



Aedeagus Morphology as a discriminant marker in two closely related Cactophilic Species of *Drosophila* (Diptera; Drosophilidae) in South America

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ABSTRACT

Drosophila serido and *D. antonietae* are sibling species belonging to the *Drosophila buzzatii* cluster. Morphologically, they can only be discriminated by quantitative traits. In this paper we analyze the length and equalized average curvature of four regions of the aedeagus of *D. antonietae* and *D. serido*. Specimens of *D. serido* and *D. antonietae* were classified correctly 96.74% of the time. Based only on the variable that most contributed to the discrimination of the groups (equalized average curvature of the arch IV of the aedeagus), we observed significant intraspecific morphological divergence in *D. serido* in relation to the *D. antonietae*, in agreement with other markers. The high morphological divergence in equalized average curvature of the arch IV of the aedeagus shows that this region evolved faster than others, since the divergence of the two species. The importance of the present study to the understanding of the genetic basis that controls the formation of the aedeagus, in the species of the *Drosophila buzzatii* cluster, is discussed.

Key words: morphometric, curvature, *Drosophila buzzatii* cluster, aedeagus.

INTRODUCTION

Morphological characters have been used historically in evolutionary and taxonomic studies. Nevertheless, the lack of qualitative morphological differences among sibling species makes it difficult to establish the diagnostic morphological characteristics among them. In many cases, quantitative variations of morphometric traits are sufficient to the discrim-

ination of sibling species (Moreteau et al. 2003, Moraes et al. 2004).

The morphology of the aedeagus (external male genitalia) is of extreme importance in the taxonomic characterization of *Drosophila* species (Vilela 1983, Silva and Sene 1991, Liu et al. 1996, Moreteau et al. 2003, Kullikov et al. 2004), as well as in other Diptera. Quantitative variations in morphological characteristics of the aedeagus have also been used for discrimination of the sibling species of *Drosophila* (Silva and Sene 1991, Kullikov et al.

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2004). In the *Drosophila repleta* group, which includes more than 90 Neotropical species, most are considered sibling species. However, a marked difference exists in the aedeagi form (shape + size) among them, which is considered the main diagnostic character in the group (Vilela 1983).

The *Drosophila buzzatii* cluster (*repleta* group, *mulleri* subgroup, *buzzatii* complex) is a monophyletic group formed by seven sibling cactophilic species: *D. buzzatii*, *D. borborema*, *D. koepferae*, *D. antonietae*, *D. gouveai*, *D. serido* and *D. seriema*. In this cluster, quantitative differences of the morphological characteristics of the wing (Moraes et al. 2004) and male genitalia (Silva and Sene 1991, Tidon-Sklorz and Sene 1995, Prado et al. 2004) have been successfully used for the discrimination of species and populations. Among the seven species of this cluster, *D. buzzatii* and *D. borborema* present aedeagi that are different in shape and size from the other species (Tidon-Sklorz and Sene 1995). However, the aedeagus morphology of the other species of the cluster is similar to the so-called *D. serido* aedeagus type (Figure 1a) and based on this observation, these species were initially classified as belonging to the same species (Vilela and Sene 1977). To study the aedeagus of these species, Silva and Sene (1991) divided it into four main regions delimited by landmarks (Figure 1b). Each region delimited by two adjacent landmarks was denominated arch. Based on the length of the arches, the researchers identified five different types of aedeagus, named from A to E, specific for each species.

Drosophila serido and *D. antonietae* are two sibling species belonging to the *Drosophila buzzatii* cluster analyzed in this study. *D. antonietae* occurs in mesophilic forests in the regions of the Paraná-Paraguay basin, in South America, associated with the *Cereus hildemannianus* cactus (Tidon-Sklorz and Sene 2001). *D. serido* is present in the Northeastern Caatinga and Atlantic Coast of Brazil, from the Northeast to the Southern region of Brazil, associated with a series of cacti genera (Pereira et al. 1983). In the southern limit of the *D. serido* dis-

tribution, there is an area of contact with *D. antonietae* (A.C. Morales et al., unpublished data). Even though *D. serido* and *D. antonietae* present distinct metaphasic plates (Baimai et al. 1983), alloenzymatic patterns (A.C. Morales et al., unpublished data, Mateus and Sene 2003), mitochondrial haplotype (Manfrin et al. 2001) and satellite DNA (Kuhn and Sene 2005), they share the same fixed chromosomal inversion $2x^7$ (Ruiz et al. 2000), which may indicate a closely phylogenetic relationship. In morphological terms, only quantitative variations can discriminate these two species (Silva and Sene 1991, Moraes et al. 2004).

