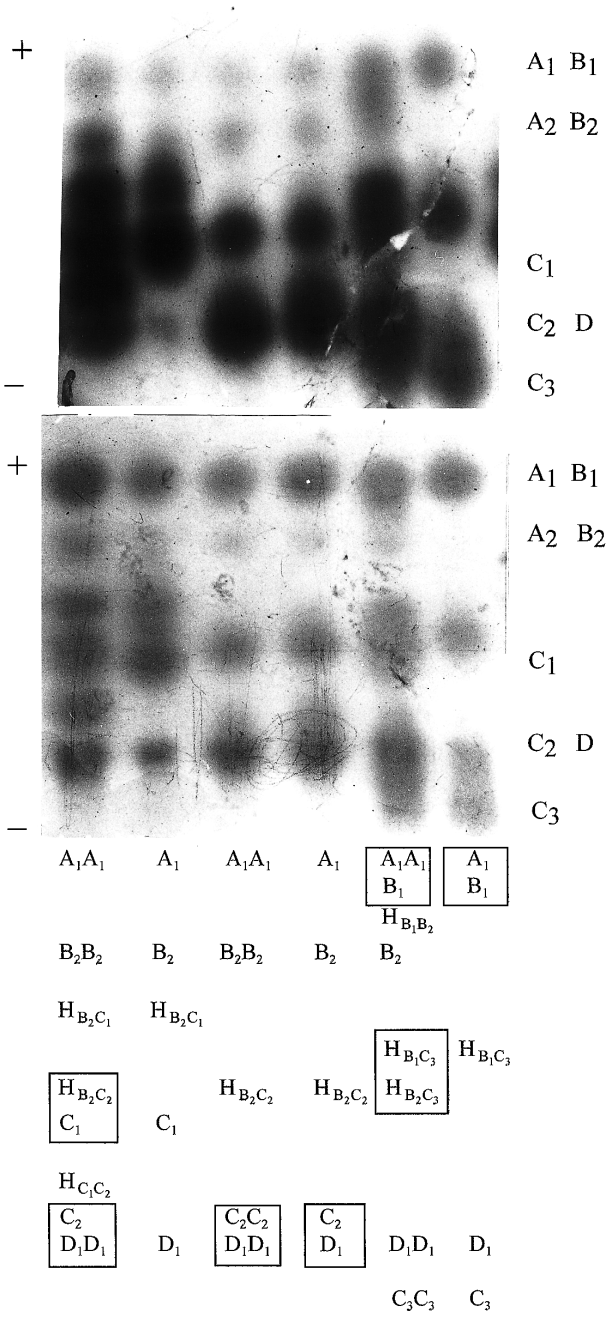

Figure 2b. – Zymograms of MDH in *P. roxburghii.*
The two gels shown originate from the same electrophoretic separation. The upper gel was stained by method 1 and the lower by method 2 (see text) which result in different staining intensities of the different zones, facilitating their interpretation. The left two lanes show the patterns of the embryo and endosperm tissues of one seed demonstrating the position of four loci and the heterodimeric inter locus hybrid band $H_{B_2C_1}$ formed between B_2 and C_1. Lane 1 also shows the inter locus hybrid band $H_{B_2C_2}$ between B_2 and C_2 and an intra locus hybrid band $H_{C_1C_2}$ between C_1 and C_2. The four lanes on the right show the banding patterns of embryos and endosperms of two seeds from a common mother tree. In the middle two lanes, the bands of C_2 and D_1 overlap and hence show only one thick band. The two lanes on the right show that the position of A_1 overlaps with that of B_1. $H_{B_1B_2}$ is an intra locus hybrid band formed between B_1 and B_2. $H_{B_1C_3}$ and $H_{B_2C_3}$ are the inter locus hybrid bands formed between B_1 and C_3, and B_2 and C_3, respectively. The migration distance of C_3 is smaller than that of D_1.

plants consists of two enzyme types: (i) non-decarboxylating MDH and (ii) oxaloacetate-decarboxylating MDH. Both MDH types using NAD as coenzyme can be stained after electrophoretic separation with the help of common tetrazolium method, where NADH + H^+ reduces to tetrazolium salt leading to the formation of blue formazan. However, the non-decarboxylating MDH which produces oxaloacetate can also be stained with a diazonium salt like fast blue BB.

As mentioned in the introduction, determination of genetic control of MDH is difficult due to a gene duplication of a certain gene locus and formation of inter locus heterodimeric hybrid bands. Hence more than four bands in endosperm extracts may indicate the presence of an inter locus heterodimeric hybrid band. Change in the position of the heterodimeric hybrid band with the changing position of certain middle bands in the zymogram indicates that the heterodimeric band was formed between two middle zones. Staining intensity and position of the heterodimeric band support our interpretation. O'MALLEY et al. (1979) and THORMANN and STEPHAN (1993) have also reported a heterodimeric hybrid band between MDH-B and MDH-C for *P. ponderosa* and *P. sylvestris* respectively. Though the staining intensities of the putatively duplicated gene loci MDH-B and MDH-C are supposed to be the same yet our results support the findings of GIANNINI et al. (1991) who have also reported the differences in staining intensities of the duplicated gene loci 6PGDH-B and 6PGDH-C in Norway spruce.

