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Genetic variability among natural populations of *Zaprionus indianus* (Drosophilidae) in the States of São Paulo and Minas Gerais, Brazil

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ABSTRACT. Random amplified polymorphic DNA (RAPD) was used to detect polymorphisms among *Zaprionus indianus* fly populations collected from six municipalities in the States of São Paulo and Minas Gerais, Brazil. This species is an important, recently introduced fruit fly pest of figs and other fruit. Among 21 primers, 16 produced 73 reproducible polymorphic fragments; primer AM-9 produced the greatest number of polymorphic bands (nine), with 52% genetic variability among populations. Genetic divergence analysis of the *Z. indianus* populations demonstrated two major groups, named Western and Eastern groups. There was greater gene flow within than between groups. The correlation coefficient for genetic and geographic distances (Mantel test) was significant, demonstrating isolation by distance.

Key words: Drosophilidae; Polymorphisms; *Zaprionus indianus*; Random amplified polymorphic DNA

INTRODUCTION

Zaprionus indianus (Gupta, 1970) fly was first reported in Brazil by Vilela et al. (1999), in the city of Santa Isabel, SP, in a persimmon (*Diospyros* sp) orchard. Since then, this fly has been found in various regions of the country, including Porto Alegre, RS (Silva et al., 2005), Valinhos, SP (Pires and Bélo, 2005), the Brazilian central savanna (Cerrado) (Tidon et al., 2003), in many cities in the State of Minas Gerais (Kato et al., 2004), and in Brazilian North and Northeast regions (Santos et al., 2003). This fly has also been reported from Panama, Florida, USA, and Uruguay (Goñi et al., 2001, 2002; Linde, et al., 2006). The rapid spread of this species in the Americas was chiefly due to the favorable conditions for its establishment and development (Stein et al., 2003).

Zaprionus indianus is the most prevalent fly of the *Zaprionus* genus in some regions of Africa, where it originated; there it has been found on 74 fruit species of 31 botanical families (Lachaise and Tsacas, 1983). In 2002 it was identified in Egypt, and it has became a dominant species in the drosophilid fauna in that country (Yassin et al., 2009). Based on studies of populations from Asia, Africa and South America, using mitochondrial DNA haplotypes of CO-I and CO-II genes, there are two phylogenetic lineages (phylads); phylad I includes some of the African populations and phylad II includes the Atlantic populations, including South and North America and also Madeira, suggesting that during its passage from endemism to cosmopolitanism, *Z. indianus* went through two independent radiations (Yassin et al., 2008).

In the State of São Paulo, Brazil, *Z. indianus* is a successful invading species and is now the second most common Drosophilidae species on fruit crops in that state, especially in mango (*Mangifera indica*), guava (*Psidium guajava*) and fig (*Ficus carica*) plantings (Pires and Bélo, 2005). Different from other Drosophilae species, *Z. indianus* can oviposit on immature fruits, which can cause additional damage to fruits of economic interest (Castro and Valente, 2001). Indeed, losses caused by this species in Brazilian fig crops were responsible for a 50% decrease in fresh fruit exports in 2000-2001 (Stein et al., 2003). This species is considered by the Brazilian Department of Agriculture to be an A2 quaternary pest and hence infected plants should be removed from orchards, as well as from domestic fruit gardens (Kato et al., 2004). Although *Z. indianus* has been detected in fig plantings, this species also attacks an array of other fruits, thus being considered an opportunistic fly with real chances of becoming a pest of great importance for orchards in Brazil (Vilela et al., 1999).

Because of its economic importance and the rapid geographical expansion of *Z. indianus*, we evaluated its genetic variability in six *Z. indianus* populations from five different municipalities in the State of São Paulo and one in the State of Minas Gerais, distributed in a geographical north-south axis, using random amplified polymorphic DNA (RAPD) techniques.

