conditions, allowing us to record variations in the responses among individuals under different intensities of desiccation. Thus, a finer gradient of desiccation stress would be an appropriate tool for assessing possible levels of adjustment in natural populations. Indeed, it would better resemble the variation of natural environmental conditions, as well as allow recording the possible plastic responses of flies.

Another objective of the experiments reported herein was to identify the most accurate variable that measures desiccation resistance. In this sense, we found that LT50 and mean survival time provided very similar information, although the former requires less effort and gives less weight to the effect of individual survival on the final measurement. Furthermore, LT50 is the most typical response variable used in desiccation studies, so we follow these criteria and choose it for further experiments.

Finally, our initial questions have been answered, and the information obtained leads us to define an experimental design that includes three desiccation treatments: control (0g), 0.5 g, and 3 g, since these treatments affected differently survival and their effect varied among populations. Both sexes provided complementary information; therefore, it is recommendable to assess desiccation resistance in females and males separately. Our study also revealed among-lines variation. Therefore, we plan to carry out a more comprehensive study assessing 10 isofemale lines per population and 5 replicates per line.

In conclusion, the results reported herein have been useful to define an experimental design to investigate desiccation resistance in natural populations of *D. buzzatii* for which there is no previous knowledge of traits related to resistance to arid conditions.

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Insights about the phylogenetic relationship between *Zaprionus* and *Drosophila* from molecular and cytogenetic data of *α-esterase7* gene of *Zaprionus indianus.*

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Introduction

Zaprionus indianus is a species that occurs throughout Africa, India, and Saudi Arabia, as well as in islands of the Indian Ocean (Comores, Madagascar, Seychelles, Mauritius, and Réunion) and the Atlantic Ocean (Canary and Saint Helena) (Chassagnard and Kraaijeveld, 1991; Chassagnard and Tsacas, 1993). In the end of the 20th and the beginning of the 21st centuries, it spreads to South America (*e.g.*, Vilela, 1999; Castro and Valente, 2001; Gõni *et al.*, 2001; Vilela *et al.*, 2001; Tidon *et al.*, 2003) and then to Central and North America (van der Linde *et al.*, 2006).

Zaprionus indianus belongs to the subgenera *Zaprionus*, which contains 44 species from the Afrotropical region (Chassagnard and Tsacas, 1993; Chassagnard, 1996), genus *Zaprionus*

Coquillett, 1901. *Zaprionus* genus also is composed by the *Anaprionus* subgenera, with 12 species from Australia and Southeastern Asia (Okada and Carson, 1983; Wynn and Toda, 1988; Gupta and Gupta, 1991). The phylogenetic relationship of *Zaprionus* genus with those of the Drosophilidae family is controversial. Throckmorton (1962, 1975), using biogeography, anatomical, and behavior data, placed *Zaprionus* as a distinctive group of the genus *Drosophila*, within the radiation *immigrans-Hirtodrosophila*. On the other hand, Grimaldi (1990), analyzing 217 morphologic data of 120 Drosophilidae species excluded *Zaprionus* from the genus *Drosophila*. This exclusion was also proposed by DeSalle (1992), using data of mtDNA, which positioned *Zaprionus* between the most basal subgenus of the genus *Drosophila – Sophophora –* and the genus *Scaptodrosophila*. However, the Grimaldi (1990) and DeSalle (1992) proposal is inconsistent with subsequent analyses using molecular markers such *Adh*, *Gpdh*, *Sod*, 18S rRNA, *Amd*, *COII*, and *Amyrel* (Pelandakis and Solignac, 1993; Thomas and Hunt, 1993; Kwiatowski *et al.*, 1994; Powell and DeSalle, 1995; Russo *et al.*, 1995; Remsen and DeSalle, 1998; DaLage *et al.*, 2007; Yassin *et al.*, 2008). Nevertheless, depending on the gene or sequence analyzed, *Zaprionus* can be positioned differently within the *Drosophila* genus. Hence, these inconsistencies justify the search for new markers, which allow reinforcing the proposed phylogenetic hypothesis.

Due to the New World recent colonization, *Z. indianus* has received recent scientific interest; however, the studies focusing on molecular variability are scarce. Among the genetic markers studied, the esterases have been analyzed just in the level of protein polymorphism (Parkash and Yadav, 1993; Parkash and Sharma, 1993; Galego *et al.*, 2006). This species presents five *loci* coding for *α-esterases*, which show variable frequencies and biochemistry functions; however, the studies have not focused on the knowledge of sequence, structure, and chromosome localization of these genes.

