# INHERITANCE OF ISOZYME PHENOTYPES IN *PTEROCARPUS INDICUS* WILLD.

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## ABSTRACT

We used horizontal starch gel-electrophoresis to observe isozyme phenotypes of narra (*Pterocarpus indicus* Willd.) at 6 polymorphic enzyme systems. An inheritance study was based on the observation of segregation within single tree progeny arrays of putatively heterozygous seed trees. Only primary leaves of young seedlings, but no material of the seed trees were available for investigation. We were able to test hypotheses on the genetic control of observed variation patterns at five enzyme systems (IDH, MNR, SKDH, MDH, PGM), but failed to explain the complicated polymorphic patterns observed at 6-PGDH.

**Key words:** *Pterocarpus indicus* Willd., isozyme, inheritance, single tree progenies

## INTRODUCTION

The investigation of the genetic system of forest tree species critically depends on the availability of easily observable genetic markers. The formal identification of a gene marker usually requires the observation of phenotypes of closely related individuals. The currently most widely used genetic markers in forest genetics are based on isozyme phenotypes.

Many isozyme systems show a high degree of developmental and environmental stability (HATTEMER 1991). However, the occurrence of completely recessively behaving "null-alleles", overlapping of zones, intra- and intergenic heterodimers, and "artefacts" or multiple bands at a zone even for homozygous individuals can complicate the interpretation of zymograms (*e.g.* FINKELDEY 1992). Furthermore, both environmental and developmental modifications have been reported for various enzyme systems (*e.g.* MÜLLER-STARCK & STARKE 1993).

The conventional approach for the identification of a gene locus involves crossing of two parents and the observation of segregation ratios in full-sib families (MENDEL 1866). At least one of the parents has to be heterozygous at the putative gene locus, and the phenotypes of both parents should be known. Another methods of genetic analysis of isozyme phenotypes is the observation of segregation in the haploid megagametophyte ("primary endosperm") of gymnosperms (BERGMANN 1974). However, the endosperm of most angiosperms is triploid. Furthermore, is not developed in the mature seed of many angiosperm forest species including *Pterocarpus indicus*.

Many forest tree species are notoriously difficult to cross. This applies in particular to most tropical species with a poorly understood reproductive biology and short viability of pollen. GILLET and HATTEMER (1989) proposed a genetic analysis, which is based on the observation of segregation in open-pollinated single tree progenies of putatively heterozygous seed trees. We used this approach to test hypotheses on the genetic control of isozyme phenotypes of *Pterocarpus indicus* Willd. The only material available for investigation were seeds and young seedlings. No material from seed trees could be investigated. The putative genotype of the seed trees had to be inferred from a sample of its open-pollinated offspring.

*Pterocarpus indicus* (local name: Narra; family: Fabaceae) is one of the most important timber species of the Philippines. The species occurs naturally throughout the Philippines and many other countries in Southeast-Asia (ABARQUEZ 1994). To the best of our knowledge this is the first study aiming at the identification of isozyme gene markers for a tree species native to the Philippines.

## MATERIALS AND METHODS

Fruits were harvested from 26 trees in the Mt. Makiling area close to Los Baños on Luzon island, Philippines,

in late July 1995. At least 200 fruits were harvested from each tree. The seeds were extracted from fruits and stored at 4 °C. Each fruit contains up to 4 seeds. Seeds from single seed trees were carefully kept separately.

The seeds were germinated and seedlings grown for approximately 2 weeks until primary leaves developed. We used young primary leaves of approximately 0.5–1 cm size for the investigation.

