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Gene Duplication and Subsequent Differentiation of Esterases in Cactophilic *Drosophila* Species

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1. Introduction

Phytophagous insects are excellent model systems to study the genetic and ecological bases of adaptation and population differentiation because the host plant constitutes an immediate environmental factor that can affect the early stages of the life cycle (Matzkin, 2005; Matzkin et al., 2006). New host plant exploitation can result in genetic and biochemical adjustments to the new resource and to chemically distinct niches, which can include potentially toxic compounds, new mating environments, parasitoids, bacteria and fungi (Kircher, 1982; Fogleman & Abril, 1990; Via, 1990; Fogleman & Danielson, 2001). These adjustments are the result of a number of physiological changes, including those related to biochemical systems associated with adaptation to the new environment.

The species of the *Drosophila repleta* group occupy different habitats, but their common feature is that they are phytophagous; that is, they lay eggs in rotting cacti cladodes. The developing larvae feed on the yeast that are part of the rotting process (Starmer & Gilbert, 1982; Pereira et al., 1983; Starmer et al., 1986), according to the cactus-*Drosophila*-yeast system; therefore, they are considered specialists. However, adults are generalists because they visit other food sources in their environment (Morais et al., 1994). This ecological specificity of cactophilic *Drosophila* directly influences species distribution, as they are always associated with the host cactus distribution (Tidon-Sklorz & Sene, 1995; Manfrin & Sene, 2006; Mateus and Sene, 2007).

Drosophila has been used as a research model for more than a century, and the first report of gene duplication was described by Bridges for the *Bar* locus in *D. melanogaster* over 70 years ago (Bridges, 1936). Since that time, mainly after the advent of biochemical and molecular biology techniques, several other examples of duplicated genes have been presented, and pathways of evolution by gene duplication have been proposed (for example, Stephens, 1951; Nei, 1969). These pathways were thoroughly discussed in 1970 in Ohno's book "Evolution by gene duplication" (Ohno, 1970). Subsequently, several other works have reviewed the mechanisms and roles of gene duplication in the evolutionary process (A. Wagner, 2002; Kondrashov et al., 2002; and Zhang, 2003).

Currently, the genomes of twelve *Drosophila* species have been completely sequenced (Tweedie et al., 2009), but many aspects of the functional divergence of the products of a

gene duplication event cannot be answered through this method alone. A deeper investigation of genetic differentiation after duplication is possible through molecular and biochemical approaches. These approaches are extremely important because gene duplication followed by functional divergence has been considered the primary mechanism of molecular evolution (Lewis, 1951; Ohno, 1970). Analyses of isozymes have been crucial in this process because they provide, along with cytological studies, evidence for the frequent occurrence of gene duplication during the evolutionary process (Gottlieb, 1982; Hart, 1983).

Esterase is a polymorphic group of isozymes that play important biochemical roles in insects. This group is composed of a heterogeneous set of hydrolytic enzymes that are widely distributed among organisms and that catalyze the hydrolysis of esters, peptides, amides and halides (Walker & Mackness, 1983). They are involved in digestive (Argentine & James, 1995) and reproductive processes (Karotam et al., 1993), the degradation of insecticides (Feyereisen, 1995) and female sex pheromones after male recognition (Vogt & Riddiford, 1981) and in the regulation of juvenile hormone levels (Gu & Zera, 1994). In *Drosophila*, esterases make up a diverse set of enzymes (G. B. Johnson, 1973, 1974), and gene duplication has been used as one explanation for their evolution (Zouros et al., 1982; Pen et al., 1990; Mateus et al., 2009).

Several studies on the changes in esterase activity during development in species of the *D. repleta* group have detected two main β-esterases that show different tissue-specific and temporal expression patterns (Zouros *et al.*, 1982; East, 1982; Pen et al., 1984; Pen et al., 1986a, 1986b; Pen et al., 1990; Mateus et al., 2009). One esterase, named EST-4, is present only in later third instar larvae and early pupae and has a high concentration in the carcass. The other esterase, named EST-5, is present throughout the insect life cycle and occurs predominantly in hemolymph and the fat body (Zouros *et al.*, 1982).

According to Zouros *et al.* (1982), who studied these enzymes in *D. mojavensis* and *D. arizonae*, the most likely hypothesis is that these enzymes are products of a gene duplication as old as the *D. repleta* group that diverged later regarding their patterns of tissue-specific and temporal expression. This hypothesis was suggested because these enzymes show interlocus heterodimers, different patterns of expression (tissue and temporal) and 82% identity in the N-terminal amino acid sequence (Pen *et al.*, 1986a; Pen et al., 1990). More recently, Robin et al. (2009) demonstrated that these enzymes are encoded by two genes that are products of a gene duplication, *Est-2a* (EST-5) and *Est-2c* (EST-4), in *D. mojavensis*.

In this study, we investigated several genetic and biochemical features of EST-4 and EST-5 in six species of the *D. repleta* group, three belonging to the *D. mulleri* cluster (*Drosophila mulleri*, *D. aldrichi* and *D. wheeleri*) and three belonging to the *D. mojavensis* cluster (*D. mojavensis*, *D. arizonae* and *D. navojoa*) of the *D. mulleri* complex, as well as hybrids from crosses involving some of these species. We aimed to establish the biochemical and genetic differences among and possible physiological roles for these enzymes in the metabolic processes of this group of *Drosophila* species.

2. Materials and methods

2.1 Species

The materials used in this study included laboratory stocks of six *Drosophila* species (*D. arizonae* - AR, *D. mojavensis* - MO, *D. navojoa* - NA, *D. mulleri* - MU, *D. aldrichi* - AL and, *D. wheeleri* - WH) and two homozygote line stocks, one for the EST-5 "slow" allele of *D. mulleri* (MU-S) and another for the EST-4 "fast" and EST-5 "slow" alleles of *D. navojoa* (NA-FS). All

stocks were multifemales, except for the NA-FS stock, which was isofemale. The laboratory stocks were obtained from Prof. Dr. Hermione E. M. C. Bicudo (Department of Biology, IBILCE/UNESP, São José do Rio Preto, Brazil), who brought them to Brazil from stocks of the Genetics Foundation (University of Texas, Austin, TX, US). The two line stocks were prepared from laboratory stocks through endogamic crosses.
All laboratory and line stocks were maintained as mass cultures at a constant temperature of 20ºC ±1ºC in culture vials with standard banana medium. The origin of each stock is listed in Table 1.

