Research Notes

Cuticle sclerotization in pharate adult and imago of *Drosophila melanogaster, Ceratitis capitata,* and *Haematobia irritans.*

Pérez, M[1]., A. Rabossi[1], J. Schachter[1], L. Pujol-Lereis[1], A. Filiberti[2], C.E. Argaraña[2], and L.A. Quesada-Allué[1].** [1]IIB, FCEyN- University of Buenos Aires, CONICET and Fundación Instituto Leloir, Patricias Argentinas 435, (1405) Buenos Aires, Argentina; [2]CIQUIBIC- University of Córdoba, CONICET. Ciudad Universitaria, Córdoba, Argentina. **corresponding author (E-mail: lualque@iib.uba.ar).

N-β-alanyldopamine synthase is the enzyme conjugating dopamine with β-alanine to generate N-β-alanyldopamine (NBAD) in the integument of insects (Pérez *et al.*, 2002). NBAD is the main cross-linking precursor of the brown insect cuticles (Hopkins and Kramer, 1992). NBAD is synthesized in the epidermis and apically exported to the cuticle to give rise to the corresponding quinone, which will crosslink proteins and eventually chitin (Hopkins and Kramer, 1992). In *Drosophila melanogaster* the locus *ebony* seems to be the coding gene for the enzyme, whereas in *Ceratitis capitata* the equivalent gene is *niger* (Pérez *et al.*, 2002). It is assumed that the enzyme expression is induced by 20-OH-ecdysone, the molt hormone, but only indirect demonstrations have been published (Pérez *et al.*, 2004). Data correlating ecdysone levels in haemolymph with epidermal NBAD synthase activity in the same insect are not available. On the other hand, we recently demonstrated that the enzyme is expressed in the neural system in a constitutive way (Pérez *et al.*, 2004). However, in terms of quantity of active enzyme, the amount in brain and ganglia is much lower than the enzyme amount in epidermis, when expressed. The knowledge of a novel brain activity, not detected previously (Pérez *et al.*, 2004), opened the possibility that different regulation of NBAD synthesis might exist in different tissues and/or insects. Since it is quite difficult to compare with precision the time of development in different insects, we focused our analysis of N-β-alanyldopamine synthase in three dipterans in which developmental time can be assessed with some accuracy, *i.e. Drosophila melanogaster,* the Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Rabossi and Quesada-Allué, 1995), and *Haematobia irritans* (the horn fly). We first wanted to

Table 1. Developmental equivalence of *Drosophila melanogaster*, *Ceratitis capitata* and *Haematobia irritans* metamorphosis markers.

Metamorphosis events	*D. melanogaster*		*C. capitata*		*H. irritans*	
	hours	cumulative % *	hours	cumulative % *	hours	cumulative % *
1- Onset of pupariation, 'Time zero'. The shaping of the puparium is completed and cuticle is still white. [a-b-c-d]	0	0	0	0	0	0
2- Pupal – adult apolysis. [b-d]	50	52	160	51.3	90	53.6
3- Ocelli visible and colored. [d]	ND	ND	168	53.8	100	59.5
4- Bristle pigmentation begins dorsally on head and thorax.[b-c-e]	70	72.9	216	69.2	120	71.4
6- Wings, ptilinum and bristles darken. [b-c-e]	86	89.5	288	92.3	144	85.7
7- Ecdysis of the imago. [b-c-e]	96	100	312	100	168	100

* The progress of metamorphosis is indicated in terms of cumulative percentage of time within the puparium. *Ceratitis* and *Haematobia* were grown respectively at 23 and 29 °C. Letters correspond to citations below: a-c, *D. melanogaster* and d-e, *C. capitata*. a) Ashburner, 1989: b) Bainbridge and Bownes, 1981: c) Bodenstein, 1951; d) Rabossi *et al*., 1991; e) Rabossi *et al*., 1992.
ND: not determined

Table 2. Overall N-β-alanyldopamine synthase activity during pharate adult instar of *Drosophila melanogaster, Ceratitis capitat*a and *Haematobia irritans.*

	Pharate adult age		NBAD synthase activity (pmol/min.mg)**
	Hours, cumulative*	% of metamorphosis	
D. melanogaster	55 hs	57.3 %	13.6
D. melanogaster	70 hs	72.9 %	20.0
C. capitata	216 hs	69.2 %	9.5
H. irritans	120 hs	71.4 %	6.7
D. melanogaster	80 hs	83.3 %	12.3
C. capitata	288 hs	92.3 %	12.7
H. irritans	144 hs	85.7 %	11.9
D. melanogaster	95 hs	98.9 %	11.5
C. capitata	312 hs	100 %	17.5
H. irritans	168 hs	100 %	16.6

(*) Age within the puparium. (**) Average of not less than 3 determinations.

Table 3. N-β-alanyldopamine synthase activity in adults of *Drosophila melanogaster*, *Ceratitis capitata* and *Haematobia irritans.*

Exarate adults		NBAD synthase activity (pmol/min.mg)*
Young imago (whole body)		
D. melanogaster	(4 hs after ecdysis)	24.6
C. capitata)	(4 hs after ecdysis	36.4
H. irritans)	(1 hs after ecdysis	30.7
Old imago (head)		
D. melanogaster	(more than 1 week after ecdysis)	43.0
C. capitata.	(more than 1 week after ecdysis)	21.1
H. irritans	(more than 3 days after ecdysis)	33.4
Old imago (thorax + abdomen)		
D. melanogaster	(more than 1 week after ecdysis)	0.0
C. capitata	(more than 1 week after ecdysis)	0.7
H. irritans.	(more than 3 days after ecdysis)	0.8

(*) Average of not less than 3 determinations

know the degree of equivalence of the three corresponding life cycles during the stages within the puparium. As shown in Table 1, using reliable markers to follow the progress of metamorphosis, the occurrence of four events differs less than 8% of the studied portion of the life cycle (from the onset of pupariation to imago ecdysis). Therefore, we may assume that a good equivalence in the time of development exists among the three flies and, therefore, that metamorphosis events may be correlated with the enzymatic activity, also assuming similarity of physiological events. NBAD synthase activity is measured and NBAD analyzed as described in Pérez *et al.* (2004). Table 2 shows that NBAD synthase activety at the time of bristles sclerotization and pigmentation in *Drosophila* is higher than during early or late metamorphosis. This is different from activity in the medfly or in the horn fly, which showed increasing activity until ecdysis. The data in Table 2 must be compared with the maximum specific activities attained by the NBAD in just-emerged imagos, as shown in Table 3. For comparison, activity values at puparium peak sclerotization are 45.2 pmoles/min.mg for *Drosophila* and 23.11 pmoles/min.mg for the medfly. As expected, the overall activity measured in epidermis of old adults is very low (see thorax + abdomen in Table 3) whereas the (constitutive) brain activity is in the range of that in recently ecdysed young imagos. Thus the apparent expression (and probably regulation) of NBAD synthase activity seems very similar in the three flies, thus allowing us to switch certain experiments from small size, short life-cycle *Drosophila* to much bigger size, longer life-cycle flies. It seems appropriate to assume that robust biochemical data obtained in such a way might be very helpful when planning the study of *ebony* expression in *Drosophila*, since little is known on gene regulation in *Ceratitis* and even much less in *Haematobia.*

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The identification of a silencer element in the *cut* locus of *Drosophila melanogaster* located 73 and 96 kb upstream from the promoters of the locus.

Sosin, D.V., O.V. Kretova, and N.A. Tchurikov. Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Laboratory of Genome Organization, Vavilov str. 32, Moscow, 119991, Russia; Author for correspondence: N.A. Tchurikov; e-mail address: tchurikov@eimb.ru

Abstract

Homeotic genes are important for early development, but they are subjected to silencing during the later stages. It is known that Polycomb group proteins are involved in this type of distant regulation. The *cut* locus, a hierarchal regulator of external sensory organs, spans about 220 kb in 7B region on X chromosome. It is important for the normal development at different stages of *Drosophila* development from embryo to imago. It follows that the chromatin organization and the regulation of the transcription inside the locus are very complex. Up-to-date silencer elements were not identified in the locus. In the present study we detected a functional silencer in the distal part of the locus. The silencer is located inside the locus control region (LCR), has long protein binding region, and possesses sequences characteristic for PRE/TRE.

Introduction

The *cut* locus of *Drosophila* is the neural selector locus encoding a homeodomain-containing protein. It is known that silencing or activation of such loci are performed through interaction between certain DNA sequences, so-called Polycomb Response Elements/Trithorax Response Elements (PRE/TRE), and proteins belonging to Polycomb group (PcG) or Trithorax group (trx), respectively (Grewal and Elgin, 2002).

However, the PRE/TRE sequences have not yet been identified in the *cut* locus. It is known that the distal area of the locus contains numerous enhancers (Jack *et al*., 1991) and an important regulatory region termed as the Locus Control Region (LCR) (Churikov *et al.*, 1998). LCR has a long region binding proteins (Churikov *et al.*, 1998). Insertion of *burdock* LTR-element about 1 kb downstream from LCR leads to *ct*-lethal phenotype (Tchurikov *et al*., 1989).

In this study we investigated the nature of regulatory sequences within LCR using the reporter genetic construct and the transfection assays on *Drosophila* Schneider 2 cell line. *Sau10* fragment from LCR was inserted about 2 kb upstream from the promoter in this construct. Our data indicate that this small proximal fragment from LCR binds abundant nuclear proteins, has a silencer that is active in Schneider 2 cells, and possesses PRE/TRE sequences.