The presence of a darker band with the highest migration rate in lane 6 (*Figure 2b*) as compared to other bands of same migration rates (upper zymogram) gives an indication of overlapping of the bands pertaining to two different zones. These two putative zones, A and B showed remarkable difference in the staining intensities to distinguish these from each other. In Lodgepole (EL-KASSABY, 1981) and Scots pine (THORMANN and STEPHAN, 1993) the bands encoded by alleles of the two loci so called MDH-A and MDH-B also overlap and because of their similar migration rates appear as a single dark band. The faintly stained slower migrating band of lane 2 (upper zymogram) reflected the overlapping of the bands of two different loci we called MDH-C and MDH-D. Appearance of a higher number of bands in embryo extracts indicate the presence of intra locus hybrid bands as MDH is dimeric structured protein.

Assuming that MDH is controlled by four loci in *P. roxburghii* as in other pines, we interpreted the allozymes of respective isozyme gene loci encoded by different bands. MDH-A, MDH-B and MDH-C loci were polymorphic. MDH-D was found monomorphic as only a single isozyme variant encoded by this locus was observed. MDH-A was variable with two allozymes whereas three allozymes were observed for MDH-B and MDH-C. For MDH-A, the faster migrating allozyme i.e., A_1 was very frequent and the allozyme A_2 very rare (0.4%). MDH-B was encoded by three allozymes with highest frequency of the allozyme B_2 followed by the allozyme B_1. The allozyme B_3 was observed very seldom. Likewise for MDH-C, the allozyme C_2 was frequent followed by the allozyme C_1 and the allozyme C_3. The presence of an intralocus hybrid band in heterozygous embryo and its absence in megagametophyte tissues at MDH-C confirms the dimeric structure of MDH.

The interpretation was tested computing χ^2 values for the allelic variants of the heterozygous mother trees. Significant value of χ^2 was though recorded for MDH-A yet it can be ascribed to the small sample size (*Table 5*). χ^2-test for MDH-B was significant, however, the difference was small. Significant χ^2 values for MDH-A and MDH-B have also been reported for other conifer species (KONNERT, 1995; HERTEL, 1997). No significant deviation from 1:1 MENDELian ratio of gametic segregation was noticed for MDH-C. The total banding pattern observed is given schematically in *figure 2a*.

Table 5. – Observed and expected gametic segregation at 17 isozyme loci of heterozygous mother trees from eight *P. roxburghii* populations.

Gene locus	No. of populations	Total number of plants	Allelic variants	Observed ratio	Expected ratio	Chi-square value
ACO-A	5	44	1:2	161:191	176:176	2.556
AAT-B	1	1	1:2	4:4	4:4	0.000
	2	2	2:3	14:2	8:8	9.000*
AAT-C	1	2	1:2	6:10	8:8	1.333
IDH-B	1	7	1:2	30:26	28:28	0.285
LAP-A	8	48	0:1	153:231	192:192	15.843*
LAP-B	2	14	1:2	48:64	56:56	2.285
MDH-A	2	2	1:2	13:3	8:8	6.250*
MDH-B	7	56	1:2	185:227	206:206	4.281*
MDH-C	8	76	1:2	283:324	303.5:303.5	2.769
	1	1	1:3	4:4	4:4	0.000
	3	17	2:3	74:60	67:67	1.462
MNR-A	1	5	1:2	14:26	20:20	3.600
MNR-B	8	86	1:2	363:325	344:344	2.098
PGI-A	2	11	1:2	36:52	44:44	2.909
PGI-B	8	129	1:2	498:534	516:516	1.258
PGM-A	2	5	0:1	16:24	20:20	1.600
	1	1	1:2	5:3	4:4	0.500
PGM-B	7	39	1:2	173:139	156:156	3.705
6PGDH-A	6	34	1:2	122:150	136:136	2.882
	2	2	1:3	8:8	8:8	0.000
	6	24	2:3	108:84	96:96	3.000
SKDH-A	1	1	1:2	3:5	4:4	0.500
	7	58	2:3	260:204	232:232	6.758*

In the context of MDH in *P. roxburghii*, we conclude that the two different methods used for staining proved useful in interpreting the zymograms. In *P. roxburghii*, MDH seems to be under the control of four isozyme loci. Similarly, genetic control of MDH by four isozyme loci in *P. sylvestris* has been suggested by MUELLER-STARCK (1985), SZMIDT and MUONA (1989), and THORMANN and STEPHAN (1993).

