Polimorfismo isoenzimático em Tetragonisca angustula (Hymenoptera; Meliponinae, Trigonini) do Sul do Brasil

Isoenzyme Polymorphism in Tetragonisca angustula (Hymenoptera; Meliponinae, Trigonini) from Southern Brazil

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Resumo
Abelhas sem ferrão são insetos nativos brasileiros e que desempenham um elevado potencial polinizador para inúmeras culturas agrícolas e plantas nativas. Tetragonisca angustula é uma das espécies mais mapeadas pelos meliponicultores, neste modo, conhecer estrutura genética dessas populações é de grande importância, principalmente, para futuros projetos de conservação. Para isso, neste estudo foi realizado a caracterização bioquímica e a relação genética por eletroforese das isoenzimas esterase (EST), isocitrate dehydrogenase (IDH), enzima málica (ME) e malato desidrogenase (MDH). Abelhas adultas foram coletadas em meliponários de Maringá e Astorga na região Noroeste do estado do Paraná, Brasil. T. angustula de Maringá apresentou dois locos polimórficos (22,22%) e oito locos polimórficos foram observados em T. angustula de Astorga (88,89%). A heterozigosidade média estimada para T. angustula foi de 0,1260. O valor de F<sub>IS</sub> foi de 0,4036 e indicou um excesso de homozigose. O valor F<sub>ST</sub> mostrou que as duas populações não foram diferenciadas, sem nenhum fluxo gênico entre elas. Os resultados sugerem a mesma origem para as duas populações de acordo com a distância genética de Nei.


1 Introduction

Tetragonisca angustula (LATREILLE, 1811) (Hymenoptera; Meliponinae, Trigonini), locally known “Jataí”, is one of the commonest neotropical Trigonini and with great importance for the ecology of several ecosystems. T. angustula is widely distributed from south of Mexico to Argentina and in the whole Brazilian territory and well adapted to a wide area of occurrence (CAMARGO; PEDRO, 2008). The Trigonini constitutes a diversified group, with many species building their nests in living or dead tree trunks (NOGUEIRA-NETO, 1997).

Although T. angustula is not endangered, the increase in environmental degradation due to the intensification of agriculture represents a serious threat (JHA, 2015). Essentially to pollinators of native flora (LICHTENBERG et al., 2017) and commercial crops (HALINSKI et al., 2020).

Human activities such as habitat destruction due to losses, degradation, fragmentation, and increased use of pesticides are causing population declines in abundance and diversity worldwide (DIRZO et al., 2014; OLLERTON et al., 2014.). Leading to a decline in pollinator populations (POTTS et al., 2010; OLLERTON et al., 2014, GIANNINI et al., 2020).

Increased environmental degradation, with consequent habitat destruction, poses a serious threat to the Meliponinae and can result in the isolation of subpopulations (LOZIER; ZAYED, 2017). Thus, a knowledge of the genetic variability and genetic structure of stingless bee species is essential for the control and preservation of these species (FRANCISCO et al., 2014, 2017).

Isoenzyme analysis provides an important source of information for species differentiation (CASTANHEIRA; CONTEL, 1995; STUCHI et al., 2012; RONQUI et al., 2016). The use of these isoenzymes in studies on the characterization
of bee population genetics (STUCHI et al., 2008) and also acts as possible bioindicators of the presence of pesticides (FERMINO et al., 2011, MOREIRA et al., 2018).

Isoenzymes studies in the Apidae have been done by several investigators (AIDAR et al., 2001; CASTANHEIRA; CONTEL, 2005; COSTA et al., 2005; RUVolo-TAKASUSUKI et al., 1997, 1998, 2006). However, only a few studies have investigated isoenzyme variability in T. angustula. Castanheira e Contel (1995) described polymorphism for the hexokinase enzyme locus and showed rare variants in two loci for glycerol-3-phosphate dehydrogenase. Castanheira e Contel (2005) observed a highly significant correlation between the amount of ferruginous color displayed in the mesepisternum of the T. angustula and the Hk88 allele frequency. Subsequently, RAPD (restriction fragment-length polymorphism) molecular markers, distinguished two groups of T. angustula based on their natural geographic distribution (OLIVEIRA et al., 2004). Despite this technique having great genetic variability between populations, just as isoenzymes were not able to separate T. angustula and T. fiebrigi in two species (BAITALA et al., 2006).