Materials and Methods

Figure 1 shows the genetic constructs that were used for the functional analysis of *Sau10* DNA fragment. The basic construct was described earlier (Kretova and Tchurikov, 2005). It has the fire fly luciferase gene driven by *hsp70* promoter, and the actin 5C 3' trailer and poly(A) addition site (*3'-act-5C*). The LCR fragment was inserted into the vector at a distance of 2215 bp upstream of the *hsp70* promoter in the same orientation as it happens in the *cut* locus. The obtained construct was used for co-transfection assays in Schneider 2 cell line along with the control construct containing *Renilla* luciferase gene flanked with the same promoter and *3'-act-5C* region (Kretova and Tchurikov, 2005).

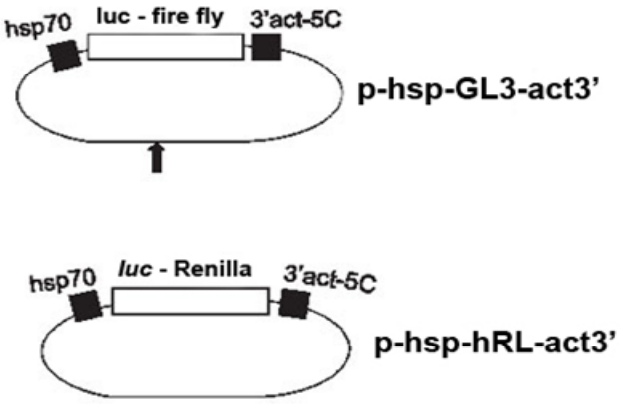

Figure 1. Genetic constructs used for co-transfection experiments. (a) - The basic vector containing the fire fly luciferase gene (*p-hsp-GL3-3'act*). (b) - Renilla luciferase gene (*p-hsp-R-hRL-act3'*). These constructs were described earlier (Kretova and Tchurikov, 2005). They contain the *hsp70* promoter and the 3'-trailer with signals of 3'-end processing from *Drosophila* actin 5C gene (*3'act-5C).* The arrow indicates the position of insertions of *Sau10* or *enh(copia)* fragments. The correctness of the genetic constructs was checked by sequencing.

For transfection experiments the Schneider 2 cells were re-plated the day before experiment to a density of 2 million of cells/ml. Liposomes containing DNA of the control and experimental constructs, 1 μg each, were prepared using the TransFast reagent (Promega) in a serum-free medium (50 μl). 0.5 ml of suspension of cells was pelleted by centrifugation at 2000 rpm for 5 min in a MiniSpin centrifuge (Eppendorf). Liposomes (50 μl) were added to the pellet, and after suspension the incubation of cells with liposomes was performed in 1.5-ml Eppendorf tubes at 27˚C for 1 h. Then the suspension of the transfected cells was transferred into the wells of 24-well plate (Nunc) filled with the complete medium (500 μl/well), and the incubation was performed for 48 h. After incubation, the cells were resuspended, pelleted by centrifugation at 3000 rpm for 5 min, and lysed. Then the luminescence of the lysate was measured according to the protocol provided by Promega (Dial-Glo Luciferase Assay System) with a Reporter Luminometer (Microplate Turner BioSystems). The *Renilla* construct was used for normalization of the data of expression of fire fly luciferase. The data were processed using the Excel and Origin software.

Results and Discussion

Figure 2 shows the genetic map of the locus. The LCR region is located at distances of 73 and 96 kb upstream from the promoters of the locus. The *Sau10* fragment (268 bp in length) is located in the proximal region of the LCR and contains a long region that binds with nuclear proteins. This was demonstrated in experiments using gel retardation assay and DNAse I footprinting (Churikov *et al*., 1998). Our experiments on gel retardation suggest that these proteins are abundant in the nucleus. The quantitation of the data obtained using a phosphoimager reveals that about 95% of labeled DNA binds to the proteins (Figure 3).

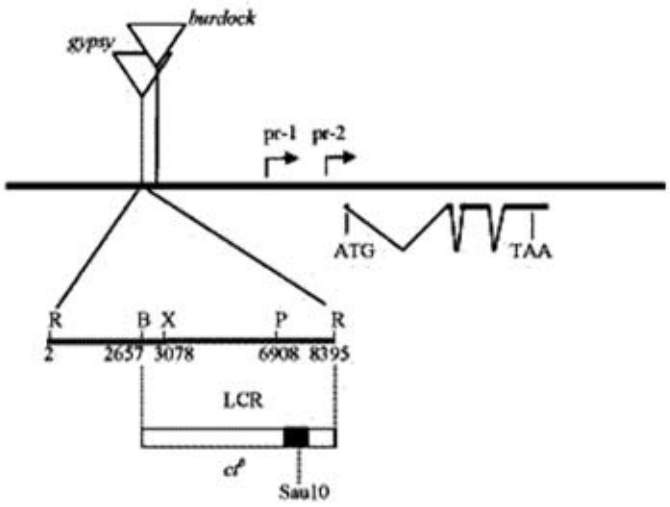

Figure 2. LCR position on the genetic map of the locus. Insertions of mobile elements *gypsy* and *burdock* induce mutations at the locus (Tchurikov *et al.*, 1989). *Eco*RI fragment approximately 8.3 kb long, in which LCR is located as shown below. Numberings are according to the sequence ACU96440.

In order to test the effect of this fragment on expression of a reporter gene, we prepared a construct containing *Sau10* fragment inserted in the basic vector expressing the fire fly luciferase. It is known that ct^6 region of the *cut* locus has a number of different enhancers (Jack *et al.*, 1991). For this reason, we expected that *Sau10* fragment could activate the expression of the reporter gene similarly to the effect of a fragment containing enhancer of *copia*, *enh(copia),* inserted in the same position.

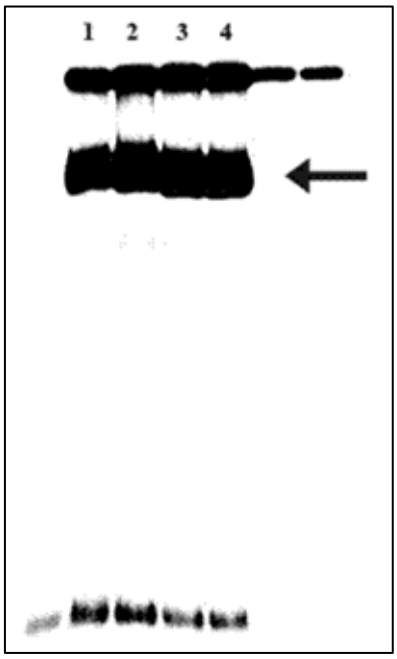

Figure 3. *Sau 10* fragment binds to abundant nuclear proteins (experiments on DNA retardation in a polyacrylamide gel). The position of the DNA-protein complex is indicated by arrow. *1-4* - indicate the independent experiments.

However, we observed the opposite effect in transfection experiments. Figure 4 shows that *enh(copia)* enhances the reporter gene expression by approximately an order of magnitude, whereas, *Sau 10* fragment suppresses it by a factor of 4-5. These well-reproducible data forced us to search for the PRE/TRE motifs in the fragment. It is known that the consensus sequence for the binding of the PHO protein is as follows: CNGCCATNDNND (nucleotides are named according to the UIPAC code) (Dejardin and Cavalli, 2004; Ringrose *et al.*, 2003). We have found such sequence in the *Sau10* region (Figure 5) that binds with nuclear proteins (Churikov *et al.*, 1998). Interestingly, the *Sau 10* fragment also contains three GAGA sequences, with which the GAGA factor may interact, a AACAA sequence, to which *trx* proteins can bind (Dejardin and Cavalli, 2004), and six poly(T) stretches, which are also characteristic for the PRE/TRE motifs (Ringrose *et al.*, 2003).

We speculate that the *Sau 10* fragment is a target for both proteins – repressors and activators of transcription in the locus. The expression of the *cut* locus is required at the early stages of development and later during the development of imago. Therefore, the presence of motifs characteristic of repressors (PcG) and activators (*trx*) may be associated with the activation of its transcription at certain stages of early and late development, as well as with its silencing at certain other developmental stages. In Schneider 2 cells, this fragment clearly exhibits the properties of a

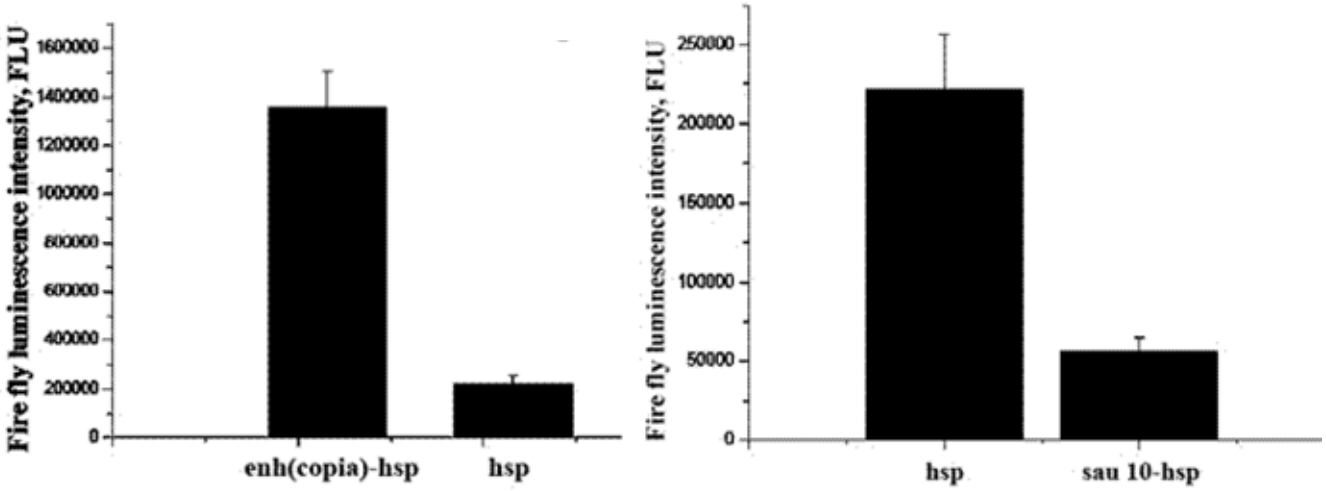

Figure 4. Results of co-transfection experiments. The effect of *enh(copia)* (a) and *Sau 10* fragment (b) on the expression of the fire fly luciferase gene driven by the non-induced *hsp70* promoter are shown.