Species	Codes	Locality
Drosophila mulleri	MU	Guayalejo, Tamazunchale, México
Drosophila aldrichi	AL	Austin, Texas, US
Drosophila wheeleri	WH	Arroyo Solloro, Baja California, México
Drosophila mojavensis	MO	Baja California, México
Drosophila arizonae	AR	Guayalejo, Tamazunchale, México
Drosophila navojoa	NA	Navojoa, México

Table 1. List of analyzed species, with the respective codes and original localities of the stocks (all stocks were obtained from the Genetics Foundation, University of Texas, Austin, TX, US).

2.2 Obtaining late third instar larvae and adult flies for electrophoresis

Late third instar larvae and adult flies were collected directly from the vials and immediately frozen at -20º C for further electrophoretic analyses. The larvae in that phase show yellowish spiraculum and maximum EST-4 activity. Female adult flies were collected at 5-10 days old and were used in electrophoresis for comparative analysis.

2.3 Obtaining hybrids

Mass crossings in both directions were performed in population boxes (16 cm^3), using 200 couples, between NA-FS and MU-S, NA-FS and MO, NA-FS and AR and MU-S and MO. After setting up a cross, the courtship behavior was observed for 10 minutes, as described by Markow (1981). The culture media were placed in Petri plates at the bottom of the boxes and were changed every three days. After every plate change, the plates were inspected to detect eggs. The plates were maintained at a constant temperature of 20ºC ±1ºC until late third instar hybrid larvae were observed. These larvae were obtained directly from the plates and frozen at -20ºC for further electrophoretic analyses.

2.4 Esterase detection

Esterase detection was performed using 10% polyacrylamide gel electrophoresis (PAGE), adapted by Ceron (1988) from Davis (1964) and Laemmli (1970). All samples were prepared in 25 µL of 0.1 M Tris-HCl (pH 8.8) buffer containing 10% glycerol, where 10 µL was used in the gels. After electrophoresis, all gels were soaked in 0.1 M phosphate buffer (pH 6.2) for 1 hour at 25ºC. After this period, the gels were stained in solution containing 100 mL of phosphate buffer, 10 ml of n-propanol and 120 mg of Fast Blue RR Salt, where 40 mg of α-naphthyl acetate and 30 mg of β-naphthyl acetate, previously dissolved in 2 ml of acetone, were added. After approximately 1 hour, the staining reactions were stopped in a solution of acetic acid:ethanol:water (1:2.5:6.5 by v:v:v). Because the esterases hydrolyze substrates

differently, the bands in the gel stain differently: black when they hydrolyze α-naphthyl acetate, red when they hydrolyze β-naphthyl acetate and magenta when they hydrolyze both α- and β-naphthyl acetates. Polyacrylamide gels were air dried at room temperature using gelatin and cellophane wound slab gels in an embroidering hoop (Ceron et al., 1992).

2.5 Characterization of esterases using inhibitors

Malathion, phenylmethylsulfonyl fluoride (PMSF), eserine sulfate, copper sulfate ($CuSO_4$), iodoacetamide (IAC), trans-epoxysuccinyl-L-leucyl-amido(4-guanidino) butane (E-64), p-chloromercuribenzoate (pCMB) and mercuric chloride ($HgCl_2$) were used as specific inhibitors, all in 1 mM concentrations (with the exception of E-64, which was used at a concentration of 5 mM) in the soaking and staining solution.

2.6 Determination of isoelectric point (I.P.)

The I.P. was determined in 10% PAGE containing 5% ampholyte solution (Sigma). The first ampholyte formed a pH gradient between 3.0 and 10.0 after 1 hour of constant 100 V pre-focusing. This experiment was performed to verify the best gradient to determine the I.P. values of all enzymes in all species. After this verification, another ampholyte was used that formed a pH gradient between 6.0 and 8.0 after 1 hour of constant 100 V pre-focusing. In both cases, ampholyte solutions were added before gel polymerization. Samples of the six *Drosophila* species and of the I.P. marker (hemoglobin) were prepared in a 10% glycerol in water solution. Esterase isoelectric focusing was performed under constant 100 V conditions in the power supply for 3 hours. After focusing, the gels were soaked in buffer for 1 hour, followed by esterase staining for the same period, as described in section 2.5. Following esterase identification, the gels were stained for total protein with Coomassie Blue G250 overnight. The staining reaction was stopped, and the gels were dried as described in section 2.5. The I.P. was estimated by comparing the positions of EST-4 and EST-5 with the position of human hemoglobin (I.P. = 7.1) after focusing.

2.7 Molecular weight (MW) estimation

The MW estimation was performed using the method adapted by Mateus et al. (2009) from Hedrick and Smith (1968). The following standard MW proteins were used: myoglobin (17.8 kD), soybean trypsin inhibitor (24 kD), carbonic anhydrase (29 kD), ovalbumin (45 kD), human serum albumin (66 kD) and phosphorylase-b (97.4 kD). All graphics were constructed using Microcal Origin software, version 3.5 (Scientific and Technical Graphics in Windows – copyright 1991 – 1994, Microcal Software Inc.).

3. Results

3.1 Esterase pattern

Figure 1 shows the esterase patterns of larvae and adults (females) from six *Drosophila* species. For all species, EST-4 always migrated slower than EST-5. The *D. navojoa* stock was the only one that had more than one band for EST-4. EST-4 was more strongly stained than EST-5 in *D. mulleri*, *D. aldrichi* and *D. wheeleri* (Figure 2A and B). The opposite was observed for *D. arizonae*, with EST-5 more strongly stained than EST-4 (Figure 2A). Differences in the staining intensity among EST-4 bands were also observed, with the *D. mojavensis* cluster species (*D. mojavensis*, *D. arizonae* and *D. navojoa*) showing fainter bands than the species of the *D. mulleri* cluster (*D. mulleri*, *D. aldrichi* and *D. wheeleri*).

