# Inheritance and Linkage Relationships of Allozyme Variants of *Ilex paraguariensis* St. Hil

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## ABSTRACT

*Eighteen enzyme systems were examined in* Ilex paraguariensis *St. Hil. using starch gel electrophoresis. Seven out of 12 active isozyme systems revealed one or more polymorphic loci (PGI, GOT, MR, G-6PDH, MDH, NDH, and 6-PGDH). However, the segregation and linkage analyses were performed only for PGI, GOT, G-6PDH and 6-PGDH systems. Gene segregation at these loci was regular, except for a few trees that showed segregation distortion. Weak linkage disequilibrium between loci was detected, but it was not enough to influence the multilocus estimate.*

**Key words:** Erva-mate (matê), electrophoresis, loci, allele and segregation

## INTRODUCTION

Biochemical marker allozymes are used in forest genetic studies since they constitute an excellent tool for breeding programs and genetic resources conservation (Bergmann and Hattemer, 1998; Finkeldey, 1998). They are mainly used in reproduction system studies, measures of population diversity and genetic structure, gene flow, migration inferences, studies of phylogeny and taxonomy and in the genomic mapping (Glaubitz and Moran, 2000). It is necessary to consider a specific gene marker to infer about inheritance mode and linkage equilibrium among loci (Gillet and Hattemer, 1989). Such studies are quite common for conifers, but they are rare for broad-leaved species (Sebbenn, 2004).

*Ilex paraguariensis* St. Hil. ("erva-mate") is a broad-leaved tree, native to South America (Oliveira and Rotta, 1985). It has several industrial applications, but is especially used for beverage preparation. Mate is a culture of important commercial value for South Brazil and neighbor countries, such as Paraguay and Argentina (Maccari Junior, 2000). Due to its strong and indiscriminate exploitation and intensive requirement, studies of its biology and genetics are necessary to guide conservation and improvement programs.

This work aimed to study the inheritance and linkage relationships of allozymes in *I. paraguariensis*, as part of a major study on the reproductive systems and genetic structure of this species.

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## MATERIAL AND METHODS

### Plant material and samples

The material was collected from a Seed Production Area located at Colombo - Parana State, Brazil (25°20' S and 49° 14' W and 920 m high). The seeds were submitted to stratification procedure for six months -. The seedling leaves from 25 mother trees and their respective progenies (30 progenies/mother tree) were analyzed.

### Electrophoresis

The enzymes were extracted from 100 mg of leaf tissues and crushed in liquid nitrogen. Subsequently, 240 μL of extraction buffer (PVP-40 6%, sucrose 5%, $Na_2EDTA.2H_2O$ 0.004 mol.$L^{-1}$, DTT 0.026 mol.$L^{-1}$, bovine serum albumin 1.5% and mercaptoethanol 1%, dissolved in Tris-HCl 0.067 mol.$L^{-1}$ buffer, pH 7.5) with 0.01 g of PVPP was added to leaf extracts. The wicks with enzymes from the mother tree and their respective progenies were positioned side by side in the gel. The protein separation was done by electrophoresis of horizontal starch gel containing 13% starch and 1.35% sucrose. Electrophoresis was conducted in three systems: Lithium-borate, Tris-citrate and Morpholine-citrate. The buffer composition and running conditions are shown in Table 1. Eighteen enzyme systems were examined (Table 2).