In this paper, morphometrical characters of the aedeagus of *D. antonietae* specimens from several different populations were analyzed, covering all the known distribution of the species, and of *D. serido* specimens coming from two different populations. We have chosen these species because they can be differentiated through genetic markers and, by presenting a secondary contact area, with a population in sympatry, with possible formation of hybrids in the southern region of Brazil. Besides the measurements of length of the arch of the aedeagus, already used in Silva and Sene (1991), curvature measurements of these arches were also used (Prado et al. 2004). Our main objectives were to introduce and test the use of the measures of curvature in the species of the *Drosophila buzzatii* cluster and to determine the regions of the aedeagus that most contributed to the discrimination of the species *D. serido* and *D. antonietae*. The data discussed here highlight the importance of these regions of the aedeagus for future research in order to understand the genetic basis of the aedeagus of the species belonging to the *Drosophila buzzatii* cluster, as well as for studies of the area of contact between the two species.

MATERIALS AND METHODS

SAMPLES

Fifty-eight aedeagi were analyzed from *D. antonietae* species from several different populations, representing the entire distribution, and 34 aedeagi

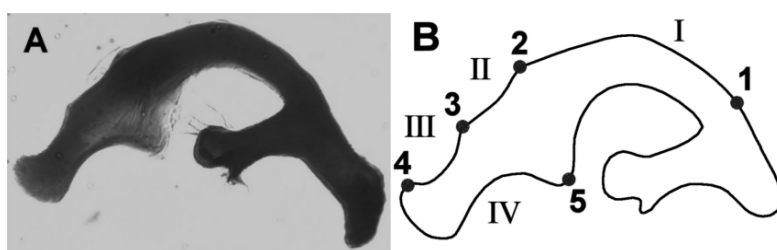


Fig. 1 – **A.** Aedeagus of a *Drosophila antonietae* specimen (magnified 200X), that represents the aedeagus form which is shared among *D. serido*, *D. antonietae*, *D. gouveai*, *D. seriema* and *D. koepferae* (Silva and Sene 1991). **B.** Delimitation through landmark, according to Silva and Sene (1991), of the regions analyzed in this work. 1–5 = landmark; I–IV = regions between two adjacent landmark denominated arches.

of *D. serido*, belonging to populations located in Junco do Seridó/PB and Milagres/BA (type localization) (Table I). All the individuals analyzed were collected in natural environments (wild-caught flies). The aedeagi were prepared in slices for optic microscopy according to Kaneshiro (1969). All aedeagi used in this paper were deposited in the Evolutive Genetics Laboratory at USP – Ribeirão Preto. The images of the aedeagi were magnified 200X and digitalized from a microscope (*Axioplan2 Zeiss*) equipped with the *Axiovision Zeiss* digital image capturing system and stored on a computer.

MEASUREMENT AND STATISTICAL ANALYSIS

Five points were defined manually as landmarks, according to Silva and Sene (1991). The landmarks were used to establish the internal outlines of the region under study; the regions between two adjacent landmarks are the arches (Figure 1b).

As in Silva and Sene (1991), the length of the arches I, II, III and IV of the aedeagus were measured (Table II). This measures the distance accumulated between the consecutive landmarks. Besides this, we also performed equalized measures of curvature of the arches. In brief, the continuous curvature is a geometric measure that expresses the rate of change of the angle between the tangent of the curve and the axis x (Costa et al. 2004). The values of the equalized curvature are generated through standardization by a sigmoid of the continuous cur-

vature to amplify the points of low curvature of the region of interest (Costa and Cesar 2000). This stage allows maximization of the information about the curvature of the structure analyzed. In order to perform the analyses, we used the averages of the equalized curvatures of the arches I, II, III and IV of the aedeagus (Table II). The measures were attained in accordance with the methodology described by Prado et al. (2004).