Aconitase (ACO)

In ACO only one zone of activity was observed and two alleles could be interpreted (*Figure 3*). There was not much difference in migration rates of the two bands representing alleles 1 and 2. The latter was frequent in distribution. The χ^2-value observed for gametic segregation was not statistically significant. A single zone of ACO activity in conifers has been report-

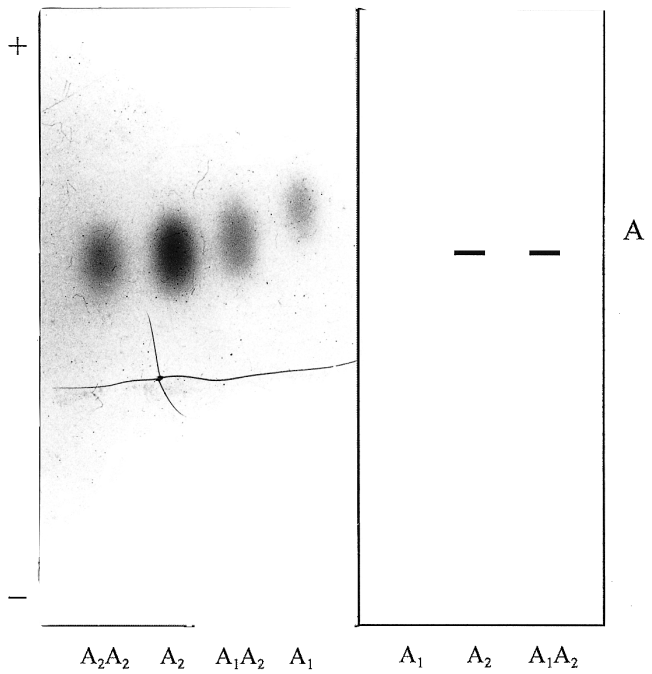

Figure 3. – Zymogram of ACO-A in *P. roxburghii* showing the relative positions of the bands representing alleles A_1 and A_2. Starting from the left, each pair of lanes represents the embryo (left) and the endosperm tissue (right) of a single seed. Below the zymogram the respective genotypes are given. On the right the positions of the bands are given schematically.

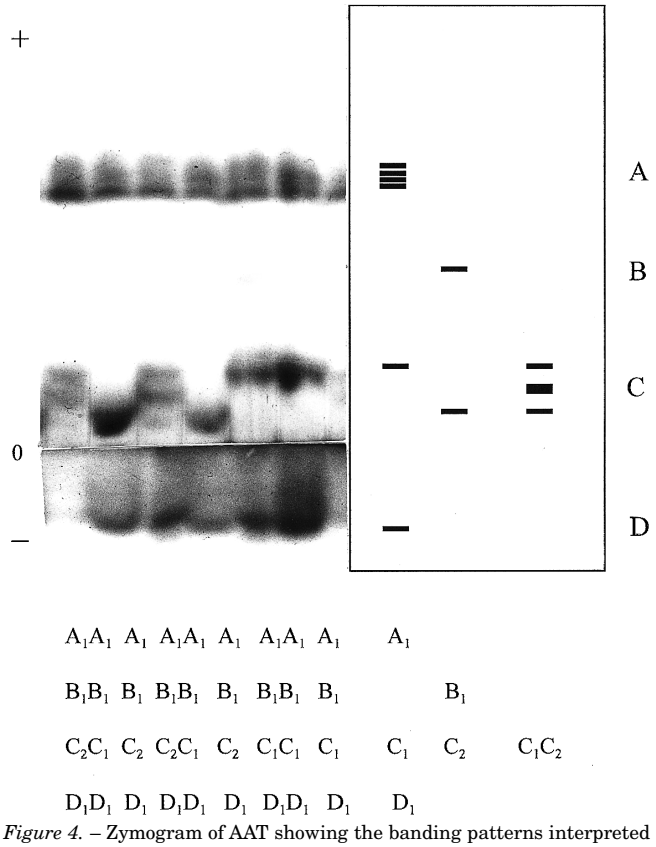

Figure 4. – Zymogram of AAT showing the banding patterns interpreted by four gene loci identified. At locus AAT-A no variation is recognisable. The variation in staining intensity observable at AAT-B locus does not follow MENDELian expectations. Because unexplainable variation in staining intensity in AAT is frequently found at AAT-B, the missing band in lane 4 is not considered to be due to a null allele. The interpretation of the variation found at AAT-C locus is given below the zymogram. The allozyme encoded by AAT-D migrates towards the cathode, the locus is monomorphic. The schematic illustration of the banding patterns is given on the right.

ed by GURIES and LEDIG (1978), ADAMS and JOLY (1980), YEH and EL-KASSABY (1980), YEH and O'MALLEY (1980), EL-KASSABY et al. (1982), and KING and DANCIK (1983).

Aspartate aminotransferase (AAT)

Four zones of activity were observed for this enzyme (*Figure 4*). The fastest and the slowest migrating zones were heavily stained. The middle zone was stained less intensively than the other two yet the resolution was enough to easily view the allozymes. The migration of AAT-C was very slow and hence found near the origin. Another zone of activity (D) migrating towards the cathodal end was also observed but without variation. In AAT-A zone, only one phenotype showing several bands of different staining intensities was observed. This occurrence of several bands might be explained by a duplication of closely linked genes. Evidence for a gene duplication in AAT was found in other tree species (KONNERT, personal communication). AAT-B and AAT-C were polymorphic with three allozymes each. The allozymes B_2 and C_2 were very frequent in their respective isozyme gene loci. Only two heterozygous mother trees out of 160 were observed for the isozyme genotype B_2B_3 of the gene locus AAT-B. The significant deviation from 1:1 simple MENDELian ratio found for the separating alleles of this genotype may be due to the small sample size.