MATERIAL AND METHODS

Zaprionus indianus collection

Adult samples of *Z. indianus* were captured with an entomological net passed over bait prepared with ripe tomato and banana fruit, placed on wooden boxes and protected with stainless steel screens in orchards located in Jaboticabal, SP (21S 15’ 17”, 48W 19’ 20”, alt. 605 m), Olímpia, SP (20S 44’ 14”, 48W 54’ 53”, alt. 506 m), Santa Cruz da Esperança, SP

(21S 17' 27", 47W 25' 48", alt. 612 m), Valinhos, SP (22S 58' 14", 46W 59' 45", alt. 660 m), Araras, SP (22S 21' 25", 47W 23' 03", alt. 629 m) and Carneirinho, MG (19S 41' 51", 50W 41' 17", alt. 431 m). Forty couples were collected from each population and were transported to the laboratory in 250-mL bottles containing 30 mL of culture medium, consisting of wheat flour (80 g), corn meal (80 g), gelatin (12 g), Agar (5 g), araruta (12 g), yeast (8 g), corn syrup (25 g), a 10% solution of nipagin in 8 mL alcohol and 2 mL propionic acid, taxonomically identified and conditioned in an environmental chamber for reproduction and oviposition at 25 ± 1°C, 70-85% relative air humidity and a 12-h constant photoperiod.

Genomic DNA extraction and quantification

DNA was isolated from five samples of each of the six populations of *Z. indianus*. Samples were constituted of 10 third-instar larvae, totaling 50 individuals per population. Larvae were macerated in 500 µL lise SET buffer (0.15 mM NaCl; 0.02 mM Tris and 1 mM EDTA, pH 8.0), 18 µL proteinase K (10 mg/mL) and 22 µL 25% SDS solution. Samples were kept at 55°C for 2 h, after which 430 µL NaCl (6 M) was added. Subsequently, samples were centrifuged at 12,000 *g* for 10 min at 4°C, the supernatant was transferred to another tube containing 40 µL sodium acetate (3 M) and 600 µL cold isopropanol. Samples were kept in a freezer at -20°C for 20 min and subsequently centrifuged at 12,000 *g*, for 15 min, at 4°C. The pellet was rinsed in 70% ethanol and resuspended in Milli-Q water. DNA was quantified with a spectrophotometer (λ = 260 and 280 nm) and its integrity analyzed by electrophoresis on a 1% agarose gel (w/v), stained with ethidium bromide (0.5 µg/mL), in 1X TBE buffer at 100 V.

Determination of variability among strains by polymerase chain reaction using random primers (RAPD)

Twenty-one random primers (Operon Life Technology and Biosynthesis Incorporated) were used to examine divergence among *Z. indianus* strains. Each of the amplifications was done to a final volume of 30 µL, containing 30 ng genomic DNA, 2 U *Taq* polymerase (Invitrogen), 10 pmol primers, 3 mM $MgCl_2$, 100 µM of each dNTP (Promega), and a specific reaction buffer. Amplifications were carried out in a PTC-100 thermocycler (MJ-Research, Inc.), under the following conditions: 2 min at 94°C for initial denaturation, annealing at 37°C for 1 min, extension at 72°C for 2 min, and 32 cycles for 10 s of denaturation at 94°C, 20 s of annealing at 40°C, an extension period of 2 min at 72°C, and finally another extension for 5 min at the same temperature. RAPD fragments were separated by electrophoresis on 1.5% ethidium bromide-stained agarose gel (0.5 µg/mL), at 100 V on 1X TBE, using a molecular weight marker of 100 bp (DNA Ladder, GE Healthcare) and photographed using Image Master VDS, Pharmacia Biotech.

Genetic variability analysis

An analysis was carried out to estimate the evolutionary relationship among populations, based on the matrix of presence (1) or absence (0) constructed from the amplification pattern observed on the agarose gel.

Binary data obtained from RAPDs were utilized to estimate allele frequencies, with a correction developed by Lynch and Milligan (1994). A descriptive analysis of total variability was next made by calculating the percentage of polymorphic loci and gene flow using the POPGENE software (Yeh et al., 1999). AMOVA was utilized for the decomposition of the total genetic variance into its components between and within the populations, allowing evaluation of variability structure, as proposed by Excoffier et al. (1992), using the programs TFPGA (Miller, 1997), AMOVA-PREP 1.01 (Miller, 1998) and WINAMOVA 1.04 (Excoffier et al., 1992).

Nei's (1978) distance was used for genetic distance calculations in a type UPGMA grouping analysis, to examine the genetic divergence among the populations; this helps determine the source of genetic variability. The correlation coefficients for genetic and geographic distance were obtained with the Mantel test using the GENES software (Cruz, 2008).