**Table 1 -** Buffers and running conditions for starch-gel electrophoresis of *I. paraguariensis* isozymes.

| Electrode buffer | Gel buffer | Running conditions |
|---|---|---|
| Lithium-borate-system<br>0.191 mol.$L^{-1}$ boric acid<br>0.042 mol.$L^{-1}$ lithium hydroxide<br>pH 8.1 | 13% starch<br>1.35% sucrose<br>0.051 mol.$L^{-1}$ Tris<br>0.009 mol.$L^{-1}$ citric acid monohydrate<br>pH 8.1<br>5% (v/v) electrode buffer | 75 mA constant<br>for 5 h |
| Tris-citrate-system<br>0.149 mol.$L^{-1}$ Tris<br>0.043 mol.$L^{-1}$ citric acid monohydrate<br>pH 7.5 | 13% starch<br>1.35% sucrose<br>0.148 mol.$L^{-1}$ Tris<br>0.043 mol.$L^{-1}$ citric acid monohydrate<br>diluted 1 part buffer: 3 parts water<br>pH 7.3 | 150 V constant<br>for 5 ½ h |
| Morpholine-citrate-system<br>0.040 mol.$L^{-1}$ citric acid monohydrate<br>Adjust pH with N-(aminopropyl)<br>pH 6.1 | 13% starch<br>1.35% sucrose<br>0.040 mol.$L^{-1}$ citric acid monohydrate<br>Adjust pH with N-(aminopropyl)<br>pH 6.1 | 65 mA constant<br>for 5 h |

**Table 2 -** Enzymes examined in this study, with their abbreviations and Enzyme Commission (E. C.) reference numbers.

| Enzyme systems | Abbreviation | E. C. no. |
|---|---|---|
| Aconitase | ACO | 4.2.1.3 |
| Alcohol dehydrogenase | ADH | 1.1.1.1 |
| Malic enzyme | ME | 1.1.1.40 |
| Acid phosphatase | ACP | 3.1.3.2 |
| Phosphoglucose isomerase | PGI | 5.3.1.9 |
| Phosphoglucomutase | PGM | 2.7.5.1 |
| 6-Phosphogluconate dehydrogenase | 6-PGDH | 1.1.1.44 |
| Formiate dehydrogenase | FDH | 1.2.1.2 |
| Glutamate dehydrogenase | GDH | 1.4.1.3 |
| Glucose-6-phosphate dehydrogenase | G-6PDH | 1.1.1.49 |
| Glutamate oxaloacetate transaminase | GOT | 2.6.1.1 |
| Isocitrate dehydrogenase | IDH | 1.1.1.42 |
| Leucine aminopeptidase | LAP | 3.4.11.1 |
| Malate dehydrogenase | MDH | 1.1.1.37 |
| Menadion reductase | MR | 1.6.99.2 |
| NADH-dehydrogenase | NDH | 1.6.99.3 |
| Peroxidase | PO | 1.11.1.7 |
| Shikimic acid dehydrogenase | SKDH | 1.1.1.25 |

## Segregation analysis

The inheritance mode of *I. paraguariensis* was inferred according to Gillet and Hattemer (1989) method, which compared the mother tree with its open-pollinated progeny. The following conditions were checked and met using this method: a) all progeny of homozygous mother tree ($A_iA_i$) possessed the allele of the maternal tree ($A_i$); in case of heterozygous mother tree ($A_iA_j$, $i \neq j$): b) ($A_iA_j$, $i \neq j$): b.1) each individual among the offsprings - contained one of the maternal alleles $A_i$, $A_j$, b.2) the number of the heterozygous progenies $A_iA_j$ ($N_{ij}$) was expected to be equal to the sum of homozygous progenies $A_iA_i$ ($N_{ii}$) and $A_jA_j$ ($N_{jj}$), $N_{ij} = N_{ii} + N_{jj}$, b.3) the number of the heterozygous progenies $A_iA_k$ ($N_{ik}$) was expected to be equal to the number of the heterozygous progenies $A_jA_k$ ($N_{jk}$), $N_{ik} = N_{jk}$ ($k \neq i, j$).