Glutamate dehydrogenase (GDH)

Only one zone of activity was observed on the gels stained for GDH (*Figure 5*). The resolution was very good. In all megagametophytes no variation was found as only one isozyme variant encoded by a sharp band was noticed. However, in a very few embryos (heterozygous), a broader band with comparatively less staining intensity was observed which indicated the presence of second variant with slow electrophoretic mobility.

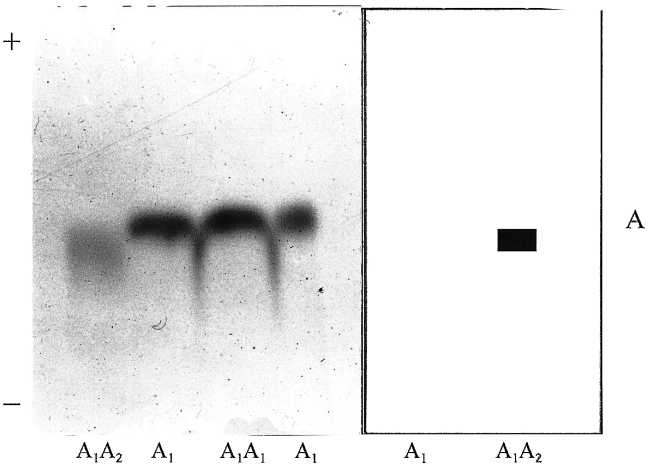

Figure 5. – Zymogram of GDH-A. The left most lane shows the banding pattern of the rare genotype. The schematic presentation of the bands is given on the right.

Isocitrate dehydrogenase (IDH)

Gels stained for IDH had two zones of activity (*Figure 6*). The faster migrating zone was found to be stained heavily in comparison to the slower one and was invariable with a single

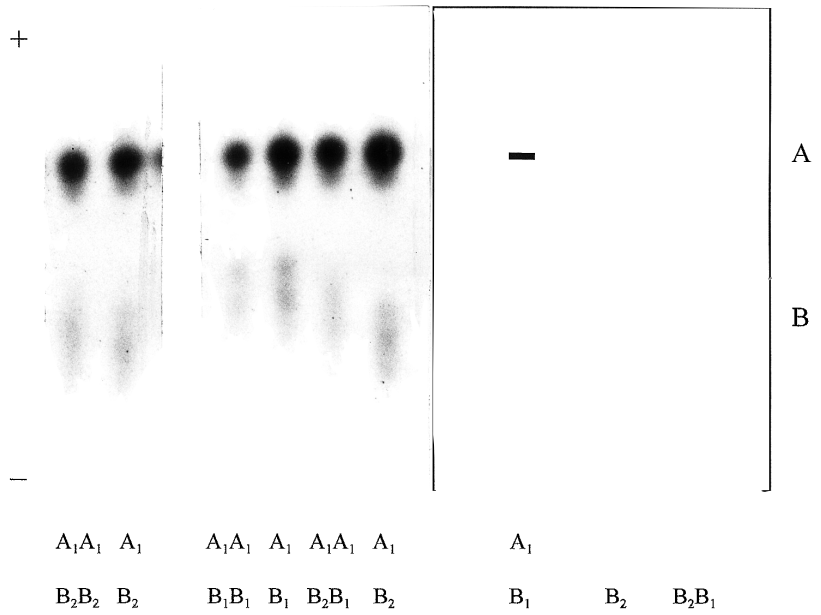

Figure 6. – Zymograms of IDH demonstrating the monomorphic banding pattern encoded by gene locus IDH-A and the variable pattern encoded by two alleles of locus IDH-B. On the right the positions of the bands are given schematically.

banded phenotype. IDH-B showed two allozymes each encoded by two faint bands. The allozyme B_1 was highly frequent whereas the allozyme B_2 was very rare. χ^2-test showed non significant deviation from the expected 1:1 segregation ratio. Many workers have reported two zones of activity for IDH in conifers (HARRY, 1986; MUONA et al., 1987; MUELLER-STARCK and LIU, 1988). However according to the one band-one gene hypothesis (NIKOLIC and BERGMANN, 1974), IDH-B should be considered to be encoded by two duplicated loci, being linked closely. For the purposes of population genetical studies it is not of much relevance because an additional locus will hardly supplement the information on the genetic structure being analysed.

Leucine aminopeptidase (LAP)

Two active zones with almost equal staining intensities were observed on gels stained for LAP (*Figure 7*). Two allozymes were found in each zone. LAP-A showed the presence of one allozyme with a single band and of other with no visible band. The absence of a band in the LAP-A zone when accompanied by the activity of an allozyme or isozyme at LAP-B zone was interpreted to reflect a null allele at LAP-A locus. Under LAP-B zone the difference between the migration distances of the bands representing the two alleles was very narrow. Hence one should be very careful while making genetic interpretation of LAP-B for *P. roxburghii*. The presence of a wide thick band from heterozygous embryos was helpful in differentiation between the two allozymes. Inheritance of allozymes of LAP-B zone was in accordance with 1:1 segregation ratio. However, segregation distortion was found for LAP-A as reported by many authors in other conifers (KONNERT, 1995; HERTEL, 1997).