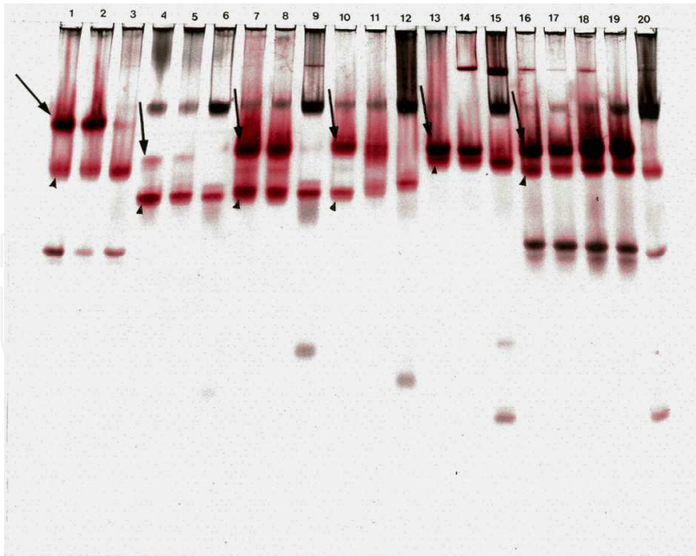

Fig. 1. Esterase pattern in 10% PAGE for late third instar larvae and adult females of *Drosophila mulleri* (1 and 2 = larvae; 3 = female), *D. arizonae* (4 and 5 = larvae; 6 = female), *D. mojavensis* (7 and 8 = larvae; 9 = female), *D. navojoa* (10 and 11 = larvae; 12 = female), *D. wheeleri* (13 and 14 = larvae; 15 = female), *D. aldrichi* (16 to 19 = larvae; 20 = female). All wells contain individual samples, except for wells 18 and 19, which contain 2 larvae of *D. aldrichi*. Arrow = EST-4; arrowhead = EST-5.

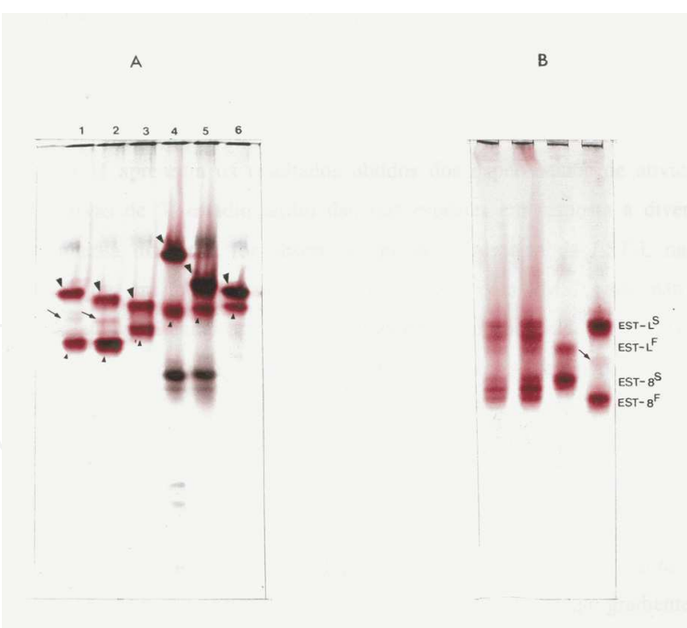

Fig. 2. A. 10% PAGE showing the electrophoretic staining differences for EST-4 and EST-5. 1- *D. mojavensis*; 2 - *D. arizonae*; 3 - *D. navojoa*; 4 - *D. mulleri*; 5 - *D. aldrichi*; 6 - *D. wheeleri*. B. 10% PAGE of late third instar larvae of *D. navojoa*, showing the different phenotypes observed. Arrows in A and B indicate the interlocus heterodimer EST-4/EST-5. Larger arrowhead = EST-4; smaller arrowhead = EST-5.

Despite the observation of homozygotes for EST-4 in five out of six species analyzed, the quaternary structure for this enzyme as a dimer could be deduced from the presence of a heterodimer between EST-4 and EST-5 in *D. mojavensis* and *D. arizonae* (Figure 2A). This dimeric structure for EST-4 and EST-5 was confirmed by hybrid analyses. In *Drosophila navojoa*, in addition to the presence of the heterodimer, three phenotypes were observed in gels for EST-4 and EST-5 (Figure 2B): homozygous for a slower band (EST-4^S and EST-5^S, respectively); homozygous for a faster band (EST-4^F and EST-5^F, respectively); and heterozygous, with a three-band pattern. These patterns reinforce the quaternary structure of these enzymes for this species. The same results were observed for EST-5 of *D. mulleri* (data not shown).

3.2 Pattern of esterase activity in the presence of inhibitors

Table 2 shows the results obtained for the esterase activity patterns of late third instar larvae of the analyzed species in the presence of different inhibitors. All species showed the same pattern for EST-4: they were inhibited only by PMSF. For EST-5, all species were inhibited only by malathion. No other inhibitor affected the activity of either esterase.

Species	Enzyme	PMSF	Malathion	$CuSO_4$	IAC	E-64	Eserine	pCMB	$HgCl_2$
D. mulleri	EST-4	++	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	EST-5	⊗	++	⊗	⊗	⊗	⊗	⊗	⊗
D. aldrichi	EST-4	++	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	EST-5	⊗	++	⊗	⊗	⊗	⊗	⊗	⊗
D. wheeleri	EST-4	++	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	EST-5	⊗	++	⊗	⊗	⊗	⊗	⊗	⊗
D. mojavensis	EST-4	++	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	EST-5	⊗	++	⊗	⊗	⊗	⊗	⊗	⊗
D. arizonae	EST-4	++	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	EST-5	⊗	++	⊗	⊗	⊗	⊗	⊗	⊗
D. navojoa	EST-4	++	⊗	⊗	⊗	⊗	⊗	⊗	⊗
	EST-5	⊗	++	⊗	⊗	⊗	⊗	⊗	⊗

Table 2. Esterase activity patterns of EST-4 and EST-5 for the six *Drosophila* species analyzed in the presence of different inhibitors. PMSF = phenylmethylsulfonyl fluoride; Eserine = eserine sulfate; $CuSO_4$ = copper sulfate; IAC = iodoacetamide; E-64 = trans-epoxysuccinyl-L-leucyl-amido(4-guanidino) butane; pCMB = p-chloromercuribenzoate; $HgCl_2$ = mercuric chloride. ++ activity inhibited; ⊗ activity not affected.

3.3 Isoelectric point (I.P.) determination

The I.P. determination was performed in two phases. In the first phase, we verified that the best range for I.P. determination was 6.0 to 8.0. In the second phase, an ampholyte solution was used for this pH range. Table 3 shows that all esterases presented I.P. between 6.0 and 7.0. As expected, the I.P. values for EST-5 in both larvae and adults of the same species were equal, ranging from 6.47 (*D. navojoa*) to 6.64 (*D. aldrichi*). EST-4 showed a wider range of I.P. variation than EST-5, with *D. mulleri* and *D. navojoa* showing the highest and lowest I.P. values (6.88 and 6.37, respectively).

