SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Phenotypic Variation of the Aedeagus of *Drosophila serido* Vilela & Sene (Diptera: Drosophilidae)

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Variação Fenotípica do Edeago de *Drosophila serido* Vilela & Sene (Diptera: Drosophilidae)

RESUMO - *Drosophila serido* Vilela & Sene é uma espécie cactofílica e politípica, com ampla distribuição geográfica no Brasil. A morfologia do edeago de indivíduos provenientes de oito populações naturais de *D. serido* foi avaliada. De acordo com as características dos seus edeagos, as populações de *D. serido* foram discriminadas com eficiência de aproximadamente 75%. O resultado do teste de Mantel sugere que a divergência morfológica de *D. serido* é correlacionada com a distância geográfica das populações. A explicação para o padrão observado não é única. Por esta razão, os resultados foram discutidos considerando-se as três principais hipóteses para explicar a evolução do edeago: *chave-fechadura*, *pleiotropia* e *seleção sexual*. Alternativamente, a variabilidade encontrada nos edeagos de *D. serido* poderia estar relacionada a fatores ambientais, tais como temperatura e/ou cacto hospedeiro.

PALAVRAS-CHAVE: Evolução, morfometria geométrica, grupo *repleta*

ABSTRACT - *Drosophila serido* Vilela & Sene is a polytypic and cactophilic species with broad geographic distribution in Brazil. The morphology of the aedeagi of eight natural populations of *D. serido* was analyzed. Based on features of their aedeagi, populations of *D. serido* were discriminated with an efficiency of nearly 75%. The analysis using the Mantel test suggests that the morphological divergence of *D. serido* is correlated with the geographic distance among populations. There is no single cause to explain the observed pattern; therefore, the results were discussed considering the three main hypotheses to explain the aedeagus evolution: *lock and key*, *pleiotropy* and *sexual selection*. Alternatively, the aedeagus variability of *D. serido* might be related to environmental causes, such as temperature and/or host cacti.

KEY WORDS: Evolution, geometric morphometric, *repleta* group

Studies of population differentiation are generally based on molecular markers, and use statistics derived from the population genetics and phylogeography (Avise 2000). However, morphological markers might supply useful alternative insights in population studies and may provide a good assessment of how the genetic variation is distributed among different entities, since morphological traits are generally under polygenic control (Falconer 1989, Garnier *et al*. 2005).

The aedeagus of insects present rapid and divergent evolution in relation to other morphological characteristics (Eberhard 1985, Garnier *et al*. 2005, Soto *et al*. 2007). For this reason, in the *Drosophila* genus, as well as other insect taxa, the aedeagus is an important organ for taxonomic purposes (Silva & Sene 1991, Kullikov *et al*. 2004, Franco *et al*. 2006). The *D. repleta* group, for example, includes more than 100 Neotropical species and the aedeagus morphology has been considered the most important diagnostic character of this group (Vilela 1983).

There are three main hypotheses to explain the genital evolution: *lock and key*, *pleiotropy* and *sexual selection* hypothesis (Arnqvist 1997). In brief, in both *lock and key* and *sexual selection* hypotheses it is expected that genital morphology is under different selection forces: stabilizing selection in the former and selection for fertilization success in the latter. On the other hand, the *pleiotropy* hypothesis assumes neutrality of genital variation, in which genetic bases can be influenced by others non genital related genes (Arnqvist 1997, Hosken & Stockley 2004).

D. serido is a Brazilian endemic species ascribed to the *buzzatii* cluster and is considered to be an important model for evolutionary biology research (Manfrin & Sene 2006). This species is associated with cacti and uses its decaying

tissues as larval breeding sites (Pereira *et al*. 1983). *D. serido* has broad geographic distribution in Brazil, occurring in the Northeastern and on the Atlantic coast from the Northeastern to the Southern regions, associated with a series of cacti genera (Pereira *et al*. 1983, Ruiz *et al*. 2000, Manfrin & Sene 2006). The geographical distribution of *D. serido* can be characterized in two ways: in northeastern Brazil, it is found in the *Caatinga* domain, an open vegetation area with a high abundance and diversity of Cactaceae, and on the Atlantic coast, *D. serido* has a fragmented distribution, occurring in environments with relatively few cacti species surrounded by the Atlantic rain forest (Manfrin & Sene 2006).