Menadione reductase (MNR)

Gels stained for MNR showed three zones. MNR-A was intensively stained as compared to MNR-B. The slowest migrating zone, MNR-C was very faint and hence not observable in *figure 8*. The MNR-A and MNR-B allozymes were found to be coded by two alleles each. Segregation of these two loci was in close conformity with MENDELian inheritance (1:1). In zone MNR-B, two bands representing the allozymes B_1 and B_2 coupled with three bands in between were observed in heterozygous embryos. This is to be expected due to the tetrameric structure of this enzyme.

Phosphoglucose isomerase (PGI)

PGI had two polymorphic zones of activity (*Figure 9*). Both the zones were found equally intensively stained. Two allozymes were observed for each zone. In PGI-A, the faster migrating allozyme was rare whereas in PGI-B, major polymorphism was observed as both the allozymes showed almost equal frequencies. No significant deviations were noticed for the observed and expected ratios of gametic segregation for the alleles of both loci. Two zones of PGI activity have usually been reported in many other conifer species by different authors (GURIES and LEDIG, 1978; ADAMS and JOLY, 1980; EL-KASSABY et al., 1982; HARRY, 1986; MUONA et al., 1987; WANG et al., 1991).

Phosphoglucomutase (PGM)

Two zones of activity were evident from the gels stained for PGM. The anodal zone was stained intensely whereas the cathodal one very faint and hence does not appear in *figure 10*. For PGM-A, two allozymes were observed. In addition, we have found a third type represented by a null allele. Whereas for PGM-B, only two variants were noticed. In heterozygous mother trees both zones segregated as single bands with observed segregation ratios not significantly different from the expected 1:1 ratio. The presence of two bands in heterozygous embryo and single band in homozygous one reflects the monomeric structure of this enzyme. Two PGM loci appear to exist in the majority of conifer species (MITTON et al.,1979; ECKERT et al., 1981; NEALE and ADAMS, 1981; LOUKAS et al., 1983; STEWART and SCHOEN, 1986).

6-phosphogluconate dehydrogenase (6PGDH)

There were two zones of activity with almost similar staining intensities on the gels stained for 6PGDH (*Figure 11*). 6PGDH-A had three allozymes whereas 6PGDH-B was monomorphic. The allozyme A_2 was frequent followed by the allozy-

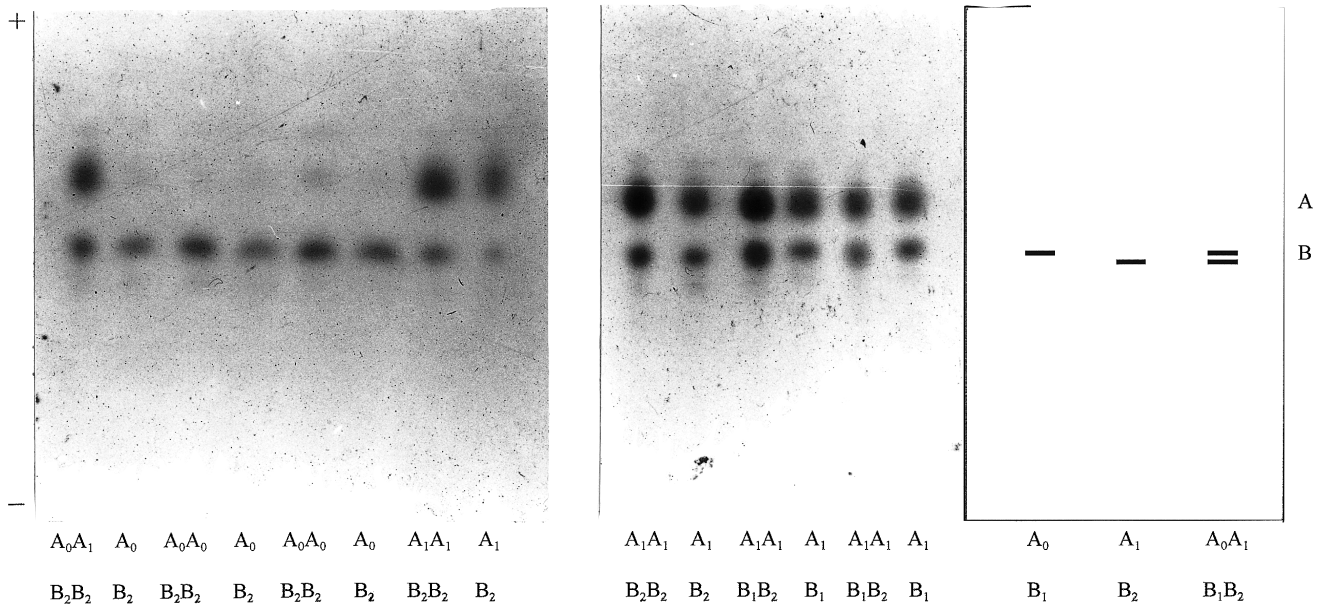

Figure 7. – Zymograms of LAP.
For LAP-A the left zymogram shows the bands representing allele LAP-A_1 whereas missing bands are interpreted as absence of enzymatic activity encoded by the allele LAP-A_0. Thus, the mother tree is heterozygous for A_0A_1. It is not known whether seeds of genotype A_0A_0 are able to grow. The right zymogram shows for LAP-B the relative positions of the bands representing alleles B_1 and B_2. The positions of the bands are drawn schematically on the right.