Figure 5. Motifs characteristic of the PRE/TRE sequences are present in *Sau 10* fragment. The sequence of the fragment (region 7441-7708 in the sequence AC U96440) and the region of insertion of the *gypsy* element in *cut* lethal mutants (Thurikov *et al*., 1989), which is located upstream of the fragment, are shown. The PHO motif is shaded. The GAGA and AACAA sequences, characteristic for TRE, are framed, and the oligo(T) regions are marked with black lines.

silencer. Thus, we conclude that LCR has silencer element which could be involved in distant silencing of the *cut* locus through interaction with PcG proteins.

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Interspecific divergence in the wing morphology of females from cactophilic species of *Drosophila buzzatii* cluster.

Franco, Fernando Faria[1*], Andre Luiz Henriques Esguicero[2], Erica Cristina Carvalho Silva-Bernardi[1], Fabio Melo Sene[1], and Maura Helena Manfrin[2*].

[1]Faculdade de Medicina de Ribeirão Preto. Universidade de São Paulo. Avenida Bandeirantes, 3900 – Departamento de Genética, Bloco A, CEP 14049-900, Ribeirão Preto, Brazil. [2]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto. Universidade de São Paulo. Avenida Bandeirantes, 3900 – Departamento de Biologia, CEP 14040-901, Ribeirão Preto, Brazil. *Corresponding author: Phone: +55-016-36023103 Fax: + 55-16-36336482 email: maura@rge.fmrp.usp.br

Introduction

The *Drosophila buzzatii* cluster (*D. repleta* group, *D. buzzatii* complex) is composed by seven cactophilic sibling species: *D buzzatii*, *D. borborema*, *D. koepferae*, *D. antonietae*, *D. gouveai*, *D. serido* and *D. seriema* (Manfrin and Sene, 2006). The main diagnostic character for these species is the aedeagus morphology (Silva and Sene, 1991). Currently, the classification of females from the *D. buzzatii* cluster, provided from the natural environment, is done by the identification of their male offspring making necessary the establishment of isofemale lines, which may be a hardy task for cactophilic *Drosophila* species.

The wing morphology is a powerful tool to study *Drosophila* species for taxonomic purposes (Klaczko and Bitner-Mathé, 1990). Particularly in the *D. buzzatii* cluster, Moraes *et al.* (2004a) found marked interspecific variation in quantitative traits of wings in these species allowing the discrimination among them. Nevertheless, in this work there was a lack of information about the wing morphology of females, which, for example, are more informative than males to discriminate two other sibling species of the *D. repleta* group, *D. mercatorum* and *D. paranaensis* (Moraes *et al.,* 2004b; Prado *et al.,* 2006).

In order to quantify the variation in the wing morphology of females from the *D. buzzatii* cluster species, we analyzed laboratory individuals of all species of this cluster. The use of the wing to discriminate the species and a comparison with the male data generated previously is presented.

Materials and Methods

Seven strains representing all species of the *Drosophila buzzatii* cluster were studied: *D. buzzatii* H86G8, *D. koepferae* B20D2, *D. antonietae* H84PM, *D. serido* J92A91M, *D. borborema* 1281.0, *D. seriema* D73C3B, and *D. gouveai* J79M3. The right wing's morphology of ten females of each species was analyzed with the ellipse method (Klaczko and Bitner-Mathé, 1990; Klaczko, 2006). The seventeen morphological measures, obtained with this method, were used to perform a discriminant analysis and a cluster UPGMA analysis based on Mahalanobis distance.

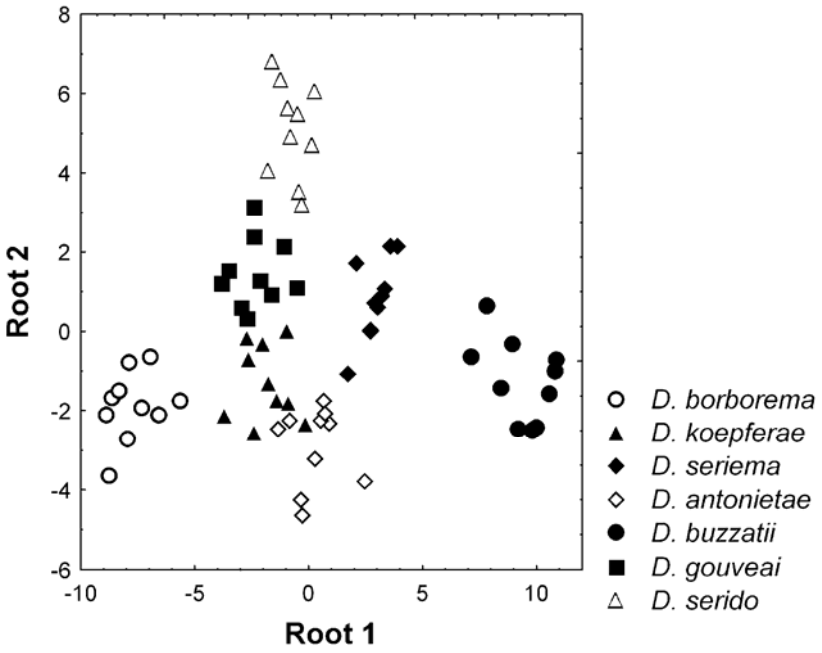

Figure 1. Plots of the individual scores for the two first discriminant roots obtained in the discriminant analyzis. Root 1 and Root 2 accounted for 57% and 15% of total variation, respectively.

Results

We detected a significant differentiation in the female's wing morphology among the species of the *Drosophila buzzatii* cluster (Wilks´Lambda = 0.00002; $p < 0.00001$), all species presented 100% of correct reclassification of cases. Except the D_F and D_H variables (not significant), all parameters were considered in the discriminant analysis. The parameters θ_E (related to the positions of the fifth longitudinal vein), θ_D (related to the positions of the fourth longitudinal vein), and W_{SH} (wing shape) were the most important for discrimination of the species. Six discriminant roots were generated on the discriminant analysis. The plot of the individual scores of the first two discriminant roots, which accounted more than 70% of total variance, can be observed in the Figure 1.

Except for *D. buzzatii*, all others species of *D. buzzatii* cluster were grouped in the same branch in UPGMA cluster analysis, indicating that *D. buzzatii* was the most morphologically divergent species. Considering the remaining species, *D. borborema* was the most morphologically differentiated. The species *D. gouveai* and *D. serido* were clustered in the same branch, whereas *D. seriema*, *D. koepferae* and *D. antonietae* where grouped in another one (Figure 2A).

Discussion

The morphometric analysis showed that the variation in the wing morphology of females of the *D. buzzatii* species cluster allows discrimination among these species with 100% of correct reclassification. Therefore, we suggest that wing morphology can be used as a taxonomic tool for female discrimination in the *D. buzzatii* cluster.

Using the same methodology as Moraes *et al*. (2004a), we obtain a more significant discrimination with female (Wilks´Lambda = 0.00002) than male individuals (Wilks´Lambda = 0.10600 in Moraes *et al.,* 2004a). Moreover, the morphological distances among the species are higher using female individuals (Figure 2), suggesting that for species discriminations and to establish the phenetic relationships among the species of the cluster *D. buzzatii*, the wings of female individuals are more informative than male specimens. Similar results were found for *D. mercatorum* and *D. paranaensis* species, where lab females and field-caught females presented discrimination higher than field-caught males (Moraes *et al.,* 2004b; Prado *et al.,* 2006).

Although the phenetic relationships among the *D. buzzatii* cluster species generated for male specimens (Moraes *et al.* 2004a) is not completely concordant with that gotten in this work with female individuals, in both phenograms, the *D. buzzatii* species is the most differentiated

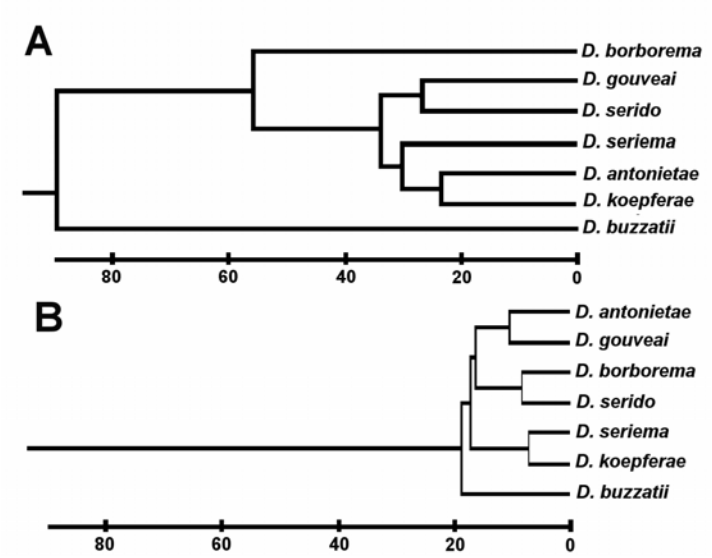

Figure 2. A, UPGMA dendrogram showing the phenetic relationships among the species of the *D. buzzatii* cluster based on female wing data. B, UPGMA dendrogram showing the phenetic relationships among the species of the *D. buzzatii* cluster based on male wing data, after Moraes *et al.* (2004). The scale bar represents the Mahalanobis distance.

morphologically (Figure 2), agreeing with others morphological, cytogenetical, and molecular studies (Manfrin and Sene, 2006).

