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# Spatial Variation of Genetic Diversity in *Drosophila* Species from Two Different South American Environments

Luciana P. B. Machado, Daniele C. Silva,
Daiane P. Simão and Rogério P. Mateus
*Universidade Estadual do Centro-Oeste – UNICENTRO*
*Brazil*

## 1. Introduction

Currently, the main factor that generates natural habitats fragmentation is the use of land that results in reduced vegetation cover and in an asymmetric distribution of the remnants, which show different sizes and shapes (Didham et al., 1996). Usually, deforestation leads to fragmentation of continuous areas, generating the occurrence of vegetation islands that are isolated from each other by areas covered with grasslands or another type of culture (Lovejoy, 1980). In addition to deforestation, cyclic global climate change, such as glacial and interglacial periods with global warming, also has effects on the distribution of several morphoclimatic plant domains in South America, also resulting in habitat fragmentation (Ab'Saber, 2000).

In the southern and southeast region of Brazil there are two types of vegetation that are fragmented. They are the relicts of xerophytic vegetation and the Atlantic Forest domain. In the first case, there is in South America an extensive area in northeast-southwest axis, including the Caatinga, the Cerrado and Chaco, named "dry diagonal", which is located between the Amazon rainforest and the Atlantic Forest (Prado & Gibbs, 1993). The morphoclimatic areas of Caatinga and Chaco, along with the Caribbean coast of Colombia and Venezuela, have high density and diversity of cactus species (Hueck, 1972). However, adjacent areas, including the area of distribution of *Araucaria* forests, cacti are also detected in isolated populations, mainly in rocky leveling or associated with vegetation formations in sandy substrates (Manfrin & Sene, 2006). These populations of cacti are remnants of the xerophytic vegetation retraction in the interglacial periods (Ab'Saber, 2000). According to Ab'Saber (2000), the Caatinga and Chaco were connected at least four times during the Quaternary glaciations due to periods where the climate was dry and cold. In the interglacial periods, the climate became warm and wet, leading to the retraction of xerophytic vegetation and expansion of tropical forests. As cacti are good indicators of dry areas, their distribution is altered with climate change. Currently, the discontinuous distribution and size of the fragments of xerophytic vegetation are the result not only of paleoclimate cycles, but also of human action. In this context, it is expected that the *Drosophila* species associated with these cacti have followed the retractions and expansions of the dry areas of the paleoenvironment, and their population structures to reflect such changes.

In the second case, the Atlantic Forest domain includes a diverse mosaic of biomes (dense ombrophylous forest, mixed ombrophylous forest (*Araucaria* forest), deciduous and semi-deciduous seasonal forests, high altitude wet forests, northeastern enclaves, riparian forests) and associated ecosystems (high altitude grasslands, marshes, mangroves (Lino, 2002).

The mixed ombrophylous forest (MOF) includes the southern Brazil typical forests, with disjunctions in southeast and in neighboring countries, Paraguay and Argentina (Kozera et al., 2006). It is one of the most diverse biome in the world despite only few fragments of the original forest remains. This fact also makes it one of the most threatened biomes in the world, being considered a biodiversity hotspot and one of the priority areas for conservation (Myers et al., 2000). The remnants of this biome are located in the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo and Minas Gerais of Brazil (Inoue et al., 1984). They are characterized by presenting high and dense phytophysionomy, being structurally composed of an upper stratum dominated by *Araucaria angustifolia* and a subforest composed of angiosperms and gymnosperms (Machado & Siqueira, 1980). In Brazil, until the beginning of the 20th century, the *Araucaria* forest was the predominant landscape of the south region, with an area of approximately 200,000 km². It is estimated that in 1870, the area covered by *Araucaria angustifolia* natural forests in the state of Paraná was approximately 73,780 km². By the year 1995, due to intense wood and other non-logging species exploitation, the area in Paraná was reduced to 2,594 km² (Sanquetta, 1999).

The semi-deciduous seazonal forest is also known as interior forest. Scattered remnants of this type of biome are found in the Brazilian plateaus, in the states of São Paulo, Paraná, Minas Gerais, Mato Grosso do Sul, Santa Catarina and Rio Grande do Sul. In the southern states of Brazil, it is often associated with the *Araucaria* forest. Some enclaves are also found in the Brazilian northeast. The semi-deciduous seazonal forest features are strongly determined by its continentality (Warren, 1996). Climate changes causes 20% to 50% of the trees to lose their leaves during the dry season. This is one of the most endangered biome within the Atlantic forest domain. What remains is confined to small and medium-sized fragments and very distant from each other, mostly of them located in protected areas (MMA-SBF, 2002). It is divided into lowland semi-deciduous seazonal forest, submountain semi-deciduous seasonal forest, and mountain semi-deciduous seazonal forest (Veloso & Góes-Filho, 1982). The ecological concept of this type of vegetation is conditioned to the dual climate seasonality: one tropical, with a time of intense summer rains followed by accentuated drought; and other subtropical, without dry season, but with physiological drought caused by the intense winter cold, with averages temperatures below 15°C (Rizzini, 1979).

The Atlantic Forest domain, as all other Brazilian biomes, is under strong fragmentation because of agricultural activities and the progressive growing the town surrounding. This drastic decrease of the vegetation cover make it as an area of extreme biological importance, priority in researches about species inventories and determination of genetic variability from species that are found in these areas. *Araucaria* forest (or Mixed Ombrophilous Forest) is one ecosystem of the Atlantic Forest biome. Since 2002, the Ministry of Environment of Brazil recommended the creation of an ecological corridor linking the remaining areas of Santa Catarina and Parana in order to avoid extinction of this ecosystem.