***D. mulleri* cluster**	**I.P.**	***D. mojavensis* cluster**	**I.P.**
D. mulleri		*D. mojavensis*	
EST-4	6.88	EST-4	6.38
EST-5 (larvae and adult)	6.51	EST-5 (larvae and adult)	6.49
D. aldrichi		*D. arizonae*	
EST-4	6.55	EST-4	6.53
EST-5 (larvae and adult)	6.64	EST-5 (larvae and adult)	6.56
D. wheeleri		*D. navojoa*	
EST-4	6.59	EST-4	6.37
EST-5 (larvae and adult)	6.53	EST-5 (larvae and adult)	6.47

Table 3. Isoelectric points for EST-4 and EST-5 of larvae and adults of the six analyzed *Drosophila* species, obtained through the comparison of esterase band mobility in gels with an I.P. marker (hemoglobin; I.P. = 7.1) in a pH range between 6.0 and 8.0.

3.4 MW determination

To determine the MW of both enzymes in all six *Drosophila* species analyzed, the technique described by Mateus et al. (2009) was applied using 6% to 12% PAGE and the same MW markers. The results presented there are part of this study. Therefore, in this study, we present the results that were not shown in Mateus et al. (2009), i.e., the MW determinations of EST-4 and EST-5 for *D. mulleri*, *D. aldrichi*, *D. wheeleri* and *D. navojoa*. After electrophoresis, the relative mobility (Rm) values for the esterases of these four species and the molecular markers were obtained. The graphs of Rm versus gel concentration for each MW marker resulted in a different slope. These slopes were plotted against the MW (Figure 1 – Mateus et al., 2009). Ferguson's plot (Log Rm versus gel concentrations) for EST-4 and EST-5 of *D. mulleri*, *D. aldrichi*, *D. wheeleri* and *D. navojoa* are shown in Figure 3.

The plots for both esterases were parallel in all species, indicating that these enzymes have different charges and/or tridimensional structures but very similar molecular weights. From these graphs, the slope was obtained for each enzyme in each species. These values were used to estimate the MW in each case, using the equation $Y = A + BX$, where A is the intercept of the Y-axis (2.18766), and B is the slope (0.09452). The slopes and molecular weights are presented in Table 4.

The slope values for both enzymes in all species were similar. EST-5 had more variation, ranging from -10.05407 ± 0.29546 for *D. navojoa* to -11.03429 ± 0.30178 for *D. mulleri*. EST-4 was less variable, ranging from -10.08361 ± 0.33581 for *D. wheeleri* to -10.52607 ± 0.44878 for *D. mulleri*. The MWs, estimated from these slope values (Table 4), were very close to each other. For EST-4, the MW ranged from 83.537 kD in *D. wheeleri* to 88.218 kD in *D. mulleri*. For EST-5, the MW ranged from 83.225 kD in *D. navojoa* to 93.595 kD in *D. mulleri*. The MWs obtained, including standard deviations, were all approximately 80 kD to 96.8 kD.

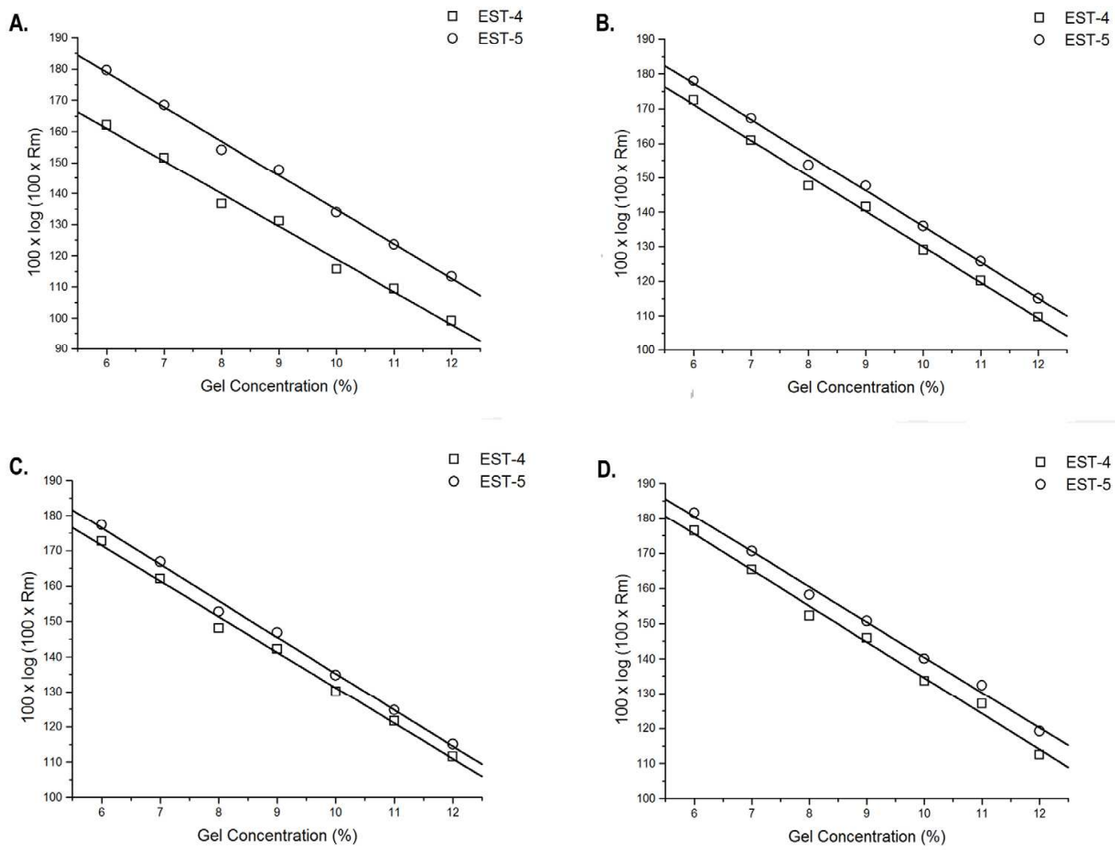

Fig. 3. Log of Rm versus gel concentrations relationship (Ferguson's plot) for EST-4 and EST-5. A – *Drosophila mulleri*; B – *Drosophila aldrichi*; C – *Drosophila wheeleri*; D – *Drosophila navojoa*.