We performed three distinct discriminant analyses, solely using the measures of length of the arch (discriminant I) and the equalized average curvature (discriminant II) and a discriminant analysis using all the measures together (discriminant III). These analyses were performed to obtain correct percentages of classification of individuals, to verify which variables contributed more to the discrimination of the groups and to compare the use of measures of curvature used with the measures of the length of the arch, previously performed by Silva and Sene (1991). The lower values of Wilk's lambda (which vary from 0 to 1) indicate a better analysis. Besides this, with the data from the discriminant analysis III, an analysis of canonic variables was performed. The discriminant and canonical variables analysis was performed with the use of the software *Splus* (version 1.8).

The absolute value of the canonical standardized coefficients, generated by the analysis of canonical variables, represent the weight of each vari-

TABLE I
Sample number of individuals of *D. serido* and *D. antonietae* and their respective collection sites.

Species	Locality	Geographic Coordinates	Locality Code	Sample N ^o
<i>Drosophila serido</i>	Milagres/BA	12°51'S, 39°53'W	J92	22
	Junco do Seridó/PB	7°00'S, 36°43'W	A3	12
<i>Drosophila antonietae</i>	Sertãozinho/SP	21°10'S, 48°05'W	H34	4
	Bocaina/SP	21°01'S, 47°18'W	H88	6
	Serrana/SP	21°26'S, 47°30'W	H84	6
	Sapopema/PR	23°50'S, 51°45'W	J15	7
	Itirapina/SP	22°16'S, 47°48'W	J9	3
	Cambreúva/SP	23°21'S, 47°20'W	H16	8
	Rio Ligeiro/PR	23°37'S, 52°31'W	D93	8
Santiago-Jaguari/RS	29°25'S, 54°45'W	H47/H46	15	

TABLE II
List of measures of the aedeagus performed in the work.

Measures	Abbreviations
Length of arch I	AL I
Length of arch II	AL II
Length of arch III	AL III
Length of arch IV	AL IV
Equalized average curvature of arch I	MKE I
Equalized average curvature of arch II	MKE II
Equalized average curvature of arch III	MKE III
Equalized average curvature of arch IV	MKE IV

able in the formation of the canonical root, where higher values correspond to the most differentiation among groups. These coefficients will be presented to show the relative contribution of each variable in the formation of the canonical root obtained and to determine which variable is most important for the discrimination of the groups.

Besides the multivariate analysis, the most important measure for discrimination of the species was used individually in order to obtain the correct percentage of classification of the individuals through the Bayesian classification method (Duda et al. 2000). For that, normal curves were established from the average and standard deviation of each group in relation to the variable, defining function density and probability used in the Bayesian analysis.

RESULTS

The results of the three discriminant analyses performed are shown in Table III. The discrimination between *D. antonietae* and *D. serido* is higher when aedeagus arch lengths are used along with the measures of curvature (discriminant III) (Wilks' $\lambda = 0.27797$; $p < 0.00001$), where 96.74% of the individuals are classified correctly (Table III), the correct classification of *D. antonietae* is 98.30% and of *D. serido* is 94.10%.

Only one canonic axis was obtained in the analysis of the canonic variables using the measures of discriminant analysis III. The canonical standardized coefficients (the weight of each variable) are shown in Table IV. The absolute values of these coefficients indicate that the most important variable

TABLE III
Values of Wilks'λ and percentage of correct classification attained by discriminate analysis performed in this work.

	Measures Used	Wilks'λ	p	Correct (%)
Discriminant analysis I	AL I, AL II, AL III e AL IV	0.56619	p< 0.00001	84.80
Discriminant analysis II	MK I, MK II, MK III e MK IV	0.42886	p< 0.00001	89.13
Discriminant analysis III	AL I, AL II, AL III, AL IV, MK I, MK II, MK III e MK IV	0.27797	p<0.00001	96.74

for the formation of the canonical root was MKE IV while the least important was the variable MKE I. Normal curves obtained by the average and standard deviation of the two groups in relation to the canonical scores are shown in Figure 2, in which we can observe the discrimination between *D. antonietae* (higher canonical scores) and *D. serido* (lower canonical scores).

TABLE IV
Standardized canonical coefficients for each variable.

Variable	Canonical Root 1
MKE I	0.078
MKE II	0.161
MKE III	0.426
MKE IV	0.646
AL I	-0.586
AL II	0.558
AL III	-0.112
AL IV	-0.285

The values for each individual obtained from the analysis of MKE IV are shown in Figure 3a. In relation to this measure, *D. serido* has higher variation around the average (standard deviation = 0.04) than *D. antonietae* (standard deviation = 0.025) (Figure 3b), mainly because there are significant differences in relation to this measure between both populations that compose the sample of *D. serido* (Figure 3a; Figure 4).

