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RAPD as genetic marker in taxonomic and evolutionary studies in the *Drosophila buzzatii* cluster.

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Introduction

Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic variability within and between species. One of these methods, the RAPD technique, requires only small amounts of DNA, is easy and rapid to perform, and exploits the use of universal primers (Williams *et. al.*, 1993). The great differentiation found with the use of different markers in the populations of the *Drosophila* species included in the *buzzatii* cluster (Baimai *et al.*, 1983; Tosi and Sene,1989; Silva and Sene, 1991; Tidon-Sklorz and Sene, 1995; Madi-Ravazzi, *et al*, 1997 and Lapenta *et al*, 1998) and the intriguing aspects of its speciation process led to the present study, elaborated mainly with the purpose of evaluating the taxonomic potential of the RAPD assay to discriminate among strains and species of *Drosophila*.

Material and Methods

Strains and geographic distribution: Three strains of *D. buzzatii*, two of *D. seriema*, two of *D. koepferae*, one of *D. serido* type B, and one of *D. serido* type D were used in the present study. As an outgroup, one strain of *D. mulleri* and one of *D. simulans* were utilized. Strains and geographical origins are shown in Table 1.

DNA extraction method: Genomic DNA was extracted from single individuals, randomly sampled from isofemale lines. Samples were stored with TE (10mM Tris, 1 mM EDTA, pH 8). Each fly was homogenized in a microtube with 160µl of cold solution I (10mM Tris, 5% sucrose, 60mM NaCl, 10 mM EDTA, pH 7.8). Then, 200 µl of solution II (300mM Tris, 5% sucrose, 1.25% SDS, 10 mM EDTA, pH 8) were added, the homogenate was mixed by inversion of the tube and the tube was placed in a water bath at 65°C for 30 min. A total of 60 µl of 3M sodium acetate was added, and the tube was cooled at -20°C for 20 min. After centrifugation for 15 min, the supernatant was transferred to an eppendorf and the sample centrifuged for another 15 min. To the pellet was added an equal volume of isopropanol at - 20°C; rinsing was with 70% ethanol. DNA was air dried overnight and resuspended in 100 µl of TE (10 mM Tris, 1mM EDTA, pH 8) and stored at - 20° C.

Table 1. Geographic region, species and strains of *Drosophila buzzatii* cluster used in present study.

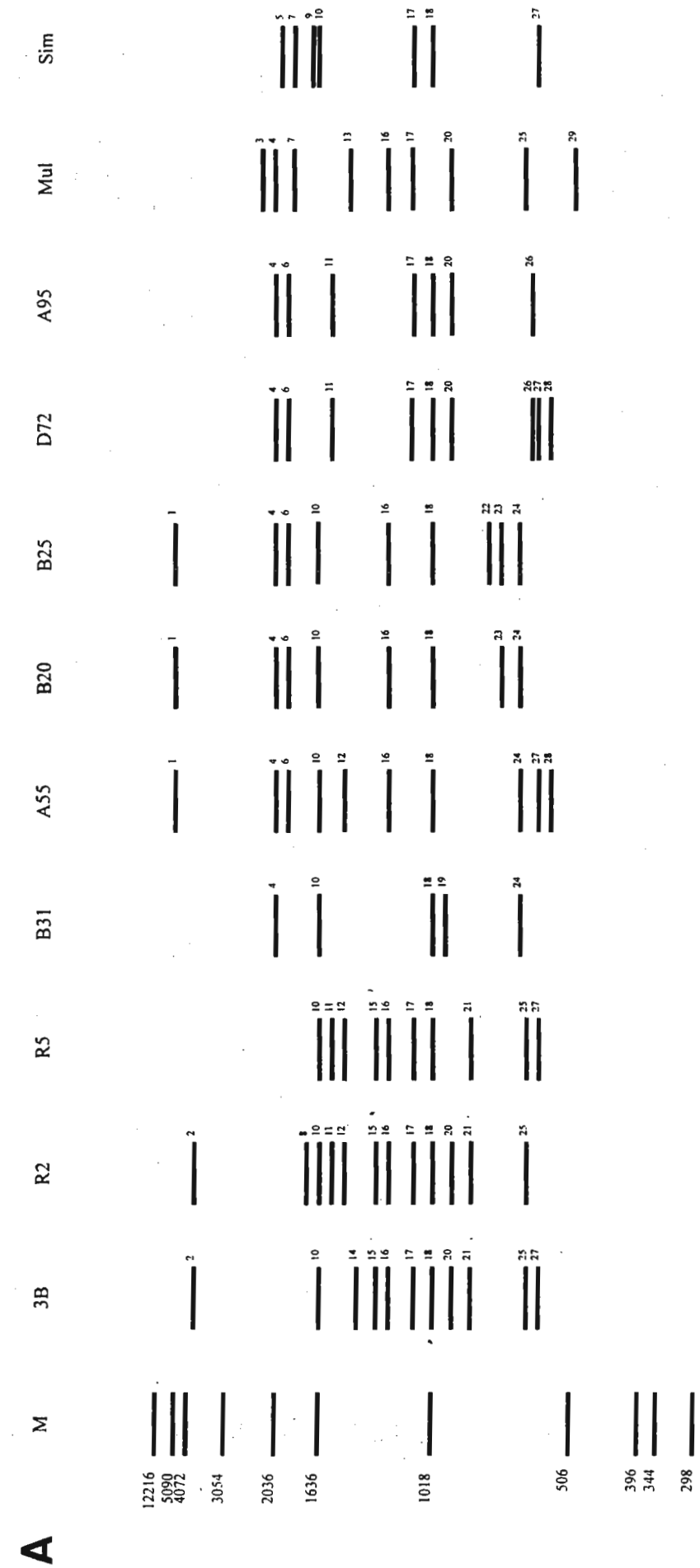
Species	Strains	Origin
Drosophila serido type B	A55F11	Bela Vista (MS, Brazil)
Drosophila serido type D	B31D1	Puerto Tirol (Chaco, Argentina)
Drosophila seriema	A95F3	Serra do Cipó (MG, Brazil)
	D72M	Morro do Chapéu (BA, Brazil)
Drosophila buzzatii	3B	Cafarnaum (BA, Brazil)
	D69R5	Bahia (Brazil)
	D69R2	Bahia (Brazil)
Drosophila koepferae	B20D2	Tapia (Tucuman, Argentina)
	B25D7	Farnatina (La Rioja, Argentina)