The homogeneity G test (Sokal and Rohlf, 1981), with one degree of freedom, was applied to compare the observed phenotypes in each progeny from heterozygous mother tree, with the expected 1:1 segregation hypothesis. Finally, all the individual tests were pooled and a G total test was obtained ($\sum G_{hypothesis1:1}$) with *n* degree of freedom, where *n* represented the analyzed progenies. Simultaneously, the observed phenotypes were pooled and a G test grouped for 1:1 segregation was obtained ($\sum G_{1:1 pooled}$) with one degree of freedom. The hypothesis of heterogeneity segregation between the progenies was tested throughout a G test ($\sum G_{heterogeneity}$), subtracting the pooled G test ($\sum G_{1:1 pooled}$) from total G test ($\sum G_{hypothesis1:1}$). These statistics were additive, so that $\sum G_{hypothesis1:1} = \sum G_{heterogeneity} + \sum G_{1:1 pooled}$, with *n*, *n-1* and *1* degrees of freedom, respectively (Sousa et al., 2002). If the test was statistically significant, trees not conforming to 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

The linkage analysis was based on linkage disequilibria of Burrows, $\Delta_{ij}$ (Weir, 1979) and estimated by GDA software (Lewis and Zaykin, 1999). The chi-square ($\chi^2$) tested the hypothesis of independent segregation (Weir, 1979).

# RESULTS AND DISCUSSION

## *Electrophoresis and enzyme polymorphism*

The extraction buffer was chosen after preliminary investigation.

The adjusted buffer efficiently preserved the activity of *I. paraguariensis* enzymes. Clear and reproducible zymograms were obtained for 12 enzymes: PGI, PGM, GOT and MR (Lithium-borate system - pH 8.1); ADH, GDH, G-6PDH, IDH, MDH, NDH, SKDH and 6-PGDH (Tris-citrate system - pH 7.1). Poor resolution and unclear bands were observed for Morpholine-citrate system and for this reason, it was excluded from genetics analysis. The loci and alleles inferred from the zymograms and investigated in this study are listed in Table 3. A locus was considered polymorphic if the frequency of the most common allele was lower than 95%. Seven from 12 active enzyme systems revealed one or more polymorphic loci.

## Description and segregation of isozyme banding patterns

### *Monomorphic enzymes*

Staining reactions for PGM, ADH, GDH, IDH and SKDH resulted in a single and uniform band. In the absence of variation, this band was empirically called locus *A*, with one allele (Table 3).

## Polymorphic enzymes

### *Glutamate oxaloacetate transaminase (GOT)*

Three polymorphic zones were identified in gels stained for GOT (Fig. 1). Each zone was controlled by two variants. The zones B and C revealed high polymorphism; however, they were excluded from the analysis because the bands were very weak and with overlapping zones and, therefore, the gel interpretation was difficult. Only GOT-A was used for genetic analysis. GOT revealed the typical pattern of dimeric enzymes, with heterozygous genotypes represented by three bands.

**Table 3 -** Enzyme polymorphism observed in Seed Production Area of *I. paraguariensis.*

| Enzyme system | Loci Scored | Alleles Scored | Polymorphism |
|---|---|---|---|
| *Lithium-borate buffer (pH 8.1)* | | | |
| PGI | A | 1 | no |
| | B | 4 | high |
| PGM | A | 1 | no |
| GOT | A | 2 | moderate |
| | B | 2 | high |
| | C | 2 | high |
| MR | A | 2 | moderate |
| *Tris-citrate buffer (pH 7.5)* | | | |
| ADH | A | 1 | no |
| GDH | A | 1 | no |
| G-6 PDH | A | 2 | moderate |
| IDH | A | 1 | no |
| MDH | A | 2 | moderate |
| | B | 2 | high |
| | C | 1 | no |
| NDH | A | 2 | low |
| | B | 1 | no |
| SKDH | A | 1 | no |
| 6-PGDH | A | 2 | moderate |
| | B | 2 | low |

**Phosphoglucose isomerase (PGI)**

Two PGI activity zones were observed and the most anodal was invariable, while the other showed four bands with high polymorphism. The phenotypes observed in B zone indicated that PGI was a dimeric enzyme (Fig. 1).In further analysis of natural populations, Wendt (2005) observed two variants in the fast migration zone and one more variant (the fifth one) in the second zone.