New methods have been developed, such as genomics, which are the most effective markers for genetic-population studies (LOZIER; ZAYED, 2017). Other simpler methods such as the use of mitochondrial DNA (mtDNA) and microsatellites (KOLING, MORETTO, 2010; FRANCISCO et al., 2014; SANTIAGO et al., 2016; Francisco et al., 2017), PCR-RAPD (polymerase chain reaction - random amplified polymorphic DNA) (OLIVEIRA et al., 2004), and PCR-RFLP (restriction fragment length polymorphism) (SANTOS et al., 2015) efficient in evaluating population genetics. Therefore, more informative systems are necessary to the understanding of the population genetic structure of these important stingless bees.

This research applied the isoenzyme analyses for the evaluation of two populations, demonstrating that it is possible to characterize and quantify genetic variability used the esterase, isocitrate dehydrogenase, malic enzyme, and malate dehydrogenase isoenzymes of T. angustula which occur in Northwest of Paraná.

2 Material and Methods

T. angustula adults were collected from two areas in the Northwest of Paraná, Southern Brazil, both areas located in semideciduous seasonal forest in the Atlantic Forest biome. Six workers were sampled from each of 10 colonies from Maringá (23° 25’ 31” S, 51° 56’ 19” W) and 23 colonies from Astorga (23° 13’ 57” S, 51° 39’ 56” W) (Figure 1). The distance between the two populations was 50 ± 10 km.

Figure 1 - Map of Brazil (right), shows the location of the state of Paraná, on the map of Paraná (left) showing the geographic sampling locations, 1 - Maringá and 2 – Astorga

Source: the authors.

2.1 Preparing the samples and electrophoresis

For electrophoretic analysis, we used the horizontal technique on 14% starch gels (Penetrose 30, Corn Products of Brazil S.A.) as described by Smithies (1955). The samples were homogenized in 30 μL of a 0.1% mercaptoethanol solution and then centrifuged at 16,000 x g for 10 minutes at 2 °C. Supernatants from the samples were then loaded singly into gel wells.

The zones of enzymatic activity visualized were the conventional ones for each isozyme studied: esterase – EST (EC 3.1.1.1), isocitrate dehydrogenase – IDH (EC 1.1.1.42), malic enzyme – ME (1.1.1.40), and malate dehydrogenase – MDH (EC 1.1.1.37), 4-Methylumbelliferyl esters (acetate and butyrate) and α-naphthyl esters (acetate and propionate) were used to study the substrate preferences of esterase. Staining with 4-Methylumbelliferyl esters and naphthol esters was performed according to the procedure described by Bitondi, Mestriner (1983). Acetate and propionate 4-methylumbelliferyl solutions were prepared immediately before use by the method of Hopkinson et al. (1973).

The population structure of the T. angustula was analyzed using the POPGENE 1.31 software (Yeh et al., 1999). Polymorphic loci were submitted to chi-square tests for deviation from Hardy-Weinberg equilibrium. The differentiation within and among subpopulations was estimated by the contingency test, F-statistics (WRIGHT, 1951, 1965; NEI, 1977, 1978).

3 Results and Discussion

3.1 Isoenzyme characterization

The number of esterase bands detected and their behavior with the substrates employed was presented in Table 1. The best substrates for the detection of esterase activity were those derived from 4-methylumbelliferyl butyrate. It can be seen that Est-1 was not detected with the α-naphthol derivatives. Est-2 presented activity in all substrates employed but presented more intense activity in the presence of 4-methylumbelliferyl acetate and α-naphthol acetate and propionate. While Est-3
was not detected with the 4-methylumbelliferyl acetate and α-naphthol derivatives, suggesting more specificity than the other esterase zones.