The *D. borborema* species was the most morphologically differentiated species within the *D. serido* sibling set species (composed by *D. borborema*, *D. koepferae*, *D. antonietae*, *D. gouveai*, *D. seriema,* and *D. serido*) (Figure 2A). This species also has aedeagus morphology qualitatively different in shape and size in relation to the remaining species (Manfrin and Sene, 2006). These data, analyzed together with the derived phylogenetic position of *D. borborema* within the *D. buzzatii* cluster (Manfrin *et al*., 2001), suggest that this species became more morphologically different than the other *D. serido* sibling set species in an interval of lesser time, indicating that the events of morphological diversification within the *D. buzzatii* cluster could have occurred at different evolutionary rates in the different lineages of this cluster.

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Submissions to Drosophila Information Service are welcome at any time. The annual issue now contains articles submitted during the calendar year of issue. Typically, we would like to have submissions by 15 December to insure their inclusion in the regular annual issue. but articles can be accepted for this volume until 31 December. Details are given in the Guide to Authors.

Altitudinal and seasonal fluctuation of *Drosophila* fauna of Chamundi hill.

Guru Prasad, B.R., and S.N. Hegde. Department of Studies in Zoology, Manasagangotri, Mysore-570006, India.

Chamundi hill, a famous tourist spot of Mysore (Karnataka: India), is situated at 11º36'N latitude and 76º55'E longitude. It has an altitude of 400m, the peak being situated at 980m above sea level. It is covered by the scrub layers with small patches of evergreen type forest with a small village located at the top of the hill with a human population of 1,500. Although the altitude is less, diverse vegetation seems to harbor rich *Drosophila* fauna. Hence the present study was undertaken to study the diversity of *Drosophila* in this region.

To study the altitudinal and seasonal variation, monthly collections were made at 680, 780, 880, and 980m altitudes from February 2004 to January 2005 using bottle trapping and net sweeping methods. In the bottle trapping method, ripe mashed bananas were put in 200ml milk bottles and tied to small bushes in each altitude. Six traps were placed in each locality. Next day these bottles were plugged with cotton and brought to the laboratory. For the net sweeping method rotten mixed fruits were placed as a lump in three spots in each altitude. After 24 hours flies were collected by sweeping with a fine net, then flies were transferred to bottles containing fresh food. The meteorological data were also noted. The flies were brought to the laboratory categorized, sexed and identified.

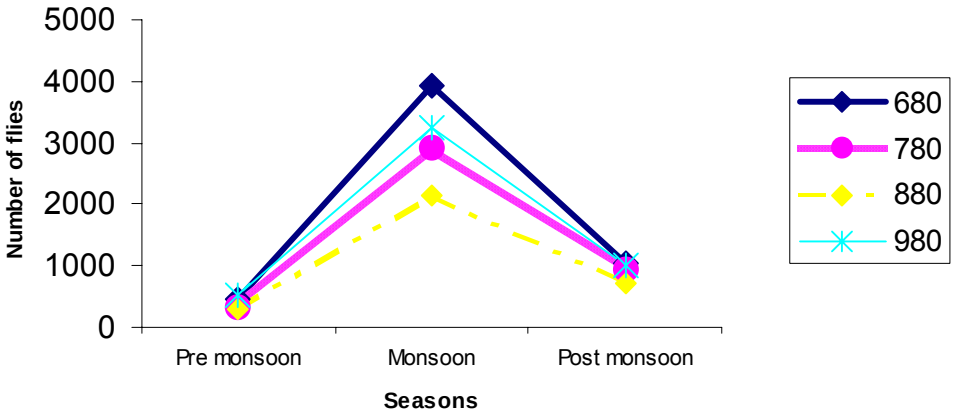

Figure 1. The altitudinal and seasonal variation of *Drosophila* fauna in different seasons in Chamundi hill.

Figure 1 shows the density of *Drosophila* at different altitudes of Chamundi hill. The number of flies decreased with increasing altitude. 980m was an exception where the density was higher than at 780 and 880m altitudes. This could be related to thicker vegetation of this locality than others and nearby human habitations.

To study seasonal variation the entire year was divided into three seasons: Premonsoon extending from February to May, Monsoon from June to September, and Postmonsoon from October to January. The density was lowest during Premonsoon, which is the hot season in India. The density declines from the middle of Postmonsoon during which cold and dry weather persists. The population size increases with the initiation of premonsoon showers and reaches peak during the monsoon season.

From the present study it is clear that population size depends on several environmental factors in addition to the colonizing ability of species (Table 1). *D. malerkotliana*, *D. rajasekari, D. jambulina, D. bipectinata, D. nasuta,* and *D. neonasuta* are the common and abundant species found in all altitudes compared to other species, such as *D. anomelani, D. coonorensis, D. punjabiensis, D.*

Table 1. List of species and numbers of *Drosophila* fauna encountered in Chamundi hill 2004-2005.

Sl.No	Species	680m	780m	880m	980m	980D	Total
	Subgenus - Sophophora						
1	*D. anomelani*	21	23	20	18	00	82
2	*D. ananassae*	00	00	00	00	748	748
3	*D. coonorensis*	00	00	15	07	00	22
4	*D. gangotrii*	16	00	12	16	00	44
5	*D. jambulina*	368	209	192	156	00	925
6	*D. kikkawai*	11	05	03	10	00	29
7	*D. malerkotliana*	1003	586	580	482	00	2651
8	*D. malenogaster*	00	00	00	00	178	178
9	*D. mysorensis*	97	80	78	73	00	328
10	*D. panjabiensis*	32	20	33	28	00	113
11	*D. rajasekari*	769	730	237	236	00	1972
12	*D. suzukii*	16	07	05	00	00	28
13	*D. takahasii*	05	33	05	06	00	49
14	D. bipectinata	254	168	139	139	00	700
	Total	2592	1861	1319	1171	926	7869
	Subgenus- Drosophila						
1	*D. nasuta*	1101	813	711	1071	00	3704
2	*D. neonasuta*	534	481	419	550	00	1984
3	*D. repleta*	85	86	51	34	00	256
4	*D. immigrans*	00	00	86	119	00	205
	Total	1720	1380	1267	1774	00	6149
	Subgenus - Drosilopha						
1	*D. buskii*	147	135	98	99	00	479
	Total	147	135	98	99	00	479
	Subgenus - Scaptodrosophila						
1	*D. brindavani*	676	691	424	583	00	2374
2	*D. nigra*	147	81	00	149	00	377
3	*D. mundagensis*	182	49	66	60	00	357
	Total	1005	821	490	792	00	3108
	Total	5464	4197	3174	3844	926	17605

*m-meters, D-domestic locality

mysorensis, D. gangotrii. Species like *D. kikkawai, D. takahashii. D. suzukii, D. repleta*, *D. immigrans, D. buskii, D. brindavani, D. nigra,* and *D. mundagensis* (Table 1) are not found in all altitudes. *D. melanogaster* and *D. ananassae* are the only two species obtained in domestic locality.

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Loss-of-function alleles of gene *garnet* appear to be lethal.

Kondrashov, Timofey A.[1], Brian Hollis[2], David Houle[2], and Alexey S. Kondrashov[3]*. [1]Dexter High School, 2200 N. Parker Road, Dexter, Michigan 48130; [2]Department of Biological Science, Florida State University, Tallahassee, FL 32306-1100; [3]Life Sciences Institute and Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-2216; *Author for correspondence (e-mail: kondrash@umich.edu).

Introduction

It has recently been shown that classical *Drosophila* loci *garnet*, *ruby*, *carmine*, and *orange* encode subunits δ, β, μ, and σ, respectively, of the tetrameric adaptor protein complex AP3 (see Ma *et al*., 2004). Although mutations at these loci have been known for many decades, only a few of them have been characterised molecularly. In particular, we know that complete loss of function of *ruby* (Figure 1 from Kretzschmar *et al*., 2000) and *carmine* (Mullins *et al*., 1999) leads to classical phenotypes and, therefore, is consistent with life. In contrast, no visible alleles of *garnet* and *orange* have been sequenced, so the nature of the corresponding functional defects remains unknown. Here, we report data suggesting that loss-of-function alleles of *garnet* are recessive lethals.

Methods

We attempted to obtain homozygous lines carrying new, EMS-induced, visible mutations of *garnet*, *forked*, *brown*, and *karmoisin*. Wild-type males were treated with 2.5 mM EMS by adding the mutagen to a 1% sucrose solution, which was then dispensed onto tissue paper that was tightly fitted into the bottom of vials. Males were placed in these vials for 24 hours, then transferred briefly to empty vials (~2 hours), then transferred to vials with medium and mated 2 days later. In the case of *garnet*, EMS-treated males were mated to $v^1\ g^2\ f^1$ females, and F_1 females with *garnet* phenotype have been isolated, with the goal of recovering new garnet mutants among their sons. The same approach has been used for the 3 other loci, and EMS-treated males were mated to $v^1\ g^2\ f^1$, $bw^1\ or^1$, or $cu^1\ kar^1$ females, respectively.