All populations of *D. serido* have one fixed polytene chromosome inversion named $2x^7$ (Ruiz *et al*. 2000) and aedeagus morphotype A (Silva & Sene 1991). However, this species is polytypic considering the karyotype pattern (Baimai *et al*. 1983), the polytene chromosomal inversions polymorphisms (Tosi & Sene 1989, Ruiz *et al*. 2000) and the mitochondrial DNA haplotypes (Manfrin *et al*. 2001, A. C. Morales, unpublished data. 2004, Manfrin & Sene 2006). The populations from the *Caatinga* domain are homogeneous considering the metaphasic plate Type I (Baimai *et al*. 1983), while on the Atlantic Coast, the populations present karyotype Type III (Arraial do Cabo, RJ locality), Type IV (Peruíbe, SP locality) or Type VI (Florianópolis, SC locality) metaphasic plates (Baimai *et al*. 1983, Manfrin & Sene 2006, Biffi F. unpublished data). Regarding the chromosomal inversions, four polymorphic inversions are restricted to northeastern populations ($2a^8$, $2b^8$, $2c^8$ and $2d^8$) and three apparently fixed inversions ($2y^9$, $2x^8$ and $2w^8$) occur in coastal populations (Tosi & Sene 1989, Ruiz *et al*. 2000). The mitochondrial DNA haplotype data also suggest differentiation among populations of *D. serido*, where the populations from *Caatinga* domain compose a distinct group of those from the Atlantic Coast, which in turn can be divided into two other groups. One enclosing populations from northern São Paulo state to southern Bahia state, while the second comprises the southern most populations, from São Paulo to Santa Catarina states (A. C. Morales unpublished data, Manfrin & Sene 2006).

Despite the high amount of cytogenetic and molecular data, there is no comparative study of morphologic traits on natural populations of *D. serido*. In order to quantify the morphological differentiation and identify some population patterns of morphological variation, we analyzed the aedeagus morphology of eight populations of *D. serido* by geometric morphometry. We chose this organ because it is considered the most important character for species identification in the *D. repleta* species group (Vilela 1983), and because its variability in different populations of a polytypic species, such *D. serido*, may provide insights on the evolution of this trait in a natural environment and if the observed variability is explained by the hypotheses of aedeagus evolution.

Materials and Methods

Samples. Eighty-seven aedeagi from eight different localities inhabited by *D. serido* were analyzed: Milagres (BA) (n = 26), Cabrália (BA) (n = 10), Mucuri (BA) (n = 9), Itaúnas (ES) (n = 10), Arraial do Cabo (RJ) (n = 8), Bertioga (SP) (n = 7), São Sebastião (SP) (n = 9) and Penha (SC) (n = 8). All analyzed individuals were collected in their natural environment. The geographic information of each locality is presented in Fig. 1.

Measurements and statistical analysis. The aedeagi of *D. serido* were prepared in slides for optic microscopy according to Kaneshiro (1969). The aedeagi were magnified 200x and acquired images were digitalized using a microscope (Axioplan2 Zeiss) equipped with Axiovision Zeiss digital image capturing system and stored on a computer. Aedeagi contours were outlined in the GIMP 2.4 image processing software.

In order to quantify the organ shape, we employed a description of aedeagus outline by the means of elliptic Fourier descriptors (EFDs) (Kuhl & Giardina 1982). In brief, in this analysis the x and y coordinates of an outline are fit separately as function of arc length by Fourier analysis, decomposing the contour into a weighted sum of sine and cosine functions, called harmonics. We performed a normalization of the descriptors based on the first harmonic ellipse that corresponds to the first Fourier approximation to

