# Inheritance and Linkage Relationships of Isozyme Variants of *Araucaria angustifolia* (BERT.) O. KTZE.

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**Abstract**

Thirteen isozyme systems were examined in *Araucaria angustifolia* using starch gel electrophoresis and megagamethophyte haploid tissue. Data from five polymorphic systems (GOT, PGM, MDH, SKDH and 6-PGDH) revealed eight loci and nineteen alleles. Gene segregation at these loci was regular in all but a few trees. Deviation from the expected 1:1 segregation ratio in heterozygous trees was more frequently observed for locus GOT-B. There was no evidence for linkage between the eight described loci.

*Key words: Araucaria angustifolia*, Paraná pine, megagametophytes, isozymes, electrophoresis, and segregation.

## Introduction

*Araucaria angustifolia* is a dioecious, anemophilous, and mostly barochoric species in the class *Pinopsida*, order *Pinales* and family *Araucariaceae* (NTIMA, 1968). This species is endemic to Brazil except for few small extant populations in Northeastern Argentina (Province of Misiones) and in Eastern Paraguay (CARVALHO, 1994). In Brazil, the species occurs in areas extending from 19°15'S to 31°30'S, and from 41°30'W to 54°30'W. Most of the populations of *A. angustifolia* are concentrated in the Southern Brazilian States: Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS). Their present northern range includes some representative forests in the States of São Paulo (SP), Minas Gerais (MG) and Rio de Janeiro (RJ). This conifer is commercially important because of the high quality of its wood, used for timber, pulp and resin. *A. angustifolia* occurs in an ecologically diversified geographic region. Thus, genetic variation among populations is expected. Knowledge of the amount and distribution of this variability is essential for the design of genetic improvement and conservation programs for this species. Differences among populations of *A. angustifolia* were reported based on conventional provenance and progeny trials (GURGEL and GURGEL FILHO, 1965; SHIMIZU and HIGA, 1979; KAGEYAMA and JACOB, 1980; GIANNOTTI et al., 1982; SHIMIZU, 1999). Contrary to the quantitative traits observed in fields trials, biochemical and molecular markers are not influenced by environmental conditions and plant age, being suitable for the measurement of genetic variability in natural stands. However, the mode of inheritance of these markers must be known prior to their use in genetic analyses (HATTEMER et al., 1993). Furthermore, most models adopted in population genetics assume regular meiotic segregation at marker loci. The haploid condition of the female gametophyte in conifers (BARTELS, 1971) is particularly useful for the determination of segregation ratios at polymorphic gene loci. Most of the published studies on population genetics in conifers were performed using isozyme markers.

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Three large natural populations of *A. angustifolia*, representing forests in Campos do Jordão (SP), Caçador (SC), and Irati (PR), were surveyed for isozyme variability (SOUSA, 2001). This paper reports on the inheritance and linkage relationships of isozymes in *A. angustifolia*, as part of a major study on the genetic structure in remnant populations of this species.

## Material and methods

*Electrophoresis*

Seeds were collected in 1998 in Campos do Jordão (22°44' S and 43° 44'W). The samples consisted of 20 seeds from each of 70 trees. The moderate numbers of seeds per tree had technical reasons. Due to uncertainty about the encountered genetic variation, a larger number of trees had to be sampled in order to get some overview. The high weight of their seeds enforced reduction in the sample size of the individual trees' seed lots. After harvesting, the seeds were stored at –30°C until enzyme extraction for electrophoresis. Embryos and female gametophytes were dissected and homogenized separately in an extraction buffer containing 5% sucrose, 9.7 mM DTT, 3% PVP-15, 1.3 mM EDTA, and 14.9 mM bovine serum albumin, dissolved in 0.1 M tris-HCl buffer, pH 7.5, as used with *Abies* seeds by BERGMANN (pers. comm.). Wicks with enzymes from the megagametophyte and from the embryo of each seed were positioned side by side in the gel. Horizontal starch gels (10.5% starch and 2.5–3.5% sucrose) were prepared following FERET and BERGMANN (1976) and CONKLE et al. (1982). Electrophoresis was conducted using a lithium-borate buffer system modified from ASHTON and BRADEN (1961) and a Tris-citrate system. Buffer composition, running conditions, and enzymes examined with each buffer are shown in *Table 1*.