In this context, drosophilids can be used as bioindicators of environment quality and diversity. These animals have the capacity to reflect ecological changes because different

species have different requirements regarding the quality of the environment (Ferreira & Tidon, 2005, Mateus et al., 2006), and most species have limited dispersal ability (Markow & Castrezana, 2000). More recently, the *Drosophila* genus has been the focus on biodiversity studies (van der Linde & Seventer, 2002; Tidon, 2006; Torres & Madi-Ravazzi, 2006, de Toni et al., 2007) because of its great diversity, morphologically and ecologically. In this sense, these insects are suitable for studies involving ecological, biogeographic and evolutionary approaches.

For the analysis of the xerophytic vegetation islands, the *Drosophila antonietae* species is a good model for study (Manfrin & Sene, 2006). On the other hand, for the analysis of the Atlantic forest biome, which includes *Araucaria* forest and semi-deciduous seazonal forest ecosystems, *Drosophila ornatifrons* was the species of choice because it is one of the prevailing species in the Atlantic forest (Sene et al., 1980; Tidon-Sklorz et al., 1994). Thus, this work aimed to characterize the genetic variability of two *Drosophila* species found in fragments of two different southern South America environments, one from the xerophytic vegetation islands of the "dry diagonal" (*D. antonietae*) and the other from Atlantic forest biome (*D. ornatifrons*), with the purpose to evaluate the effect of differetnt types of habitat fragmentation over this genetic feature.

## 2. Materials and methods

### 2.1 Species

*Drosophila antonietae* specimens were collected in three areas of *Cereus hildmaniannus* occurrence in the Iguassu river basin, located in the middle portion of the Parana-Uruguay rivers basin, named as Cantagalo-PR (25º25'00.0" S, 52º04'14.9" W), Rio do Poço/Guarapuava-PR (25º17'29.8" S, 51º53'08.8" W) and Segredo (25º46'27,2" S, 52º06'55,6" W), all in Parana State, Brazil. Following the protocol proposed by Mateus et al. (2005), thorax and abdomen were individually dry stored at -20ºC and used for the allozymic analyses in another work (Lorenci et al., 2010). The respective heads were stored in 70% ethanol at -20ºC and were used for DNA extraction, which were amplified for 7 microsatellite loci (AluRSAIanto-1, HaeIIIanto-2, HaeIIIanto-3, HaeIII400anto-4, HaeIII400anto-5, AluRSAIanto-6, AluRSAIanto-7), according to Machado et al. (2003).

*Drosophila ornatifrons* were collected in four Atlantic forest fragmented areas in the south and southeast regions of Brazil, two *Araucaria* forest fragments, and two semi-deciduous seazonal forest fragments, named as Parque das Araucárias/Guarapuava-PR - PA (25º23'36" S, 51º27'19" W), Salto São Francisco/Guarapuava-PR – SSF (25º03'49.1" S, 51º17'29.8" W), Cajuru/SP - CAJ (21º19'10.2" S, 47º16'14.7" W) and Sertãozinho/SP – SRT (21º09'07.8" S, 48º04'58.0" W), respectively. Following the protocol proposed by Silva et al. (2010), thorax and abdomen were individually dry stored at -20ºC for the isoenzymatic analyses using six allozymic systems (EST, 1-GPDH, IDH, MDH, PGM and ME) according to Mateus and Sene (2003, 2007). The respective heads were stored in 70% ethanol at -20ºC and will be used in further analyses (not performed yet) using DNA markers (microsatellite).

### 2.2 Population genetics analyses

The population genetic analyses were performed, using two different softwares. The allele frequency, mean heterozigosity (observed – Ho, and expected – He), polymorphic loci

percentage, Wright's F statistics (Weir & Cockerham, 1984), genetic distance (Nei, 1972), Hardy-Weinberg equilibrium test (using the exact test with the conventional method of Monte Carlo and the Markov Chain test – 10 batches and 2,000 permutations per batch) and the UPGMA grouping analysis (using Nei's *D*) were obtained through the TFPGA (Miller, 1997) software. The genetic distance (Reynolds et al., 1983), the presence of exclusive allele and the Neighbor-Joining grouping analysis (Saitou e Nei, 1987) were performed using the GDA (Lewis e Zaykin, 2001) software. The correlation between *D. antonietae* populations genetic distances and both geographical (straight distances between populations) and ecological (distances between populations through the rivers) distances, according to Mateus et al. (2007), were tested using the Mantel test in the TFPGA software. In this test, the pairwise populations were compared and the genetic distances applyed were Reynolds et al. (1983), obtained in the GDA software.

The Fst values obtained were used for: (1) Classification of the genetic differentiation among populations (spatial analyses) using the qualitative guide proposed by Wright (1978) as 'low' (0 – 0,05), 'moderate' (0,05 – 0,15), 'high' (0,15 – 0,25) and 'very high' (> 0,25); (2) Gene flow estimate among populations using the formula described by Wright (1931), $Fst = 1/(4Nm + 1)$, where $Nm$ represents the efective number of migrant gametes among populations per generation, and the verification of the balance between genetic drift and gene flow according to Kimura & Weiss (1964). In this case, it was assumed that the populations were in equilibrium according to the island model of gene flow, in which the equation is based.

## 3. Results and discussion

### 3.1 Environment 1: Xerophytic vegetation islands

The amplification of the seven microsatellite loci described by Machado et al. (2003) was conducted in 75 *Drosophila antonietae* specimens (25 from each natural population analyzed). The HaeIIIanto-2 locus was amplified only in four of these samples, two from Cantagalo and two from Rio do Poço. Thus, this locus was not used in the populational analyses. The allele numbers detected for each locus were: five in both AluIRSAIanto-1 and HaeIIIanto-3 loci; four in HaeIII400anto-4, AluRSAIanto-6 and AluRSAIanto-7; and three in HaeIII400anto-5.

