Table 6. — Comparison of different layouts for minimum number of clones with 4 ramets in each case.

Type of design	Number of clones	Repeatable design
Rectangular	60	No
Rectangular	50	No (Outer ring corners ignored)
Triangular	36	No
Hexagonal	20	Yes
Hexagonal	16	No

point A could in certain cases end up within the crown area of the plant at point G. Point B and similarly located points D, E, F on the outer ring could, therefore, be ignored in adjacency tests the same way as B.

In square layout it is not possible to give equal chance to all clones in the inner and outer rings due to the inherent inequality of the distances between centre and corners, and centre and midpoints.

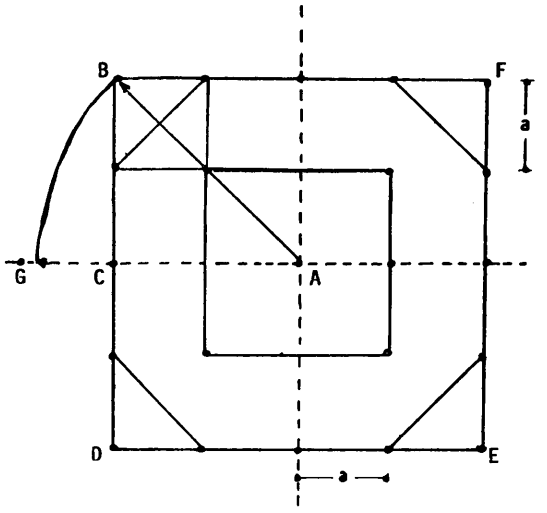

Fig. 1. — Schematic diagram showing corner plants on outer ring.

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Interpretation of Isozyme Patterns of Malate Dehydrogenase in Scots Pine Using Two Different Staining Methods

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(Received 21st April 1992)

Summary

The isozyme system malate dehydrogenase was analysed by means of horizontal starch gel electrophoresis using crude extracts of megagametophytes and embryos from Scots pine seeds. Two different staining methods were used to distinguish the loci coding for the enzyme system. It was shown that genetics of malate dehydrogenase in Scots pine can be explained with the presence of 4 coding loci. It is proposed to use both staining methods in studies of the enzyme system.

Key words: Pinus sylvestris, malate dehydrogenase, gene duplication, enzyme gene marker.

Zusammenfassung

Das Isoenzymsystem Malat-Dehydrogenase wurde bei Kiefer (*Pinus sylvestris* L.) mittels Stärkegelelektrophorese von Rohextrakten aus Megagametophyten und Embryonen untersucht. Zur Unterscheidung der einzelnen Genloci wurden 2 verschiedene Färbemethoden verwendet. Es wird gezeigt, daß die Genetik der Malat-Dehydrogenase mit 4 kodierenden Genloci erklärt werden kann. Es wird vorgeschlagen bei Untersuchungen des Enzymsystems beide Färbemethoden einzusetzen.

Introduction

Isozyme studies have become a useful tool in several areas of genetics. They possess practical importance for studies of population genetics and phylogenetic traits (e. g. papers in Tanksley and Orton, 1983). The basis for a correct interpretation of isozyme patterns obtained from electrophoretical studies is a complete knowledge of the modes of inheritance (Hattemer, 1991).

In Scots pine (*Pinus sysvestris* L.) the enzyme system of malate dehydrogenase is coded by genes at more than one locus. Due to the difficulty of interpreting many bands the number of loci considered to be responsible for the genetic control of the enzyme system varies between 3 (Rudin and Ekberg, 1978; Yazdani *et al.*, 1985; Muona and Harju, 1989; Prus-Glowacki and Siwecki, 1990) and 4 (Müller-Starck, 1985; Szmidt and Muona, 1989). Although the same isozyme patterns were observed by the different authors, they were interpreted in different ways.

The present paper reports an interpretation of the genetics of malate dehydrogenase in Scots pine using two different staining methods.

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Material and Methods

The study bases on the analysis of seed samples from 189 open pollinated *Pinus sylvestris* trees of a non-autochthonous stand in northern Germany (described in THORMANN *et al.*, 1991) and further samples from 175 open pollinated trees of 7 Spanish native stands (for details of provenances see PARDOS and STEPHAN, 1988).