### Table 1 - Specificity of *Tetragonisca angustula* esterases for substrates derived from 4-Methylumbelliferyl and α-Naphthyl Esterase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Est-1 (++)</th>
<th>Est-2 (+)</th>
<th>Est-3 (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylumbelliferyl acetate</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Methylumbelliferyl butyrate</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-Naphthyl acetate</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-Naphthyl propionate</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*++* maximum esterase activity; + 50% esterase activity; - no esterase activity

**Source:** Research data

Seven loci of isozyme activity were analyzed in *T. angustula* collected from Maringá and nine loci were observed in *T. angustula* from Astorga. For all of the enzymatic systems that presented polymorphism, the observed alleles were denominated according to electrophoretic mobility starting from the anode (Figure 2).

**Figure 2** - Starch gel electrophoretic pattern of *Tetragonisca angustula* isoenzymes; (A) Esterase activity stained with 4-Methylumbelliferyl Butyrate of the Astorga population; (B) Isocitrate dehydrogenase of the Astorga population; (C) Malic enzyme of the Maringá population; (D) Malate dehydrogenase of the Astorga population

The quaternary structure of the enzymes can be often inferred from the electrophoretic pattern of acceptable heterozygotes. The electrophoretic analysis of extracts of heterozygous *T. angustula* for EST, IDH, ME presented a two bands pattern, suggesting that these enzymes possess a quaternary monomeric structure.

The staining techniques revealed the esterase isozyme patterns of the *T. angustula* adults and indicated three esterase loci, referred to as Est-1, Est-2, and Est-3. Est-1 was monomorphic for individuals collected from Maringá and Est-3 presented activity only in *T. angustula* collected from Astorga.

*T. angustula* populations collected from two localities (Maringá and Astorga) presented one activity zone for IDH, with two alleles (Idh-1<sup>1</sup>, Idh-1<sup>2</sup>) being detected, with the Idh-1<sup>1</sup> allele being the most anodic. Two zones of ME activity were detected in both populations, with Me-1 the most anodic and Me-2 the least anodic. The population of Maringá was monomorphic for Me-1 and Me-2. While the population from Astorga presented Me-2 polymorphism, with two alleles being detected and Me-1 considered monomorphic.

Three activity zones for MDH, with Mdh-1 being the most anodic and Mdh-3 exhibited cathodic migration. Population from Maringá does not present polymorphism for the Mdh loci, but in populations from Astorga were detected polymorphism for all Mdh loci. Mdh-1 zone was only observed in individuals collected from Astorga.

### 3.2 Population genetics

The allele frequencies of each subpopulation are given in Table 2. *T. angustula* collected in Maringá presented two polymorphic loci (Est-2 and Idh) out of seven analyzed, totaling 22.22%. While *T. angustula* collected in Astorga presented 88.89%, with eight polymorphic loci.

Estimates of genetic variability for each subpopulation and for the total population were calculated on the basis of polymorphic loci (Table 2) and mean heterozygosity (Table 3). The two populations are in Hardy-Weinberg equilibrium for Idh (\(\chi^2\) = 3.309, \(p = 0.0689\)), Me-2 (\(\chi^2\) = 0.004, \(p = 0.9484\)), Mdh-1 (\(\chi^2\) = 0.047, \(p = 0.8277\)), Mdh-2 (\(\chi^2\) = 1784, \(p = 0.1816\)). The observed heterozigosity for *T. angustula* populations was 0.0775 and expected heterozygosity was 0.1632 (Table 3).