	EST-4		**EST-5**	
D. mulleri cluster	Slope	M.W.	Slope	M.W.
D. mulleri	–10.52607 ± 0.44878	88.218 ± 4.748	–11.03429 ± 0.30178	93.595 ± 3.193
D. aldrichi	–10.29768 ± 0.30168	85.802 ± 3.192	–10.34904 ± 0.28362	86.346 ± 3.000
D. wheeleri	–10.08361 ± 0.33581	83.537 ± 3.553	–10.32114 ± 0.30252	86.050 ± 3.201
D. mojavensis cluster				
[1]*D. mojavensis*	–10.45339 ± 0.27581	87.450 ± 2.918	–10.33036 ± 0.27437	86.148 ± 2.903
[1]*D. arizonae*	–10.27775 ± 0.32146	85.591 ± 3.401	–10.16554 ± 0.30570	84.404 ± 3.234
D. navojoa	–10.24157 ± 0.38515	85.209 ± 4.074	–10.05407 ± 0.29546	83.225 ± 3.126

Table 4. Slopes of Log Rm versus gel concentration relationships and MW estimates for EST-4 and EST-5 of the six cactophilic *Drosophila* species analyzed.[1] Data obtained from Mateus et al. (2009).

3.5 Interspecies crosses

3.5.1 Crosses between *D. mulleri* and *D. mojavensis*

The cross between *D. mulleri* and *D. mojavensis* showed asymmetric isolation, with many descendents only in the direction of *D. mulleri* females and *D. mojavensis* males. The reciprocal cross did not produce offspring despite the presence of courtship among couples and eggs in the plate. The hybrid larvae were analyzed in 10% PAGE and showed three-band patterns for EST-4 and EST-5 (Figure 4). For both enzymes, the slower band corresponded to the enzyme encoded by *D. mulleri* and the faster to the enzyme encoded by *D. mojavensis*. The intermediate band represented a hybrid enzyme, indicating that EST-4 and EST-5 are dimeric in both species.

For EST-4, the hybrid intermediate bands were located closer to the band encoded by *D. mulleri*, which could be a result of differences in the I.P. values of theses enzymes (Table 3). The same was not observed for EST-5, as this enzyme has nearly the same value for both of these species.

3.5.2 Crosses between *D. navojoa* and *D. mojavensis*

Asymmetric isolation was also observed in the cross between *D. navojoa* and *D. mojavensis.* No offspring were obtained in the direction of *D. mojavensis* females and *D. navojoa* males, despite the fact that courtship between couples and eggs on the plate were observed. The cross between *D. navojoa* females and *D. mojavensis* males was very fertile.

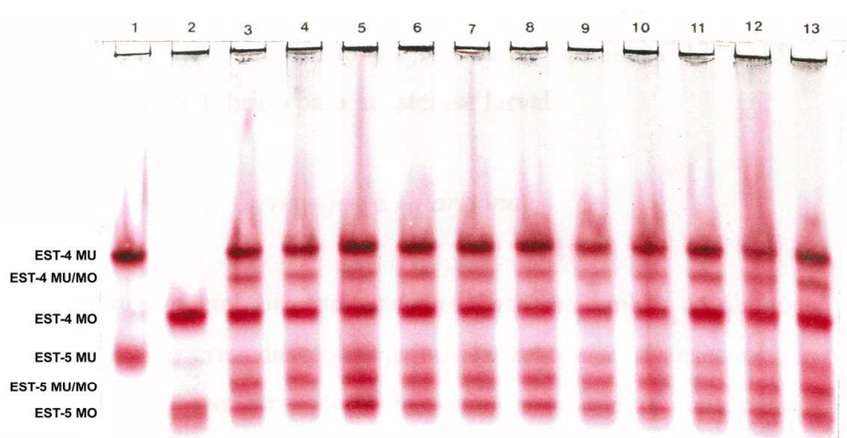

Fig. 4. Esterase pattern in 10% PAGE of late third instar larvae from the parental lines and the hybrids obtained from the cross of *D. mulleri* females and *D. mojavensis* males. 1 = *D. mulleri*; 2 = *D. mojavensis*; 3-13 = hybrid larvae.

The hybrid larvae from this cross were analyzed in 10% PAGE and showed the same three-band patterns observed for *D. mulleri* and *D. mojavensis* (Figure 5). For EST-4, the slower band corresponded to the enzyme encoded by *D. mojavensis*, the faster band corresponded to the enzyme encoded by *D. navojoa*, and the intermediate band corresponded to a hybrid enzyme. The opposite was observed for EST-5: the slower band was from *D. navojoa*, the faster band was from *D. mojavensis*, and an intermediate band was a hybrid enzyme. Again, these results confirm the quaternary structure of both enzymes of these species. An interesting observation was the absence of EST-5 expression in two samples (samples 12 and 13; Figure 5).

3.5.3 Crosses between *D. navojoa* and *D. arizonae*

This cross was very fertile in both directions. However, larvae from the cross in the direction of *D. navojoa* females and *D. arizonae* males had very slow development and took much longer to achieve the late third instar stage; they also had a high mortality rate. The larvae analyzed in 10% PAGE from both cross directions presented the same three-band patterns for EST-5. For EST-4, as in both species of the cross, the enzymes had almost the same migration speed under these electrophoretic conditions. One thicker band was observed in the hybrid larvae, which must be the agglomeration of the three bands expected for this enzyme (Figure 6).

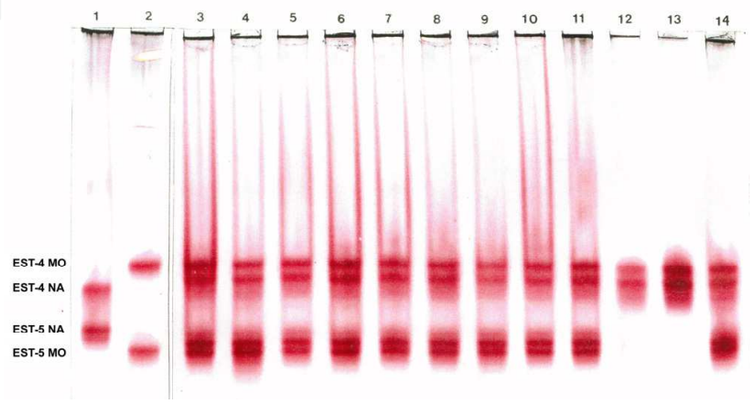

Fig. 5. Esterase pattern in 10 % PAGE of late third instar larvae from the parental lines and the hybrids obtained from the cross of *D. navojoa* females and *D. mojavensis* males. 1 = *D. navojoa*; 2 = *D. mojavensis*; 3-14 = hybrid larvae.