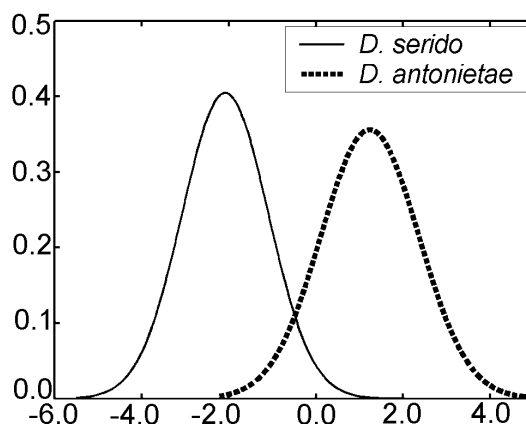


Fig. 2 – Normal curves representing the distribution of the individuals of *D. antonietae* and *D. serido* in relation to the canonic scores.

In accordance with the Bayesian analysis, based only on the MKE IV measure, 69.8% of the individuals were classified correctly and over 90% of the individuals of *D. serido* from Milagres/BA were classified correctly. Nevertheless, a high percentage of mistakes were observed in the individuals of the *D. serido* from Junco do Seridó/PB and *D. antonietae* (Table V). The same analysis was performed without the individuals of *D. serido* from Junco do Seridó/PB and the percentage of correct classifications was 94.83% (Table VI), showing extreme divergence between the individuals of the population of *D. serido* from Milagres/BA and *D. antonietae* based on MKE IV.

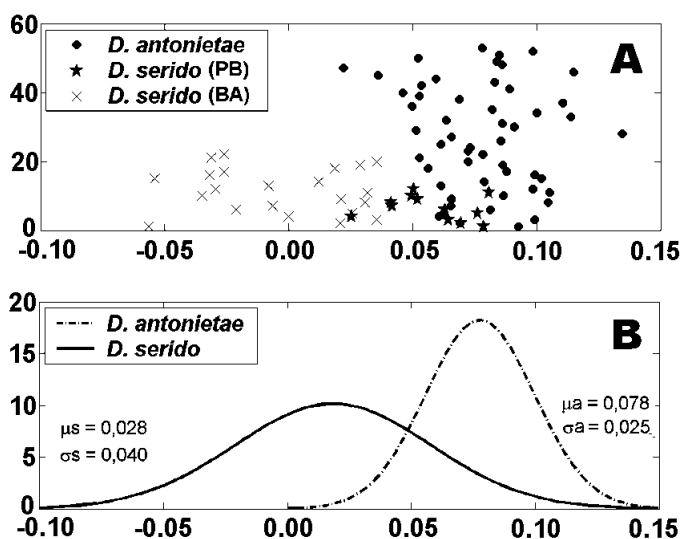


Fig. 3 – A. Graph showing the distribution of the individuals of *D. antonietae*, *D. serido* (Junco do Seridó/PB) and *D. serido* (Milagres/BA) in relation to MKE IV values. B. The Normal curves generated from the average and standard deviation of individuals of *D. serido* and *D. antonietae* in relation to the MKE IV values. μs = average value of MKE IV from individuals of *D. serido*. μa = average value of MKE IV from individuals of *D. antonietae*. σs = standard deviation for MKE IV in *D. serido*. σa = standard deviation for MKE IV in *D. antonietae*.

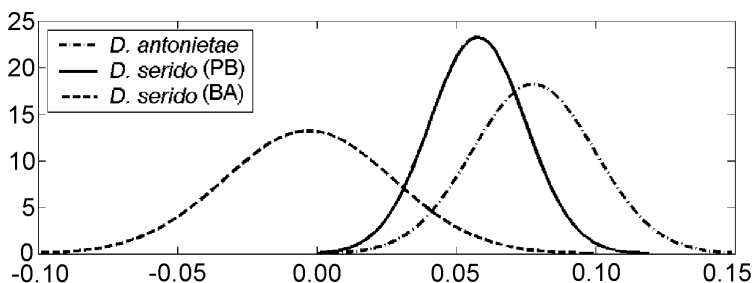


Fig. 4 – Normal curves generated from the average and standard deviation of the groups represented by the individuals of *D. antonietae* and of the individuals of the *D. serido* belonging to populations from Milagres/BA and Junco do Seridó/PB, in relation to the values of the MKE IV measures.