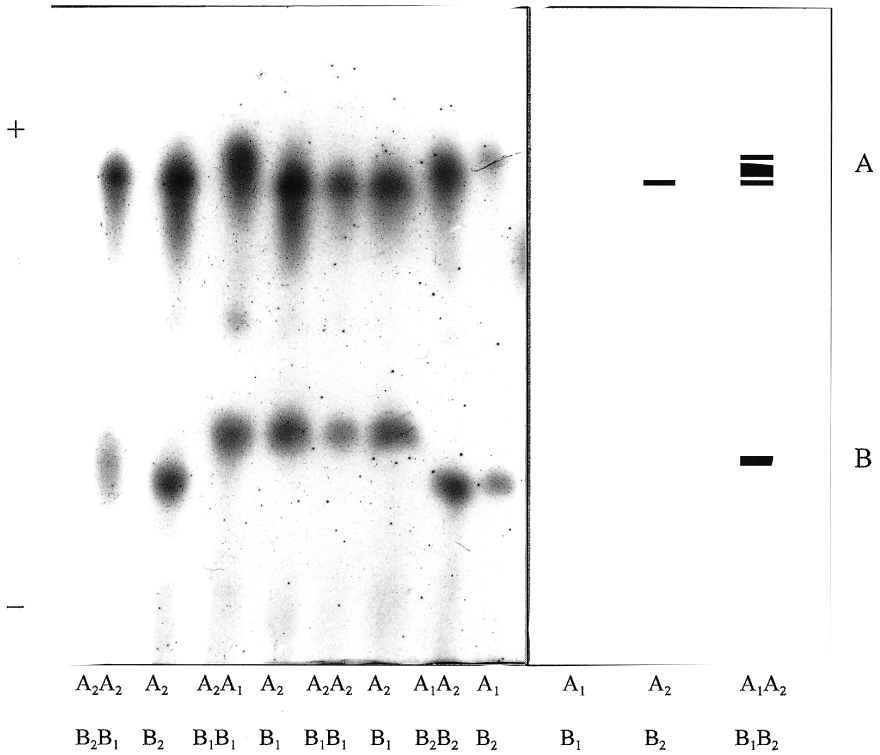

Figure 8. – Zymogram of MNR demonstrating the relative positions of the bands representing the MNR-A and -B locus. The banding patterns are given schematically on the right.

me A_1. The allozyme A_3 was found to be very close to the slower migrating zone (6PGDH-B). The observed segregation was in accordance with the MENDELian inheritance of 1:1 ratio.

Shikimic acid dehydrogenase (SKDH)

Two zones of activity were observed on the gels stained for SKDH. Whereas clear resolution was observed for the faster migrating zone, the slower one showed very weak staining which is hardly visible in *figure 12*. Three allozymes were recorded for SKDH-A locus. The allozyme A_1 was rare while the allozyme A_2 occurred frequently followed by the allozyme A_3. Segregation distortion was observed for this locus which is also well documented in the literature for *P. sylvestris* (HERTEL, 1997) and *Abies alba* (KONNERT, 1995).

These results contribute to an understanding of the inheritance of enzyme gene loci of *P. roxburghii* and thus have