*Table 1.* – Buffers and running conditions for starch-gel electrophoresis of *A. angustifolia* isozymes.

| Electrode buffer | Gel buffer | Running conditions | Enzyme Systems |
|---|---|---|---|
| Ashton-system (modified)<br>0.192 M boric acid<br>0.042 M lithium hydroxide<br>pH 8.6 | 10.5% starch<br>2.5% sucrose<br>0.050 M Tris<br>9.5 mM citric acid<br>pH 8.6 | 30 V $cm^{-1}$<br>for 5 h<br><br>maximum<br>80 mA | AAP,<br>GOT<br>LAP,<br>PGI<br>PGM |
| Tris-citrate-system<br>0.148 M Tris<br>0.047 M citric acid<br>pH 7.3 | 10.5 % starch<br>3.5% sucrose<br>0.038 Tris<br>0.012 M citric acid<br>diluted 1 part buffer: 3 parts water<br>pH 7.3 | 20 V $cm^{-1}$<br>for 5 ½ h<br><br>maximum<br>180 mA | IDH,<br>GDH,<br>G6-PDH<br>MDH,<br>MNR<br>NADH,<br>SKDH<br>6-PGDH |

*Segregation analysis*

The female gametophyte in mature conifer seeds is a haploid tissue, which contains the maternal genes. The same genes are also present in the embryo tissue (MÜLLER-STARCK, 1976). Since the genotypes of the maternal gametes can be identified, open pollinated seeds can be used for determining the inheritance of isozyme variants. The mode of inheritance of *A. angustifolia* isozymes was inferred from the segregation ratios found in female gametophytes produced by putative heterozygous trees (BARTELS, 1971). This method allows to assign gene loci to the isozyme activity zones observed in the gel. Bands within zones are attributed to alleles of the identified locus if they conform to the 1:1 segregation ratio expected in meiosis. First a homogeneity G test (WEIR, 1996) was applied. The null hypothesis for this test is equal proportions of the gamete types in the female gametic arrays produced by different trees sharing a heterozygous genotype. If these arrays were considered homogeneous (P>0.05), the goodness of fit to the expected 1:1 ratio was tested in data pooled over all arrays. These statistics are additive, so that $\Sigma G^2_{1:1} = \Sigma G^2_{\text{homogeneity}} + G^2_{1:1\ \text{pooled}}$, with *n , n-1,* and *1* degrees of freedom, respectively. If the homogeneity test was statistically significant (P<0.05), trees not conforming with the 1:1 segregation hypothesis were eliminated from the data and the statistical tests were repeated. Letters and numbers designated the loci and alleles identified in the gel, respectively, in the order of their migration rates towards the anode.

*Linkage analysis*

In this study, genetic linkage is defined as the association of genes located on the same chromosome. For the linkage analysis, the $\chi^2$ test was performed for pairs of loci, *A* and *B*, segregating in the megagametophytes of double heterozygous trees. The first $\chi^2$ tested the conformity of observed genotypic counts to the 1:1 ratio of balanced segregation of alleles at each locus individually. The second $\chi^2$ tested for the 1:1:1:1 ratio of independent segregation of the two loci, with three degrees of freedom. Trees showing significant deviation of the 1:1 segregation at individual loci were excluded from the analysis for independent segregation. Gene segregation and linkage analyses for all loci were performed with seeds collected in Campos do Jordão, the population with the highest number of heterozygous genotypes. The *A. angustifolia* populations in Caçador and Irati were far less polymorphic (SOUSA, 2001). Four trees from the Caçador population, heterozygous at *SKDH-A,* where included in the segregation study to increase the sample for this locus.

## Results and Discussion

*Electrophoresis and isozyme polymorphism*

The extraction buffer described above efficiently preserved the activity of *A. angustifolia* enzymes. The electrophoresis buffer systems described in *Table 1* were chosen after preliminary experiments. The quality of the zymograms obtained with ASHTON's system was improved by raising the pH of the buffer from 8.1 to 8.6. Clear and reproducible zymograms were obtained for 13 enzyme systems. The loci and alleles inferred from the zymograms are listed in *Table 2*. A locus was considered polymorphic if the frequency of the most common allele was lower than 99%. Five of the 13 active isozyme systems showed one or more polymorphic loci. Gels stained for ADH (alcohol dehydrogenase) and FDH (formiate dehydrogenase) showed no bands. Polymorphism in PGI was very low, while moderate to high for enzymes in the GOT, PGM, MDH, SKDH and 6-PGDH systems.