DISCUSSION

Our data confirm the existence of quantitative differences in morphological characteristics of the aedeagus of the individuals analyzed, making possible the discrimination of the *D. serido* from *D. antonietae*. Furthermore, the equalized average cur-

vature of arch IV (MKE IV) was the variable that most contributed to the discrimination of the groups (Table IV).

The length of the first four arches of the aedeagus had already been used with success in the discrimination of species of the *Drosophila buzzatii* cluster, including among them *D. serido* and *D. an-*

TABLE V

Classification of the specimen based on the values of MKE IV. A = *D. antonietae*. B = Junco do Seridó/PB population (*D. serido*). C = Milagres/BA population (*D. serido*).

Group	Correct	Mistake	Correct (%)
A	30	28	51.72
B	8	4	66.67
C	20	2	90.91
Total	58	34	69.77

TABLE VI

Classification of the specimens based on the values of MKE IV. A = *D. antonietae*. B = Milagres/BA population (*D. serido*).

Group	Correct	Mistake	Correct (%)
A	52	6	89.66
B	22	0	100.00
Total	74	6	94.83

tonietae (aedeagus Type A and D, respectively in Silva and Sene 1991). In this paper we found a considerable rise in the discrimination of the groups adding measures of equalized average curvature in the morphometric analysis, where 96.74% of the individuals are correctly classified (Table III). Thus, the measurements of equalized average curvature are important for the discrimination of *D. antonietae* and *D. serido* and may be useful in the study of a zone of sympatry among these species in the southern region of Brazil. As measures of curvature do not take into consideration the size of the structure, it is possible to infer that small quantitative alterations in the shape of the aedeagus, especially in the region of arch IV, may have occurred since the event of cladogeneses of the *D. serido* and *D. antonietae* species.

There is a significant morphological divergence in relation to MKE IV between *D. antonietae* and *D. serido*, since *D. antonietae* shows relatively less variation around the average than *D. serido* (Fig-

ure 3b). Individuals from different populations, representing the entire distribution area of *D. antonietae* populations (Table I), were analyzed; in this sample, the data suggest that there is homogeneity among the *D. antonietae* populations. Monteiro and Sene (1995) analyzed the morphology of the aedeagus of individuals from several populations of *D. antonietae* through the truss network morphometric method and also did not observe the morphological differences among them. Alloenzymatic (Mateus and Sene 2003) and microsatellites patterns (L.P.B. Machado et al., unpublished data) also showed homogeneity among the different populations of *D. antonietae*. These results suggest gene flow among the populations of *D. antonietae*, favored by the association of this species and its host cactus, *Cereus hildmaniannus* that occurs in mesophile gallery forests along the rivers of the Paraná-Paraguay basin, which form corridors of migration for individuals of the species *D. antonietae* (Monteiro and Sene 1995).

Drosophila serido has, comparatively, a higher interspecific variation in relation to MKE IV than to *D. antonietae* (Figure 3b), as can be observed in the differences found among the individuals from the populations of Junco do Seridó/PB and Milagres/BA. The individuals of *D. serido* from the population of Junco do Seridó/PB have intermediate values for MKE IV of those found for the populations of *D. serido* in Milagres/BA and the populations of *D. antonietae* analyzed (Figure 4). These data suggest a morphological differentiation between both populations of *D. serido*, probably due to the restriction of the gene flow among them. There is a large river between the Brazilian states of Bahia and Pernambuco, the São Francisco river, which could be acting as a geographic barrier, preventing the gene flow between the populations of *D. serido* analyzed in this paper. Morphological divergences among the populations of *D. serido* are in agreement with other works, being that this species is polytypical in relation to other markers. The northeastern populations of *D. serido* have Type I metaphasic plates, while some coastal populations have Type

III (Arraial do Cabo/RJ) and Type IV (Peruíbe/SP) metaphasic plates (Baimai et al. 1983). Regarding the chromosomal inversions, although all populations of *D. serido* share the fixed inversion $2x^7$, four polymorphic inversions are restricted to northeastern populations ($2a^8$, $2b^8$, $2c^8$ and $2d^8$) and two fixed inversions ($2x^8$ and $2w^8$) occur in coastal populations (Tosi and Sene 1989, Ruiz et al. 2000). Furthermore, the *D. serido* populations from northeastern Brazil are partially isolated reproductively from the population of Arraial do Cabo/RJ along the Brazilian coast (N.M.V. Bizzo, unpublished data). Recent studies also suggest discrimination among the populations of *D. serido* in the Northeast and coastal Brazil based on differentiation of mitochondrial haplotypes (A.C. Morales et al., unpublished data).