Fresh leave tissue was ground in 3 drops of an extraction buffer (0.08M Tris-HCl, pH 7.2, with 4% polyvinyl-pyrrolidone (PVP 40), 1% polyethylene glycol (PEG), 0.2% (Triplex-II) ethylenediaminetetraacetic acid (EDTA), 0.08% cysteine-HCl, 0.05% dithiotreitol (DTT), (all % are w/v), and 1% (v/v) mercaptoethanol). Paper wicks saturated with the homogenate were inserted into starch gels (12% starch concentration; SIGMA starch). The electrode buffer was 0.135 M Tris – 0.043 M citric acid, the gel buffer 0.042 M Tris – 0.013 M citric acid. The pH of both electrode- and gel-buffer was adjusted to 6.7 for PGM, MNR and MDH, and to 7.3 for IDH, SKDH and 6-PGDH. Electrophoresis was conducted for 5 ½ hours at approximately 20 $V \cdot cm^{-1}$ (max. 130 mA). Staining procedures were slightly modified from those described by CHELIAK and PITEL (1984).

The following enzyme systems were investigated (in brackets: abbreviations; E.C. Nos.): isocitrate dehydrogenase (IDH; 1.1.1.42); menadione reductase (MNR; 1.6.99.2); phosphoglucomutase (PGM; 2.7.5.1); shikimic acid dehydrogenase (SKDH; 1.1.1.25); malate dehydrogenase (MDH; 1.1.1.37); 6-phosphogluconate dehydrogenase (6-PGDH; 1.1.1.44).

We observed isozyme phenotypes at these 6 enzyme systems of a total of 810 progenies from 13 seed trees. We investigated 29 to 115 progenies from each seed tree. The heterogeneous sample sizes were due to fungal infection during seed storage and low germination of several progeny arrays.

Based on experience from other tree species we formulated hypotheses on the genetic control of the observed polymorphic variation patterns. Vegetative material from the seed trees was not available for investigation. Thus, we inferred the putative genotype of the seed trees from an investigation of a sample of its open-pollinated offspring.

The occurrence of two different putative homozygotes ($A_iA_i$ and $A_kA_k$) at a particular gene locus in the offspring of a seed tree indicates heterozygosity of the seed tree ($A_iA_k$) at that locus. The occurrence of the same allele $A_i$ in all progeny genotypes (all progeny genotypes $A_iA_x$ with $x = i,j,k,...$) usually indicates homozygosity $A_iA_i$ of the respective seed tree. However, if an otherwise rare allele $A_r$ was observed in high frequencies in the offspring of a particular seed tree, we assumed this tree to be heterozygous $A_iA_r$, even if the homozygous genotype $A_rA_r$ was not observed in the offspring (progeny genotypes either $A_iA_x$ or $A_rA_y$ with $x = i,j,k,...,r,...$ and $y = j,k,...; y \neq r$).

Let $P(A_xA_y)$ denote the absolute frequencies of the genotype $A_xA_y$ in the progeny of a single seed tree. Assuming co-dominance of all alleles it holds for the segregation in the open-pollinated offspring of a heterozygous seed tree ($A_iA_k$): $P(A_iA_k) = P(A_iA_i) + P(A_kA_k)$, and $P(A_iA_x) = P(A_kA_x)$ for $x \neq i,k$ (GILLET & HATTEMER 1989). We tested the deviation of the observed segregation in the progenies of putatively heterozygous seed trees from these expectations for statistical significance using the $\chi^2$-test (SOKAL & ROHLF 1981: 701ff.) and exact probabilities $p$ to obtain deviations from an expected 1:1 ratio equal to or greater than the observed ratio based on a binomial distribution (two-tailed test; SOKAL & ROHLF 1981: 70ff.).

## RESULTS

The observed isozyme phenotypes for the 6 enzyme systems are depicted in Figure 1. The putative genotypes at a polymorphic gene locus are given below each banding pattern for 5 enzyme systems. Positions of zones (A, B, C) and alleles (1, 2, 3, 4) are indicated at the left of the drawings. The phenotypes of putatively homozygous genotypes are shown on the left of the dashed line, phenotypes of putatively heterozygous genotypes are depicted on the right side for each gene locus. The position of rare alleles, which were observed only in heterozygotes (allele $A_1$ and $A_3$ for IDH, $C_3$ for MNR), are also included on the left with their unobserved homozygous genotypes in brackets.