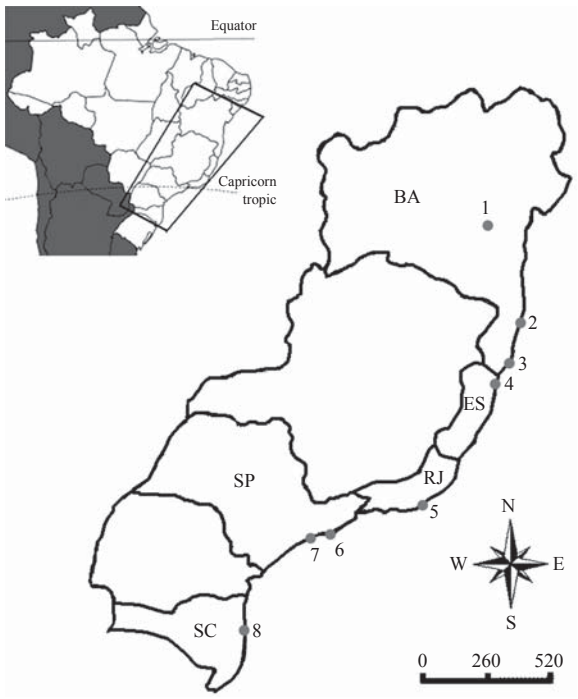

Fig. 1. Collection sites (1 through 9) of *D. serido* in Brazil. (1) Milagres, BA (12º52'12.0''S, 39º51'32.0''W); (2) Cabrália, BA (16º18'50.2''S, 39º01'26.1''W); (3) Mucuri, BA (18º05'28.5''S, 39º32'58.9''W); (4) Itaúnas, ES (18º24'26.0''S, 39º41'52.8''W); (5) Arraial do Cabo, RJ (22º59'30.0''S, 42º00'45.0''W); (6) São Sebastião, SP (23º49'00.0''S, 45º25'00.0''W); (7) Bertioga, SP (23º51'16.0''S, 46º08'19.0''W) and (8) Penha, SC (26º45'00.0''S, 48º40'00.0''W). BA, Bahia state. ES, Espírito Santo state. RJ, Rio de Janeiro state. SP, São Paulo state. SC, Santa Catarina state. The scale bar is in km.

the contour information (reviewed in Lestrel 1997). Thus, size, orientation and starting position of the contours were standardized accordingly with the size and alignment of the major axes of the first ellipse, leading to representations of the organs that are only based on internal properties of the outlines (i.e. shape) (Kuhl & Giardina 1982).

The number of harmonics considered in the analysis defines the precision of the outline description (Liu *et al*. 1996). Therefore, the number of variables generated is directly proportional to the number of harmonics considered. We considered 25 harmonics and, consequently, 100 coefficients (4 per harmonic) were generated. The variance-covariance matrix of the 100 estimated EFD coefficients was used as input in a principal components analysis. This procedure allowed us to summarize the information assessed in the coefficients and to reduce the dimensionality of the variables (Rohlf & Archie 1984) in a lower number of principal components (PC). The PC scores generated can be considered as quantitative shape variable and can be used as an independent variable in subsequent analyses. The EFDs and the shape PC scores were obtained using the SHAPE package (Iwata & Ukai 2002), available at http://cse.naro.affrc.go.jp/iwatah/shape. The area of contour was used as a size measurement.

The significance of morphological differences within *D. serido* was tested through a Discriminant Analysis (DA), considering each population as a group. To estimate the phenetic relationship among these populations, a Mahalanobis distance matrix was used to build an UPGMA (Unweighted Pair Group Method with Arithmetic mean) phenogram. The DA was performed on STATISTICA software (StatSoft 2001) and the UPGMA was performed on MEGA 3.0 (Kumar *et al*. 2004). In order to detect relationships between morphology and geographic distances among populations of *D. serido*, matrices of averaged Mahalanobis distances and geographical distances in kilometers were compared using the Mantel test, which calculates the correlations between matrices with a permutation procedure (Mantel 1967, Smouse *et al*. 1986). As the test assumes a linear relationship between divergence and geographical distance, the latter were log-transformed before the analyses. The Mantel test was performed with the TFPGA software with 2000 permutations.

Results

The variation of the harmonic coefficients was reduced to 11 independent PC, which together explained more than 94% of the total variation and were further considered as quantitative traits (Fig. 2).