*Table 2.* – Isozyme polymorphism observed in *Araucaria angustifolia* seed tissues

| Isozymes | Abbr. | E.C Number | Loci Scored | Alleles Scored | Polymorphism |
|---|---|---|---|---|---|
| **Ashton buffer pH 8.6** | | | | | |
| Alanine aminopeptidase | AAP | 3.4.11.2 | A | 1 | no |
| Glutamate oxaloacetate transaminase | GOT | 2.6.1.1 | A | 3 | moderate |
| | | | B | 3 | moderate |
| | | | C | 2 | low |
| Leucine aminopeptidase | LAP | 3.4.11.1 | A | 1 | no |
| | | | B | 1 | no |
| Phosphoglucose isomerase | PGI | 5.3.1.9 | A | 1 | no |
| | | | B | 3 | very low |
| Phosphoglucomutase | PGM | 2.7.5.1 | A | 2 | moderate |
| | | | B | 1 | no |
| **Tris-citrate buffer pH 7.3** | | | | | |
| Isocitrate dehydrogenase | IDH | 1.1.1.42 | A | 1 | no |
| Glutamate dehydrogenase | GDH | 1.4.1.3 | A | 1 | no |
| Glucose-6-phosphate dehydrogenase | G-6-PDH | 1.1.1.49 | A | 1 | no |
| Malate dehydrogenase | MDH | 1.1.1.37 | A | 1 | no |
| | | | B | 2 | high |
| | | | C | 1 | no |
| | | | D | 1 | no |
| Menadione reductase | MNR | 1.6.99.2 | A | 1 | no |
| NADH dehydrogenase | NDH | 1.6.99.3 | A | 1 | no |
| | | | B | 1 | no |
| Shikimate dehydrogenase | SKDH | 1.1.1.25 | A | 2 | low |
| | | | B | 2 | low |
| 6-Phosphogluconate - dehydrogenase | 6-PGDH | 1.1.1.44 | A | 1 | no |
| | | | B | 3 | high |

*Table 3.* – Test for homogeneity of segregation ratios among megagametophytic arrays produced by *Araucaria angustifolia* ($G^2$ values) and test for the hypothesis of regular meiotic segregation ($\chi^2$ values) of isozyme markers. Tree genotypes were inferred from gametic arrays showing regular segregation ratios.

| Enzyme locus/ Genotype | | Number of trees (n) | $\Sigma N_i$ | $\Sigma N_j$ | $\Sigma G^2$ 1:1 hypothesis d.f. = n | | $\Sigma G^2$ equal proportions d.f.= n-1 | | $G^2$ 1:1 hypothesis pooled d.f.=1 | | Trees with unbalanced allele ratios |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***GOT-B*** $B_1B_2$ | a) | 26 | 223 | 285 | 68.6738 | *** | 61.0879 | *** | 7.5858 | ** | 6 |
| | b) | 20 | 189 | 204 | 18.7362 | ns | 18.1635 | ns | 0.5727 | ns | - |
| ***GOT-C*** $C_1C_2$ | a) | 5 | 44 | 45 | 1.1400 | ns | 1.4437 | ns | 0.0112 | ns | 0 |
| ***PGM-A*** $A_1A_2$ | a) | 22 | 246 | 192 | 59.4296 | *** | 52.7551 | *** | 6.6745 | ** | 2 |
| | b) | 20 | 210 | 190 | 22.4151 | ns | 21.4147 | ns | 1.0004 | ns | - |
| ***MDH-B*** $B_1B_2$ | a) | 39 | 346 | 420 | 53.2685 | ns | 46.2095 | ns | 7.1600 | ** | 2 |
| | b) | 37 | 340 | 386 | 28.2507 | ns | 25.3341 | ns | 2.9166 | ns | - |
| ***SKDH-A*** $A_1A_2$ | a) | 6 | 28 | 20 | 15.3208 | * | 13.9812 | * | 1.3396 | ns | 2 |
| | b) | 4 | 14 | 18 | 5.1967 | ns | 4.6954 | ns | 0.5013 | ns | |
| ***SKDH-B*** $B_1B_2$ | a) | 15 | 136 | 151 | 32.4169 | ** | 53.9763 | *** | 0.7843 | ns | 3 |
| | b) | 12 | 21 | 13 | 9.2819 | ns | 9.1778 | ns | 0.2736 | ns | |
| ***6-PGDHB*** $B_1B_2$ | | 1 | 1 | 19 | --- | --- | --- | --- | 19.7853 | *** | 1 |
| ***6-PGDHB*** $B_2B_3$ | a) | 24 | 251 | 209 | 43.0107 | ** | 39.1706 | * | 3.8401 | * | 2 |
| | b) | 22 | 216 | 204 | 17.4707 | ns | 17.1278 | ns | 0.3429 | ns | |