**Glucose-6-phosphate dehydrogenase (G-6PDH)**

Gels stained for G-6PDH showed a single activity zone with two bands. The variants found in G-6PDH revealed a monomeric structure in *I. paraguariensis* (Fig. 1).

**Malate dehydrogenase (MDH)**

Three MDH activity zones were identified. Zones A and B were polymorphic showing two variants in each one. Zone C was monomorphic. A triple-banded variant when heterozygous suggested a dimeric structure for this enzyme. The patterns of zymograms were very complicated to understand. Therefore, this system was excluded from the data analysis. Konnert et al. (2001) also excluded MDH enzyme from their study with *Acer pseudoplatanus* as consequence of a complicated enzymatic pattern.

**Menadion reductase (MR)**

One zone with two variants appeared in gels stained for MR. This enzyme showed a tetrameric structure. However, it was not possible to identify all heterodimers. Therefore, this system was excluded from the genetic analysis. In most plant species, MR is a multimeric enzyme with a number of intragenic heterodimers in phenotypes of heterozygotes that makes their identification difficult (Finkeldey et al., 1998). Fallour et al., (1997) and Finkeldey et al., (1998) working with *Pinus pinea* and *Pterocarpus indicus,* respectively, also excluded the MR enzyme, as consequence of very complex banding patterns and difficulties in zymograms interpretation.

**NADH-dehydrogenase (NDH)**

Two enzyme activity zones were identified on gels stained for NDH. The faster migrating zone (A) stained weakly and showed two variants. These bands in the progenies were not clear enough to be evaluated. NDH revealed the typical pattern of monomeric enzyme. The B zone was monomorphic. However, studies in natural populations revealed polymorphism in the second zone (two variants) being utilized in the analyses of genetic structure (Wendt, 2005).

## 6-Phosfogluconate dehydrogenase (6-PGDH)

Two polymorphic zones were identified in gels stained for 6-PGDH. Each zone was controlled by two variants. The observed phenotypes showed that 6-PGDH isozyme in *I. paraguariensis* was a dimer (Fig. 1).
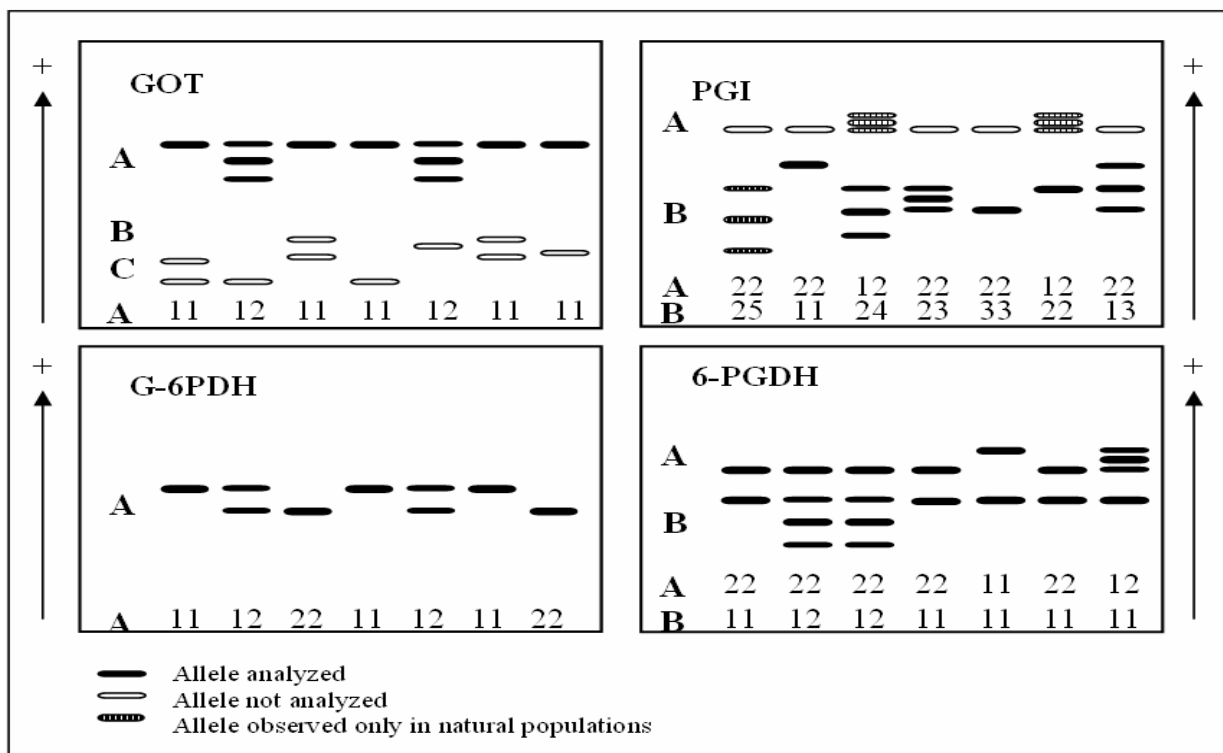