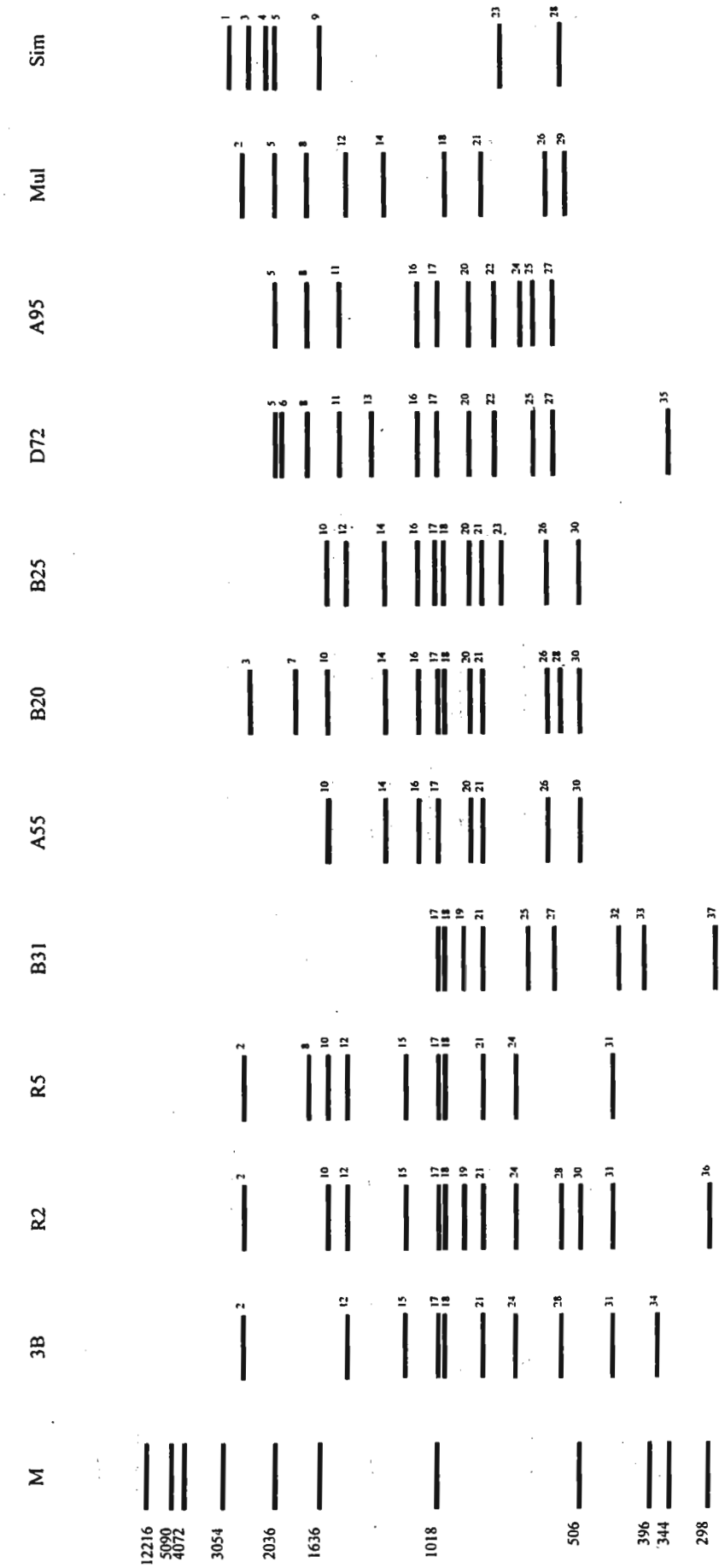
RAPD reactions and electrophoresis: The DNA from thirty individuals from each of the strains was amplified, *i.e.*, fifteen females and fifteen males. A negative control (without template DNA) was included in each reaction set amplified. The amplification reactions were performed following the protocol of Williams *et al* (1990) with minor modifications. The final reaction volume was 25 µl containing 50 ng of genomic DNA, 0.2 mm of dNTP, 0.2 mM of primer, 1.0 unit of Taq polymerase (GIBCO BRL) and 2 mM of $MgCl_2$. Each reaction mixture was overlaid with 40 µl of mineral oil. DNA amplification was carried out for 45 cycles, each comprising denaturation (1 min, 94°C), annealing (1 min, 35°C), and elongation (2 min, 72°C), in an MJ Research Minicycler. One additional cycle of 5 min at 72°C was used for final extension. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in 1x TAE (4 mm Tris-acetate, 1mm EDTA, pH 8.5), visualized by ethidium bromide staining under UV, and photographed using a polaroid system.