Double bands for putatively homozygous individuals were observed for the upper zone of both IDH and SKDH. The staining intensity of these double bands varied among the seedlings, the lower band being either weaker than the upper band or of the same staining intensity. The heterozygous genotype $A_1A_4$ at the *Idh-A* locus showed two clearly separated bands between the position of the alleles $A_1$ and $A_4$. The bands at the *Mnr–C* zone were very broad and of intense staining. In most plant species MNR is a multimeric enzyme, but we failed to count the number of intragenic heterodimers in phenotypes of heterozygotes. Both PGM and SKDH are monomeric systems according to the observed phenotypes. MDH phenotypes showed only three bands for homozygotes and five bands for heterozygotes. No genetic interpretation is suggested for the zones *Mnr–A* and *–B*, *Pgm–B*, and *Skdh–B*, which were monomorphic and of much weaker staining intensity

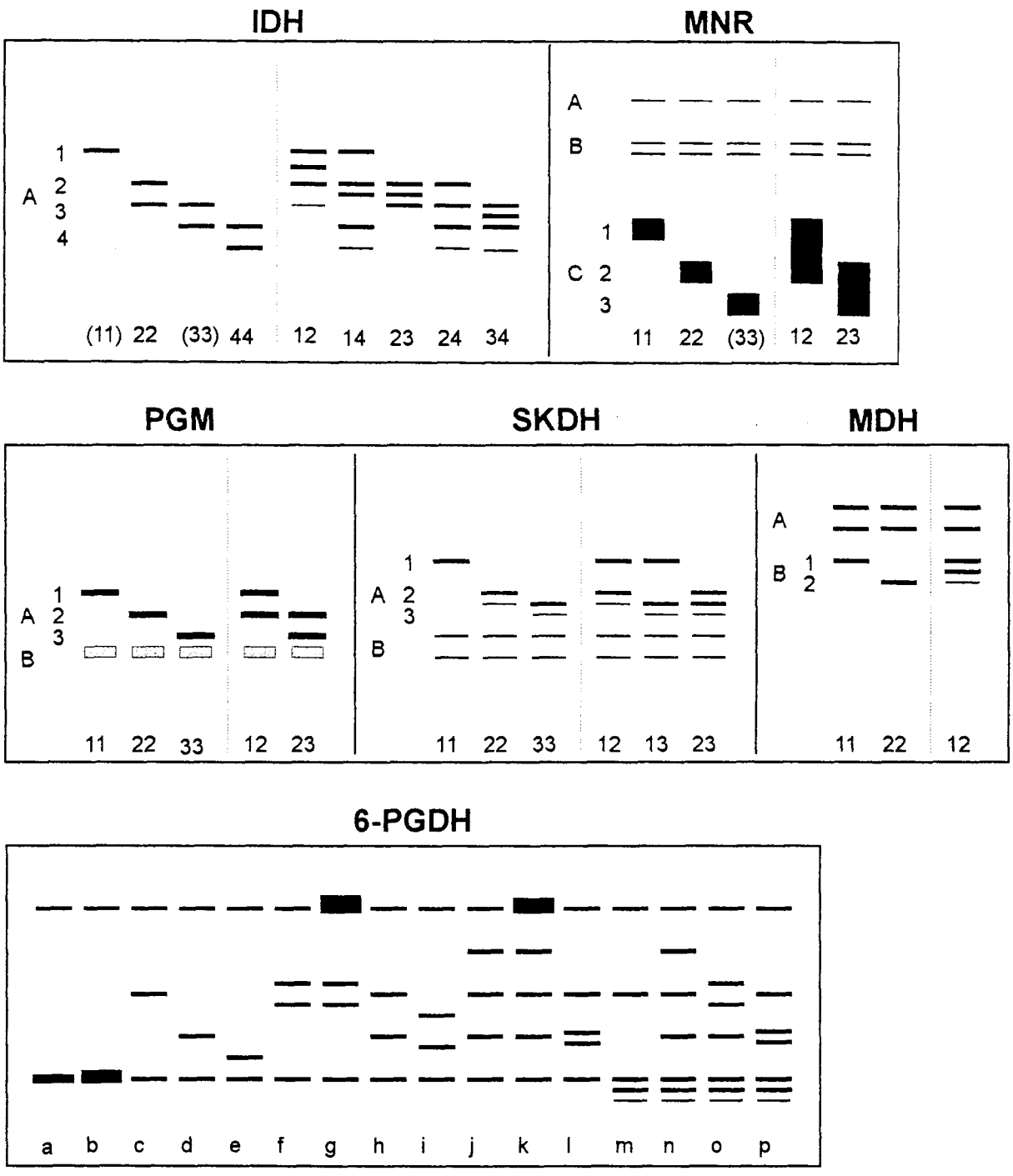


**Figure 1** Isozyme phenotypes of *Pterocarpus indicus* at 6 enzyme systems. Further explanations in the text.

than the polymorphic zones. No variation was also observed at the *Mdh–A* zone (two bands in all investigated progenies), which might be controlled by either one or two gene loci.