The isozyme system malate dehydrogenase (E.C.1.1.1.37) was analysed by means of horizontal starch gel electrophoresis using crude extracts of megagametophyte and embryo tissue isolated from seeds. The tissue was homogenised in a Tris/HCl pH 7.5 buffer. Two staining methods were used: Method 1: staining of NADH, the co-substrate of the enzymatic reaction (SHAW and PRASAD, 1970, slightly modified). Method 2: staining of oxaloacetate, the end product of the enzymatic reaction, with a solution containing 90 mg L-malic acid, 20 mg NAD and 220 mg Fast Blue dissolved in 60 ml Tris/HCl pH 9.5 buffer solution. The specifity of MDH for the co-substrate was tested with method 2 and a staining solution where the co-substrate NAD was replaced by NADP.

Results

On gels stained for NADH (method 1) between 2 and 5 bands could be distinguished in megagametophytes of Scots pine seeds (*Fig. 1a*). The band with the fastest migration rate was clearly visible in all cases. The most intensive staining could be observed in some cases for the band with the slowest migration rate.

In the corresponding embryos the number of bands varied between 4 and 9 bands (*Fig. 2a*). In some embryos a blurred band appeared in the most cathodal zone of the gel.

Slices of the same gel stained for oxaloacetate (method 2) show equal numbers of bands in megagametophytes compared to those stained with method 1. However, in some cases the fast migrating band could be distinguished from other bands by very weak staining patterns (see A1 + A2 in

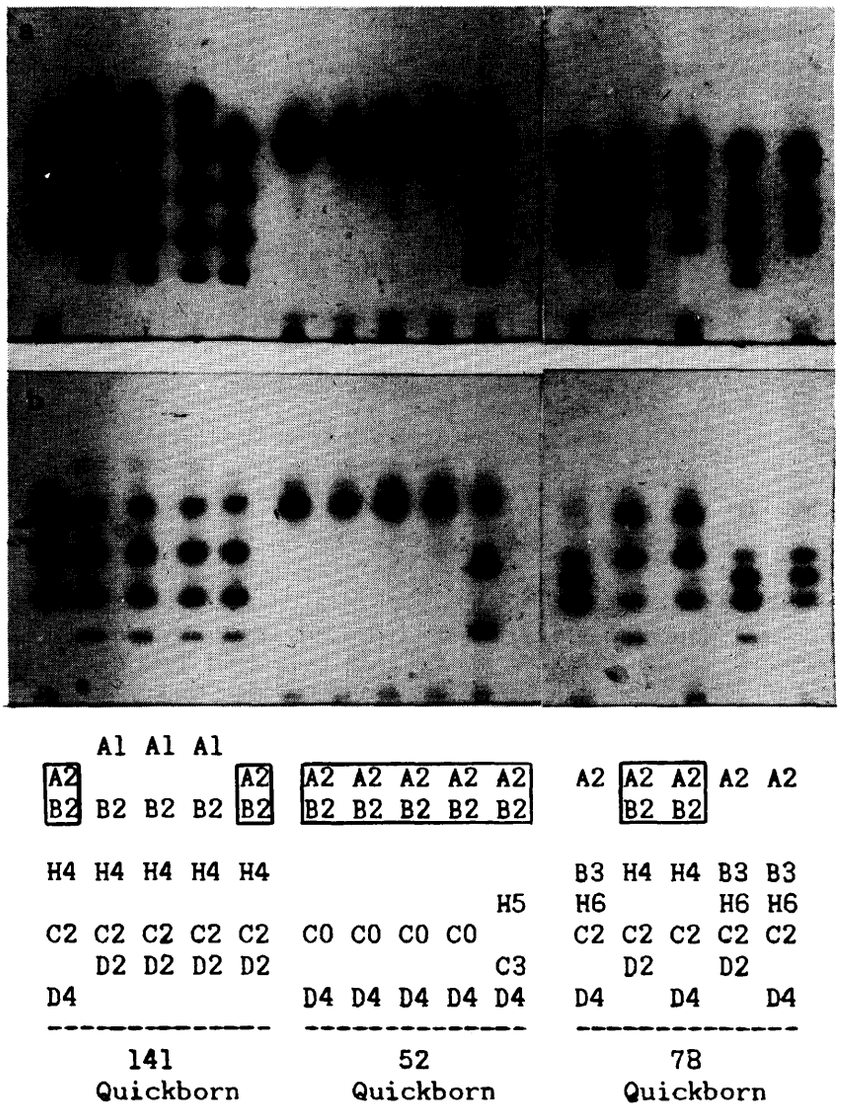

Figure 1. — Electrophoretic patterns of MDH extracted from megagametophytes of Scots pine. Slices of the same gel were stained with 2 methods. 1a: megagametophytes stained with method 1. 1b: megagametophytes stained with method 2. Origin of seed samples, tree numbers and phenotypes are indicated below the zymograms. Alleles with the same migration rate are depicted in rectangles. Interlocus hybrid bands are: H1= B1/C2; H2= B1/C3; H3= B2/C1; H4= B2/C2; H5= B2/C3; H6= B3/C2.