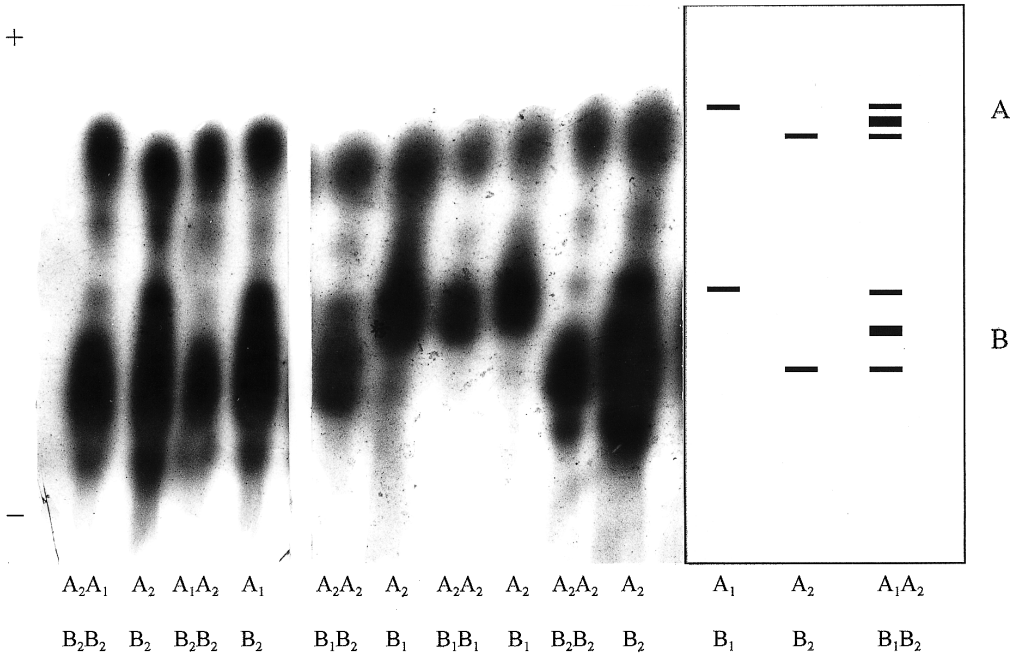

Figure 9. – Zymograms of PGI showing the banding patterns representing loci PGI-A and -B. The left four lanes belong to seeds from a different tree than those six on the right. The endosperm tissue seems to contain higher amounts of the enzyme which lead easily to overstanding. The schematic presentation of the bands is given on the right.

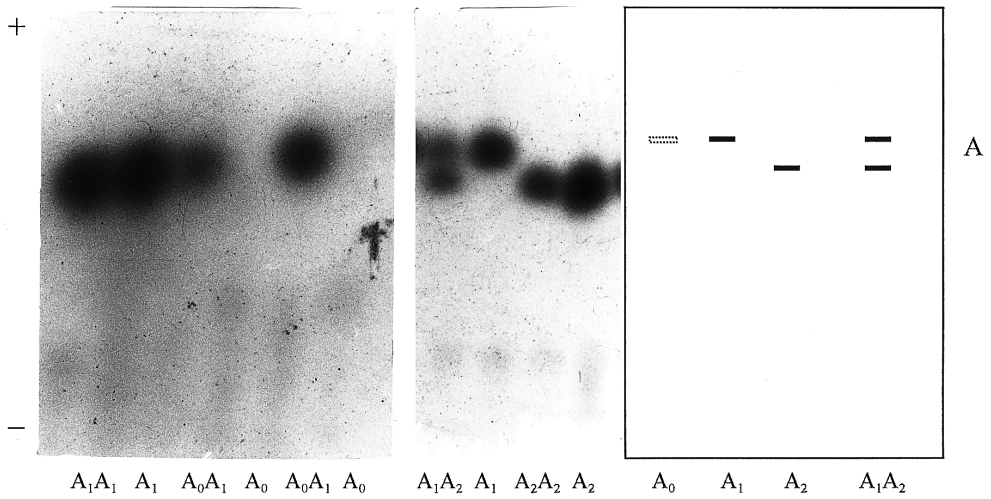

Figure 10. – Zymograms of PGM-A demonstrating the relative positions of the bands pertaining to alleles A_1 and A_2. The presence of null allele (A_0) is obvious by the absence of bands in lane 4 and 6 of the left zymogram. The schematic illustration of the bands is shown on the right.

important implications to study the genetic structure of the species. The interpretation of zymograms is the first and foremost step for generating data to estimate genetic variation present in a species, at isozyme level. The confirmation of genetic control of the allozymes with simple MENDELian inheritance facilitates their use as gene markers in other studies. The two staining methods proved useful to determine the genetic control of MDH. With method 1, zone 3 was stained intensely as compared to other three zones viz., 1, 2 and 4 whereas with method 2, all zones except zone 2 were stained heavily. Overlapping bands of zone 3 and 4 can be separated with staining method 1 because of different staining intensities of these two zones with this method which was not possible with staining method 2 which showed almost equal staining intensities for these zones. Different staining intensities of different zones with two staining methods facilitated identification of four loci of MDH in *P. roxburghii* as in *P. sylvestris*. It is advisable to employ the two staining methods for further studies on genetics of MDH in *P. roxburghii*, or other species.

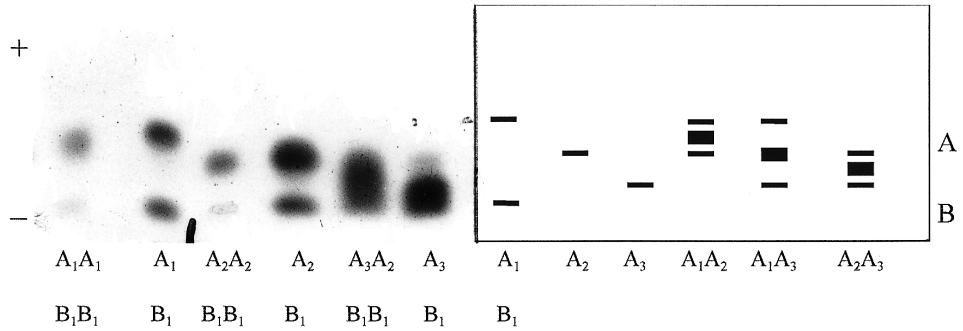

Figure 11. – Zymogram of 6PGDH demonstrating the relative positions of the bands pertaining to 6PGDH-A and -B loci, the latter being monomorphic. The left two lanes belong to one seed from a different mother tree than those four to the right. On the right the banding patterns are drawn schematically.