We detected a significant differentiation among the *D. serido* populations (Wilks´Lambda = 0.0608; $P < 0.0001$), presenting 74.6% of correct case reclassification (Table 1). The parameters PC2 ($P = 0.5324$), PC10 ($P = 0.3666$) and Area ($P = 0.1683$) were not significant to discriminate the populations. All other parameters were considered in the DA. The most important quantitative variables for the discrimination of the populations were PC1 (Partial Wilks´Lambda = 0.6964; $P = 0.0006$), PC5 (Partial Wilks´Lambda = 0.7177; $P = 0.0015$) and PC7 (Partial Wilks´Lambda = 0.7291; $P = 0.0023$), respectively.

Two population groups were formed in UPGMA cluster analysis: one that comprises populations from the Northern distribution of *D. serido* (Milagres, BA; Mucuri, BA; Cabrália, BA and Itaúnas, ES) and the other one with populations from the Southern distribution of *D. serido* (Bertioga, SP and Penha, SC). Populations from São Sebastião (SP) and Arraial do Cabo (RJ) did not group with any other population (Fig. 3a). Fig. 3b shows the plot of the individual scores for each population group, as defined in UPGMA cluster analysis, in relation to the first two discriminant roots which accounted for more than 70% of total variance.

The Mantel test showed a significant correlation between the geographic and morphological distance matrices ($r = 0.3917$; $P = 0.0005$), indicating the greater the geographic distance among the populations the higher is the morphologic differentiation.

Discussion

We presented morphometric data to discriminate populations of *D. serido* with an efficiency of nearly 75% (Table 1). The differentiation among the *D. serido* populations was an expected result if we considered the fact that this species is polytypic for chromosomal (Baimai *et al*.

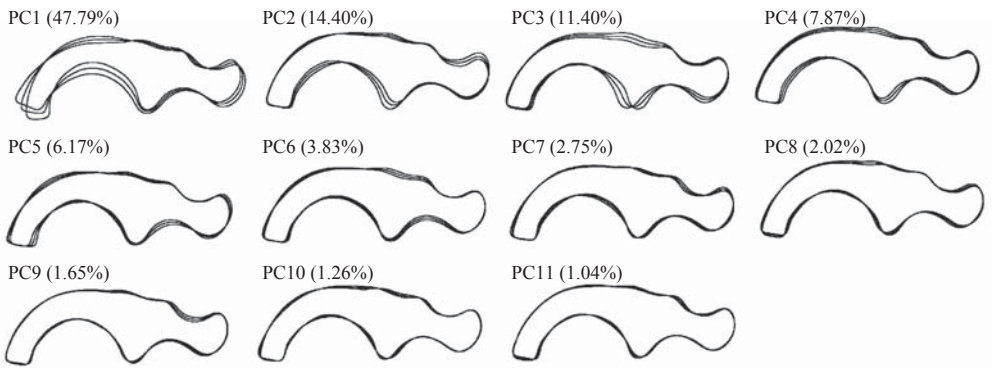

Fig. 2. Overlap of average shapes and standard deviations of each Principal Component (PC) generated through Fourier analysis, revealing the regions of variability for each quantitative variable. The variability percentage, determined by PC, is presented in parenthesis.

Table 1. Correct reclassification of *D. serido* accordingly to population origin based on discriminant analysis (Wilks´ Lambda = 0.0608; P < 0.00001). BER - Bertioga (SP), PEN - Penha (SC), MIL - Milagres (BA), SSB - São Sebastião (SP), ARC - Arraial do Cabo (RJ), ITA - Itaúnas (ES), MUC - Mucuri (BA), and CAB - Cabrália (BA).

Observed classifications	Predicted classifications								
	BER	PEN	MIL	SSB	ARC	ITA	MUC	CAB	Percent
BER	6	0	0	0	0	0	0	1	85.7
PEN	0	8	0	0	0	0	0	0	100.0
MIL	0	0	19	2	1	1	1	2	73.1
SSB	0	0	1	6	0	1	1	0	66.7
ARC	0	0	2	0	6	0	0	0	75.0
ITA	0	0	2	1	0	7	0	0	70.0
MUC	0	0	2	0	0	0	6	1	66.7
CAB	0	1	1	0	1	1	0	6	60.0
Total	6	9	27	9	8	10	8	10	74.6

1983, Tosi & Sene 1989, Ruiz *et al*. 2000) and molecular markers (Manfrin *et al*. 2001, Manfrin & Sene 2006). Moreover, *D. serido* has a scattered distribution due to its association with cacti that are discontinuously distributed in dry vegetation areas throughout its geographic distribution range (Manfrin & Sene 2006), allowing the isolation and consequent differentiation of *D. serido* populations.