$N_i$ and $N_j$ = number of megagametophytes with allele *i* and *j* , respectively.
a) includes all trees heterozygous at the given locus.
b) excludes the trees with segregation distortion at that locus.
(***) P<0.001; (**) P<0.010; (*) P<0.050; ns not significant

*Description and segregation of isozyme banding patterns*
*Monomorphic enzymes*

Staining reactions for alanine aminopeptidase (AAP), isocitrate dehydrogenase (IDH), glutamate dehydrogenase (GDH), leucine aminopepetidase (LAP), menadione reductase (MNR), NADH dehydrogenase (NDH) and glucose-6-phosphate-dehydrogenase (G-6-PDH) resulted in a single and uniform band. In the absence of variation, this band was empirically called locus *A*, with one allele (*Table 2*). Gels stained for PGI (phosphoglucose isomerase) revealed two enzyme activity zones, the most anodal being monomorphic. Rare variation found in the embryo lanes suggested that zone B of PGI is controlled by one gene with three alleles in the sampled populations. Besides being rare, the variant isozymes were restricted to the pollen pool, so that no segregation analysis could be performed for PGI. Several monomorphic loci were also found among the enzyme systems described next.

*Polymorphic enzymes*

Ordered genotypes, with respect to maternal and paternal gene contributions, were deduced from the comparison of bands in the paired zymograms of megagametophyte and embryo of each seed. *Table 3* summarizes the tests of regular segregation ratios applied to isozyme bands to confirm the inferred genotypes. These include the G test for the hypothesis of equal proportions of gamete types among samples, and the G test for the 1:1 segregation ratio expected in Mendelian inheritance. The $\chi^2$ value for the hypothesis of Mendelian segregation is also shown. The first row referring to each enzyme locus (*Table 3*) shows the pooled number of megagametophytes carrying each putative allele and the G values calculated for data pooled for all trees identified as heterozygous at that locus. In the second row, the trees with statistically significant deviation from the 1:1 segregation hypothesis (P<0.05) were excluded from the data. The number of trees eliminated in this procedure is given in the last column of *Table 3*. Their female gametic arrays are shown in *Table 4*. Allele frequencies at polymorphic loci are shown in *Table 5*.

*Glutamate oxaloacetate transaminase (GOT)*

Three polymorphic zones were identified in gels stained for GOT (*Figure 1*). Embryos heterozygous at GOT loci were recognized by their three-banded phenotypes. In zone GOT-A, the bands associated with heterozygous embryos were blurry and phenotypes could not be clearly distinguished. The observed patterns suggested that zone GOT-A is controlled by a locus with two alleles but this locus was not used in this study. Zone GOT-B is controlled by one locus with three alleles. The segregation of alleles $B_1$ and $B_2$ at locus *GOT-B* was regular in 20 out of 26 trees (*Table 3*). A rare allele $B_3$ was detected in embryos only, meaning that it was present in the pollen pool.

Six of the trees with genotype $B_1B_2$ produced gametic arrays with deviation from the expected 1:1 ratio. As shown in *Table 4*, the deviation was not allele-dependent. It favored allele $B_1$ in one case and allele $B_2$ in five. Bands in zone GOT-C migrated towards the cathode when the buffer pH was 8.1. These bands became anodal when the pH was raised to 8.6. One locus with two alleles, $C_1$ and $C_2$, was identified in the GOT-C zone. The segregation at *GOT-C* was regular in the five examined trees. GOT isozymes were described for several conifers (ADAMS and JOLY, 1980a and SIREGAR, 2000). As in *A. angustifolia*, three zones of GOT activity were reported for *Pinus sylvestris* (RUDIN and EKBERG, 1978), *Pinus roxburghii* (HUSSAIN, 1995), and *Pseudotsuga menziesii* (NEALE et al., 1984).