Table 1 shows the allele frequencies for 7 loci in three populations analyzed. A locus was considered polymorphic when the most frequent allele did not have frequence above 95%.

The allele frequencies analysis (Table 1) demonstrated that all populations showed polymorphism in all loci. Exclusive alleles were found for the AluIRSAIanto-1 (allele 1 in Rio do Poço) and HaeIIIanto-3 (allele 5 in Rio do Poço) loci. All loci showed significant departure from the expected by the Hardy-Weinberg equilibrium in at least one population, with Rio do Poço (PR) presenting only one and Cantagalo and Segredo presenting four loci out of the Hardy-Weinberg expectations. These results indicated that the frequencies and the genetic diversity observer can not be maintained by recurrent mutation alone.

A very high within and among populations heterozygote deficiency were detected (Fis = 0.2561 and Fit = 0.2927), however they were not statistically different from zero (Table 2), confirming the observation that all mean expected heterozygosities (He) were higher than all mean observed heterozygosities (Ho) in all populations (Table 1). However, the overall mean observed heterozygosity, considering the three analysed populations, was 0.4614. This

| ***Loci*/Alleles** | **Rio do Poço** | **Cantagalo** | **Segredo** |
|---|---|---|---|
| *AluRSAIanto-1* | | | |
| 1 | **0.24** | - | **-** |
| 2 | **0.37** | - | **0.42** |
| 3 | **0.27** | 0.59 | **0.14** |
| 4 | **0.04** | 0.35 | **0.33** |
| 5 | **0.07** | 0.06 | **0.11** |
| *HaeIIIanto-3* | | | |
| 1 | 0.52 | **0.48** | **0.43** |
| 2 | 0.19 | **0.29** | **0.28** |
| 3 | 0.17 | **0.17** | **0.22** |
| 4 | 0.10 | **0.06** | **0.07** |
| 5 | 0.02 | **-** | **-** |
| *HaeIII400anto-4* | | | |
| 1 | 0.50 | **0.37** | 0.47 |
| 2 | 0.11 | **0.07** | 0.08 |
| 3 | 0.34 | **0.41** | 0.36 |
| 4 | 0.04 | **0.07** | 0.09 |
| 5 | - | **0.02** | - |
| 6 | - | **0.06** | - |
| *HaeIII400anto-5* | | | |
| 1 | 0.46 | **0.48** | **0.40** |
| 2 | 0.46 | **0.48** | **0.34** |
| 3 | 0.08 | **0.04** | **0.26** |
| *AluRSAIanto-6* | | | |
| 1 | 0.42 | **0.07** | 0.31 |
| 2 | 0.46 | **0.60** | 0.53 |
| 3 | 0.04 | **0.19** | 0.16 |
| 4 | - | **0.14** | - |
| 5 | 0.08 | **-** | - |
| *AluRSAIanto-7* | | | |
| 1 | 0.54 | 0.43 | **0.10** |
| 2 | 0.29 | 0.50 | **0.32** |
| 3 | 0.15 | 0.07 | **0.46** |
| 4 | 0.02 | - | **0.12** |
| **$P_{0.95}$** | 100 | 100 | 100 |
| **Ho** | 0.4926 | 0.5664 | 0.4132 |
| **He** | 0.6389 | 0.6112 | 0.6396 |

Table 1. Allelic frequencies for seven microsatellite loci in three populations of *Drosophila antonietae*. Ho = mean observed heterozygosity; He = mean expected heterozygosity; $P_{0.95}$ = polymorphic loci percentage; numers in bold indicate loci that showed departure from the Hardy-Weinberg equilibrium.

value is higher than the mean observed heterozygositiy obtained for cactophilic (Ho = 0.087) and non cactophilic (Ho = 0.160) *Drosophila*, using allozyme data (revision from Zouros, 1973; Johnson, 1974; Barker & Mulley, 1976; Moraes & Sene, 2002). It was also higher than

the previous calculated for *D. antonietae*, also with allozymes (Ho = 0.2242 – Mateus & Sene, 2003; Ho = 0.319 – Mateus & Sene, 2007), it was higher than the Ho obtained for 5 populations of this species by Machado et al. (2003) using microsatellite DNA (Ho = 0.2543), and even slightly higher than the Ho obtained for 10 populations of this species analyzed by Machado (2003) also using microsatellite DNA (Ho = 0.3835).

| ***Locus*** | Fis | Fit | Fst |
|---|---|---|---|
| *AluRSAIanto-1* | 0,4313 | 0,5303 | 0,1742 |
| *HaeIIIanto-3* | 0,4861 | 0,4749 | -0,0217 |
| *HaeIII400anto-4* | -0,3963 | -0,4144 | -0,0130 |
| *HaeIII400anto-5* | 0,3030 | 0,2991 | -0,0056 |
| *AluRSAIanto-6* | 0,2379 | 0,2318 | -0,0080 |
| *AluRSAIanto-7* | 0,4196 | 0,4889 | 0,1194 |
| **All *loci*** | 0,2561 | 0,2927 | 0,0491 |
| **I.C. 95%** - minimum | -0,0301 | -0,0295 | -0,0142 |
| - maximum | 0,4296 | 0,4823 | 0,1135 |

Table 2. Wright F statistics for seven *loci* in three *Drosophila antonietae* populations.