**Table 2** - Isozyme allele frequency in *Tetragonisca angustula* populations from Maringá and Astorga

<table>
<thead>
<tr>
<th>Locus</th>
<th>Maringá Population</th>
<th>Astorga Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele A</td>
<td>Allele B</td>
</tr>
<tr>
<td>Est-1</td>
<td>1.0000</td>
<td>---</td>
</tr>
<tr>
<td>Est-2</td>
<td>0.4595</td>
<td>0.5405</td>
</tr>
<tr>
<td>Est-3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Idh</td>
<td>0.7250</td>
<td>0.2750</td>
</tr>
<tr>
<td>Me-1</td>
<td>1.0000</td>
<td>---</td>
</tr>
<tr>
<td>Me-2</td>
<td>1.0000</td>
<td>---</td>
</tr>
<tr>
<td>Mdh-1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mdh-2</td>
<td>1.0000</td>
<td>---</td>
</tr>
<tr>
<td>Mdh-3</td>
<td>1.0000</td>
<td>---</td>
</tr>
</tbody>
</table>

**Source:** Research data.
Population differentiation was also examined by calculating $F_{ST}$, $F_{IT}$, and $F_{ST}$ values for each locus and the mean value across all loci (Table 4). $F_{IS}$ values correspond to mean deviation from random mating within subpopulations (0.4036), $F_{IT}$ values correspond to mean deviation from random mating over all subpopulations (0.5827) and $F_{ST}$ values correspond to the measure of the degree of genetic differentiation among subpopulations (0.5827). There are also three loci (Me-2, Mdh-1, Mdh-2) in heterozygosis.

Table 4 - Fixation index ($F_{IS}$), $F_{IT}$, $F_{ST}$ of *Tetragonisca angustula* populations. Nm = Gene flow estimated from $F_{ST} = 0.25(1 - F_{ST})/F_{ST}$

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sample Size</th>
<th>Observed heterozygosity</th>
<th>Expected heterozygosity</th>
<th>Average heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Est-1</td>
<td>148</td>
<td>0.0000</td>
<td>0.1023</td>
<td>0.068</td>
</tr>
<tr>
<td>Est-2</td>
<td>137</td>
<td>0.0000</td>
<td>0.3252</td>
<td>0.3220</td>
</tr>
<tr>
<td>Est-3</td>
<td>79</td>
<td>0.0380</td>
<td>0.3154</td>
<td>0.1577</td>
</tr>
<tr>
<td>Idh</td>
<td>149</td>
<td>0.1946</td>
<td>0.2275</td>
<td>0.2713</td>
</tr>
<tr>
<td>Me-1</td>
<td>150</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>Me-2</td>
<td>121</td>
<td>0.0165</td>
<td>0.0164</td>
<td>0.0110</td>
</tr>
<tr>
<td>Mdh-1</td>
<td>110</td>
<td>0.0455</td>
<td>0.0444</td>
<td>0.0222</td>
</tr>
<tr>
<td>Mdh-2</td>
<td>150</td>
<td>0.2000</td>
<td>0.1800</td>
<td>0.1178</td>
</tr>
<tr>
<td>Mdh-3</td>
<td>138</td>
<td>0.2029</td>
<td>0.2580</td>
<td>0.1635</td>
</tr>
<tr>
<td>Mean</td>
<td>131.5</td>
<td>0.0775</td>
<td>0.1632</td>
<td>0.1260</td>
</tr>
<tr>
<td>St. Dev</td>
<td></td>
<td>0.0927</td>
<td>0.1269</td>
<td>0.1146</td>
</tr>
</tbody>
</table>

Source: Research data.

Genetic identities, for the two populations, were calculated using the gene frequencies (Table 2), and Nei’s genetic distance (1978) was determined from the genetic identity values. Nei’s unbiased measures of genetic identity and genetic distance were 0.8401 and 0.1742, respectively (Table 5).

Three esterase activity zones were detected in *T. angustula* using 4-methylumbelliferyl butyrate. However, Est-3 was specific for a population from Astorga. Differences in several zones of esterase activity and substrate specificity detected here showed that 4-methylumbelliferyl butyrate was the best substrate for the detection of esterase activity since they permitted the detection of a larger number of esterase activity zones.