*Table 4.* – Trees of *Araucaria angustifolia* showing statistically significant deviation from the expected 1:1 ratio in single locus meiotic segregation of isozyme markers. Observed proportions of megagametophytes and $\chi^2$values.

| Enzyme/ Tree I.D. | $N_i$ | $N_i$ | $\chi2$ d.f.=1 | Enzyme/ Tree I.D. | $N_i$ | $N_i$ | $\chi2$ d.f.=1 |
|---|---|---|---|---|---|---|---|
| ***GOT-B*** | | | | ***SKDH-A*** | | | |
| NF18 | 16 | 4 | 7.200** | CjNF10 | 7 | 1 | 4.500* |
| NF33 | 2 | 14 | 9.000*** | CaNF12-5 | 7 | 1 | 4.500* |
| NF34 | 2 | 18 | 12.800*** | ***SKDH-B*** | | | |
| EF15 | 5 | 14 | 4.263* | NF10 | 1 | 19 | 16.200*** |
| EF30 | 4 | 16 | 7.200** | NF15 | 2 | 18 | 12.800*** |
| EF33 | 5 | 15 | 5.000* | EF21 | 12 | 1 | 9.307*** |
| ***PGM-A*** | | | | ***6-PGDH-B*** | | | |
| NF24 | 5 | 15 | 5.000* | ***$B_1B_2$*** | | | |
| EF14 | 1 | 19 | 16.200*** | EF31 | 1 | 19 | 16.200*** |
| ***MDH-B*** | | | | ***$B_2B_3$*** | | | |
| EF22 | 5 | 15 | 5.000* | EF02 | 18 | 2 | 12.800*** |
| EF26 | 1 | 19 | 16.200*** | EF28 | 17 | 3 | 9.800*** |

(***) P<0.001; (**) P<0.010; (*) P<0.050

*Table 5.* – Allele frequencies at seven polymorphic loci of *A. angustifolia* in the population Campos do Jordao.

| **Locus** | ***GOT-B*** | | | ***GOT-C*** | | ***PGM-A*** | |
|---|---|---|---|---|---|---|---|
| **Alleles** | ***$B_1$*** | ***$B_2$*** | ***$B_3$*** | ***$C_1$*** | ***$C_2$*** | ***$A_1$*** | ***$A_2$*** |
| Seed-trees | 0.257 | 0.743 | 0.000 | 0.043 | 0.957 | 0.836 | 0.164 |
| Embryos | 0.189 | 0.811 | <0.001 | 0.037 | 0.963 | 0.852 | 0.148 |
| Effective pollen | 0.166 | 0.834 | <0.001 | 0.034 | 0.966 | 0.851 | 0.149 |
| **Locus** | ***MDH-B*** | | ***SKDH-B*** | | ***6-PGDH-B*** | | |
| **Alleles** | ***$B_1$*** | ***$B_2$*** | ***$B_1$*** | ***$B_2$*** | ***$B_1$*** | ***$B_2$*** | ***$B_3$*** |
| Seed-trees | 0.436 | 0.564 | 0.135 | 0.865 | 0.007 | 0.829 | 0.164 |
| Embryos | 0.398 | 0.602 | 0.148 | 0.852 | 0.181 | 0.683 | 0.136 |
| Effective pollen | 0.378 | 0.622 | 0.169 | 0.831 | 0.361 | 0.511 | 0.128 |

*Phosphoglucomutase (PGM)*

Two PGM activity zones were detected. The faster-migrating PGM zone in *A. angustifolia* is controlled by a single polymorphic locus, *PGM-A*, with two alleles. Heterozygous embryos present a two-banded isozyme phenotype (*Figure 1*). Departure from the 1:1 segregation ratio was observed in two samples (trees NF24 and EF14, *Table 4*), only one with P<0.001. Two PGM activity zones were also reported for *Abies pinsapo* (PASCUAL et al., 1993) and *Pinus attenuata* (STRAUSS and CONKLE, 1986).

*Malate dehydrogenase (MDH)*

Gels stained for MDH revealed four zones of enzyme activity. According to HERTEL (1996) most conifer species have four MDH loci. In *A. angustifolia*, isozyme bands stain stronger in zones A and B than in C and D. Zones A, C and D were monomorphic. Zone B is controlled by one gene with two alleles. Embryos heterozygous at *MDH-B* show a three-banded isozyme phenotype. Two trees (EF22 and EF26) out of the 39 did not conform with the 1:1 ratio hypothesis, only one with a strong deviation.