The positive correlation between morphological and geographical distances found for *D. serido* populations, as pointed by Mantel test, indicates that the morphological variation of *D. serido* is related to the geographic space. There is no single cause to explain the obtained results. For this reason, we will discuss the morphological gradient in *D. serido* in the light of hypotheses to explain genital evolution (Arnqvist 1997), considering the variation in aedeagus morphology as neutral and/or under selection. Moreover, we will also discuss some environmental condition that could generate the morphologic gradient found.

If the variability of aedeagus would be neutral, as supposed by the *pleiotropy* hypothesis of genital evolution (Hosken & Stockley 2004), the balance between gene flow and genetic drift could have shaped the variation found in *D. serido*, once the positive correlation among geographic and morphologic distances may be explained by both isolation-by-distance and stepping-stone models (see Congdon *et al*. 2000, Telles & Diniz-Filho 2005). The former model estimates that individuals of a metapopulation are distributed more or less uniformly through its geographic distribution range, and due to low migratory capacity of the individuals a structuration is formed throughout a continuum (Wright 1969). The stepping-stone isolation model was defined by Wright (1969) as a "*continuum* with scattered clusters of high density, within a

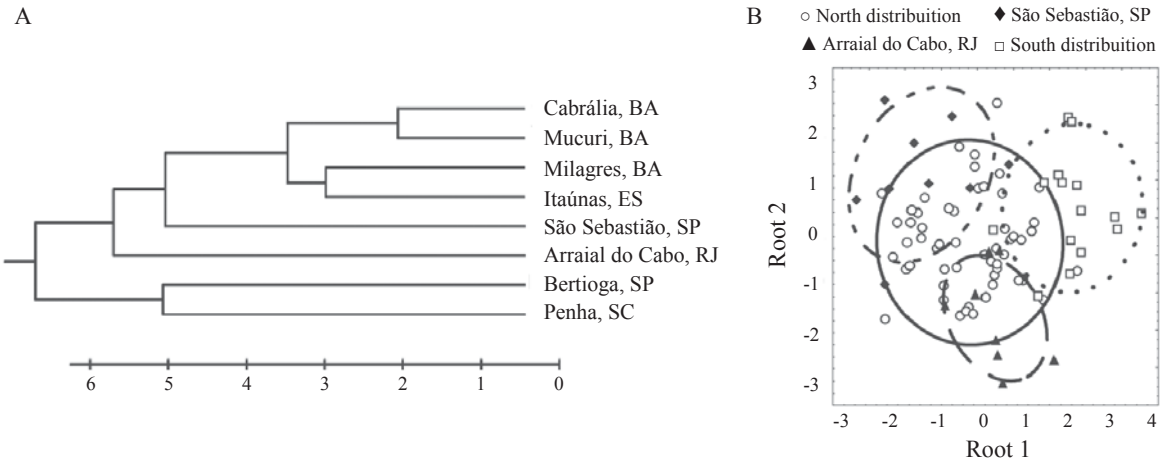

Fig. 3. A. UPGMA dendrogram showing the phenetic relationships among the populations of *D. serido*, according to aedeagus morphology. The scale bar represents the Mahalanobis distance. B. Plots of the individuals scores for the two first discriminant roots obtained in the discriminant analysis. The northern distribution group is composed by Cabrália, BA, Mucuri, BA, Milagres, BA and Itaúnas, ES populations and the southern group is composed by Bertioga, SP and Penha, SC populations, as defined in UPGMA cluster analysis.

model of a uniform *continuum*", i.e., populations are distributed throughout a geographic *continuum*, but with fragmented population distribution. Due to the scattered distribution of *D. serido,* we infer that the stepping-stone model is a better approach to explain the population structure for this species, considering the aedeagus genetic bases as neutral.