**Figure 1** - Schematic diagram of enzyme phenotypes observed in *I. paraguariensis.*

## Segregation analysis

Table 4 summarizes the tests of regular segregation ratios applied to enzyme bands to confirm the inferred genotypes.

GOT showed three activity zones; however, only GOT-A was utilized for segregation analysis, since the others were very complicated to analyze. The *GOT-A* is controlled by one locus with two alleles. Although $A_2$ was not detected in homozygous form, the three analyzed trees did not show segregation deviation for both *G* total ($\sum G_{hypothesis1:1}$), heterogeneity ($\sum G_{heterogeneity}$) and pooled segregation hypothesis 1:1 ($\sum G_{1:1 pooled}$). These results allowed considering that this zone was controlled by a codominant locus (Table 4).

The segregation analysis from $B_1$ and $B_2$ alleles in *PGI-B* locus showed significant deviation segregation for all the tests performed. These results could be attributed to high deviations from C51 progeny.

When it was excluded from analysis, the data were perfectly adjusted (Table 4). Progenies from C51 tree showed an excess of heterozygote genotype ($N_{jk}$) prevailing the $B_2B_3$ type (25 individuals) (Table 5). The natural selection might explain these results since those deleterious alleles could be eliminated favoring this type. Linkage between gene markers and deleterious alleles was described by Furnier et al. (1986) and Strauss and Conkle (1986) as a reason for segregation distortion. Recessive deleterious alleles linked with gene markers are normally frequent in tree species of crossed pollination (Willians and Savolainen, 1996).

The three heterozygous trees $B_2B_3$ at *PGI-B* locus, showed regular segregation for both $B_2$ and $B_3$ alleles. The genotypes $B_1B_3$, $B_1B_4$ and $B_2B_4$ were observed for just one tree. The segregation for $B_1$ and $B_3$ alleles was according to expected ratio ($G_{1:1 pooled}$).