*Shikimate-dehydrogenase (SKDH)*

Two enzyme activity zones stained for SKDH (*Figure 1*). Enzyme activity is stronger in zone B. Two loci, *SKDH-A* and *SKDH-B*, were assigned to these zones. Embryos heterozygous at locus *SKDH-A* or *SKDH-B* displayed a two-banded isozyme phenotype. Only two trees were heterozygous at locus *SKDH-A* in the Campos do Jordão population. Four additional trees, heterozygous at this locus, were found in Caçador (SC). The two deviant arrays (P<0.050) found for *SKDH-A* (*Table 3*) are best explained by their small samples (8 seeds per tree). Segregation at the locus *SKDH-B*, scoring 20 seeds per array, fit the 1:1 ratio in 12 out of 15 heterozygous trees found in Campos do Jordão.

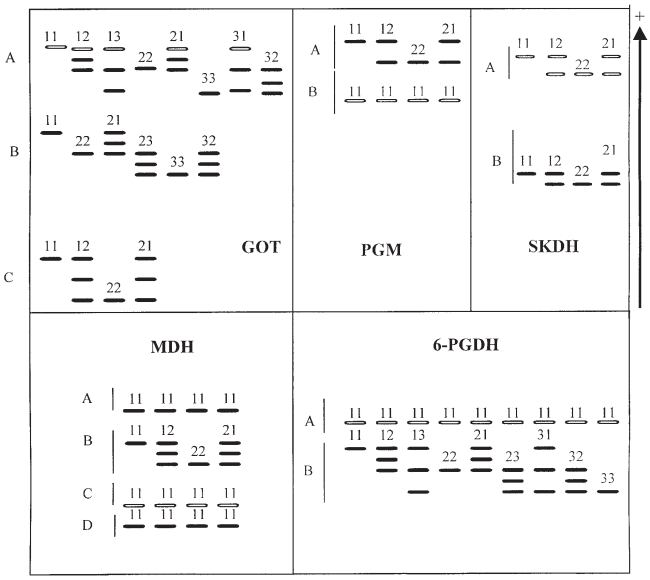


*Figure 1.* – Schematic diagram of isozyme phenotypes observed in embryos of *Araucaria angustifolia* and corresponding ordered genotypes.

*6-Phosphogluconate dehydrogenase (6-PGDH)*

Gels stained for 6-PGDH presented two enzyme activity zones with close migration rates. Zone 6-PGDH-A was monomorphic (*Figure 1*). The phenotypes observed in the 6-PGDH-B zone revealed one locus with 3 alleles. Heterozygous embryos show three-banded isozyme phenotypes, typical of dimeric enzymes. A single tree (EF31) represented genotype $B_1B_2$ at locus *6-PGDH-B*. Allele $B_1$ was found in only one out of its 20 female gametophytes scored at this locus (*Table 4*). The presence of this allele was confirmed in the genotype of the corresponding embryo. Genotype $B_2B_3$ was represented by 24 trees. Deviation from the expected segregation ratio was observed in 2 of these trees (EF02 and EF28, *Table 4*). Genotype $B_1B_3$ was not found among the seed trees. Two loci coding for 6-PGDH are also reported for other conifers (GURIES et al., 1978; ADAMS and JOLY, 1980b; EL-KASSABY et al., 1982; HARRY, 1986; HUSSENDÖRFER et al., 1995).

*Interpretation of segregation ratios*

This first study on the genetics of isozymes in *A. angustifolia* aimed to identify useful genetic markers for the description of genetic structure and gene flow in remnant populations of the species. The samples consisted of 20 seeds per tree. Regular segregation of the alleles in heterozygous trees was confirmed for most of the segregating loci when examined in multiple trees. The pooled data provided sufficient evidence for Mendeli-