According to Prout & Barker (1993), there are several possible reasons for a positive Fis: positive assortative mating, inbreeding resulting from sib mating, null alleles, and temporal Wahlund effect. Besides those, selection against heterozygotes is another possible cause. Kimura & Crow (1963) proposed that the Fis should be negative under a random mating system. Our results evidenced one locus with negative Fis (HaeIIanto-4). For all others, a variation between high and very high deficiency of heterozygotes within populations occurred for all populations (Table 2). These results diverge from what it is expected for endogamic populations as different Fis values were observed for each locus and inbreeding should affect all loci at the same rate. Furthermore, Mateus & Sene (2003) showed that *D. antonietae* do not display an inbreeding behavior analysing the allozymic pattern of flies emerged from different rotting cacti.

Wahlund effect can be a possible cause of heterozygote deficiency in populational genetics studies (Johnson e Black, 1984) and, in the present case, it can not be discarded. The most plausible scenario is that our samples are compoused by flies from different generations (temporal Wahlund effect). A spatial Wahlund effect is less possible as there is population structure and geographic isolation among *D. antonietae* populations.

Another plausible cause of heterozygote deficiency is the presence of null alleles, which could be quite commom in microsatellite samples (see Van Treuren, 1998; and McGoldrick et al., 2000, as examples). The fixation os a null allele is responsable for most of the failure in amplification experiments (Callan et al., 1993). Null alleles are represented by segments that do not amplify and segregates with other amplifying alleles, generating a false homozygote. The amplification of only two alleles for HaeIIIanto-2 locus in only four specimens (two from Rio do Poço and two from Cantagalo) is an evidence of null allele presence for this locus. According to Machado (2003), the presence of null alleles can explain the non amplification of *Drosophila buzzatii* microsatellite loci in *D. antonietae*. Machado et al. (2010), analyzing the same seven microssatellite loci of *D. antonietae*, detected the presence of null allele in the AluRSAIanto-6 locus and size homoplasy in four out of seven loci. However,

they concluded that null allele and size homoplasy do not appear to represent significant problems for the population genetics analyses because the large amount of variability at microsatellite loci can compensate the low frequency of these problems in the populations investigated. In our case, the presence of null alleles was important as the HaeIIIanto-2 locus was excluded from the populational analyses.

Natural selection against heterozygotes is another event that could be generating the heterozygosity deficiency observed. However, the data present here do not allow the evaluation of such event as the fitness of the heterozygotes over time was not measured. In *D. antonietae*, Mateus & Sene (2003) verified the possible action of natural selection using temporal and spatial approaches of the allozymic variation. Later, Mateus & Sene (2007) pointed out that natural selection is a possible factor preventing genetic divergence among *D. antonietae* populations. Machado (2003) tested the occurrence of a hitchiking effect between 7 microsatellite and 10 allozymatic loci in *D. antonietae* and no correlation was found between these markers. However, the hitchiking hypothesis with another genetic system was not discarded.

Natural populations can present heterozygotes deficience because of assortative mating. For the *D. buzzatii* cluster, Machado et al. (2002), analysing courtship behavior, observed that males always court females no matter they are the same species or not. Kelly & Noor (1996) also observed the same pattern with other *Drosophila* species. Therefore, there are evidences that assortative mating is not occurring not only in the *D. buzzatii* cluster but in the *Drosophila* genus in general.

The Wright F statisctics (Table 2) also showed low and statistically not different from zero genetic differentiation among populations (Fst = 0.0491). The highest Reynolds et al. (1983) genetic distance (Table 3) was found between Cantagalo and Segredo (0.0725) and the lowest was between Cantagalo and Rio do Poço (0.0321). The Neighbor-Joining analysis using Reynolds et al. (1983) distances did not show any correlation between ecological and genetic distances among populations (Figure 1). Rio do Poço population, which is located in the head of the Cavernoso river and therefore is the first in the sequence of this river transection (frow headwater to the mouth), was grouped in the middle of Cantagalo (the second downstream) and Segredo (the third and the last before Cavernoso river flows into Iguassu river). The Mantel test did not result in a statistically sigficative correlation between genetic and ecologic distances (r = 0.63; p = 0.33) and genetic and geographic distances (r = 0.82; p = 0.32). As these populations are in the same river system and because no correlation was found among distances, it was not possible to assume that they were in regional equilibrium (Hutchison and Templeton, 1999) and thus the number of migrantes (*Nm*) was not calculated.

| **Populations** | Rio do Poço | Cantagalo |
|---|---|---|
| Cantagalo | 0.0321 | **** |
| Segredo | 0.0425 | 0.0725 |

Table 3. Reynolds *et al.* (1983) distances between all pairwise populations of *D. antonietae*.

The genetic differentiation (Fst) found for *D. antonietae* (Table 2) was lower than the observed in a microgeographic analysis realized with *D. mediopunctata* from *Araucaria* forest fragments in the Parana state, Brazil (Fst = 0.066 in the winter; Cavasini, 2009). However, it

was higher than the Fst detected by Mateus & Sene (2003) for *D. antonietae* in the within population spatial and temporal allozyme variation approaches (Fst = 0.0355 and 0.0023, respectivelly). Considering a among population approach, the Fst obtained here was lower (almost half the value) than those obtained using allozymes (Fst = 0.0723; Mateus & Sene, 2007) and microsatellite (Fst = 0.0730; Machado, 2003). In the same way, Reynolds et al. (1983) genetic distances (Tabela 3) showed values consistents with the Fst, sugesting that despite the low genetic differentiation there is population structure among the populations studied.