The *α-esterase* cluster of *Drosophila* comprises a superfamily of hydrolytic enzymes that share a common structure called α/β hydrolase fold (Ollis *et al.*, 1992). In *D. melanogaster*, the *α-esterase* cluster is one of the largest gene families so far described (Robin *et al.*, 1996). It is localized in the 3R chromosome and comprises 11 genes in a region of 60 kb. These genes are irregularly distributed and share high conservation in the coding sequences (Robin *et al.*, 1996). Biochemical analysis suggested the occurrence of five loci encoding for *α-esterases* in *Z. indianus* (Parkash and Yadav, 1993; Parkash and Sharma, 1993), but no molecular data regarding these genes have been accumulated up to now.

Since *α-esterases* are one of the few genes studied in the *Zaprionus* genus, one gene of the *α-esterase* cluster, the *α-esterase7* gene (*α-E7*), was analyzed in this study aiming at determining its nucleotide sequence and cytogenetic localization to evaluate whether this gene can be used as a molecular marker to infer the phylogenetic relationships of *Zaprionus* with the other *Drosophila* species.

Material and Methods

Species and DNA Extraction

Two strains of *Zaprionus indianus* were used in this study: Mirassol and 725-*Standard*. The first was collected in Mirassol (SP, Brazil, 2002) and has been maintained as a laboratory culture since then, and the second is an inbred homokaryotypic strain, used to make the chromosomic photomap of the species (Ananina *et al.*, 2007). The Mirassol strain was used to carry out the molecular analysis, and both strains have been used for cytogenetics studies. DNA was extracted from a group of 30 flies, according to Jowett (1986).

PCR, Cloning, and Sequencing

PCRs were performed using 100 ng of genomic DNA in a solution of 200 μM of each dNTP, 2.5 mM $MgCl_2$, 0.4 μM of each primer, and 1 U of Taq polymerase (Invitrogen) in 1× buffer. The reactions were heated to 95°C for 3 min and then submitted to 35 cycles of 1 min at 95°C, a 1 min step at 55°C, and a 2 min step at 72°C. An additional extension step of 5 min at 72°C was performed after the last cycle. DNA from *D. melanogaster* and ultrapure water were used as positive and negative controls, respectively. The amplification of partial sequence of *α-E7* gene in *Z. indianus* was carried out using two pairs of primers (Zi2a/Zi2b and Zi4a/Zi4b) constructed based on a consensus sequence of the *D. melanogaster*, *D. buzzatii*, and *L. cuprina* sequences (Figure 1). PCR fragments with the expected size were extracted from agarose gels (GFX PCR DNA and Gel Band Purification Kit; GE Healthcare) and were cloned with a TOPO TA Cloning Kit (Invitrogen). Both strands of three clones, chosen randomly, were automatically sequenced.

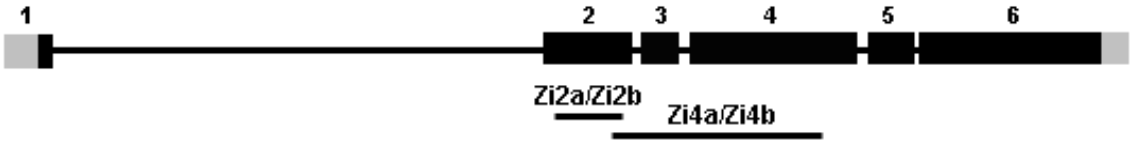

Figure 1. Schematic representation of *D. melanogaster α-esterase*7 gene and the fragments amplified in *Z. indianus*. The black boxes, the gray boxes and the black lines correspond to the ORFs, UTRs and introns sequences, respectively. The two black lines below the gene schema represent the sequences amplified with the primers Zi2a/Zi2b and Zi4a/Zi4b. (Zi2a: 5'AGCATAAAGTCCAGCAGTATCGCC 3', position in *DmαE*7 gene: 2081-2105; Zi2b: 5' TATCGAAGACGAACTGCACCTGGA 3', position: 2312-2336; Zi4a:5' TSCAGGTGMADTTCGTYYTCGAYA 3', position: 2317-247 and Zi4b: 5'RRAACTSYAGCACATCCTTSTCKT 3', position: 2988-3012).