The segregation within the offspring of putatively heterozygous seed trees is analysed in Tables 1 and 2. Table 1 contains 31 comparisons of the frequencies of the heterozygous genotype $A_iA_k$ with the frequencies of both homozygous genotypes $A_iA_i$ and $A_kA_k$ in the offspring of putatively heterozygous seed trees ($A_iA_k$). It also shows the results of a goodness-of-fit test of observed and expected segregation ($\chi^2$) and the exact probabilities $p$ to obtain deviations from the expected ratio equal to or greater than the observation. In Table 2 the frequency of the heterozygotes $A_iA_x$ and $A_kA_x$ ($x \neq i,k$) in the offspring of seed trees of the genotype $A_iA_k$

**Table 1 Segregation within the progenies of putatively heterozygous seed trees of *Pterocarpus indicus*. Genotype of the seed tree ($A_iA_k$), frequency of putatively homozygous progenies $P(A_iA_i)$ and $P(A_kA_k)$), frequency of putatively heterozygous progenies ($P(A_iA_k)$), expected frequency of heterozygous ($A_iA_k$) and homozygous ($A_iA_i + A_kA_k$) progenies ($E$), results of a goodness of fit test for statistical significance of the deviation between observed and expected segregation frequencies ($\chi^2$-test; $K_{0.05;\,1df} = 3.841$; $K_{0.01;\,1df} = 6.63$), and exact probabilities $p$ .**