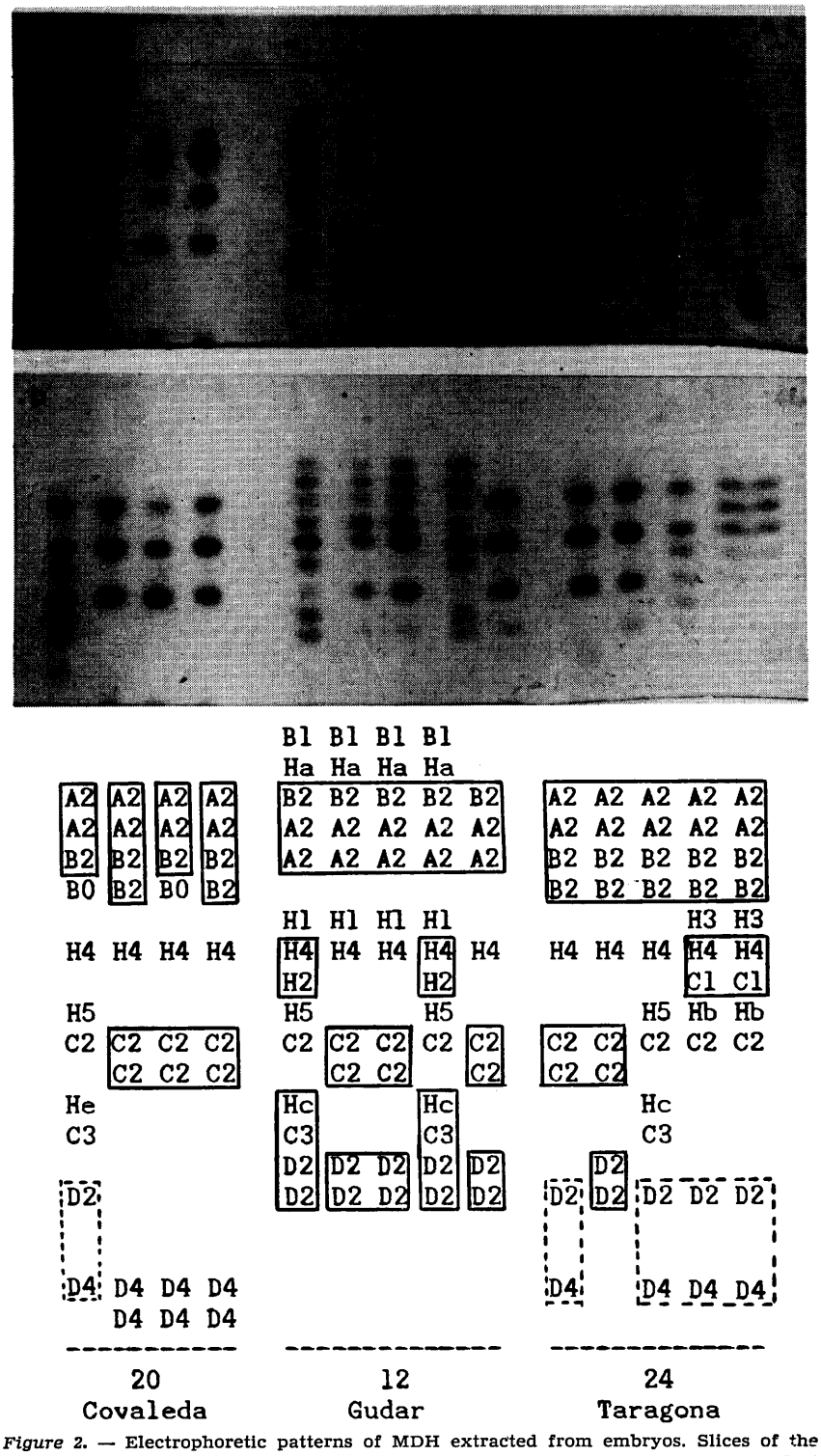

Figure 2. — Electrophoretic patterns of MDH extracted from embryos. Slices of the same gels were stained with two staining methods. 2a: embryos stained with method 1. 2b: embryos stained with method 2. Blurred bands of heterozygotes at locus MDH-D are depicted in hatched rectangles. Hybrid bands are: Ha= B1/B2; Hb= C1/C2; Hc= C2/C3. For further explanation and designation of interlocus hybrid bands see *figure 1.*

Fig. 1b). A weaker staining intensity was also observed for the most cathodal band (D2 + D4 in *Fig. 1b*), but the differences between both staining methods were less obvious compared to the most anodal bands. In megagametophytes with more than 3 bands a strongly stained band always appeared in a middle position between a fast and a slow migrating band, showing the characteristics of an interlocus hybrid band between locus B and locus C (see hybrid bands H4—H6 in *Fig. 1*).