Sequence and Phylogenetic Analyses

The sequences obtained were manipulated into the BioEdit sequence Alignment Editor (Hall, 1999) and assembled into consensus sequences, based on the high similarity between them. The two sequences obtained from *Z. indianus α-E7* gene were assembled into a consensus sequence that was deposited in GenBank database (EU583135). Nucleotide sequences of *D. melanogaster α-E7* (genomic: NT033777 and mRNA: NM079537), *L. cuprina* (U56636) and *M. domestica* (AF133341) from GenBank were used in the analysis. The predict mRNAs of *D. simulans* (chr3R_1.594, start:3397163, end:3401020), *D. sechellia* (super_6.621, start:3441104, end:3444918), *D. yakuba* (chr3R_2.277, start:6968131, end:6972118), *D. erecta* (scaffold_4770.633, start:3605365, end:3609522), *D. ananassae* (scaffold_13340.544, start:3116468, end:3121382), *D. pseudoobscura* (chr2_5.251, start:26643434, end:26646667), *D. persimilis* (super_6.345, start:2056967, end:206090210), *D. virilis* (scaffold_12855.1054, start:6242945, end:6248593), *D. mojavensis* (scaffold_6540.992, start:6589403, end:6596391) and *D. grimshawi* (scaffold_25013.1241, start:8083938, end:8089229) were extracted from the BLAT tool (http://genome.brc.mcw.edu/cgi-bin/hgBlat) using the *α-E7* mRNA of *D. melanogaster* as query.

The phylogenetic reconstruction was inferred based on the predicted RNA sequences by maximum-parsimony method (branch-and-bound algorithm) and the p-distance was used to construct the distance matrix, both as implemented in PAUP 4.0b10 (Swofford, 1997). Branch support was calculated by bootstrap analysis consisting of 1000 replicates (Felsenstein, 1985).

In situ *hybridization*

The chromosomic localization of the *Z. indianus α-E7* gene was performed by *in situ* hybridization (ISH). Polytene chromosomes of five individuals for each strain were obtained from salivary glands of third-instar larvae in Ringer buffer, fixed in 60% acetic acid and spread in lactic acid:water:acetic acid (1:2:3). Slides were dried, dehydrated, pretreated in 2X SSC at 37 °C and stored at 4 °C until use. The plasmid containing the 691 bp sequence of *Z. indianus α-E7* gene amplified with the Zi4a/Zi4b primers was used as probe. It was labeled with biotin 16-dUTP by nick translation using the Nick Translation System (Invitrogen), according the manufactur's intructions.

Chromosomes and DNA probe were denatured for 90 s in NaOH 0.07 M and 5 min at 94°C, respectively. Hybridization was carried out overnight at room temperature in a humidified chamber. After removing the glass coverslip, the slides were washed two times for 10 min in 2X SSC solution, one time for 90 s in 0.1% triton/1X PBS and then two times for 10 min in 1X PBS, always at room temperature. Detection consisted of incubating the slides for 30 min with ExtrAvidin (Sigma) and 5 min with DAB – H_2O_2. Finally, the slides were counterstaining with Giemsa (Merck) and mounting in Entellan (Merck). Slides were observed under a Zeiss Axioplan 2 microscope. Chromosome images were generated using a highly sensitive CCD camera (Princeton Instruments, Evry, France) and the Metaview 4.1.7 image-analysing system (Universal Imaging Corporation).

Results and Discussion

Characterization of Z. indianus *α-esterase7*

The sequence of the *α-esterase7* of *Z. indianus* (*ZiαE7*) is 912 nt long and presents the fundamental molecular structure of the *DmαE7* gene of *D. melanogaster* (Figure 1). Based on the *DmαE7* mRNA of *D. melanogaster*, the partial coding sequence embraces part of the exon 2 (nucleotide 1 to 309), complete sequences of intron 2 (nucleotide 310 to 372), exon 3 (nucleotide 373 to 521), intron 3 (nucleotide 522 to 579) and a partial sequence of exon 4 (nucleotide 580 to 912). There are no stop codons, the open reading frame begins in the third nucleotide and the partial protein predicted is 262 aa long. The *ZiαE7* DNA sequence, coding sequence and amino acid sequence are 72.4%, 78.2% and 86.6% similar to the *DmaE7*, respectively. In *D. melanogaster* this gene encodes two mRNA isoforms characterized by an alternative splicing of the third and fourth exons (GENE database, NCBI). The *ZiaE7* sequence does not maintain these splicing sites (data not shown).