Table 2. Specific-species and specific-strains fragments obtained with #54, #69 and #86 primers in RAPD technique. * : Fragment observed too in *Drosophila mulleri* and *D. simulans*; ** : fragment observed too in *D. simulans*.

	Fragments						
	Species-specific				Strain-specific		
Species	#54	#69	#86	Strains	#54	#69	#86
D. buzzatii	15, 21	15,31	26	R2	8	36	23
				3B	14	34	1, 3
				R5	-	-	-
D. seriema	26	11, 22	6, 27	D72	27, 28	6, 13, 35	8*
				A95	-	-	12**
D. koepferae	23	-	-	B20	-	3** and 7	
				B25	22	23	
D. serido tipo D	-	32,33, 37	28	B31	-	-	-
D. serido tipo B	-	-	16	A55	-	-	-

Figure 1 (next three pages). Pattern of bands obtained utilizing RAPD technique. A, Primer 54. B, Primer 69. C, Primer 86.


A
M
3B
R2
R5
B31
A55
B20
B25
D72
A95
Mul
Sim
12216
5090
4072
3054
2036
1636
1018
506
396
344
298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3B
R2
R5
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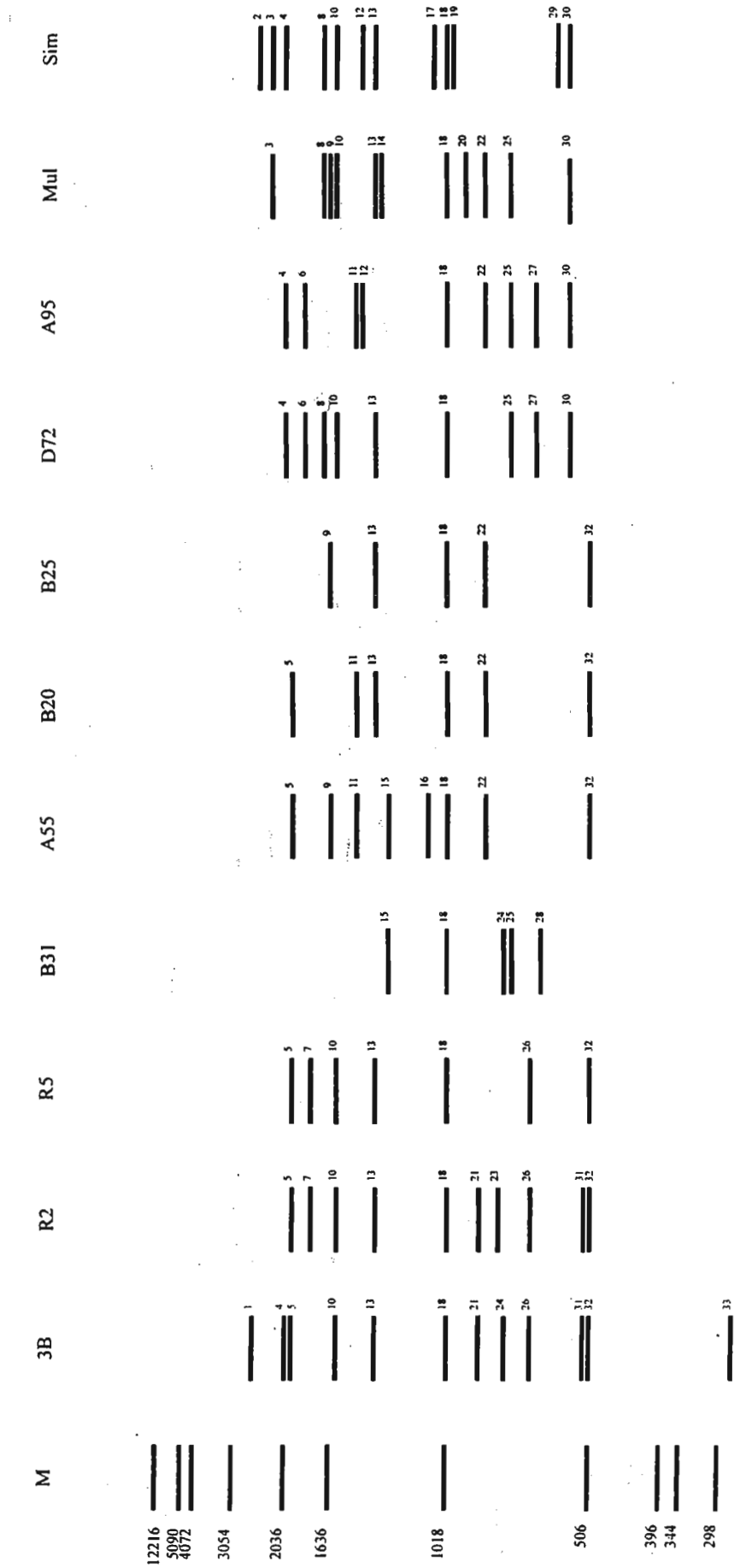
C
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Primers: The arbitrary decamer oligonucleotides were obtained from Biotechnology Laboratory-Vancouver, Canada. An initial survey of 16 primers was perfomed, and 3 were selected based on consistency among individuals, pattern of amplified fragment reproducibility, and potential for strain discrimination. The primers selected were: 54 (GTC CCA GAG C); 69 (GAG GGC AAG A) and 86 (GGG GGG AAG G).

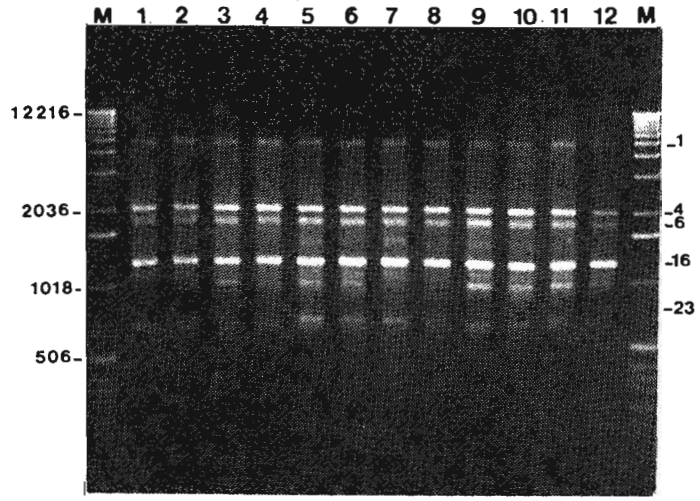

Figure 2. Amplification products of the genomic DNA of A55 strain (1-4) of *D. serido* type B, B20 (5-8) and B25 (9-12) of *D. koepferae* with primer 54. Band 1 specific to 3 strains, band 23, specific to *D. koepferae* and bands 4, 6 and 16, monomorphic in 3 strains, are shown to the right of the gel. M = size marker (1kb DNA ladder).