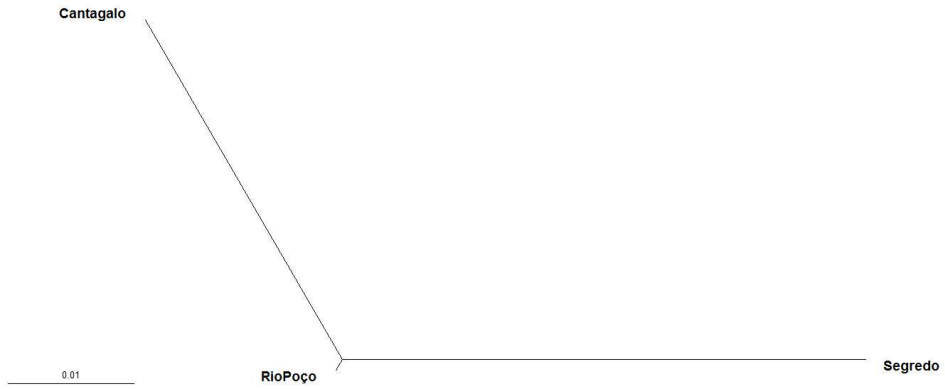


Fig. 1. Neighbor-Joining analysis for three *Drosophila antonietae* populations using Reynolds et al. (1983) genetic distances.

Gene flow is a factor that could decrease among population differentiation and the allele sharing could increase the genetic variability within populations. All that is possible considering that population size is not as small as it should be to make the genetic drift to become an important force. As detected, there is high polymorphism and diversity within and low genetic differentiation among the analyzed populations. Gene flow was suggested by Monteiro & Sene (1995) as the promoter of the low morphological differentation in the aedagi of flies from geographic isolated *D. antonietae* populations. However, the geographic distances between populations of this species raises the question of the possibility of a real and current gene flow among them. Mateus & Sene (2007) with allozymes and Manfrin et al. (2001) and de Brito et al. (2002) with mDNA indicated a certain degree of differentiation among the populations from the high portion and from the low portion of the Parana-Uruguay rivers basin. Because of the association between *D. antonietae* and the cactus *Cereus hildmaniannus*, which is distributed mainly along the rivers basin, it has been proposed that there is gene flow among *Drosophila* populations through these rivers "corridors" following the cacti distribution, preventing genetic differentiation among them (Monteiro & Sene, 1995; Machado, 2003; Mateus & Sene, 2007). Our results did not support this hypothesis.

In this ecological specificity context, Manfrin & Sene (2006) argue that this cactophilic association of the *D. buzzattii* cluster is an important aspect of this group evolution. Cactophilic *Drosophila* populations might have followed the xerophitic vegetation expansions and retractions caused by the paleoclimate cyclic changes, with the actual distribution being a result of all the glaciation cycles that occured in the Terciary and

Quaternary periods (Bigarella et al., 1975; Ab'Saber, 2000), associated with recent fragmentation caused by human activities. These events might have acted as a vicariant agent contributing to this group evolutionary history (Sene et al., 1988). A possible hypothesis to explain this pattern is the historical maintenance of shared polymorphisms, as proposed by Mateus & Sene (2007). After each retraction, the remaining populations could have retained the ancestral population polymorphism through several non-exclusive factors: 1) by having large effective population size (Mateus & Sene, 2003), decreasing the genetic drift performance; 2) by a non-sufficient divergent time among populations (Manfrin & Sene, 2006; Mateus & Sene, 2007), and; 3) the microsatellite markers could be hitchhiking a natural selection on other genetic systems, with the exception of allozymes (Machado, 2003). Current or recent gene flow among populations is unlikely to be the explanation as the Mantel test performed here did not showed correlation between geographic positioning and genetic differentiation.

In spite of the low genetic differentiation among populations obtained, exclusive alleles were detected for two out of the seven evaluated loci. Cantagalo and Segredo were the most different populations regarding allele composition and also Reynolds et al. (1983) genetic distance. Rio do Poço and Cantagalo showed the lowest distance (Table 3). In the Neighbor-Joining grouping analysis Rio do Poço population was placed bewteen Cantagalo and Segredo populations, despite it is located upstream in the Cavernoso river (Figure 1). This absence of pattern between geographical positioning and genetic distances was corroborated by the Mantel test. These data seems to indicate that an incipient diversification is occuring in the populations analysed, process that could be in response to a regional adaptation or simply by stocastic events.

Thus, the results obtained using microsatellite loci diversity analyses of *D. antonietae* populations from the Iguassu river basin, which flows into the Parana river, are in agreement with other markers for this species, that is, high within population diversity and low among population differentiation, however with the presence of an ancipient diversification.

### 3.2 Environment 2: Atlantic forest fragments

The electrophoretic analyses of 9 isoenzymatic systems of 145 *Drosophila ornatifrons* specimens (58 from PA, 55 from SSF, 17 from CAJ and 15 from SRT) resulted in 7 loci and 46 alleles. The allele frequencies for all *D. ornatifrons* populations are presented in Table 4.

The allele numbers detected for each locus were: ten in *Est*; and six in *Gpdh*, *Idh*, *Mdh-1*, *Mdh-2*, *Me* and *Pgm*. A locus was considered polymorphic when the most frequent allele did not have frequence above 95%. Three out of four populations (PA, SSF e SRT) showed all loci polymorphic. The CAJ population showed only one monomorphic locus (*Mdh-1*). These results demonstrated that there is high allele diversity for this species, higher than most of the microstellite loci previous tested for this species (Laborda et al., 2009) and other species of this genus. For example, Mateus et al. (2008), analysing the allele diversity of 4 isoenzymatic systems (IDH, MDH, ME e1-GPDH) in 11 species of the *tripunctata* group (that belongs to the same *quinaria-tripuncata* section where the *guarani* group is found) observed higher number of alleles only for *Idh* and *Mdh-1*. Saavedra et al (1995) studied the polymorphism for 4 allozymatic loci (*Sod*, *Odh*, *Est-2* and *Est-3*) in natural populations from

south of Brazil of *D. maculifrons*, another species os the *guarani* group, and also found high allele diversity (32 alleles), however lower than the one found here.