Aiming at searching the cytogenetic localization of *ZiαE7* gene we have performed *in situ* hybridization analysis in polythene chromosomes using the fragment Zi4a/Zi4b (Figure 1) as probe. The results (Figure 2 A) showed that *ZiαE7* is encoded by a single-copy gene at chromosome IV, section 70BC on the polytene map (Figure 2 B) based on the *Z. indianus* chromosome photomap of Ananina *et al.* (2007). The localization is 100% frequent in all individuals analyzed and no additional hybridization was found. According the first *Z. indianus* photomap (Gupta and Kumar, 1987; Figure 2 C), the *ZiαE7* gene could be localized around the limits of the regions 26 and 27 of the IV chromosome, but it is impossible precisely localize it in this photomap since it is not enough resolved and presents different divisions or bands compared to the Ananina *et al.* (2007)'s photomap. Thus, in despite of the original polytene chromosomes classification, we will discuss the data obtained based on the localization done by Ananina *et al.* (2007).

Campos *et al.* (2007) discussed the chromosome synteny between *Z. indianus* and *D. melanogaster*. The authors have performed *in situ* hybridization of six genes and compared their localization with the *D. melanogaster* chromosomes. The authors concluded that the chromosome 3R, which harbors the *D. melanogaster α-esterase* cluster, could be orthologous to the V chromosome of