Considering the demographic events, the phylogeographic data based on mitochondrial DNA suggests that *D. serido* colonized the Atlantic Coast of Brazil from the Brazilian Northeast with population expansion events (A. C. Morales unpublished data, Manfrin & Sene 2006). These events could play an important role in the stochastic genetic differentiation of the *D. serido* populations in a gradient manner because migrating individuals carry only a percentage of genetic variability of the original population (Hartl & Clark 1997, Avise 2000). The phylogeographic analyzes also detect the existence of three population groups for *D. serido*: northwestern, northern coast and southern coast (A. C. Morales unpublished data, Manfrin & Sene 2006). Our UPGMA analysis is also in partial agreement with mitochondrial DNA data as two groups were clearly isolated (Fig. 3), indicating the existence of historical events, such as fragmentation of the original distribution area, associated with differentiation of *D. serido* populations.

The *pleiotropy* hypothesis for genital evolution also suggests that natural selection acting in other non genital related genes may also influence genital development (Hosken & Stockley 2004). In cactophilic *Drosophila* the host cactus seems to be the main ecological factor involved in adaptive responses and in species evolution (Ruiz *et al*. 2000). *D. serido* is associated with different cacti species throughout its geographic distribution (Manfrin & Sene 2006), which could drive the differentiation of its populations by local adaptation to different environments. This differentiation related to host cacti could influence, by pleiotropy for example, aedeagus morphology.

The gradient found in aedeagus morphology of *D. serido* could also be a consequence of natural selection acting in aedeagus morphology, resulting in a cline distribution of the variation among populations. Despite natural selection, the genital characters could also be governed by a sexual selection regime, which is related to variability in reproductive success (Arnqvist 1997). Although the causes of genital evolution are still in discussion, empirical data have supported the strong role of sexual selection for evolution of genital traits (reviewed in Hosken & Stockley 2004), and this evolutionary force can not be discarded as an explanation for morphological variation observed in *D. serido*, even so we can not argue how. Among the natural selection types, we can discard stabilizing selection because high levels of phenotypic variability in the aedeagus of *D. serido* were found (Table 1, Figs. 2, 3). This result is in disagreement with the *lock and key* hypothesis, which predicts low levels of phenotypic variation in genital structures (Arnqvist 1997, Soto *et al*. 2007).

Finally, there are two environmental factors that could also explain the divergence in aedeagus morphology of *D. serido*: temperature and host cactus. Temperature is an abiotic factor that affects many morphological characters in *Drosophila* species, as well as in other insects (Bitner-Mathé & Klaczko 1999, Andrade *et al*. 2005). In relation to the aedeagus morphology, it was reported that temperature changes in initial stages of development affects shape and size of *D. mediopunctata* Dobzhansky & Pavan aedeagi (Andrade *et al*. 2005). *D. serido* is found at different latitudes (Fig. 1), being susceptible to different climatic and temperature conditions, which could influence aedeagus morphology of this species. Considering the host, a recent laboratory study suggests that the cactus species used to prepare the medium culture affects the genital morphology of cactophilic *Drosophila* species (Soto *et al*. 2007) and, in the same way, could induce a plastic response of aedeagus morphology in *D. serido*, which is associated to different cacti species throughout its geographical range.

The morphological variability found in the aedeagus of *D. serido* species could be interpreted by both *pleitropy* and *sexual selection* hypotheses because the distribution of this variability is explained considering the genetic bases of aedeagus as neutral or under sexual selection. Moreover, the natural selection and phenotypic plasticity could also explain the pattern found, as observed in other morphological traits. On the other hand, we can discard the *lock and key* hypothesis to interpret our data because the variability found can not be explained considering stabilizing selection influencing the aedeagus morphology. Indeed, the same results have been found in several others groups (Hosken & Stockley 2004), including two others species of the *D. buzzatii* cluster: *D. buzzatii* Patterson & Wheeler and *D. koepferae* Fontdevila & Wasserman (Soto *et al*. 2007).

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