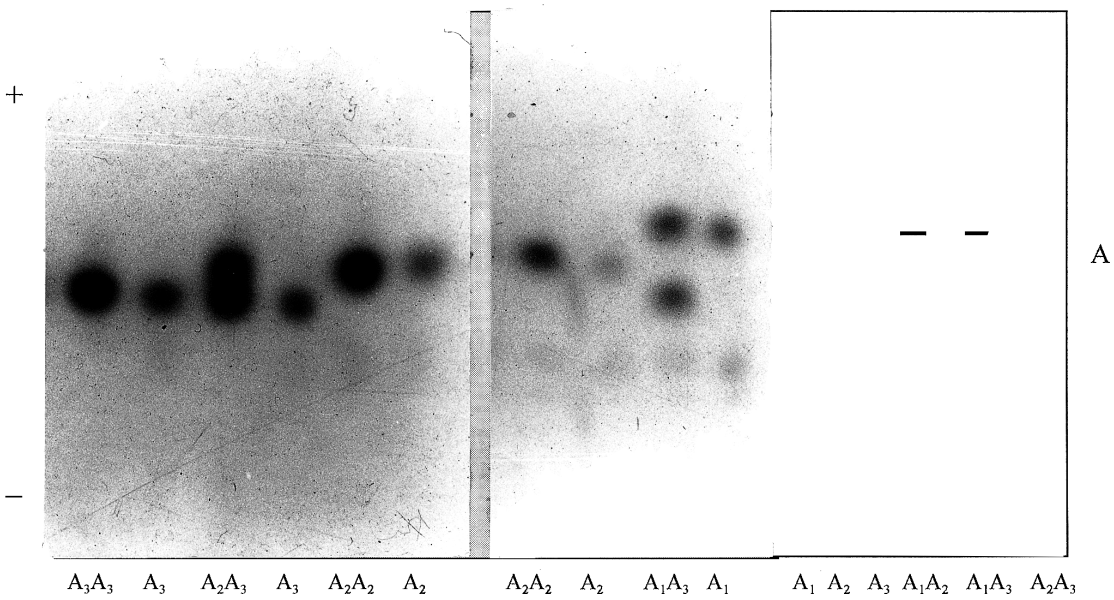

Figure 12. – Zymograms demonstrating relative positions of the different allozymes found to be encoded by the SKDH-A locus. Each gel contains samples from a different tree. The schematic illustration of the bands is shown on the right.

Acknowledgment

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Application of Ovary and Ovule Culture in *Populus alba* L. x *P. euphratica* OLIV. Hybridization

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Summary

An *in vitro* method was used to develop *Populus alba* x *P. euphratica* hybrid plants. Developed ovaries and ovules were isolated from *P. alba* female branches which were pollinated with *P. euphratica* pollen grains by using twig and pot breeding technique. Isolated ovaries and ovules were then transferred to growth regulator free half concentrated MS agar medium for embryo germination. Maximum three plantlets were observed per ovary and more than 90% of cultured ovules successfully produced plantlets, whereas the efficiency of ovary culture to produce plantlets was 70%. Plantlets were cultured in the same medium within jars, before being transferred to potting soil. Seventy five interspecific *P. alba* L. x *P. euphratica* OLIV. hybrid plants were successfully acclimatized in greenhouse.

Key words: Ovule culture, embryos, ovary, *Populus euphratica, Populus alba,* interspecific hybridization.

FDC: 165.442; 165.72; 163; 176.1 *Populus alba;* 176.1 *Populus euphratica.*

Introduction

There has been a long sustained interest in the hybridization of poplars largely by the benefits derived from capturing heterosis and combining desirable traits ultimately expressed in the amount and quality of wood production (STETTLER, 1980). Owing to the resistance to drought and salinity, *Populus euphratica* OLIV. was chosen as parental species in Populus breeding, but the incompatibility was observed between this species with some of other poplar species (WILLING and PRYOR, 1976). During last decade, a number of observations have been made on pollen-stigma interactions in interspecific crosses or Populus, with emphasis on the sections Aigeiros, Leuce and Tacamahaca (GAGET *et al.,* 1984; GURIES and STETTLER, 1976; HESLOP-HARRISON, 1975; KNOX *et al.,* 1972). Except for interspecific combination involving Leuce poplars and Aigeiros-Tacamahaca poplars, most hybridization experiments were reported to be successful with methods of bottle grafting and the technique of twig and pot (technique described in KOUIDER *et al.,* 1984; see also RAJORA and ZSUFFA, 1984). The reasons for incompatibility in trees can be typically due to premature abscission of flowers, early dehiscence of the capsules, pollen mortality, grafting failure and some unknown physiological disorders occurring within the flowering branches (reviewed in RAMMING, 1990 for fruit trees). In addition, with interspecific crosses, intergeneric and crosses between diploids and tetraploids, the endosperm often develops poorly or not at all. By aseptically culturing the embryo in a nutrient medium, this problem may be overcome. This technique was used in many different crosses and seems to be amenable to large-scale application in poplar hybridization (LI *et al.,* 1983; KOUIDER *et al.,* 1984; LI and LI, 1985; NOH *et al.,* 1986 and SAVKA *et al.,* 1987).