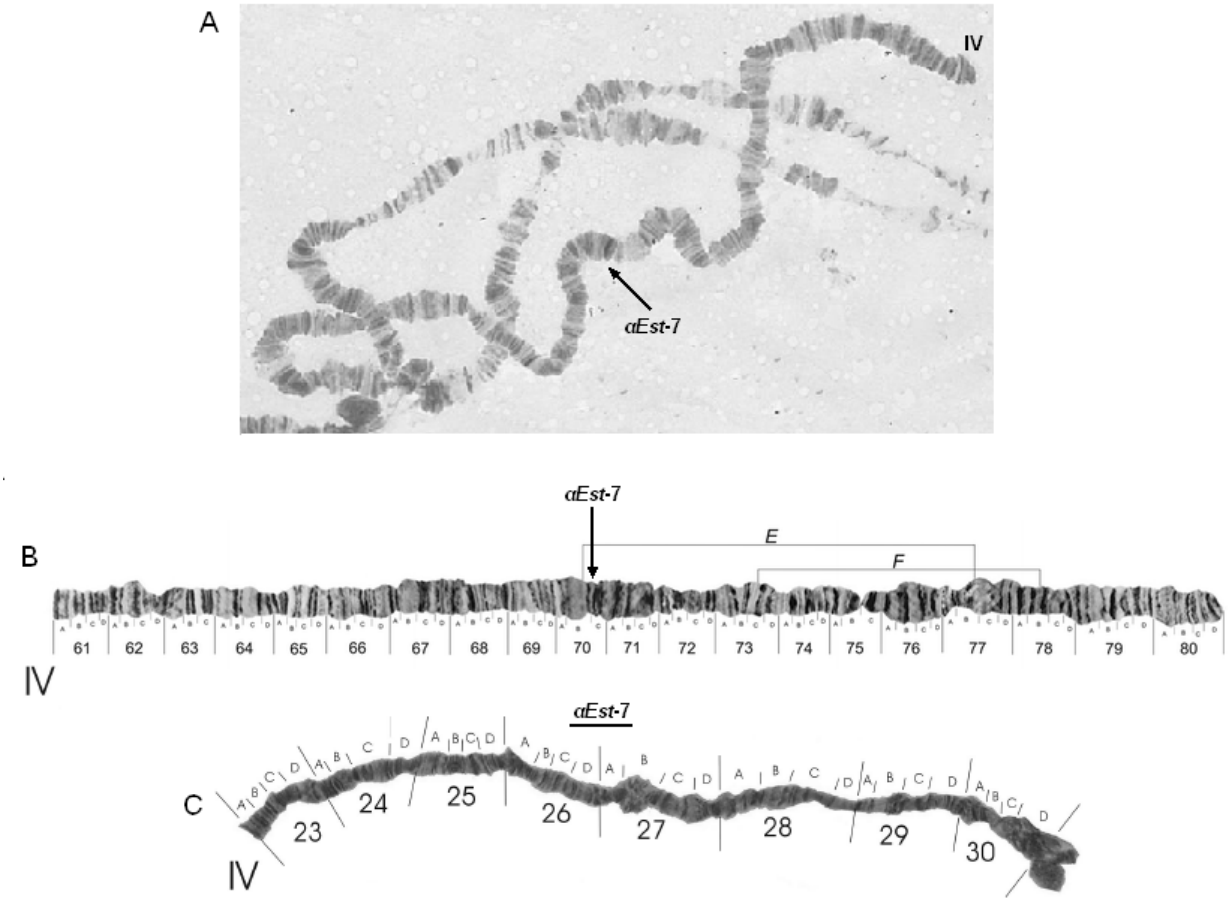

Figure 2. Polytene chromosomes from *Z. indianus* salivary glands. A: *in situ* hybridization demonstrates the cytogenetic position of *Zi*α*E7* gene in the chromosome IV, section 70BC (black arrow); B: Chromosome IV photomap of Ananina *et al.* (2007) illustrating the exact position (black arrow) of the *Zi*α*E7*; C: Chromosome IV photomap of Gupta and Kumar (1987) showing the probably region (black line) of the *Zi*α*E7* in the oldest photomap of *Z. indianus*.

Z. indianus based on the localization of *Hsr-*ω and *Hsp70* genes. They also pointed out that a putative rearrangement in the distal part of chromosome V of *Z. indianus* could have occurred, due to the presence of the *Dpp* gene which is located in the distal region of chromosome 2L of *D. melanogaster* (GENE database, NCBI). Since we identified the *α-esterase7* gene in the 84D9 section of the 3R chromosome of *D. melanogaster*, the location of *Zi*α*E7* gene in the chromosome IV suggests that an additional rearrangement have occurred during the *Z. indianus* chromosome V evolution. The rearrangement might involve the median region of the chromosome IV and the distal region of the chromosomes V (Figure 3), which correspond, respectively, to chromosomes 2R and 3R of *D. melanogaster*. This additional rearrangement indicates that migth have occurred a complex pattern of chromosomic changes in the ancestral lineages that originated *Z. indianus* and *D. melanogaster* species. Since the *α-esterase* cluster is composed by 11 genes dispersed over 60 Kb of the chromosome 3R in *D. melanogaster* (Robin *et al.* 1996), it is interesting to verify if they are all

localized in the *Z. indianus* chromosome IV or if the rearrangement between the chromosomes IV and V of *Z. indianus* disrupted the *α-esterase* cluster in this species.