Table 5 - Genetic distance (NEI, 1978) for two populations of *Tetragonisca angustula*. Superior and inferior diagonals indicate respectively genetic identity and genetic distance

<table>
<thead>
<tr>
<th>Population</th>
<th>Maringá</th>
<th>Astorga</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maringá</td>
<td>*****</td>
<td>0.8401</td>
</tr>
<tr>
<td>Astorga</td>
<td>0.1742</td>
<td>*****</td>
</tr>
</tbody>
</table>

Source: Research data.

Three esterase activity zones were detected in *T. angustula* using 4-methylumbelliferyl butyrate. However, Est-3 was specific for a population from Astorga. Differences in several zones of esterase activity and substrate specificity detected here showed that 4-methylumbelliferyl butyrate was the best substrate for the detection of esterase activity since they permitted the detection of a larger number of esterase activity zones.
were observed: EST-1 (β-esterase, cholinesterase I), EST-2 (α-esterase, cholinesterase II), and EST-4 (αβ-esterase, carboxylesterase). Two more esterases were detected in *T. angustula*: EST-3 (β-esterase, acetylesterase) and EST-4 (αβ-esterase, carboxylesterase) (STUCHI et al., 2012). And the electrophoretic profile of *T. weyrauchi* showed six stereo regions (RONQUI et al., 2016), exceeding those observed for *T. angustula* and *T. fiebrigii*.

Allele variation for *Idh* did not get observed in a previous study (FALCÃO; CONTEL, 1991b). *Idh* polymorphism was detected in three species of Brazilian stingless bees: *Partamona helleri*, *Scaptotrigona bipunctata* and *Friesella schrottkyi* and three electrophoretic phenotypes were detected in *F. schrottkyi* females (FALCÃO; CONTEL, 1991b). Analysis of the electrophoretic profiles of IDH and genetic segregations obtained for the hives analyzed indicate that IDH molecule has a dimeric structure and that the control of this polymorphic system depends on the activity of two alleles at one locus. *Idh* allele frequencies indicate a high polymorphism degree in *T. angustula*.

Considering the ME isoenzyme, two loci were detected, but only the Astorga Me-2 was polymorphic. The Me polymorphism did not detect by Falcão and Contel (1991b) in studies with 10 species of Brazilian stingless bees, including *T. angustula*.

No genetic polymorphism for *Mdh* was observed in *T. angustula* from Maringá, and these results were observed by Falcão and Contel (1991b). The variation in MDH observed in *T. angustula* from Astorga indicates that two alleles at three loci are responsible for these polymorphisms. The MDH data obtained in the present study were not sufficient to permit inferences about the structure of the molecule of this enzyme, despite the complexity of electrophoretic patterns. The MDH isozyme study in the *P. droryana* showed individual variation which suggests the presence of four alleles at a single locus (MACHADO et al., 1992).

Population genetics research conducted is helping to explain general patterns of population structure and gene flow, mating systems and effective population sizes of common species (JHA, 2015). Genetics also played a key role in conservation biology, since the molecular tools allowed studies of the main conservation parameters that were previously very difficult, including species identity, gene flow levels, genetic diversity, inbreeding and kinship (ALLENDORF et al., 2013).

The heterozygosity observed (0.077) in this study, despite the few loci analyzed, was higher than that observed in other eusocial hymenopterans (0.05) (METCALF et al., 1975; PAMILO et al., 1978). However, it was considered low when compared to studies with *T. angustula* from different regions. Where population samples from Ivatuba (PR) were considered high (0.247), whereas, for the population of Dracena (SP) average heterozygosity (0.099) was observed, despite the distance of one population from the other, they were considered as a single population (STUCHI et al., 2006).