**Table 4 -** Test for hypothesis of regular segregation (*G*) of isozyme markers in *I. paraguariensis.*

| Locus | Maternal genotype | Case | Number of trees (*n*) | $\sum N_{ij} : \sum (N_{ii} + N_{jj})$ | $\sum N_{ik} : \sum N_{jk}$ | $\sum G_{hypothesis1:1}$ GL = *n* | | $\sum G_{heterogenity}$ GL= *n*-1 | | $\sum G_{1:1\,pooled}$ GL = 1 | | Trees with unbalanced allele ratios |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *GOT-A* | $A_1A_2$ | a | 3 | 46:43 | - | 0.30 | | 0.20 | | 0.10 | | |
| *PGI-B* | $B_1B_2$ | a | 10 | 106:133 | 14:43 | 56.12 | ** | 37.81 | ** | 18.31 | ** | 1 |
| | | b | 9 | 105:131 | 13:18 | 28.55 | | 24.87 | | 3.68 | | |
| | $B_1B_3$ | a | 1 | 10:9 | 5:6 | - | | - | | 0.90 | | |
| | $B_1B_4$ | a | 1 | 2:3 | 18:7 | - | | - | | 13.33 | ** | 1 |
| | $B_2B_3$ | a | 3 | 37:40 | 6:7 | 7.19 | | 7.07 | * | 0.12 | | |
| | $B_2B_4$ | a | 1 | 0:12 | 17:0 | - | | - | | 40.20 | ** | 1 |
| *6-PGDH-A* | $A_1A_2$ | a | 11 | 79:194 | - | 77.92 | ** | 27.93 | ** | 49.99 | ** | 8 |
| | | b | 3 | 34:40 | - | 6.27 | | 5.79 | | 0.49 | | |
| *6-PGDH-B* | $B_1B_2$ | a | 1 | 16:14 | - | - | | - | | 0.13 | | |
| *G-6PDH-A* | $A_1A_2$ | a | 11 | 111:212 | - | 42.39 | ** | 10.28 | | 32.12 | ** | 6 |
| | | b | 5 | 63:83 | - | 3.27 | | 0.52 | | 2.75 | | |

$N_{ij}$ and $N_{ii}+N_{jj}$ numbers observed of heterozygous and homozygous genotypes, respectively.
a includes all heterozygous trees at the given locus.
b excludes the trees with segregation distortion at that locus. (**) *P*<0.010; (*) *P*<0.050.

However, there was segregation deviation for the two last progenies ($B_1B_4$ and $B_2B_4$) (Table 4). The progeny of heterozygous mother tree ($B_1B_4$) revealed an excess of genotype $N_{ik}$ (15 plants $B_1B_2$ and three $B_1B_3$, among 30 plants). The progeny T54 ($B_2B_4$) showed an excess of $B_2$ allele. From 30 analyzed genotypes, 12 were homozygous ($B_2B_2$) and 16 heterozygous ($B_1B_2$) (see Table 5). The segregation distortion for both genotypes was allele-dependent and in this case disfavoring allele $B_4$. The more frequent allele at *PGI-B* locus was $B_2$, followed by $B_1$. According to Sousa (2000), segregation distortion can also be attributed to selective advantage from the most frequent allele at locus.
*6-PGDH-A* showed significant (1% level) segregation deviation (Table 4) as consequence of heterozygous excess. Eight samples showed segregation deviations, where two of them were statistically significant at 0.1% level, one at 1% level and five at 5% level (Table 5). However, the data were adjusted to expected ratio when the progenies with deviation were excluded. The observed homozygotes excess for many trees, in particular in relation to $A_2A_2$ type, suggested a null allele segregating at this locus. The allele segregation for $B_1$ and $B_2$, from *6-PGDH-B* was regular for just one analyzed progeny (Table 4). Segregation distortion has been reported by many authors such as Cheliak et al. (1984); Strauss and Conkle (1986); Sousa et al. (2002).

*G-6PDH-A* locus showed segregation distortion for both tests: total ($\sum G_{hypothesis1:1}$) and pooled segregation 1:1 ($G_{1:1\,pooled}$). Individual segregation deviation was detected for six progenies: two at 5% significance level and four at 1% significance level. When the progeny with deviation were excluded, the segregation ratio 1:1 was established (Table 5). Segregation deviation for some trees was consequence of homozygous excess; however, it was not allele-dependent. The $A_1$ allele was favored in five progenies while $A_2$ only in one progeny (Table 5). The reason for this unbalanced value could be attributed to a null allele or sampling problem for this system. Cheliak and Pitel (1985) described a null allele at *6-PGDH* gene loci in *Larix laricina*.
In the present work, segregation distortion at some loci was observed for a few individual trees. When these trees were excluded, the segregation was according to expected, as observed in *G* test realized (*G* total, *G* pooled and *G* heterogeneity). Therefore, the analyzed loci were genetically controlled and segregating according to mendelian ratio (1:1).
Many authors described the segregation distortion for a few species in individual trees: Ying and Morgenstern (1990) for *Larix laricina*; Murillo and Hattemer (1997) for *Alnus acuminata*; Finkeldey et al. (1998) for *Pterocarpus indicus*; Konnert et al. (2001) for *Acer pseudoplanatus* and Sousa et al. (2002) for *Araucaria angustifolia*.