Results and Discussion

Surprisingly, we failed to isolate new, mutant alleles of garnet from the offspring of 3 F_1 *garnet* females. As it is often the case with EMS-induced mutation (*e.g.*, Jenkins, 1967), two of these females were 50/50 mosaics, with one eye (mostly) wild-type, and the other eye (mostly) garnet. The third female, having a complete *garnet* phenotype, turned out to be a gonadal mosaic. Thus, we expected ~50% of cells in these females to have a $v^+\ g^*\ f^+ / v^1\ g^2\ f^1$ genotype, and the remaining ~50% to have a $v^+\ g^+\ f^+ / v^1\ g^2\ f^1$ genotype, where g^* is an EMS-induced allele of *garnet*.

However, in the offspring of these females we observed many *v g f* and $v^+\ g^+\ f^+$ sons (132 totally), and some number of $v^+\ g^+\ f$, $v^+\ g\ f$, $v\ g^+\ f^+$, and $v\ g\ f^+$ recombinants (65 totally; *v*, *g*, and *f* are located at 33.0, 44.4, and 56.7 of the X chromosome recombination map, respectively), but no $v^+\ g\ f^+$ or $v\ g^+\ f$ sons. The most plausible explanation for the absence of $v^+\ g\ f^+$ sons is that g^* hemizygotes are lethal.

In contrast, our attempts to isolate homozygous EMS-induced mutations at the 3 other loci have been successful. We obtained over 20 v^+ g^+ f^*, bw^* or^+, and cu^+ kar^* lines, and the *de novo* nature of the corresponding f^*, bw^*, and kar^* alleles has been confirmed by sequencing.

If correct, our hypothesis that loss-of-function alleles of *garnet* are recessive lethals has two implications. First, the function of the AP3 protein complex must be essential, which is consistent with its high degree of evolutionary conservation. Second, the absence of β (encoded by *ruby*) and μ (encoded by *carmine*) subunits must affect the function of AP3 less than the absence of δ (encoded by *garnet*). Indeed, complete loss of function of one of the two human orthologs of *ruby*, AP3B1 (ADTB3A) causes a severe, but not lethal, form of Hermansky-Pudlak syndrome type 2 (Huizing *et al*., 2002). Accordingly, one may expect that loss of function of the human ortholog of *garnet*, AP3D1, is lethal. The importance of the σ (encoded by *orange*) subunit of AP3 remains to be elucidated.

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First record of *Zaprionus indianus* Gupta, 1970 (Diptera, Drosophilidae) in Argentina.

Soto, I., C. Corio, J.J. Fanara, and E. Hasson. Laboratorio de Evolución. Departamento de Ecología, Genética y Evolución. Facultad de Ciencias Exactas y Naturales. Universidad de Buenos Aires. Ciudad Universitaria, Pab. II. (C1428EHA) Buenos Aires. Argentina. email: ehasson@ege.fcen.uba.ar

The genus *Zaprionu*s, Coquillet, 1901 is composed of two subgenera with 56 species (Vilela *et al*., 2001) from the Old World. *Zaprionus indianus* (Gupta, 1970) is a drosophilid originally from the Afrotropical region and recently introduced in America (reviewed by Vilela *et al*., 2001). It was registered in this continent for the first time in Brazil (Santa Isabel, State of São Paulo) on March 1999 (Vilela, 1999) and has rapidly spread southwards. It was recorded with relative frequencies as high as 45% in Southern Brazil (De Toni *et al*., 2001, Castro and Valente, 2001). In Uruguay it was detected in 2000 (Goñi *et al.,* 2001). Nowadays it is considered a pest species by Brazilian agricultural authorities (Vilela *et al*., 2001)

Our sampling site, Cañadon de Profundidad Provincial Park (27º 34' S, 55º 43' W, Candelaria Department) is located at 30 km south-east of Posadas, capital city of the province of Misiones. Due to antropic influence, the park presents two environments, a forest with Urunday (*Astronium balansae*), and grasslands. In the ecotone, the vegetation is characterized by the presence of *Schinus molle* (aguaribay), *Anadenanthera macroearpa* (curupay), and *Lithraea molleoides* (chichita).

We used a mix of commercial yeast and mashed banana as bait. The baits were located in the forest portion of the park in rocky terrain and near a creek bank.

In the locality sample, constituted by nearly 50 specimens of drosophilids, we found 3 females of *Zaprionus indianus*. Once in the laboratory, they laid fertile eggs proving that they were inseminated in the field.

Figure 1. Left laterodorsal view of *Zaprionus indianus* (a collected female at 25x magnification). Note the white stripes flanked by thin black stripes along the dorsal surface of the head and the thorax.

Zaprionus indianus specimens were distinguished from other captured drosophilids by a distinctive trait: a pair of conspicuous silvery-white stripes flanked by thin black stripes running along the submedian area of the dorsal surface of the head and the thorax (Figure 1). There are no Neotropical species of drosophilids presenting this distinctive trait (Vilela, 1999). They will be deposited in the entomological collection of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia".

In conclusion, we are reporting for the first time the presence of *Zaprionus indianus* in Argentine territory with the consequent widening of the former distributional range westwards. Future work in Argentina will further elucidate the new distributional range of this pest.

Acknowledgments: This study was partly supported by CONICET, ANPCyT and Universidad of Buenos Aires.

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Nine new records of drosophilids in the Brazilian savanna.

Roque, Francisco, Roberta Figueiredo, and Rosana Tidon. Instituto de Biologia, Universidade de Brasília, Brasília, DF – BRAZIL. Corresponding author: rotidon@unb.br

Introduction

In a previous study (Chaves and Tidon, 2005), one of us reviewed the published records of drosophilids in the Brazilian savannas, which consists of 81 species primarily associated with rotting fruits. Here, we add to the former list nine species collected on fungi, between 2005 and 2006.

The Brazilian savanna, locally known as Cerrado biome, covers approximately 2 million km^2 of the South America (Ratter *et al.,* 1997; Oliveira and Marquis, 2002), and is one of the 25 biodiversity hotspots of the world (Myers *et al.*, 2000). Climate in this region is tropical, and precipitation is highly seasonal, characterized by a well-defined dry season from May to September. The typical vegetation consists of savanna of very variable structure on the well-drained interfluves, with gallery forests or other moist vegetations following the watercourses (Oliveira-Filho and Ratter, 2002). This heterogeneity reflects in a high diversity of the vertebrate and invertebrate faunas, as well as of microorganisms and fungi (Viegas, 1943; Dianese *et al.,* 1997).

Material and Methods

Specimens of *Pleurotus sp.* (Pleurotaceae) were collected from living trees, in the city of Brasília, the capital of Brazil. The remaining fungi were gathered from rotting trunks, in natural vegetation fragments located in the neighborhoods of Brasília. They were initially classified by us into morphospecies, but further identification of these samples revealed several taxa inside each group recognized by us. For this reason, it was not possible to associate species of drosophilids to their hosts, with the exception of *Pleurotus*.

The fungi were labeled by collection site and then placed on moist, sterile sawdust or sand in plastic containers sealed with fine gauze. Containers were held in a laboratory, where temperatures varied between 22°C and 26°C. Water was added, when necessary, to prevent desiccation. Emerged insects were removed daily for two weeks, until no further emergence took place.

All drosophilids were identified to species (Freire-Maia and Pavan, 1949; Frota-Pessoa, 1954; Magalhães, 1962; Wheeler, 1981; Vilela and Bächli, 1990; Chassagnard and Tsacas, 1993; Bächli *et al*., 2000), with the exception of one species from the *Drosophila repleta* group and one from the *D. willistoni* group, which are possibly undescribed. Voucher specimens of each species were lodged in the University of Brasilia Insect Collection.

Results

The identification of the fungi revealed several undetermined species of basidiomycetes from the class Homobasidiomycetae, belonging to the orders Agaricales and Boletales. From these hosts, 626 drosophilids have emerged and were classified in 20 species from 5 genera (Table 1). Nine of these species were recognized as new records for the Brazilian savanna: *Hirtodrosophila pleuralis, Leucophenga maculosa, L. ornativentris, L. bimaculata, L. montana*, *L. varia,* and *Zygothrica poeyi,* besides two undetermined species probably belonging to the *Drosophila repleta* and *D. willistoni* groups.

Discussion

Although we have been collecting drosophilids in the Brazilian savanna since 1998 (Ferreira and Tidon, 2005; Tidon, 2006), we have never trapped species from the *Hirtodrosophila*, *Leucophenga*, and *Zygothrica* genera. These flies are primarily mycophagous and rarely attracted to fruit baited traps.

The cosmopolitan genus *Hirtodrosophila* currently includes 158 species, 37 of them (36 extant plus 1 fossil) are known to occur in the New World (Vilela and Bächli, 2004). The dominant species in our samples, *H. pleuralis* Williston, was previously known only from the West Indies.

The genus *Leucophenga* has a worldwide distribution, but the majority of the species are tropical (Lin and Wheeler, 1972). Of the five species of this genus collected by us, only *L. maculosa*

is widespread in the New World. *L. ornativentris* is known only from Bolivia, and the remaining species were recorded in North and Central America (*L. bimaculata*: Mexico and Cuba; L. *montana*: Canada and USA; *L. varia*: Canada, USA and México) (Wheeler, 1981).

Table 1. Drosophilid species that emerged from fungi samples. Other basidiomycetes refer to undetermined species from the orders Agaricales and Boletales (Homobasidiomycetae).

species	Pleurotus sp.	*other basidiomycetes*	*total*
Hirtodrosophila pleuralis	279	164	443
Drosophila sp.A (*repleta* group)	48	2	50
Zygothrica poeyi	0	26	26
Drosophila cardini	14	10	24
Drosophila nebulosa	0	13	13
Leucophenga maculosa	0	12	12
Drosophila cardinoides	0	9	9
Drosophila paraguayensis	0	7	7
Leucophenga ornativentris	0	7	7
Leucophenga bimaculata	0	6	6
Leucophenga varia	0	6	6
Drosophila mediostriata	2	2	4
Drosophila ornatifrons	0	4	4
Drosophila willistoni	0	4	4
Drosophila sturtevanti	0	3	3
Hirtodrosophila morgani	2	0	2
Leucophenga montana	0	2	2
Zaprionus indianus	0	2	2
Drosophila shildi	0	1	1
D. sp. B (*willistoni* group)	0	1	1

Zygothrica poeyi was the only species of this genus found in this study. It was previously known from Central America (Cuba, México and Costa Rica) (Wheeler, 1981), and Southern of South America, in the Atlantic Forest (Dödge, 2006).