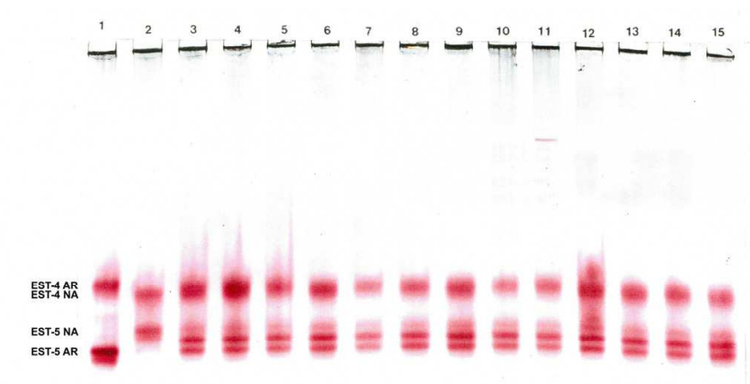

Fig. 6. Esterase pattern in 10% PAGE of late third instar larvae from the parental lines of *D. arizonae* and *D. navojoa* and the hybrids obtained from crosses in both directions. 1 = *D. arizonae*; 2 = *D. navojoa*; 3-15 = hybrid larvae.

3.5.4 Crosses between *D. navojoa* and *D. mulleri*

The cross between *D. navojoa* and *D. mulleri* was fertile in both directions. The larvae analyzed by 10% PAGE showed three-band patterns for both EST-4 and EST-5, with the slower enzyme from *D. mulleri* and the faster band from *D. navojoa.* The intermediate band was a hybrid enzyme. These results confirm the dimeric quaternary structure of these

enzymes in these species (Figure 7). Again, the EST-4 hybrid band migrated closer to the slower band from *D. mulleri*, which could be a consequence of the different I.P. values of these enzymes. The same was not observed for EST-5, as they show similar I.P. values for both species.

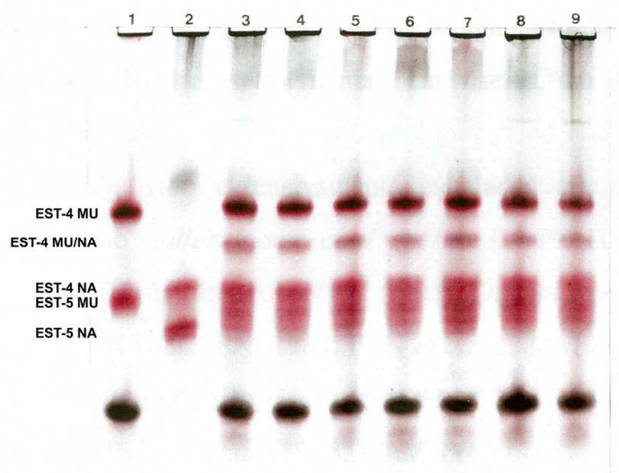

Fig. 7. Esterase pattern in 10% PAGE of late third instar larvae from the parental lines of *D. mulleri* and *D. navojoa* and the hybrids obtained from crosses in both directions. 1 = *D. mulleri*; 2 = *D. navojoa*; 3-9 = hybrid larvae.

4. Discussion

Isozymes are very important in insects and have been used to understand biological problems in several fields of research, including population genetics and systematics, tissue organization, development, metamorphosis, gene regulation and protein synthesis and gene duplication (R. P. Wagner & Selander, 1974). The set of proteins known as esterases constitute one of the most heavily studied groups of isozymes. In the *Drosophila mulleri* complex, which is the subject of this study, esterases have been extensively studied in several species, including *D. serido* (Lapenta et al., 1995, 1998), *D. buzzatii* (East, 1982; Barker, 1994; Lapenta et al., 1995, 1998), *D. mojavensis* (Zouros et al., 1982; Zouros & Van Delden, 1982; Pen et al., 1984, 1986a, 1986b; Mateus et al., 2009), *D. arizonae* (Zouros et al., 1982; Ceron, 1988; Mateus et al., 2009), *D. aldrichi* (F. M. Johnson et al., 1968; Kambysellis et al., 1968) and *D. mulleri* (F. M. Johnson et al., 1968; Kambysellis et al., 1968; Richardson & Smouse, 1976; Ceron, 1988).

Zouros et al. (1982) detected two esterases with different patterns of temporal and tissue-specific expression in *Drosophila mojavensis* and *D. arizonae* (formerly *D. arizonensis*). They detected a specific β-esterase of the late third instar phase of larval development and in the carcass, named EST-4, in contrast to another β-esterase, named EST-5, which is expressed during all developmental phases and is found predominantly in hemolymph and the fat body. They proposed that the most likely hypothesis is that both enzymes are products of a gene duplication that occurred prior to the speciation of the *D. repleta* group, and their patterns of tissue-specific and temporal expression diverged more recently. This hypothesis was suggested because the enzymes show interlocus heterodimers, different patterns of expression (Zouros et al., 1982) and 82% identity in their N-terminal amino acid sequences

(Pen et al., 1986a; Pen et al., 1990). More recently, Robin et al. (2009) proposed that, in *D. mojavensis,* these enzymes are most likely encoded by two genes, Est-2a (EST-5) and Est-2c (EST-4), which are products of one gene duplication out of a total of eleven duplications that explain the evolution of the catalytic β-esterase cluster in the *Drosophila* genus (five in the *Sophophora* and 6 in the *Drosophila* subgenus).

Our results reinforce the hypothesis proposed by Zouros et al. (1982), extending the knowledge about these enzymes as products of a gene duplication to other *D. mulleri* complex species. All six analyzed species show distinct temporal expression patterns for EST-4 and EST-5, with EST-4 showing activity only at the end of the third instar larval stage (Figure 1). The inhibition experiments (Table 2) showed that EST-4 has the same pattern for all six species: it is inhibited by PMSF and not affected by malathion. The opposite was observed for EST-5 in all six species: it was inhibited by malathion and not affected by PMSF. The other inhibitors tested (eserine sulfate, copper sulfate, iodoacetamide and E-64) had no effect on the activity of either enzyme. Moreover, the presence of homozygotes and heterozygotes for EST-5 independent of the EST-4 genotype in *D. navojoa* (Figure 2) and *D. mulleri* (data not shown) support the idea of an independent origin of these enzymes from two distinct loci. Despite these differences, these enzymes display similar features, such as structural similarities (Pen et al., 1986a; Pen et al. 1990) that allow the formation of dimers in *D. mojavensis*, *D. arizonae* and *D. navojoa* (Figure 2).