| Gene locus | Tree No. | Genotype ($A_iA_k$) | $P(A_iA_i)$ | $P(A_kA_k)$ | $P(A_iA_i)+P(A_kA_k)$ | $P(A_iA_k)$ | $E$ | $\chi^2$ | p |
|---|---|---|---|---|---|---|---|---|---|
| *Idh–A* | 21 | $A_2A_4$ | 38 | 14 | 52 | 45 | 48.5 | 0.505 ns | 0.543 |
| *Idh–A* | 25 | $A_2A_4$ | 36 | 12 | 48 | 55 | 51.5 | 0.476 ns | 0.555 |
| *Idh–A* | 23 | $A_3A_4$ | 0 | 7 | 7 | 7 | 7 | 0 ns | 1 |
| *Idh–A* | 14 | $A_2A_4$ | 11 | 3 | 14 | 16 | 15 | 0.133 ns | 0.855 |
| *Idh–A* | 20 | $A_2A_4$ | 9 | 14 | 23 | 14 | 18.5 | 1.256 ns | 0.188 |
| *Idh–A* | 17 | $A_2A_4$ | 10 | 5 | 15 | 12 | 13.5 | 0.333 ns | 0.701 |
| *Idh–A* | 16 | $A_2A_4$ | 3 | 7 | 10 | 7 | 8.5 | 0.529 ns | 0.629 |
| *Idh–A* | 19 | $A_2A_4$ | 5 | 6 | 11 | 11 | 11 | 0 ns | 1 |
| *Mnr–C* | 21 | $C_1C_2$ | 20 | 40 | 60 | 46 | 53 | 1.849 ns | 0.206 |
| *Mnr–C* | 25 | $C_1C_2$ | 20 | 36 | 56 | 59 | 57.5 | 0.078 ns | 0.852 |
| *Mnr–C* | 24 | $C_1C_2$ | 10 | 24 | 34 | 35 | 34.5 | 0.014 ns | 1 |
| *Mnr–C* | 12 | $C_1C_2$ | 16 | 23 | 39 | 39 | 39 | 0 ns | 1 |
| *Mnr–C* | 2 | $C_1C_2$ | 15 | 26 | 41 | 36 | 38.5 | 0.325 ns | 0.649 |
| *Mnr–C* | 23 | $C_1C_2$ | 19 | 12 | 31 | 38 | 34.5 | 0.710 ns | 0.470 |
| *Mnr–C* | 20 | $C_1C_2$ | 10 | 20 | 30 | 14 | 22 | 5.818 * | 0.023 |
| *Mnr–C* | 17 | $C_1C_2$ | 4 | 7 | 11 | 17 | 14 | 1.286 ns | 0.345 |
| *Mnr–C* | 16 | $C_1C_2$ | 7 | 3 | 10 | 7 | 8.5 | 0.529 ns | 0.629 |
| *Mnr–C* | 14 | $C_1C_2$ | 9 | 3 | 12 | 16 | 14 | 0.571 ns | 0.572 |
| *Pgm–A* | 17 | $A_1A_2$ | 0 | 17 | 17 | 12 | 14.5 | 0.862 ns | 0.458 |
| *Pgm–A* | 4 | $A_2A_3$ | 5 | 3 | 8 | 9 | 8.5 | 0.059 ns | 1 |
| *Skdh–A* | 25 | $A_1A_3$ | 2 | 37 | 39 | 21 | 30 | 5.400 * | 0.013 |
| *Skdh–A* | 24 | $A_1A_3$ | 5 | 25 | 30 | 22 | 26 | 1.231 ns | 0.332 |
| *Skdh–A* | 23 | $A_2A_3$ | 6 | 22 | 28 | 28 | 28 | 0 ns | 1 |
| *Skdh–A* | 14 | $A_2A_3$ | 6 | 11 | 17 | 13 | 15 | 0.533 ns | 0.585 |
| *Skdh–A* | 20 | $A_2A_3$ | 9 | 3 | 12 | 21 | 16.5 | 2.455 ns | 0.080 |
| *Skdh–A* | 17 | $A_2A_3$ | 1 | 12 | 13 | 9 | 11 | 0.727 ns | 0.523 |
| *Skdh–A* | 9 | $A_2A_3$ | 3 | 13 | 16 | 19 | 17.5 | 0.257 ns | 0.736 |
| *Skdh–A* | 19 | $A_2A_2$ | 5 | 6 | 11 | 12 | 11.5 | 0.043 ns | 1 |
| *Mdh–B* | 14 | $B_1B_2$ | 12 | 0 | 12 | 17 | 14.5 | 0.862 ns | 0.458 |
| *Mdh–B* | 17 | $B_1B_2$ | 14 | 0 | 14 | 16 | 15 | 0.133 ns | 0.856 |
| *Mdh–B* | 16 | $B_1B_2$ | 6 | 3 | 9 | 9 | 9 | 0 ns | 1 |

are listed. Only four tests were performed, since alleles $x$ ($x \neq i,k$) were observed in less than 4 progenies in all other cases.

Out of the 35 tests performed 3 tests showed significant deviations between observation and expectation at the 5% level of significance, and no test showed significant deviations at the 1% level. Exact probabilities were below 0.05 for only two tests. Tree No. 25 showed significant deviations between observation and expectation according to the goodness of fit test at the *Skdh–A* locus both in Table 1 ($P(A_1A_1)$ + $P(A_3A_3)$ significantly higher than $P(A_1A_3)$; $p = 0.013$) and Table 2 $P(A_2A_3)$ significantly higher than $P(A_1A_2)$; $p = 0.064$). We assume that segregation distortion favouring the allele $A_3$ in this tree may be responsible for these results.

In general, the observed frequencies are in good agreement with the expectations. Thus, we did not find evidence forcing us to reject the hypotheses on the genetic control of the polymorphic zones of the five enzyme systems IDH, MNR, PGM, SKDH, and MDH, which are controlled by one gene locus.