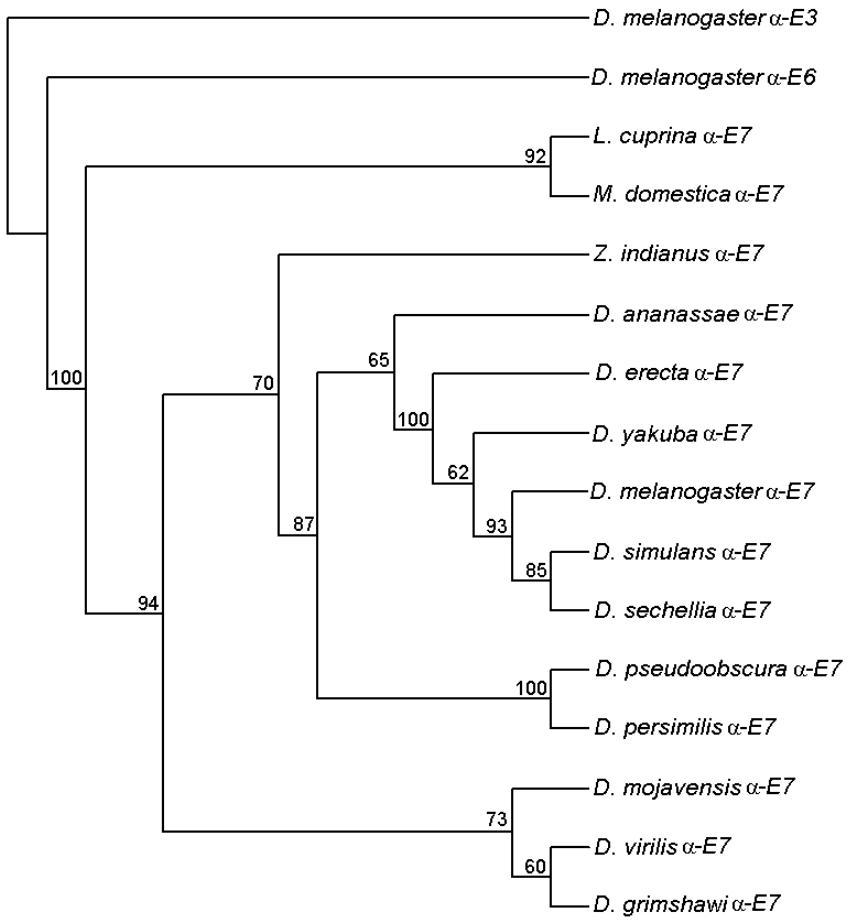

Figure 3. Phylogenetic relationships of the *α-E7* sequence of *Z. indianus*, *L. cuprina*, *M. domestica* and the 11 species of *Drosophila* sequenced. The tree was generated by maximum-parsimony method as implemented by PAUP 4.0 b10 (Swofford, 2000). Bootstrap confidence levels (1000 replications) are indicated in each node. *D. melanogaster α-E3* and *α-E6* were used as outgroups. Of 835 total characters analyzed, 403 characters are parsimony informative. The consistency index was 0.6127 and the retention index was 0.5423.

Phylogenetics inference of the Drosophilidae from α-esterase7

The partial DNA sequence described above was used to infer the phylogenetic relationships of *Zaprionus* genus using the *α-esterase 7* sequences of the 11 *Drosophila* species and two other Diptera (*L. cuprina* and *M. domestica*). We have used the mRNA predicted sequences to carry out the analysis due the absence of complete gene sequences for *M. domestica* and *L. cuprina* in the GenBank.

The tree reconstructed from the alignment of the 14 sequences revealed a main monophyletic clade that contains all sequences from *α-E7* (Figure 3). The *M. domestica*/*L. cuprina* and

Drosophilidae were separated into two distinct monophyletic groups. The Drosophilidae family is also separated into two branches. The first contains all sequences belonging to species of the *Sophophora* subgenus (*melanogaster* group: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta*, *D. yakuba* and *D. ananassae*; and *obscura* group: *D. pseudoobscura* and *D. persimilis*) and the *Z. indianus* sequence basally separated from the *Sophophora* species. *D. pseudoobscura* and *D. persimilis* (*obscura* group) branch together and all sequences of the *melanogaster* species group are grouped following the classification proposed by Lachaise and Silvian (2004). The second subgroup includes the two sequences of the *Drosophila* subgenus (*D. mojavensis* and *D. virilis*) and *D. grimshawi* (Hawaiian species). There is incongruence in this clade according the consensual *Drosophila* phylogenetic relationships, *D. virilis* branches with *D. grimshawi* instead of *D. mojavensis*, however with a relatively low bootstrap value (60%).

Table 1. Nucleotide divergence (p-distance) of *α-E7* mRNA sequences of *Z. indianus*, *M. domestica*, *L. cuprina* and the *Drosophila* species. The distances of the *D. melanogaster α-E3* and *α-E6* sequences, used as outgroups in the phylogenetic tree, were also included.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *D. melanogaster α-E3*	-														
2. *D. melanogaster α-E6*	0.445	-													
3. *L. cuprina α-E7*	0.485	0.497	-												
4. *M. domestica α-E7*	0.495	0.489	0.277	-											
5. *Z. indianus α-E7*	0.464	0.493	0.315	0.355	-										
6. *D. melanogaster α-E7*	0.444	0.484	0.325	0.338	0.214	-									
7. *D. simulans α-E7*	0.448	0.492	0.325	0.334	0.210	0.033	-								
8. *D. sechellia α-E7*	0.445	0.489	0.321	0.327	0.208	0.036	0.016	-							
9. *D. yakuba α-E7*	0.449	0.485	0.316	0.330	0.229	0.084	0.074	0.073	-						
10. *D. erecta α-E7*	0.442	0.484	0.325	0.336	0.222	0.073	0.066	0.063	0.071	-					
11. *D. ananassae α-E7*	0.459	0.507	0.299	0.332	0.234	0.166	0.160	0.163	0.173	0.168	-				
12. *D. pseudoobscura α-E7*	0.455	0.488	0.312	0.338	0.226	0.170	0.166	0.167	0.186	0.178	0.186	-			
13. *D. persimilis α-E7*	0.452	0.488	0.315	0.336	0.226	0.167	0.163	0.164	0.185	0.174	0.184	0.008	-		
14. *D. mojavensis α-E7*	0.447	0.489	0.304	0.338	0.204	0.205	0.201	0.201	0.214	0.197	0.204	0.185	0.182	-	
15. *D. virilis α-E7*	0.459	0.493	0.274	0.342	0.211	0.234	0.230	0.232	0.234	0.232	0.218	0.204	0.204	0.141	-
16. *D. grimshawi α-E7*	0.467	0.471	0.307	0.329	0.216	0.238	0.241	0.242	0.245	0.241	0.227	0.215	0.214	0.163	0.162