The gene duplication process is considered one of the most important mechanisms of the generation of new genes and functions during the evolutionary process. Jeffreys & Harris (1982) suggested gene duplication mechanisms that could happen to genes during evolution. Among the mechanisms presented, the most likely mechanism that could have generated the EST-4 and EST-5 loci is the same mechanism that might have generated the globin family, that is, *in tandem* gene duplication by pairing errors during meiosis that cause unequal crossing-over because of the presence of short repeat sequences located in the 3′ and 5′ ends of the unduplicated ancestral gene.

The EST-5 gene in *D. pseudoobscura* is a good example of gene duplication with later divergence (Brady & Richmond, 1992). The EST-5 enzyme is encoded by the *Est-5B* gene, which is expressed during the life cycles of all insects and is linked to two other genes, *Est-5A* and *Est-5C*, on the X chromosome (Brady et al., 1990). In *D. melanogaster*, the homologous gene is *Est-6*, which codes for the enzyme EST-6 during the insect's life cycle and has only one grouped gene, *Est-P* (Collet et al., 1990). Both *Est-5A* of *D. pseudoobscura* and *Est-P* of *D. melanogaster* are expressed only at the third instar larval stage, producing only one transcript. On the other hand, *Est-5C* of *D. pseudoobscura* is not expressed in any developmental phase (Brady et al., 1990). According to Brady & Richmond (1992), who compared the DNA sequences of coding and flanking regions of all three *D. pseudoobscura* and two *D. melanogaster* genes, only two genes, which are already products of a gene duplication, were present before these two species diverged. These two ancestral genes were probably *Est-5A* and *Est-5B* in the first species and *Est-6* and *Est-P* in the second species. A second duplication occurred later in *D. pseudoobscura*, giving rise to the *Est-5C* gene. However, the findings of Robin et al. (2009) contrast with the evolutionary model proposed by Brady & Richmond (1992); in their analyses, the *Est-5A*/*Est-5B* duplication (which they call *Est6*/*7*) occurred after the melanogaster/obscura group divergence, whereas Brady & Richmond (1992) place this duplication prior to the divergence.

In our case, Zouros et al. (1982) proposed that the genes coding for EST-4 and EST-5 (*Est-2c* and *Est-2a*, respectively, according to Robin et al., 2009) were also products of a duplication event prior to the divergence of the species that belong to the *D. repleta* group and that the EST-4 gene was later inactivated in some species of this group, including *D. tira*, *D. hydei* and *D. eohydei*. Moreover, the lower activity of EST-4 in *D. mulleri*, *D. aldrichi*, *D. repleta* and *D. peninsularis* could indicate this EST-4 inactivation process. However, our results showed that *D. mulleri*, *D. aldrichi* and *D. wheeleri* had high EST-4 activities compared to the other species (Figure 2A). This difference in the level of activity of EST-4 for the same species in these studies could be the result of differences in the origins of the lines used in each study. Therefore, the populations of *D. mulleri* and *D. aldrichi* that were analyzed by Zouros et al. (1982) could have a certain degree of EST-4 inactivation that was not observed in the present study.

The enzymes analyzed in this study had biochemical differences compared to other esterases of other *Drosophila* species. For example, the I.P. values for EST-4 and EST-5 for the six *Drosophila* species analyzed were between 6.0 and 7.0 (Table 3). These values were different from those of *D. melanogaster* obtained by Healy et al. (1991), as only 2 out of 15 esterases had I.P. values close to the values obtained in this study (between 6.0 and 7.0). All others showed values below 6.0, with the majority of values between 4.0 and 5.0.

Regarding the MWs of these enzymes, our results are in agreement with previous studies that estimated this parameter. EST-4 had MW values between 83 and 89 kD, and EST-5 had MW values between 83 and 94 kD (Table 4), which are very close to the MWs obtained by Pen et al. (1984), which were between 85 and 95 kD for a variant of the EST-4 (with altered specificity to α-naphthyl acetate) using gel filtration chromatography. Pen et al. (1984) also used denaturing gel electrophoresis (SDS-PAGE) and obtained the MWs of the subunits of EST-4 as 62-64 kD. In another study, Pen et al. (1986a) determined the MWs for the subunits of EST-5 as 64-66 kD. Regardless of the method used and the different results obtained (for the entire protein or for subunits), EST-4 had a smaller MW than EST-5, as observed in this study.

The interspecies crosses performed in this study had results that were in accordance with the known phylogenetic relationships among the species analyzed. This information is based on the morphological work of Throckmorton (1982) and Vilela (1983), the cytological work of Wasserman (1982, 1992 for reviews), several allozyme studies (Zouros, 1973; Richardson et al., 1975; Richardson and Smouse, 1976; Richardson et al., 1977; Heed et al., 1990), molecular studies (Sullivan et al., 1990; Russo et al., 1995; Spicer, 1995, 1996) and an analysis using multiple sources of characters (Durando et al., 2000). The crosses between *D. mulleri* and *D. mojavensis* showed the same results of those of Patterson & Crow (1940) and Bicudo (1982), with offspring obtained only in the direction of *D. mulleri* females and *D. mojavensis* males. For *D. navojoa* crossed with *D. mojavensis,* an F1 was produced only in the direction of *D. navojoa* females and *D. mojavensis* males. In this case, Ruiz et al. (1990) observed descendants in both directions but a very low percentage of offspring, depending on the geographic lineage used, in the direction in which we detected isolation. In crosses between *D. navojoa* and *D. arizonae*, both directions were fertile, which was also found by Ruiz et al. (1990). Finally, in crosses between *D. navojoa* and *D. mulleri,* we detected descendants in both directions, in contrast to the results of Bicudo (1982), who found fertility only in the direction of *D. mulleri* females and *D. navojoa* males.

In all of these crosses, the phenotypic observations of the esterase patterns from late third instar hybrid larvae produced three bands for both EST-4 and EST-5 (Figures 4, 5, 6 and 7), except for larvae from the cross between *D. navojoa* and *D. arizonae,* which produced a thicker band because the parental bands have almost the same migration pattern under the electrophoretic conditions used in this study. These results indicate that in all six *Drosophila* species, EST-4 and EST-5 have dimeric quaternary structures. Another important observation from some of these crosses was the presence of hybrid larvae with no EST-5 activity (*D. navojoa* x *D. mojavensis* – Figure 5; *D. navojoa* x *D. arizonae* – data not shown). These results indicate that some hybrid larvae had problems with the regulation of *Est-2a* gene expression, which most likely codes for the EST-5 enzyme, without affecting the expression of its homologous gene, *Est-2c*, which most likely codes for the EST-4 enzyme (Robin et al., 2009). These results reinforce the idea that these two loci are independent.