**Table 5 -** Trees of *I. paraguariensis* showing deviation of segregation from the expected 1:1 ratio, for different enzyme loci.

| Enzyme loci/Tree | Maternal genotype | Sample size | Observed segregation | | | | | $\chi^2$ (A) | | $\chi^2$ (B) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $N_{ii}$ | $N_{jj}$ | $N_{ij}$ | $N_{ik}$ | $N_{jk}$ | $N_{ii}+N_{jj}=N_{ij}$ | | $N_{ik}=N_{jk}$ | |
| *PGI-B* | | | | | | | | | | | |
| C51 | $B_1B_2$ | 28 | | 1 | 1 | 1 | 25 | 0.00 | | 22.15 | *** |
| T67 | $B_1B_4$ | 30 | 2 | 1 | 2 | 18 | 7 | 0.20 | | 4.84 | * |
| T54 | $B_2B_4$ | 29 | 12 | 0 | 0 | 17 | 0 | 12.00 | *** | 17.00 | *** |
| | | | | | | | | | | | |
| *6-PGDH-A* | | | | | | | | | | | |
| S36 | $A_1A_2$ | 27 | 4 | 15 | 8 | - | - | 4.48 | * | --- | |
| CM39 | $A_1A_2$ | 23 | 3 | 19 | 1 | - | - | 19.17 | *** | --- | |
| CM44 | $A_1A_2$ | 27 | 4 | 16 | 7 | - | - | 6.26 | * | --- | |
| C51 | $A_1A_2$ | 25 | 0 | 21 | 4 | - | - | 11.56 | *** | --- | |
| T52 | $A_1A_2$ | 21 | 3 | 12 | 6 | - | - | 3.86 | * | --- | |
| T67 | $A_1A_2$ | 26 | 6 | 13 | 7 | - | - | 5.54 | * | --- | |
| T75 | $A_1A_2$ | 28 | 7 | 15 | 6 | - | - | 9.14 | ** | --- | |
| T78 | $A_1A_2$ | 22 | 4 | 12 | 6 | - | - | 4.72 | * | --- | |
| | | | | | | | | | | | |
| *G-6PDH-A* | | | | | | | | | | | |
| CM34 | $A_1A_2$ | 30 | 19 | 4 | 7 | - | - | 9.63 | ** | --- | |
| S36 | $A_1A_2$ | 30 | 20 | 2 | 8 | - | - | 7.50 | ** | --- | |
| C42 | $A_1A_2$ | 29 | 7 | 13 | 9 | - | - | 4.97 | * | --- | |
| T66 | $A_1A_2$ | 30 | 14 | 7 | 9 | - | - | 5.63 | * | --- | |
| T75 | $A_1A_2$ | 28 | 11 | 10 | 7 | - | - | 8.04 | ** | --- | |
| T80 | $A_1A_2$ | 30 | 18 | 4 | 8 | | | 7.50 | ** | --- | |

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

**Linkage analysis**

Linkage disequilibrium was not performed for all the combinations in the five observed loci, since only few mother trees were heterozygous for many loci simultaneously. From ten possible combination pairs, only six were analyzed in relation to segregation independence of loci (Table 6).

Only three trees showed significant deviation from independent segregation hypothesis: CM39 (*GOT-A:6-PGDH-A*), T52 (*GOT-A:PGI-B*) and C66 (*PGI-B:G-6PDH-A*). Although these loci seemed physically linked, the low value of linkage disequilibrium of Burrows ($\hat{\Delta}_{ij}$) showed a weak linkage, considering the value range observed (-0.25 a 0.25). The more expressive measure of linkage disequilibrium ($\hat{\Delta}_{ij}$), statistically significant at 5% level, was observed for tree T66 between the loci *PGI-B:G-6PDH-A* (0.131) (Table 6).