All the nominal species of *Drosophila* recorded here have already been collected in the Brazilian savanna (Chaves and Tidon, 2005), using fruit baited traps. Some of them are versatile and widely distributed in South America (*e.g.*, *D. cardini*, *D. sturtevanti*), whereas others are restricted to gallery forests (*D. ornatifrons* and *D. paraguayensis*). Additional information about these species may be found in Tidon (2006). The two undetermined species of the genus *Drosophila* are possibly undescribed species. Species A probably belongs to the *D. fasciola* subgroup of the *D. repleta* group, and species B was represented by only one male, whose external morphology and terminalia are similar to *D. willistoni*.

These nine new records of drosophilids in the Brazilian savanna correspond to an increase of more than 10% on the known richness of drosophilids in this biome. This probably happened because we surveyed these insects on fungi, instead of rotting fruits. Therefore, we strongly recommend that new collections of drosophilids should focus on the diversity of substrata that may represent potential hosts for these flies in South America.

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Drosophila melanogaster **lack R7/8 mediated phototaxis in mutants lacking rhodopsin in R1-6 photoreceptors in the compound eye.**

Haskins, Michael, and William S. Stark. Department of Biology, Saint Louis University, St. Louis, MO 63103. e-mail: starkws@slu.edu

In the *Drosophila* compound eye, there are three receptor types in each ommatidium, R1-6, R7 and R8. Comparing white-eyed otherwise wild-type with white-eyed retinal degeneration B (w rdgB), it had been found that R7/8 dominate phototaxis at photopic light levels (Hu and Stark, 1977). RdgB flies have degeneration in R1-6, but R7/8 remain functional. By contrast, w;ora (outer rhabdomeres absent) lack phototaxis (Harris *et al*., 1976; Hu and Stark, 1977) even though R7/8 should work; ora is a nonsense allele (Washburn and O'Tousa, 1989) of the gene coding for Rh1 (R1-6 opsin) (O'Tousa *et al*., 1985; Zuker *et al*., 1985). That gene is named ninaE where nina stands for neither inactivation nor afterpotential based on the electrophysiological defect in mutants. An allele of ninaE called oI17, a big deletion (O'Tousa *et al*., 1985), has been used in many studies.

The purpose of this study was to determine whether ninaE^{oI17} also lacks phototaxis. One reason this is not a trivial question is that ora was found to carry a mutation, in addition to the ninaE

nonsense mutation, called ort (O'Tousa *et al*., 1989) which could, theoretically, be the cause for the absence of phototaxis in ora.

Since R7 sensitivity is highest in the ultraviolet (UV), we decided to determine phototaxis at 365 nm, abundant in the mercury (Hg) arc lamp we used. We compared phototaxis in w;ninaE^{oI17}, w;ninaE^{ora}, w rdgB and w. At the time of this study, we lacked the w rdgB^{KS222} stock, used in previous studies (Hu and Stark, 1977), so we substituted w rdgB^{EE170} (Harris and Stark, 1977).

We have confidence in our methods that utilize a phototaxis arena (Hu and Stark, 1977), a Y-shaped box with a window on each side. The control window was illuminated with 525 nm light from a tungsten filament and interference and heat filters. The variable intensity window was illuminated by 365 nm light from a mercury arc, a monochromator and a UV filter. A calibrated photodiode (Stark *et al*., 1985) was placed at the location of the window of the arena, and the voltage was fed to a readout. The intensity of the unfiltered UV light was calculated to be 14.7 log quanta cm^{-2} s^{-1}. Intensity was varied with neutral density filters subtracted from the 14.7 value. The control light intensity was 14.1. We noted that the UV stimulus flickered, presumably because the mercury arc power supply was not regulated or DC; this may be important because flies have a very high flicker-fusion frequency (Autrum, 1958).

We examined live w rdgB^{EE170} eyes in the microscope to see if the stock was still good and because there had been very few studies with the EE170 allele. Pseudopupil techniques allowed us to visualize receptor integrity in the whole eye, in each ommatidium and by the visual pigment conversion (Harris *et al*., 1976; Stark *et al*., 1977; Stark and Johnson, 1980). All three diagnostics showed normal structure and function in newly-eclosed w rdgB^{EE170}; ten day post-eclosion flies lost all signs of R1-6 while R7/8 were intact. Since we wanted R7/8 but not R1-6 to be functional, we tested phototaxis in w rdgB^{EE170} flies aged one week.

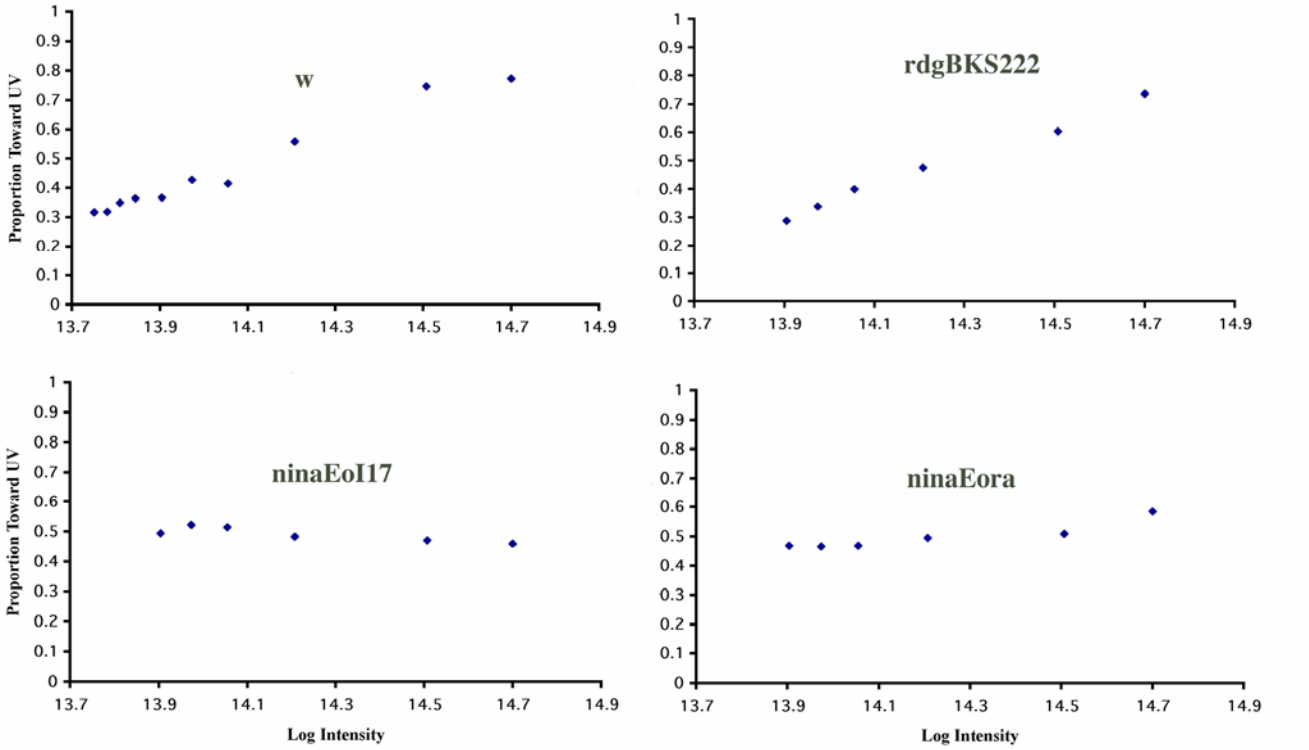

Figure 1 (top left); Figure 2 (top right); Figure 3 (bottom left); Figure 4 (bottom right).

About 20 flies from each strain were anesthetized and placed in the arena. Once awakened, they were shaken to the base of the Y shaped box, placed between the two light sources, and given five seconds to move down one of the two paths. Then a sliding metal door was shut to finalize the choice. Measurements were taken 25 times at each of 6 intensities, so it was as if 500 flies were run for each intensity-response function.

A direct relationship was found between the percentage of w flies on the UV side and its intensity. Figure 1 (top left) shows that the percent choosing the UV side increased as the intensity of the 365 nm light increases ($r = 0.988$, $P < 0.001$, $df = 9$). As shown in Figure 2 (top right), the $rdgB^{EE170}$ flies tested also yielded a direct relationship between the intensity and the UV phototaxis ($r = 0.859$, $P < 0.05$, $df = 5$). There was no phototaxis, *i.e*. the response was 50% no matter what the UV intensity, for $ninaE^{ora}$ and $ninaE^{oI17}$ (Figures 3 and 4, bottom left and right, respectively). The lack of phototaxis for $ninaE^{oI17}$ is an important new finding, because many researchers use this allele. It was also important to replicate the previous finding of no phototaxis in $ninaE^{ora}$. The importance of these findings is that they rule out the theory that the ort mutation was responsible for the lack of phototaxis in $ninaE^{ora}$.