Differentiation among populations of the same species is an important requirement in the process of speciation, thus, studies involving a variety of populations of a polytypical species, such as *D. serido*, are important in order to quantify the morphological divergence in relation to the genetic divergence in natural populations.

Not all the arches of the aedeagus are equally informative in the discrimination of the species. According to Silva and Sene (1991) arches II and III (Figure 1) are the most informative for the discrimination of the species in the *Drosophila buzzatii* cluster. In comparison pair-by-pair, using the truss network method, the measures related to arch III were the most significant in the discrimination between *D. antonietae* and *D. gouveai* (Monteiro and Sene 1995). Through the analysis of the geometric morphometry using the measures of curvature, Prado et al. (2004) observed that the curvature and the normalized length of arch III are important measures in the discrimination between the *D. gouveai* and *D. antonietae* species. Our data show that MKE IV is the most important one in the discrimination of the species *D. serido* and *D. antonietae*; and based solely on this measure, it is possible to discriminate more than 94% of the individuals of *D. serido* from the population of Milagres/BA (type localiza-

tion) from those of *D. antonietae* (Table VI). These results show that the distal portion of the aedeagus (arch III + arch IV) present the highest interspecific differences, independent from other parts of this organ, suggesting that this region can be considered a "hot evolutionary spot" (Kullikov et al. 2004) for the aedeagus during events of cladogeneses in the *Drosophila buzzatii* cluster, but this question still remains open for discussion.

The morphometric variations between and within the groups of organisms reflect the expression of a phenotype resulting from an integrated polygenic control, which is altered during cladogeneses and the evolution of groups (Falconer 1989). In addition, several epigenetic and environmental factors can affect the formation of a structure (Atchley et al. 1992), which make it difficult to identify the causes of morphologic divergence among populations and species. Nevertheless, comparative morphometric studies of the homologous regions of both species, such as the arches of the aedeagus, are important requisites for the understanding of the genetic basis that controls the formation of that region. Although the genetic basis of the aedeagus is being discovered for other groups of *Drosophila* (Liu et al. 1996), the genes that control the formation of the aedeagus in the species of the *Drosophila buzzatii* cluster are not yet established. The high percentage of correctness of individuals analyzed in this article, based solely on the MKE IV, indicates that the arch four region may be useful for future studies to the understanding of aedeagus genetic basis in these species, since establishing informative morphological characters among distinct taxonomic groups is a preliminary step in the study of QTL (Quantitative Trait Loci), a methodology for the detection, mapping and estimate of effects of some loci in the metric characteristics.

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RESUMO

Drosophila serido e *D. antonietae* são espécies crípticas pertencentes ao “cluster” *Drosophila buzzatii*. Morfológicamente, elas podem ser discriminadas apenas por diferenças quantitativas. Neste trabalho, nós analisamos o comprimento e a média da curvatura equalizada de quatro regiões do edeago de indivíduos de *D. antonietae* e *D. serido*. Os espécimes de *D. serido* e *D. antonietae* foram discriminados com 96,74% de eficiência. Apenas com base na variável que mais contribuiu para discriminação dos grupos (média da curvatura equalizada do arco IV do edeago), nós observamos significativa divergência morfológica intraespecífica em *D. serido* em relação a *D. antonietae*, o que está em concordância com outros marcadores. A alta divergência morfológica apenas na média da curvatura equalizada do arco IV do edeago mostra que essa região do edeago evoluiu mais rápido que as demais desde a divergência entre as duas espécies. A importância do presente estudo para o entendimento das bases genética que controlam a formação do edeago, nas espécies do “cluster” *Drosophila buzzatii*, é discutida.

Palavras-chave: morfometria, curvatura, cluster *Drosophila buzzatii*, edeago.

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