The disequilibrium was significant at 5% level, just for one single tree in two loci combinations:

*GOT-A:6-PGDH-A* and *PGI-B:G-6PDH-A* (see Table 6). These results suggested that the observed disequilibrium was not a consequence of physical linkage between loci, but it was due to gametic disequilibrium as result of selection. If the disequilibrium resulted from physical linkage, all the analyzed trees should show absence of independent segregation between alleles and loci. Linkage disequilibrium was identified throughout chi-square test at 1% level only for the combination *GOT-A:PGI-B*. However, the observed value was based in one progeny only. To be sure about the strong dependency relationships between alleles, other studies involving more progenies would be necessary. Linkage disequilibrium between loci *GOT-A:PGI-B* was observed in *Pinus taeda* by Adams and Joly (1980). However, the authors could not attest if these loci were in the same chromosome. Linkage disequilibrium for *GOT-A:6-PGDH-A* and *PGI-B:G-6PDH-A* combination has not yet been described for tree species.

**Table 6** - Burrows values for gametic disequilibrium ($\hat{\Delta}_{ij}$) and chi-square test ($\chi^2$) for independent segregation hypothesis for different isozyme loci pairs of *I. paraguariensis*

| Locus combination/Trees | $\hat{\Delta}_{ij}$ | $\chi^2$ | |
|---|---|---|---|
| *6-PGDH-A: G-6PDH-A* | | | |
| S36 | 0.009 | 0.04 | |
| CM39 | -0.082 | 2.68 | |
| T67 | -0.035 | 0.34 | |
| T75 | 0.033 | 0.21 | |
| *GOT-A:6-PGDH-A* | | | |
| CM39 | -0.058 | 4.39 | * |
| T52 | 0.017 | 0.18 | |
| *PGI-B:6-PGDH-A* | | | |
| S36 | 0.018 | 0.21 | |
| C51 | -0.006 | 0.20 | |
| T52 | 0.024 | 0.68 | |
| T78 | 0.056 | 1.15 | |
| *GOT-A:G-6PDH-A* | | | |
| CM39 | -0.064 | 3.63 | |
| *GOT-A:PGI-B* | | | |
| T52 | 0.057 | 305.09 | *** |
| *PGI-B:G-6PDH-A* | | | |
| S36 | -0.043 | 2.06 | |
| C42 | 0.042 | 0.57 | |
| T66 | 0.131 | 5.88 | * |
| T78 | -0.030 | 0.57 | |

(*) $P < 0.050$; (***) $P < 0.001$.

Exclusion of a locus from each linkage pair allows breaking the association between them and can be used to study breeding system and paternity analysis. Linkage disequilibrium measure of Burrows indicated that the linkage was probably weak and, therefore, the multilocus parameters must not be affected. Linkage disequilibrium was not observed for combination between loci; therefore, the alleles from different loci were not linked and segregate randomly.

Inheritance and relationships studies indicated the loci *GOT*, *PGI*, *6-PGDH* and *G-6PDH* could be useful as genetic marker in population genetics of *Ilex paraguariensis* species.

## ACKNOWLEDGEMENT

## RESUMO

Dezoito sistemas enzimáticos foram analisados em *Ilex paraguariensis,* utilizando eletroforese em gel de amido. Sete dos 12 sistemas que apresentaram atividade enzimática revelaram um ou mais locos polimórficos (PGI, GOT, MR, G-6PDH, MDH, NDH e 6-PGDH). Entretanto, as análises de segregação e desequilíbrio de ligação foram realizadas somente nos sistemas PGI, GOT, G-6PDH e 6-PGDH. A segregação destes locos foi regular, exceto para algumas árvores, que apresentaram distorções de segregação. Foram detectados indícios de desequilíbrio de ligação entre alguns locos, mas que não devem influenciar as estimativas multilocos.

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