The possible role of EST-4 in these *Drosophila* species remains an open question. According to Holmes & Masters (1967, as cited in Oakeshott et al., 1993), esterases can be classified into four types through their specific inhibition patterns. Carboxylesterases are esterases that are inhibited only by organophosphates, such as paraoxon, fenitrooxon and DFP (diisopropylfluorophosphate). Cholinesterases are inhibited by organophosphates and carbamates, such as eserine sulfate. Arylesterases are inhibited only by sulfhydrylic agents, such as p-chloromercuribenzoate (pCMB). Acetylesterases are not inhibited by any of these agents. Inhibition of EST-5 only by malathion, an organophosphate, suggests that this enzyme belongs to the class of carboxylesterases. Inhibition of EST-4 by PMSF and the absence of inhibition in the presence of all other inhibitors tested suggest that this enzyme probably belongs to the class of acetylesterases.

According to Augustinsson (1968), esterases are closely related to the class of serine-proteases, forming a multigenic family of serine-hydrolases. The main features that support this hypothesis are the three consensus amino acid residues that are present in the active site of esterases and serine-proteases, including an invariant serine, enzymatic inactivation by DFP, which binds irreversibly to the serine residue of both enzymes, inhibition by organophosphates and carbamates and the superposition of substrate preference (Augusteyn et al., 1969; Krisch, 1971; Dayhoff et al., 1972; Heymann, 1980; Previero et al., 1983; as cited in Myers et al., 1988). However, Myers et al. (1988) showed that some esterases cannot be included in this multigenic family because they do not have the same amino acid residues in the charge exchange system of the enzyme active site.

The absence of EST-4 and Est-5 inhibition by copper sulfate and iodoacetamide, combined with data for E-64, which is a diagnostic inhibitor of cysteine-proteases, indicate that neither enzyme has an essential cysteine residue in its active site. On the other hand, the inhibition of EST-4 by PMSF, which is a diagnostic compound for serine-proteases and other enzymes with a serine residue in the active site, and of EST-5 only by malathion indicated that both enzymes have an important serine residue in the active site, suggesting that they belong to the class of serine-hydrolases. As these enzymes display high esterase activity, we can postulate that they are serine-esterases (Holmes & Masters, 1967). The multigenic family of serine-esterases includes several enzymes with a wide range of functions, including cholinesterases, lipases, lysophospholipases, cholesterol-esterases, non-specific carboxylesterases and juvenile hormone esterases (Ryger et al., 1989; Doctor et al., 1990; Shimada et al., 1990; as cited in Myers et al., 1993). Therefore, EST-4 and EST-5 probably belong to this multigenic family, with EST-4 as an acetylesterase (E.C. 3.1.1.6) and EST-5 as a non-specific carboxylesterase (E.C. 3.1.1.1).

To establish the possible role of EST-4, the following information must be considered. Healy et al. (1991) observed that all *D. melanogaster* acetylesterases are inhibited by OTFP (3-octylthio-1,1,1-trifluoropropan-2-one), which is a powerful inhibitor of juvenile hormone esterase activity in Lepidoptera, suggesting that all acetylesterases from this species have similar properties as juvenile hormone esterase. Moreover, East (1982) proposed that esterase-J from *D. buzzatii*, which is supposedly the enzyme from this species that corresponds to EST-4 in this study, is a juvenile hormone esterase, acting together with EST-1 in the larval phase to control the levels of this hormone. In the adult phase, only EST-1 would be responsible for this control. On the other hand, EST-2 could be the enzyme responsible for digestive and detoxification processes and ester absorption in adults. EST-4 has a very tissue-specific and temporal pattern of expression, which indicates that there is a specific regulatory system that controls its expression at a specific tissue (carcass) and period of time (at the end of the larval phase, when all of the processes for pupation have been initiated). Therefore, as an acetylesterase with a very specific temporal expression pattern, EST-4 could be involved in these transformation processes, acting as an auxiliary enzyme for the degradation of juvenile hormone esterase. The degradation of this hormone in this phase allows the liberation of prothoracicotropic hormone by the brain, which stimulates ecdysone production by the prothoracic gland, initiating metamorphosis (Coundron et al., 1981). However, analyzing the EST-4 inhibition data alone could lead to the hypothesis that this enzyme could be a serine-protease that also has esterase activity and is involved in a proteolytic activity during the larva-pupae conversion process; it is likely to be involved in this process. Regarding EST-5, considering the fact that it is expressed during the entire life cycle of the insect and is found mainly in the hemolymph and fat body, it is a non-specific carboxylesterase that is probably involved in digestive processes.

5. Conclusions

This study contributes to a better understanding of the differentiation of two enzymes that are products of a gene duplication in six cactophilic *Drosophila* species. We present additional evidence to support the gene duplication event that gave rise to the genes responsible for the EST-4 and EST-5 enzymes, which are the main β-esterases found in several species of the *D. mulleri* complex of the *D. repleta* group. We also contribute to the elucidation of the possible physiological roles of these esterases in this group. Further steps in this investigation will be to determine specific biochemical parameters of both enzymes after purification. We are also interested in identifying the changes that occur in the regulatory system of gene expression that lead to differentiation in the patterns of tissue-specific and temporal expression of these enzymes; that is, understanding what triggers EST-4 expression only in the late third instar larvae and at the larval carcass. We are also interested in determining the intra- and/or extracellular processes in which these enzymes are involved and their interacting molecules. Thus, we will be able to complement this initial step with an increased understanding of the differentiation of these two genes that result from a gene duplication event.

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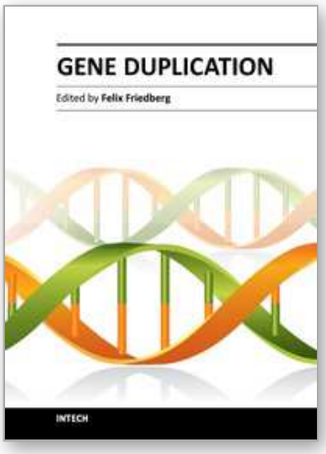

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The book Gene Duplication consists of 21 chapters divided in 3 parts: General Aspects, A Look at Some Gene Families and Examining Bundles of Genes. The importance of the study of Gene Duplication stems from the realization that the dynamic process of duplication is the "sine qua non" underlying the evolution of all living matter. Genes may be altered before or after the duplication process thereby undergoing neofunctionalization, thus creating in time new organisms which populate the Earth.

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