Data analysis: Only distinct bands were included in the data set. Each individual was scored for the presence (1) or absence (0) of every such band. This was done by initially scoring against a molecular weight (BRL 1 kb ladder) and assigning tentative identification numbers to each band based on approximate molecular weight. Band identification numbers were verified by running an aliquot of representative individuals from each gel that contained the band of interest side by side on a subsequent gel (verification gels not shown). Bands that ran at the same molecular weight were scored as the same band. The largest fragments (1 and 2) obtained with 54 primer were not included in this analysis, because fragments of such size were not observed, which not observed in amplification products by the RAPD technique. More refined studies are necessary .

Results and Discussion

In total, 97 RAPD bands that varied from 290 to 2936 bp were produced. Strain-specific and species-specific DNA fragments generated by the different primers produced a characteristic pattern of RAPD bands that allowed us to recognize the different strains and species (Figure 1 and Table 2).

Similarity index and the cluster analysis obtained through RAPD patterns (Castro and Madi-Ravazzi, unpublished data) confirmed the relationships given by other markers in the same strains, reinforcing the idea of a differentiation between the strains of *D. serido* types B and D and the other species in the cluster (Monteiro, 1997; Machado,1998; Lapenta, 1998). However, strain A55 (*D. serido type* B), from Brazilian cerrado, showed to be closer to *D. koepferae* of the Argentinean chaco (Figure 2). This finding was not observed in further studies. These data may be useful as geographic markers and help us to understand the origin(s) of the Brazilian founder populations of this *Drosophila* complex. Reys and Ochando (1998) showed that the RAPD technique was a powerful approach for detecting the geographical origin of populations of *Ceratitis capitata*.

The current study confirmed the utility of the RAPD method, as supplementary too to other methods for obtained intraspecific and interspecific genetic markers, even without any previous DNA sequence information on the species studied.

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Breeding sites of Neotropical Drosophilidae (Diptera). II. Fallen fruits of *Citharexylum myrianthum* Cham. (Verbenaceae).

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One hundred fallen fruits of *Citharexylum myrianthum* Cham. (Verbenaceae) in several stages of rotting were collected by Dr. J.S. Morgante on the ground under one single tree growing on the border of a secondary Forest Reserve located at the Cidade Universitária "Armando de Salles Oliveira", within São Paulo City, state of São Paulo, Brazil. The reserve, which is composed of native and exotic trees (Rossi, 1994), represents a fragment of the Atlantic Forest. The fruits were all collected on 14 February 2000 and were kept in ¼ liter vials (20 fruits per vial) containing a layer of wet sand, under controlled temperature (23 ± 1 °C) and photoperiod (13 h : 11 h; L : D). From February 20 (first emergence) through March 21 (last emergence), the emerged imagoes were daily aspirated and they are listed in Table 1. The observations were made until April 7 , when the remainings of the fruits were discarded.

A total of 416 adult insects (Diptera, Coleoptera and Hymenoptera) emerged, of which 82 % belonged to the family Drosophilidae (n = 341), and of these 56.9 % are *Zaprionus indianus* (n = 194) and the remaining 43.1 % belonged to the following, alphabetically ordered, 13 species of *Drosophila* (in parentheses the number of emerged flies): *D. arauna* (1) , *D. capricorni* (2), *D. cuaso* (3), *D. kikkawai* (5), *D. griseolineata* (43), *D. maculifrons* (4), *D. nebulosa* (2), *D. paulistorum* (1), *D. parabocainensis* (1), *D. paraguayensis* (3), *D. polymorpha* (1), *D. trifilum* (1), and *D. willistoni* (80). It should be pointed out that two of the listed species *(D. kikkawai* and *Z. indianus*) are introduced to the neotropics and together they represented most (58.4 %) of the drosophilid flies that emerged from fruits of *Citharexylum myrianthum*.

Zaprionus indianus Gupta, 1970 is an invading species of Afrotropical origin, which is also widespread in the Australasian, Oriental and Palearctic regions, in addition to several Islands of the Atlantic and Indian Oceans. It is the most widespread fly (Tsacas, 1985; Chassagnard and Kraaijeveld, 1991; Chassagnard and Tsacas, 1993) of its genus (described by Coquillet in 1901),