an inheritance of the polymorphic isozymes here described. Some seed samples did not conform with the 1:1 null hypothesis. Unbalanced gametic arrays were found more often in trees heterozygous at *GOT-B* (6 out of 26). Unbalanced gametic arrays were also found, but less frequently, in *PGM-A* (2 out of 22), *MDH-B* (2 out of 39), *SKDH-B* (3 out of 15), and *6-PGDH-B* (2 out of 24). Larger seed samples need to be examined for an interpretation of the unbalanced gametic arrays found in *A. angustifolia*. The unbalance could be due to random sampling variation. However, detailed isozyme studies performed with other conifer species have detected a persistent segregation distortion when large samples of female gametophytes are scored. According to STRAUSS and CONKLE (1986), certain enzyme loci, including the GOT, MDH and 6-PGDH isozymes, frequently show some degree of segregation distortion. The same was observed for GOT by RUDIN and EKBERG (1978) and ADAMS and JOLY (1980a), and for 6-PGDH by these authors and CHELIAK et al. (1984). For loci controlling MDH, SIMONSEN and WELLENDORF (1975) and CHELIAK et al. (1984) reported segregation distortion. Segregation distortion has been attributed to selection occurring between meiosis and fertilization, or to strong linkage between the marker alleles and a deleterious gene (FURNIER et al., 1986; STRAUSS and CONKLE, 1986). The frequency of recessive deleterious alleles in outcrossing forest tree species is generally high (WILLIAMS and SAVOLAINEN, 1996). Inbreeding, as a potential explanation for segregation distortion, was investigated in some conifers with the reasoning that selection against homozygous embryos carrying a recessive deleterious allele would result in a deficiency of the associated allozyme phenotype among both embryos and female gametophytes. Consequently, if meiotic segregation is studied using megagametphytes, only the seeds with a viable embryo are examined, i.e. after selection has taken place (STRAUSS and CONKLE, 1986). There was no evidence for inbreeding in the Campos do Jordão population of *A. angustifolia* (SOUSA, 2001). Another explanation for segregation distortion calls for a selective advantage of the most common allele at the locus, providing higher fitness to the haploid gamete. HUSSAIN (1995) found the most frequent allele in the population to be consistently in excess among female gametes in *Pinus roxburghii*. In *A. angustifolia*, segregation distortion is suggested, but not proven, by the high frequency of trees with unbalanced allele ratios in their gametophytes. The most frequent allele found in the Campos do Jordão population was favored in all but one of these arrays. Interesting however, is the fact that the loci represented in the unbalanced megagametophyte samples in *A. angustifolia*, except for *SKDH-B*, correspond to those cited in the literature on segregation distortion in conifers. *Araucariaceae* is an ancient family within conifers (SETOGUCHI et al., 1998). If segregation distortion comes to be confirmed in *A. angustifolia* and other species in the family, the hypothesis of a conserved linkage relationship between certain isozyme loci and a gene active during the gametic phase would certainly deserve further investigation.

*Independent segregation between loci*

Eleven out of 21 possible two-loci combinations of 7 polymorphic loci were found in this survey (*Table 6*).

Four of the combinations, including 5 loci, were represented by just two trees, EF07 (*GOT-B:GOT-C* and *GOT-C:SKDH-B*) and EF31 (*GOT-B:6-PGDH-B* and *MDH-B:6-PGDH-B*), which were heterozygous at multiple loci. The sample for EF07 (15 seeds) was less than minimal for a $\chi^2$ with 3 degrees of freedom. The allelic ratio at *6-PGDH-B* was strongly unbalanced in the sample of gametes of EF31 as already shown in *Table 4*. Thus, independent segregation of these four combinations of genes was not proven in spite of the non-significant $\chi^2$ values shown in *Table 6*. Seven gene combinations were found in multiple trees. Again, unbalanced samples of female gametophytes explain the significant $\chi^2$ values obtained in the single locus segregation tests. Tree EF14 participates in combinations *GOT-B:PGM-A, PGM-A:MDH-B,* and *PGM-A:SKDH-B*. The unbalanced gametic sample of tree EF14 at locus *PGM-A* explains the $\chi^2$ values for single-locus segregation tests in *GOT-B:PGM-A* and *PGM-A:MDH-B,* and for independent segregation in the last combination. Combination *PGM-A: MDH-B* also includes trees NF24 and EF26, which show deviant allelic ratios at *PGM-A* and *MDH-B,* respectively (*Table 4*). The $\chi^2$ value for *GOT-B:PGM-A* is non-significant ($\chi^2$ = 5.835, P>0.050) when tree EF14 is eliminated from the pooled data. Likewise, the $\chi^2$ value for *PGM-A:MDH-B* is non-significant ($\chi^2$ = 3.511, P>0.050) when trees EF14 and EF24 are eliminated. Finally, the combination *PGM-A:SKDH-B* includes tree NF10 and EF14, with unbalanced gametic samples for locus *SKDH-B* and *PGM-A*, respectively (*Table 4*). The effect of these gametophytic arrays is compensated by other trees in the pooled data. The $\chi^2$ value is 0.053, with 3 degrees of freedom, when the two trees are eliminated. Excluding the unbalanced samples reduces total counts but does not change the evidence for independent segregation of loci *GOT-B, PGM-A, MDH-B,* and *SKDH-B*. However, the absence of linkage between any of these loci and *GOT-C* or *6-PGDH-B* remains to be tested with larger samples of heterozygous trees and seeds per tree.