Although we were able to observe repeatable polymorphic banding patterns at 6-PGDH, we refrained

**Table 2 Segregation for genotypes $A_iA_x$ and $A_kA_x$ ($x \neq i,k$) within the progenies of putatively heterozygous seed trees ($A_iA_k$) of *Pterocarpus indicus*. Genotype of the seed tree ($A_iA_k$), designation of allele ($x; x \neq i,k$), observed frequency of putatively heterozygous progenies ($P(A_iA_x)$ and $P(A_kA_x)$), expected frequency of heterozygous $A_iA_x$ and $A_kA_x$ progenies ($E$), results of a goodness-of-fit test for statistical significance of the deviation between observed and expected segregation frequencies ($\chi^2$-test; $K_{0.05;1df} = 3.841$; $K_{0.01;1df} = 6.63$), and exact probabilities $p$.**

| Gene locus | Tree No. | Genotype ($A_iA_k$) | $x$ | $P(A_iA_x)$ | $P(A_kA_x)$ | $E$ | $\chi^2$ | $p$ |
|---|---|---|---|---|---|---|---|---|
| *Idh–A* | 21 | $A_2A_4$ | 1 | 3 | 3 | 3. | 0 ns | 1 |
| *Idh–A* | 23 | $A_3A_4$ | 2 | 3 | 4 | 3.5 | 0.143 ns | 1 |
| *Skdh–A* | 25 | $A_1A_3$ | 2 | 5 | 14 | 9.5 | 4.263 * | 0.064 |
| *Skdh–A* | 24 | $A_1A_3$ | 2 | 14 | 8 | 22 | 1.636 ns | 0.286 |

from a genetic interpretation of the observed variation. The system is highly variable. We observed 16 different phenotypes with 2 to 7 clearly separated bands. 6-PGDH may be controlled by 3 variable gene loci as reported for several other tree species including *Picea abies* (GIANNINI *et al.* 1991), *Fagus sylvatica* (MÜLLER-STARCK & STARKE 1993), and *Pterocarpus macrocarpus* (LIENGSIRI *et al.* 1995). The upper zone may be controlled by a single gene locus, the lower zone by two overlapping loci forming complex banding patterns due to the occurrence of intra- and intergenic heterodimers. However, the banding patterns observed within open-pollinated single tree progenies did not allow us to formulate and test a hypothesis on the genetic control of this enzyme system.

## DISCUSSION

The banding patterns observed after electrophoretic separation and biochemical staining of enzymes are phenotypes. The identification of gene loci, which control the observed phenotypic variation, requires the formulation of hypotheses on the genetic control and the statistical test of these hypotheses. The test usually involves the observation of segregation within related individuals (full-sib families or single tree progenies) and its comparison to expected segregation ratios.

Crossing techniques have not been developed for many tree species. In these cases the production of full-sib families is impossible. However, open-pollinated single-tree progeny arrays can be easily obtained for most tree species. Thus, the method proposed by GILLET and HATTEMER (1989) is easily applicable to the majority of tree species, which have not yet been investigated at isozyme gene loci. It can be combined with genetic studies *e.g.* aimed at inferring aspects of the mating and gene flow system within populations and requires little additional experimental effort.

Although the observation of segregation within open-pollinated progeny arrays is frequently used to formulate hypotheses on the genetic control of isozyme variation (*e.g.* MURAWSKI & HAMRICK 1991), a statistical test of the hypotheses is usually omitted. A formal inheritance analysis of isozyme phenotypes is rarely performed for tropical tree species in spite of possible complications in their interpretation *e.g.* described by HATTEMER (1991).

The unavailability of vegetative material from seed trees seriously impedes the formulation of hypotheses on the genetic control of complex isozyme phenotypes. It might have been possible to identify the genetic control of the complicated 6-PGDH system if the phenotypes of the seed trees were known. However, our results show that hypotheses on the genetic control of simple isozyme phenotypes can be tested based on the segregation in open-pollinated single-tree progenies even if the phenotypes of seed trees are unknown.

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