| ***Loci*/Alleles** | **PA** | **SSF** | **CAJ** | **SRT** | ***Loci*/Alleles** | **PA** | **SSF** | **CAJ** | **SRT** |
|---|---|---|---|---|---|---|---|---|---|
| *Mdh-1* | | | | | *Est* | | | | |
| 1 | **0.10** | **0.23** | 1.00 | **0.07** | 1 | **0.02** | **0.02** | **-** | **-** |
| 2 | **0.42** | **0.09** | - | **0.63** | 2 | **-** | **0.06** | **-** | **-** |
| 3 | **0.22** | **-** | - | **0.17** | 3 | **0.07** | **0.12** | **-** | **0.04** |
| 4 | **0.22** | **0.39** | - | **0.13** | 4 | **0.10** | **0.14** | **0.04** | **0.43** |
| 5 | **0.02** | **0.29** | - | **-** | 5 | **0.22** | **0.15** | **0.23** | **0.21** |
| 6 | **0.01** | **-** | - | **-** | 6 | **0.14** | **0.18** | **0.18** | **0.21** |
| *Mdh-2* | | | | | 7 | **0.14** | **0.08** | **0.14** | **0.07** |
| 1 | **0.09** | **0.37** | - | - | 8 | **0.03** | **0.20** | **0.14** | **0.04** |
| 2 | **0.17** | **-** | - | - | 9 | **0.17** | **0.04** | **0.27** | **-** |
| 3 | **0.04** | **0.63** | | - | 10 | **0.11** | **0.01** | **-** | **-** |
| 4 | **0.33** | **-** | - | - | *Gpdh* | | | | |
| 5 | **0.30** | **-** | - | - | 1 | **0.04** | - | **-** | - |
| 6 | **0.07** | **-** | - | - | 2 | **0.06** | - | **-** | - |
| *Me* | | | | | 3 | **0.20** | - | **-** | 0.07 |
| 1 | **0.19** | **0.33** | **0.29** | **0.21** | 4 | **0.46** | 0.77 | **0.83** | 0.68 |
| 2 | **0.09** | **0.22** | **0.29** | **0.57** | 5 | **0.20** | 0.21 | **0.17** | 0.25 |
| 3 | **0.43** | **0.37** | **0.36** | **0.71** | 6 | **0.04** | 0.02 | **-** | - |
| 4 | **0.12** | **0.06** | **0.03** | **-** | *Idh* | | | | |
| 5 | **0.12** | **0.01** | **0.03** | **0.14** | 1 | **0.36** | **0.36** | **0.21** | **0.15** |
| 6 | **0.05** | **0.01** | **-** | **-** | 2 | **0.20** | **0.37** | **0.46** | **0.11** |
| *Pgm* | | | | | 3 | **0.25** | **0.25** | **0.08** | **0.54** |
| 1 | **-** | **-** | 0.81 | **0.20** | 4 | **0.12** | **0.02** | **-** | **0.04** |
| 2 | **0.02** | **-** | 0.06 | **0.55** | 5 | **0.07** | **-** | **0.21** | **0.08** |
| 3 | **0.20** | **0.09** | 0.13 | **0.25** | 6 | **-** | **-** | **0.04** | **0.08** |
| 4 | **0.09** | **0.16** | - | **-** | **$P_{0.95}$** | 100 | 100 | 83 | 100 |
| 5 | **0.64** | **0.60** | - | **-** | **Ho** | 0.3609 | 0.4060 | 0.1489 | 0.2640 |
| 6 | **0.05** | **0.16** | - | **-** | **He** | 0.7326 | 0.6308 | 0.4881 | 0.6229 |

Table 4. Allelic frequencies for nine allozyme loci in four populations of *Drosophila ornatifrons*. Ho = mean observed heterozygosity; He = mean expected heterozygosity; $P_{0.95}$ = polymorphic loci percentage; numers in bold indicate loci that showed departure from the Hardy-Weinberg equilibrium.

The mean observed heterozygosity (Ho) was 0.3609 for PA, 0.4060 for SSF, 0.1489 for CAJ and 0.2640 for SRT. The mean expected hetreozygosity (He) was 0.7326 for PA, 0.6308 for SSF, 0.4881 for CAJ and 0.6229 for SRT. These values were higher than the Ho obtained for other *Drosophila* species (Zouros, 1973; Johnson, 1974; Barker & Mulley, 1976; Moraes & Sene, 2002; and Cavasini, 2009) and the São Paulo populations showed lower Ho than the Parana populations. This result could be due to the fact that *D. ornatifrons* is a species restricted to non-disturbed environments (Ferreira & Tidon, 2005), fact that could also explain the Ho similarity between semi-deciduos forest populations and between *Araucaria* forest populations.

All loci showed significant departure from the expected by the Hardy-Weinberg equilibrium in at least two populations: PA presented all nine polymorphic loci out of the Hardy-Weinberg expectations; SSF and SRT showed only *1-Gpdh* in equilibrium; and CAJ showed only *Pgm* in equilibrium. These results indicated that the frequencies and the genetic diversity observed can not be maintained by recurrent mutation alone and is expected in natural populations. According to Falconer & Mackay (1996) the changes in the allele frequencies in natural populations could be due to systematic process as mutation, natural selection, gene flow, inbreeding or a dispersive process as genetic drift. All these will be discussed here.