The variation of the isoenzyme polymorphism observed in this study was different from the polymorphism value of other bees such as *Plebeia remota*, *Partamona mulata*, and *Partamona helleri*, which have reduced numbers of polymorphic loci (FRANCISCO et al., 2006). Information similar to this study was observed with markers using mtDNA and microsatellites, where fifteen of the 21 loci proved to be polymorphic, with heterozygosities estimates observed ranging from 0.00 to 0.89 for *T. angustula* (BRITO et al., 2009). These polymorphic variations observed in Tetragonisca may be related to factors such as low genetic dispersion, different ecological conditions, and remote isolation shape the population structure of this species (FRANCISCO et al., 2017).

They may be associated with queens philopaties, interspecific reproductive parasitism and or artificial maintenance of hives, because evaluations with the species *Melipona mondury* Smith and *Melipona quadrifasciata* Lepeletier using heterospecific markers observe low levels of genetic variability for both species within the same meliponary (KOSER et al., 2014). About queens philopaties, the formation of new colonies leads to a reduction in genetic variability, consequently a greater genetic relationship between females (LÓPEZ-URIBE et al., 2015). This explains why the increase in nest density in a meliponary due to nest divisions can reduce genetic variability, leading to the production of diploid males and nest losses (SANTIAGO et al., 2016). This is due to the division of the colony keeping the same genetics in the meliponary and the reduced area of dispersion of the new nests. Making male dispersion a potentially important mechanism of genetic exchange between populations and prevention of inbreeding (LÓPEZ-URIBE et al., 2015). Genetic variation is reported with great importance in natural populations and these populations have a certain degree of genetic heterogeneity that provides adaptive flexibility (FRANCISCO et al., 2017).

The positive value of the *Fst* (0.4036) indicates that there is a homoyzgous excess in these populations. Through the value of *Fst* = 0.3002, we can observe that the populations present a high differentiation since the estimated value of *Fst* = above 0.25 indicates a high genetic differentiation (WRIGHT, 1978). Likewise, it is observed that there may be gene flow between populations, due to the values of *Nm*. Where *Nm* = 0.5827 is greater than *Nm* = 0.5 indicating gene flow (WRIGHT, 1978).

As the *T. angustula* analyzed in this study was collected from meliponaries, this difference can be divided by inbreeding, which would explain the high homoyzygous values detected. Microsatellite analyzes showed similar genetic variability between wild and captive populations. However, captive populations showed less mitochondrial genetic variability (SANTIAGO et al., 2016).

Genetic studies have shown variation both between
colonies within populations and between populations, and the increase in heterogeneity can have positive effects on colonial performance (survival and productivity) (PAMIILO et al., 1997). For the analyzed populations of T. angustula, small genetic distances were observed. The same behavior was observed when evaluating populations in the cities of Maringá and Cianorte, they observed the smallest genetic distances between the populations T. fiebrigi (BAITALA et al., 2006). The evaluation of populations of T. angustula from Ivatuba-PR and Dracena-SP, was not reported the separation of the two populations, forming a single population, despite the geographical distance between them (STUCHI et al., 2008).

Thus, due to variations in polymorphic loci added to the adaptation to similar habitats, it may have produced reduced genetic distance between the population analyzed, because about 50 years ago, the Atlantic Forest covered all of these areas, and adaptation to similar habitats may have caused a low genetic distance between populations (BAITALA et al., 2006).

More promising studies are being carried out since the discovery of the A. mellifera genome (WHITFIELD et al., 2006), and are producing contributions to address questions about the evolutionary history of native bees, providing tools to estimate points about species conservation (LOZIER; ZAYED, 2017). This information will be important for the control, preservation, and handling of these stingless bees and to alert about the importance of natural environment preservation for the maintenance of high levels of genetic variability to preserve pollination and production.

4 Conclusion

The application of the isoenzymes established a profile for T. angustula, producing good results since genetic variability was observed for the studied loci. The analyzed loci allowed to observe the genetic variability for both populations, showing that both populations have the same origin.

References