It seemed remarkable that ninaE (both alleles), lacking function in R1-6, should be completely non-phototactic while R7/8 have normal electrophysiological function (Harris *et al*., 1976). This is why we thought it was important to replicate the finding that rdgB flies (with R1-6 degenerated and R7/8 functional) do, by contrast, have phototaxis. The direct relationship found between UV intensity and phototactic response indicates that the $rdgB^{EE170}$ flies do have phototaxis. This extends previous findings since it was a different allele, $rdgB^{KS222}$, that had been shown to have R7/8-mediated phototaxis.

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Courtship sound analysis in *Drosophila mercatorum* subspecies.

Prado, Pedro R.R.[1,3], Maura H. Manfrin[2], Luciano F. Costa[3], and Fabio M. Sene[1].
[1]Faculdade de Medicina de Ribeirão Preto/USP; [2]Faculdade de Filosofia Ciências e Letras de Ribeirão Preto/USP; [3]Instituto de Física de São Carlos/USP.
*Corresponding author: Fabio de Melo Sene, Departatmento de Genética, FMRP-USP, 14049-900-Ribeirão Preto-SP-BR. Email: famesene@usp.br

Abstract

Qualitative and quantitative aspects of sexual courtship are studied to define the sound patterns from the *Drosophila* species. The current work proposes the automated acquisition of

measurements by applying the Hilbert Transform methodology. This methodology allows the analysis of a complete courtship sound, and not only part of the signal. The application of this methodology to comparative studies of the subspecies *D. mercatorum* allowed the correct classification of the individuals and, above all, showed a greater variability within the measurements obtained than results previously obtained. This factor is important for a better understanding of the standards of the sexual courtship of *Drosophila.*

Introduction

The courtship sound in *Drosophila*, produced by the male through the vibration of its wings, is considered an important feature for species identification during mating and is consequently associated with sexual isolation among species (Bennet-Clark and Ewing, 1967).

Qualitative and quantitative aspects have been considered for the definition and determination of sound patterns for different species (Chang and Miller, 1978; Ikeda and Maruo, 1982; Ewing and Miyan, 1986; Tomoru and Okuma, 1994). Whereas the qualitative analysis generally involves the visual description of graphic patterns, quantitative analyses traditionally consider the following four parameters: *pulse* – pure sound unit consisting of one or more cycles; *frequency of pulse* – sound frequency within the pulses, measured in cycles per second; *interpulse interval* – measured from the beginning of a pulse to the beginning of the next; and *burst* – a series of pulses. This group of measurements has been used to describe the differences in the courtship sound between species and among populations of the same species in *Drosophila* (Miller *et al.*, 1975; Ritchie *et al.*, 1994; Manfrin *et al.*, 1997).

Generally, the sound signals obtained from sexual courtship, represented graphically, allow the characterization of the species in terms of the distribution and presence or absence of bursts and of the length of the pulses. However, such patterns may show a high degree of similarity, making species identification by visual inspection a particularly challenging and subjective procedure (Hoikkala and Lumme, 1987; Noor *et al.*, 2002).

In these cases, the quantitative measurements of the parameters mentioned above are necessary in order to define the species and oftentimes to describe an inter-population variation. The most commonly adopted methodologies to obtain these measurements are often based on manual methods (Costa *et al.*, 2000), which severely limit the speed, accuracy, and objectivity of the measurements, implying constraints on the number of considered individuals. At the same time, the conclusions concerning a specific sound pattern can have little significance, especially when more objective and informative measurements are required, as is the case for population analysis and evolutionary investigation of the species. Therefore, it is necessary to devise and apply automated approaches capable of addressing such situations.

The methodology of the current work can be divided into the following four stages: (i) the sound produced by male individuals is recorded and stored on a computer; (ii) the obtained signals are pre-processed in order to reduce noise and interference; (iii) the position of the pulses and bursts is automatically determined by using the Hilbert Transform (Long *et al.*, 1995), which allows for the precise identification of the envelopes of pulses and bursts; and (iv) the pre-processed sound elements are measured in order to obtain the length of bursts and individual pulses (PI), as well as the distance between each individual pulse (IPI). The Fourier Transform is also applied in order to obtain the fundamental frequency characterizing each of these structures. This mathematic-computational procedure for sound characterization and analysis was applied in order to validate the analysis in two subspecies of *Drosophila mercatorum*: *D. m. pararepleta* and *D. m. mercatorum*.

The sound pattern of these two subspecies is composed of two groups of signals with distinct characteristics (Ikeda *et al.*, 1980). The first one, called sound A, is produced by the male at the

beginning of the sexual courtship. The second, hence sound B, is produced by the male immediately before copulation. Although they produce different graph patterns, these sounds have a similar frequency composition and vary only in their harmonics (Manfrin *et al.*, 1997). Intra-specific analyses performed by Manfrin *et al.* (1997) have shown the occurrence of quantitative variation in IPI values and the fundamental frequency for both types of sounds between the subspecies.

The choice of these particular subspecies in the present work was motivated by the fact that their sound patterns are complex and well-studied, presenting quantitative variation in their sound parameters (Ikeda *et al.,* 1980; Manfrin *et al.*, 1997). It contributes to the validation of the mathematic-computational methodology, which is aimed at a faster and more accurate definition of sound signals in terms of IPI and PI measurements, in such a way that it is compatible to what is normally expected in the literature.

Material and Methods

Fly Preparation

Sound signals were recorded from males of the *Drosophila mercatorum mercatorum* - lineage 152118 - New York, USA, and *Drosophila mercatorun pararepleta* - lineage J93M52 - Bahia, Br. Virgins flies were isolated in groups of the same gender six hours after hatching, stored in bottles containing culture medium, and kept for a period of seven to ten days until they were sexually mature (Ikeda *et al.*, 1980).

Sound Recording

To record the sound, a couple of virgin flies were put into a sound recording chamber (Sene and Manfrin, 1998). The chamber was placed over an ultra-sensitive microphone diaphragm (Bennet-Clark, 1984) inside an acoustically-isolated chamber, and the sound was recorded on audio tapes digitized by using an audio capture card. Courtships lasting longer than 10 minutes, without copulation, were discarded. The sampling frequency remained at 8,000 Hz. Figure 1 shows a complete sound sequence of a *Drosophila mercatorum* courtship. The two first bursts are called sound A, and the last one, sound B.

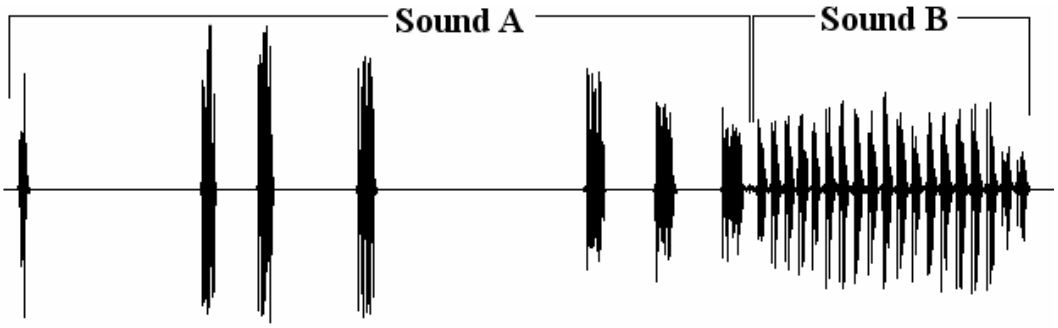

Figure 1. Sound patterns of the sexual courtship of the *Drosophila mercatorum* species. A sequence showing seven bursts of sound A and one burst of sound B. (Manfrin *et al.*, 1997).

Mathematical analysis
Measurements of sequential structures.

Detection of sound envelopes by using the Hilbert Transform

The sound A signal recorded from the sexual courtship of *D. mercatorum* consists of a set of packets of oscillations called bursts. The typical pattern of such signals is illustrated in Figure 2a, which shows eight pulses that make up a burst, and Figure 2b, which shows the zoomed version of the third pulse of the burst. In order to speed up the delimitation of the bursts and its internal oscillations (pulses), we applied the Hilbert Transform (Long *et al*, 1995) to extract the envelopes from oscillating signals. Such envelopes are transformed into frames of pulses identifying the beginning and the end of each burst. A similar procedure is used for the identification of the individual pulses inside the bursts.

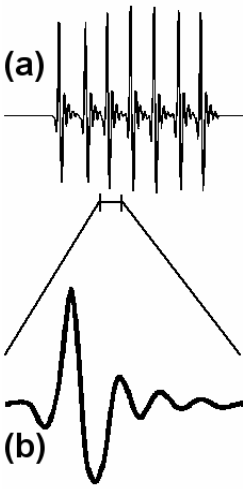

Figure 2. One burst of sound A of the sexual courtship of *Drosophila mercatorum*. (a) – The expanded burst showing 8 pulses. (b) – The amplified version of the third pulse.

An example of envelope extraction is presented in Figure 3, which shows a train of pulses with the detected envelopes represented by dashed lines (a). By thresholding such envelopes, it is possible to isolate the bursts, as illustrated in (b). Figure 3(c) shows the application of the Hilbert Transform to detect pulses internal to a burst.

The fundamental frequency of the spectrum are obtained by applying the discrete version of the Fast Fourier Transform (FFT), namely the Discrete Fourier Transform (DFT), over the digitized sound elements (Costa and Cesar, 2000).