Mutation have a very little impact in the populational genetic diversity over time and could be disregarded. Natural selection caused by environmental factors is possible because several authors already reported that this genus is sensitive to environmental changes (Barker et al., 1986; Moraes, 2000; Mateus, 2001; Moraes & Sene, 2002; Mateus & Sene, 2003; Ferreira & Tidon, 2005; Cavasini, 2009). Several works suggested some association of the allozyme genetic variation with environment; however whether the variation is maintened by natural selection still remains as an important question in evolutionary biology (Lewontin, 1974; Nei, 1975; Kimura, 1983; Koehn et al., 1983). Zapata et al. (2000) detected that natural selection is an evolutionary force operating in the allozyme-chromossomic inversion association in *D. subobscura*. In *D. buzzatii*, the analysis of allozyme variation in colonizing populations in Australia and Spain suggested a significant role of natural selection shaping the allele frequency distribution for several loci (Barker & East, 1980; Barker et al., 1986; Rodriguez et al., 2000), which are strictly linked to chromossomal inversions rearrangements (Schaffer et al., 1993; Betrán et al., 1995; Rodriguez et al., 2000). In the *guarani* group would be necessary to verify the association between allozymes and chomossomic inversions that were already detected in some of its species (Brncic, 1953; Salzano, 1954).

In the present work was not possible to directly associate the genetic diversity with environmental changes because abiotic data were not collected to perform this type of analysis. However, the possible action of natural selection caused by environmental factor can not be discarded because the areas of collection show different characteristics, such as different sizes, vegetation (*Araucaria* forest in Parana and Seazonal semideciduos forest in São Paulo), conservation level, climate, altitude, average temperature and pluvisosity, which could result in different microhabitats and selection pressures.

Table 5 shows the results of the Wright F statistics. The Fis indicated a very high and statistically significant heterozygote deficiency both within (Fis = 0.4896) and among populations (Fit = 0.5621). All loci presented a very high heterozygote deficiency.

These values were higher than the heterozygote deficiency found for other species of the genus, such as *D. mediopunctata* (Cavasini, 2009), *D. antonietae* (Mateus & Sene, 2003, 2007, and the present work), *D. gouveai* (Moraes & Sene, 2002). According to Kimura & Crow (1963), a negative Fis is expected under random mating. There are several reasons to detect a positive Fis: null alleles, temporal Wahlund effects, selection against heterozygotes, assortative mating and inbreeding (Prout & Barker, 1993). The frequency of null alleles in allozymes is low and not sufficient to explain a positive Fis, remaining inbreeding, Wahlund effect and selection against heterozygotes as the most likely hypothesis. Inbreeding should affect all loci in the same way and similar Fis values are expected for all loci. However, the data presented here showed different values of Fis for each locus, which is inconsistent with inbreeding. Assortative mating seems unlikely because this behavior was never detected in

*Drosophila* (Kelly & Noor, 1996) and some works already described the existence of hybrids in the *guarani* group (King, 1947; Kastritsis, 1969). A Wahlund effect is possible if it is considered that more than one generation was sampled in each collection (overlapping generations). Selection against heterozygotes is difficult to be tested as discussed above.

| ***Locus*** | Fis | Fit | Fst |
|---|---|---|---|
| *Est* | 0,4753 | 0,4902 | 0,0284 |
| *Gpdh* | 0,2625 | 0,3007 | 0,0518 |
| *Idh* | 0,5322 | 0,5540 | 0,0465 |
| *Mdh-1* | 0,5407 | 0,6086 | 0,1480 |
| *Mdh-2* | 0,2611 | 0,5010 | 0,3246 |
| *Me* | 0.6729 | 0.6904 | 0.0534 |
| *Pgm* | 0,6246 | 0,7301 | 0,2811 |
| **All *loci*** | 0,4896 | 0,5621 | 0,1420 |
| **I.C. 95%** - minimum | 0,3740 | 0,4764 | 0,0583 |
| - maximum | 0,5810 | 0,6437 | 0,2338 |

Table 5. Wright F statistics for nine loci in four *Drosophila ornatifrons* populations.

The overall Fst (0.1420) indicated a moderate genetic differentiation and the existence of a population structure among the four analysed populations as already detected for other *Drosophila* species: *D. pavani* (Kojima et al., 1972), *D. mediopunctata* (Cavasini, 2009), *D. antonietae* (Mateus & Sene, 2007). This result was corroborated by the Nei (1972) genetic distance analyses (Table 6) that showed variation between 0.3145 (between PA and SSF) and 0.7166 (between PA and CAJ). The Nei (1972) genetic identities ranged between 0.4884 (between PA and CAJ) and 0.7302 (between PA and SSF). These results showed that a possible correlation between geographic and genetic distance exist for *D. ornatifrons* as the Parana populations (SSF and PA) presented the higher identity and the lower distance. However, this possible relation was not confirmed in the Neighbor-Joining analysis (Figure 2).

| **Populations** | PA | SSF | CAJ | SRT |
|---|---|---|---|---|
| PA | - | 0.3145 | 0.7166 | 0.4643 |
| SSF | 0.7302 | - | 0.4902 | 0.5343 |
| CAJ | 0.4884 | 0.6125 | - | 0.6642 |
| SRT | 0.6286 | 0.5861 | 0.5147 | - |

Table 6. Nei (1972) distances (above diagonal) and identities (below diagonal) between all pairwise populations of *D. ornatifrons*.