In the diploid tissue of the corresponding embryos additional bands were observed like on gels stained with method 1 (*Fig. 2b*). According to results found in megagametophytes some of these additional bands can be explained as interlocus hybrid bands (H1-H5 in *Fig. 2*). In

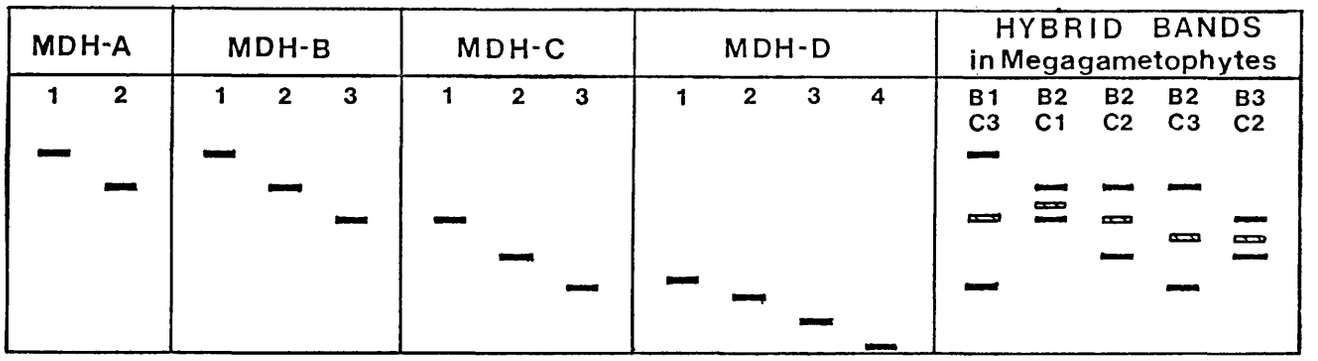

Figure 3. — Allozyme patterns, designation of gene loci and alleles of malate dehydrogenase in Scots pine. The most anodal locus is designated as A; the most anodal allel of each locus as 1. Hybrid bands found in megagametophytes between the putative duplicated loci B and C are indicated by hatched bars.

some cases additional bands in the fast migrating zone appeared only faintly after staining with method 2. Obviously this band is a hybrid band between alleles A1 and A2 of a locus which was only weakly stained with this method, while another band with the same migration rate (Ha in *Fig. 2b*) is an interlocus hybrid band between alleles B1 and B2 of a locus which is clearly visible with method 2. A schematic drawing of alleles, inter- and intralocus hybrid bands found in megagametophytes is given in *figure 3.*

Staining for oxaloacetate (method 2) with a staining solution where the co-substrate NAD was replaced by NADP shows no staining activity. No bands were found as well after staining for NADH (method 1) when using a solution which contained NAD but not the substrate L-malic acid.

Null alleles were found at loci B and C. In some cases embryos with the double null phenotype MDH C0/C0 were found as well.

Discussion

The isozyme patterns of MDH have been used for numerous genetic studies on pine species. However inconsistency in the reported number of loci coding for the enzyme still exists and could lead to different interpretations obtained from the same data (e.g. estimates of heterozygocity). The present study shows that the genetics of MDH in Scots pine can be explained by the presence of 4 coding loci (see also El-Kassaby, 1981; Müller-Starck, 1985; Szmidt and Muona, 1989) better than with the hypothesis of double and triple bands in the zone called locus MDH B by Rudin and Ekberg (1978).

The presence of interlocus hybrid bands between loci MDH B and MDH C in megagametophytes of Scots pine is an important criterion for duplication of gene loci (Gottlieb, 1982; Weeden, 1983; Jones et al., 1986). Duplication of genes coding for isozyme systems has been documented for several species (see Gottlieb, 1982), but evidence for a gene duplication in gymnosperms has only been reported for Norway spruce (*Picea abies* (L.) Karst.) by Giannini *et al.* (1991). Null alleles were only found at the putatively duplicated gene loci MDH B and MDH C.

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