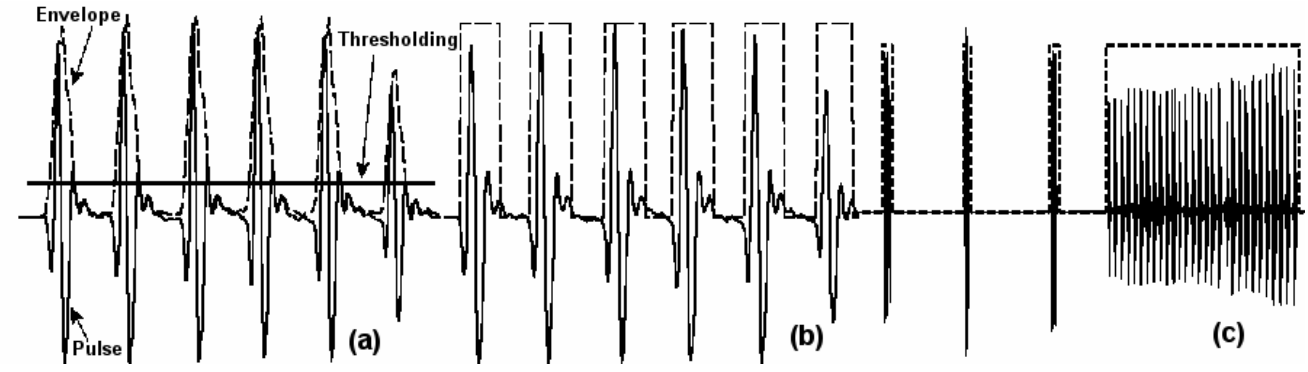

Figure 3. Sound signal and envelopes (dashed lines) obtained by using the Hilbert Transform. A – The separation of the pulses can be performed by thresholding the envelopes, producing results such as those illustrated in (b). A group of pulses defines a Burst, as illustrated in (c).

Measurements

The measurements obtained in this work to characterize the sexual courtship sound were IPI (interpulse interval) and FFP (Fundamental Frequency of Pulses). These measurements characterize the sound patterns of the sexual courtship of each individual within the species. Each investigated individual has its own features, which present some dispersion with respect to the average values obtained for all experiments. Once the descriptive statistics have yielded the average and standard deviation for each individual, the multivariate statistical method was applied in order to compare the different lineages. It was considered that the obtained measurements are represented by a one-dimensional Gaussian probability density (Costa and Cesar, 2000; Duda and Hart, 2000). The normal probability density function was weighted by the respective mass probabilities (Costa and Cesar, 2000).

Results

We analyzed 77 individuals of *Drosophila mercatorum mercatorum* and 83 individuals of *Drosophila mercatorum pararepleta* from laboratory lineages. The sexual courtship signals from the males were recorded and transferred to a microcomputer, where the Hilbert Transform was applied in order to delimit the bursts and pulses, as well as the measurements concerning their definition. The comparison between lineages was restricted to the average of FFP (Fundamental Frequency of Pulses) and interpulse interval (IPI) values, for the sound A, for each individual within a lineage. The results of the average of FFP are shown in Figure 4a. The respective normal density function was estimated in terms of the average and standard deviation for each lineage and is shown in Figure 4b.

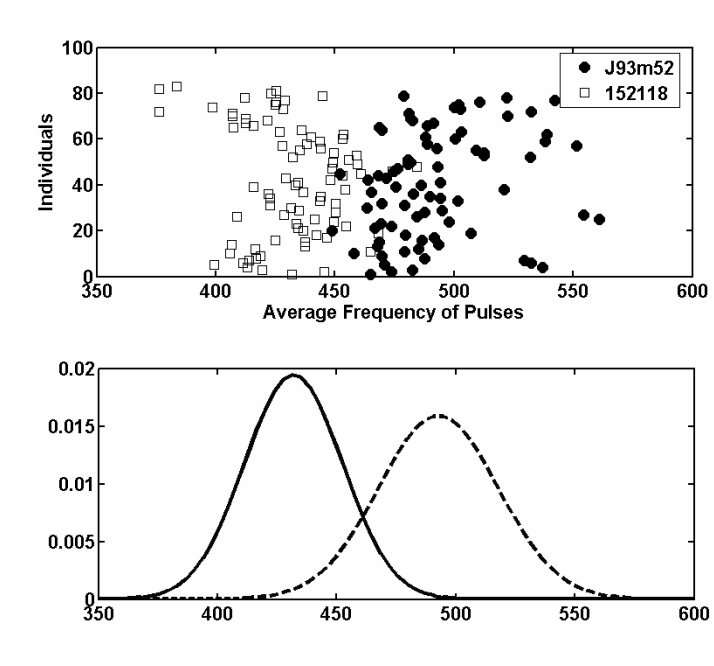

Figure 4. Scatterplot of the average values of the Fundamental Frequency of Pulses (FFP) for each individual from the J93M52 lineage from the subspecies *D. m. pararepleta* and from the 152118 lineage from the *D. m. mercatorum* subspecies (a), and the normal density functions fitted for the frequency distribution for each lineage (b).

Table 1. Comparative values for analyzed individuals considering their lineages using the Bayesian classification and of the average of Fundamental Frequency of Pulses.

Species	J93M52	152118
J93M52	74	3
152118	4	79

Correct matches: 95.62%

The individual discrimination was performed using the Bayesian decision, and the number of correct and incorrect classifications of each individual in relation to its respective lineage is shown in Table 1. Results of the IPI are shown in Figure 5a. The respective normal density function in Figure 5b and the classifications are shown in Table 2.

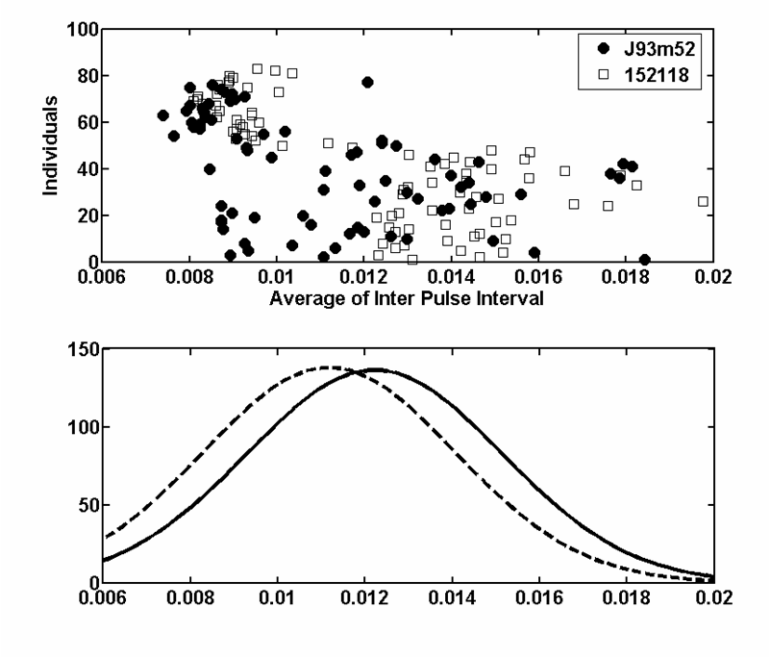

Figure 5. Scatterplot of the average values of the interpulse interval for each individual from the J93M52 lineage from the *D. m. pararepleta* subspecies and from the 152118 lineage from the *D. m. mercatorum* subspecies (a), and the normal density functions fitted for the frequency distribution for each lineage (b).

Table 2. Comparative values for analyzed individuals considering their lineages using Bayesian classification and IPI average.

Species	J93M52	152118
J93M52	46	31
152118	35	48

Correct matches: 60.00%

Discussion

In this work, the sound patterns of lineages representative of the two subspecies of *Drosophila mercatorum* were analyzed using the Hilbert Transform technique, which made it possible to isolate the individual bursts and pulses, measurements used to discriminate the subspecies. The results obtained show that, despite the similarities in the considered patterns, which does not allow for the visual distinction of the subspecies, it was possible to distinguish the subspecies based on the quantitative values of the frequency of pulses. *D. m. mercatorum* had smaller values in relation to the *D. m. pararepleta.* Similar results were obtained by Manfrin *et al*. (1997).

In addition to the measurements of the frequency of pulses, the IPI measurements were analyzed as well, mainly due to the fact that this measurement is considered to be the most relevant parameter to characterize the sound of the species (Ritchie and Kyriacou, 1996). Considering the IPI values for sound A, the obtained results do not allow for a discrimination of the lineages representative of the two subspecies of *D. mercatorum* (Table 2). The result obtained is different from the one previously obtained by Manfrin *et al.* (1997), in which *D. mercatorum mercatorum* were defined by having greater IPI in relation to *D. mercatorum parerepleta*. The hypothesis is that this discrepancy was caused by differences in the methodology used in these analyses. The methodology proposed in this work uses the Hilbert Transform, which employs envelopes to capture automatically data of components of the courtship sounds, burst and pulses, over the complete sexual courtship period. This approach allows one to increase the number of IPI values over a complete sexual courtship that can be analyzed, thereby increasing the knowledge about the variability of data. In this work, the non-definition of *Drosophila mercatorum* subspecies by IPI, when compared to previous work, may be related to its variability over the entire sexual courtship, which is not detected when part of the signal is analyzed.

The methodology, employing the Hilbert Transform to delimit pulses in sound signals, can be considered important for characterizing inter- and intra-specific variations as its use enabled and enhanced the acquisition of the measurements. Another advantage of this methodology is that it can be used not only for the sexual courtship of *Drosophila*, but also in other models of biological signals.

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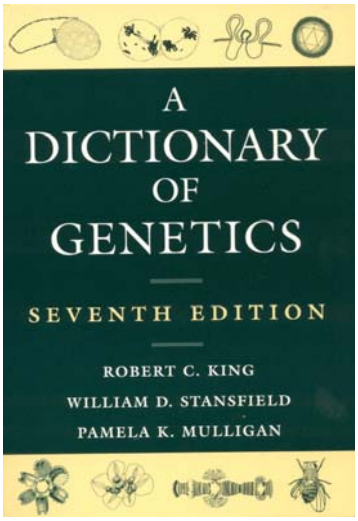

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