Through the Fst obtained for *D. ornatifrons* (0.1420), the effective number of migrants (*Nm*) was calculated as 1.51, indicating moderated levels of gene flow and genetic drift according to Kimura e Weiss (1964). However, the Nei (1972) distances and identities obtained were also an indication of the presence of more than one evolutionary lineage in the samples of this species. According to Avise & Smith (1997) and Thorpe (1983), populations of the same species tend to show Nei's identity above 0.9 and distance below 0.1, and congeneric species show identity between 0.25 and 0.85 and distances between 0.16 and 1.39. Similar results were found by Mateus et al. (2010) in two criptic species of the *buzzatii* cluster: *D. antonietae* and *D. gouveai*. In fact, a recent aedeagi analysis of flies identifed as *D. ornatifrons* resulted in at least three different aedagi present in the sample (N. P. Heinz, personal communication).

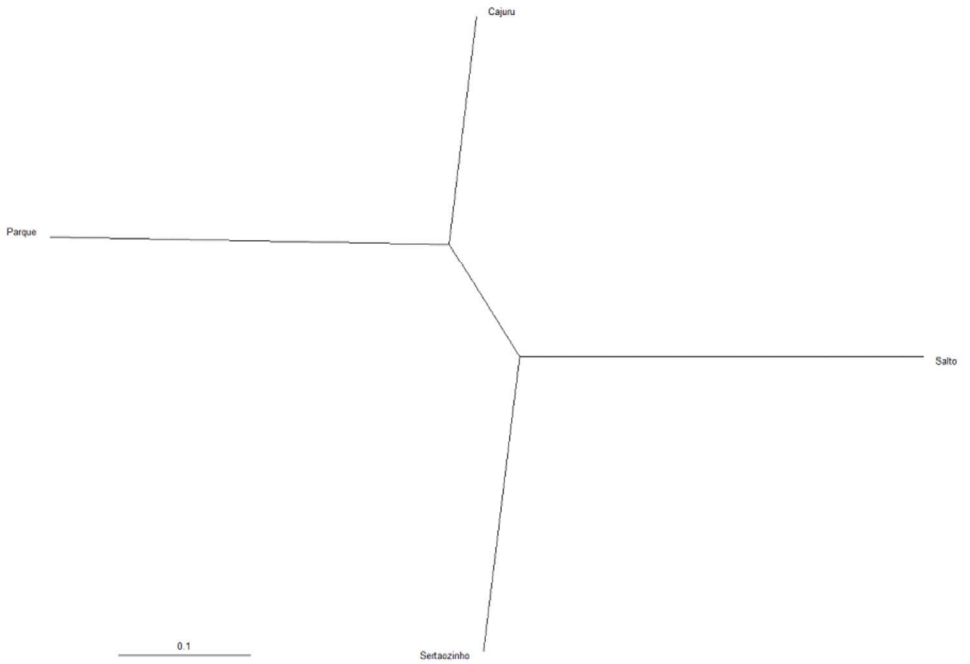


Fig. 2. Neighbor-Joining analysis for four *Drosophila ornatifrons* populations using Nei (1972) genetic distances.

There are several reasons that could explain the results obtained so far for *D. ornatifrons*. The characteristics of each collection area, such as type of vegetation, climate, altitude and mainly the conservation status. All four areas of collection worked here are conservation units that present different degrees of perturbation. It seems that the PA and SSF areas are bigger and better preserved than CAJ and SRT, which are smaller and relatively more isolated fragments. This could explain the moderate genetic differentiation among these populations. The spatial analysis of the allozymatic variability distribution for *D. ornatifrons* revealed that the PA (43 alleles and Ho = 0.3609) and SSF (33 alleles and Ho = 0.4060) populations showed higher genetic variability than the CAJ (22 alleles and Ho = 0.1489) and SRT (26 alleles and Ho = 0.2640). These results probably also reflect the characteristics of each fragment as described above, and also because *D. ornatifrons* is considered ecologicaly more restricted and should suffer more drastically with its habitat fragmentation. Nevertheless, the process of fragmentation of the Atlantic forest biome is relatively recent when compared to other biomes, which could lead to a greater and stronger inbalance over the genetic diversity distribution. This fact reinforces the urgency for new conservation program in these areas.

## 4. Conclusions

These results show that the fragmentation in each of the environments sampled in this work has differentiated effects over the *Drosophila* species analyzed. It seems that the xerophytic

vegetation, which has a more ancient fragmentation process when compared to the Atlantic Forest, implies over the *Drosophila* species a more constant genetic diversity distribution. On the other hand, the *Drosophila* species that occurs in the Atlantic Forest biome seems to be under a more conspicuous pressure because of the fragmentation, maybe because this fragmentation is recent and lead to an imbalance greater and stronger over the genetic diversity distribution.

Our results also showed that the microsatellite loci diversity analysis of *D. antonietae* populations from the Iguassu river basin, which flows into the Parana river, are in agreement with other markers for this species, that is, high within population diversity and low among population differentiation, however with the presence of an ancipient diversification. Regarding *D. ornatifrons* populations, a high genetic differentiation was detected, which could due to the presence of the new species in the populations analyzed. However, new data should be added to corroborate this hypothesis as this is the first of a series of works on the genetic diversity distribution for this species. Currently, our laboratory is producing new data regarding the genetic diversity distribution for this species using other molecular and morphological data, besides a philogeographical approach. In the future, we hope to shed some light and better understand the effect of forest fragmentation on the genetic diversity distribution in *D. ornatifrons*.

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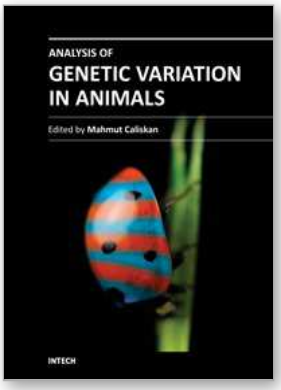


**Analysis of Genetic Variation in Animals**

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Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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Phone: +86-21-62489820
Fax: +86-21-62489821