*Table 6.* – Two-locus segregation patterns and chi-square tests for independent segregation of seven isozyme loci of *Araucaria angustifolia.*

| Locus combination | Observed numbers Of allelic combination | | | | | $\chi^2$ (df = 1) | | Joint (df=3) | |
|---|---|---|---|---|---|---|---|---|---|
| | $A_iB_i$ | $A_iB_j$ | $A_jB_i$ | $A_jB_j$ | Sum | Locus *A* | Locus *B* | | |
| **Data from single trees** | | | | | | | | | |
| *GOT-B:GOT-C* | 3 | 5 | 3 | 3 | 14 | 0.286 | 0.286 | 0.286 | |
| *GOT-B:6PGDH-B* | 0 | 6 | 1 | 13 | 20 | 3.200 | 16.200 *** | 1.800 | |
| *GOTC:SKDHB* | 4 | 2 | 6 | 3 | 15 | 0.600 | 1.667 | 2.333 | |
| *MDH-B:6PGDH-B* | 0 | 13 | 1 | 6 | 20 | 1.800 | 16.200 *** | 21.200 | *** |
| **Pooled data from multiple trees** | | | | | | | | | |
| *GOT-B:PGM-A* | 37 | 14 | 32 | 27 | 110 | 0.582 | 7.127 ** | 10.650 | * |
| *GOT-B:MDH-B* | 53 | 50 | 39 | 51 | 193 | 0.876 | 0.420 | 2.461 | |
| *GOT-B:SKDH-B* | 43 | 37 | 45 | 46 | 171 | 0.708 | 0.146 | 1.140 | |
| *GOT-C:PGM-A* | 14 | 13 | 15 | 12 | 54 | 0.000 | 0.296 | 0.370 | |
| *PGM-A: MDH-B* | 60 | 82 | 39 | 58 | 239 | 8.473 ** | 7.033 ** | 15.540 | *** |
| *PGM-A:SKDH-B* | 22 | 32 | 15 | 26 | 95 | 1.779 | 4.642 * | 6.432 | |
| *MDH-B:SKDH-B* | 32 | 32 | 35 | 44 | 143 | 1.573 | 0.566 | 2.706 | |

(*) $P \leq 0.05$; (**) $P \leq 0.01$; (***) $P \leq 0.001$


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# Linkage of Random Amplified Polymorphic DNA Markers in *Pinus Halepensis* MILL.

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**Abstract**

A genetic linkage analysis involving 60 random decamer primers in Aleppo pine (*Pinus halepensis* MILL.), one of the most important Mediterranean conifers, is reported. Five trees originating from five natural Spanish populations and 40 haploid megagametophytes per tree were investigated based on joint segregation and independent assortment. Twenty-two decamers were selected for their stable and repeatable banding patterns and 10 of these produced 24 polymorphic loci that presented Mendelian inheritance. Some degree of segregation distortion was evident in 16% of the loci tested. A total of 155 linkage tests were executed based on LOD-scores and $\chi^2$ contingency tables. Six linkage groups that include 13 loci were detected: $OPA01_{750}$ : $OPP04_{1200}$ : $OPP04_{930}$, $OPA11_{2500}$ : $OPA11_{950}$, $OPA19_{1150}$ : $OPA19_{1090}$, $OPN06_{690}$ : $OPN06_{420}$, $OPN12_{450}$ : $OPN12_{300}$ and $OPP10_{640}$ : $OPP10_{600}$. Recombination frequencies were homogeneous across trees and chromosomal interference was found to be negative. Total consensus genetic map length ranged from 175 cM to 225 cM depending on the mapping function used. This is the first linkage study in this species using RAPD markers and single tree megagametophytes.

*Key words: Pinus halepensis,* RAPD, Mendelian inheritance, linkage test, gene mapping.

## 1. Introduction

Linkage analysis is the basis for the establishment of a genetic linkage map. Linkage maps are useful for different genetic studies: location of genes controlling important traits in plants, analysis of genetic variation in natural populations, taxonomy, evolution, etc. Traditionally, genetic linkage maps have been based on biochemical markers such as isoenzymes

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