The nucleotide divergence (Table 1) between the sequences corroborates the topology obtained by the phylogenetic reconstruction. The *α-E7* sequences of all species searched are in average 0.4736 (SE: 0.0038) divergent of *α-E6* and *α-E3*. The *Sophophora*/*Z. indianus* branch is

0.2196 (SE: 0.0035) divergent from the *D. virilis/D. mojavensis/D. grimshawi* branch. However, the *Z. indianus α-E7* sequence is equally divergent from the *Sophophora α-E7* (0.2211 ± 0.0033) and from the *D. virilis/D. mojavensis/D. grimshawi* (0.2103 ± 0.0035) sequences. In a complementary neighbor-joining search, *Z. indianus α-E7* branches with *D. virilis/D. mojavensis/D. grimshawi* clade but with no bootstrap support (data not shown).

The sequence analysis, the phylogenetic reconstruction and the nucleotide divergence shows that the *ZiαE7* gene belongs to the *α-esterase* cluster and it is monophyletically related to the *Drosophila* species *α-esterases*. In the phylogenetic analysis, the *ZiαE7* gene branches inside the *Drosophila* genus (bootstrap 94%) and is closer related to the *Sophophora* species, although the nucleotide divergence and the neighbor-joining search show that the *ZiαE7* sequences are equally divergent from the *Sophophora* and the *Drosophila* subgenera sequences.

The molecular studies using sequences of the *Adh* gene (Thomas and Hunt, 1993; Russo *et al.*, 1995), *Adh*, *Gpdh* and *Sod* (Kwiatowski and Ayala, 1999), the 18S RNAr (Pelandakis and Solignac, 1993), the *Amd* and *COII* (Robe *et al.*, 2005) and the gene *Amyrel* (DaLage *et al.*, 2007; Yassin *et al.*, 2008) reinforced the Throckmorton (1962, 1975)'s proposition of inclusion of *Zaprionus* within the *Drosophila* genus. Thus, Kwiatowski and Ayala (1999) proposed that *Zaprionus* must be downgraded to the status of subgenus of the genus *Drosophila*. Despite several molecular studies have included *Zaprionus* species within the *Drosophila* genus, their exact relationships with the other *Drosophila* species is uncertain, since that depending on the gene or sequence analyzed, *Zaprionus* can be positioned differently. Thomas and Hunt (1993) proposed that *Zaprionus* genus could form a clade between the subgenera *Drosophila* and *Sophophora* and Pelandakis and Solignac (1993), Kwiatowski *et al.* (1994), Russo *et al.* (1995), Powell and DeSalle (1995), Remsen and DeSalle (1998) and Tatarenkov *et al.* (1999) inferred that *Zaprionus* genus is closely related to species of the *Drosophila* subgenus. Our results also positioned *Z. indianus* within the *Drosophila* genus but closer to the *Sophophora* species.

Even with the morphologic differences (Grimaldi, 1990) and the uncertainty about the exact relationships between the species of the *Drosophila* and *Zaprionus* genus, our results corroborate the proposed close relationship between the two genera and point out for the necessity of downgrade *Zaprionus* as a group of species belonging to the *Drosophila* genus. Nonetheless, all the results reinforce to the need of further studies for properly place *Zaprionus* as a new subgenus in the genus *Drosophila* or as a group of the *Drosophila* subgenus or even the *Sophophora* subgenus.

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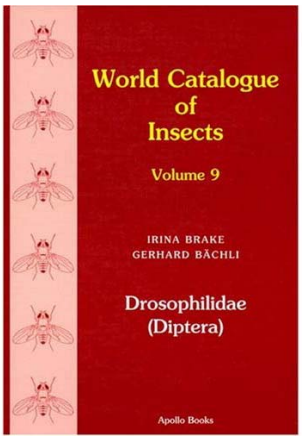

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