RESULTS

Twenty-one random primers were used to analyze the *Z. indianus* strain variability, of which 16 produced reproducible and well-defined polymorphic bands (Table 1). The nucleotide sequence and the total number of bands (mono- and polymorphic) produced by amplification were also determined for each primer (Table 1). Seventy-four bands were obtained, of which 73 were polymorphic and 1 monomorphic. The number of bands amplified by each primer varied from three to nine, with a mean of 4.6, and variable sizes of 400 to 1300 bp. Primer AM-9 produced nine amplified bands and was followed by primers AM-6 with seven and AM-1, AM-4, AM-11, which produced five polymorphic bands each.

Table 1. Primers used in the amplification experiments, their nucleotide sequences, and the individual and total number of polymorphic and monomorphic bands.

Primers	Nucleotide sequences (5' to 3')	Bands		
		Polymorphic	Monomorphic	Total
AM-1	AATCGGGCTG	5	0	5
AM-2	CAGGCCCTTC	4	0	4
AM-3	AGGGGTCTTG	4	0	4
AM-4	TGCCGAGCTG	5	0	5
AM-5	GTGACGTAGC	6	0	6
AM-6	AGGGGTCTTG	7	0	7
AM-7	GTGAGCTAGG	3	1	4
AM-8	AGGTGACCGT	3	0	3
AM-9	GGACTGGAGT	9	0	9
AM-10	GTTGCGATCC	4	0	4
AM-11	TGGGGGACTC	5	0	5
AM-14	AGCGTCACTC	5	0	5
AM-16	GTCCACACGG	3	0	3
AM-17	CCCCGATGGT	3	0	3
AM-19	TCGCCCAGTG	4	0	4
AM-21	TGCCGAGCTG	3	0	3
Total		73	1	74

A sample pattern of the random amplified fragments obtained for all populations using different primers is shown in Figure 1. The profile of the bands produced by amplification allowed the development of a dendogram (Figure 2).

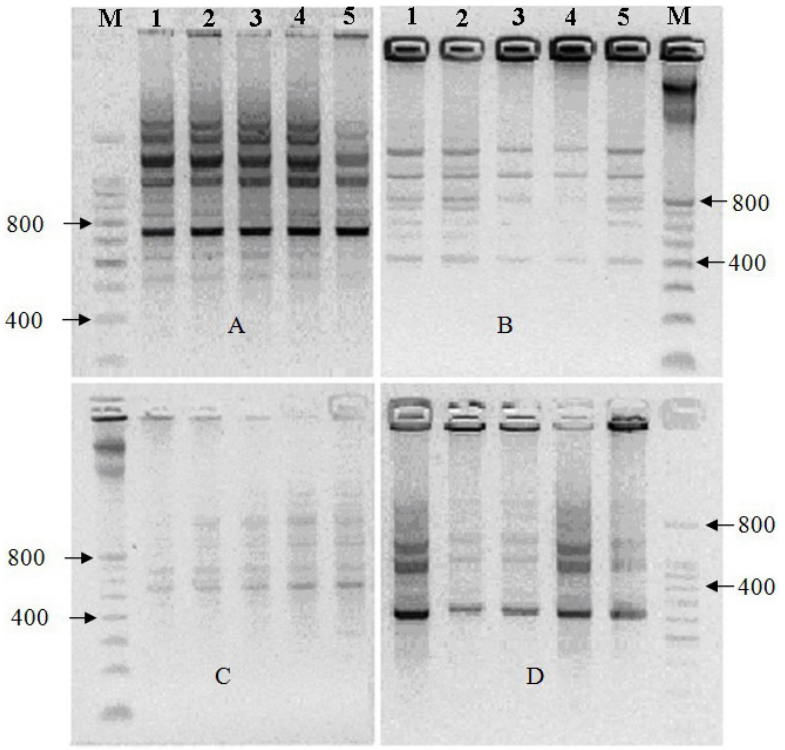

Figure 1. Electropherogram of the randomly amplified fragments in DNA samples (*lanes 1-5*) of the four populations using random primers. **A.** Jaboticabal, SP, using the AM-1 primer. **B.** Santa Cruz da Esperança, SP, using the AM-6 primer. **C.** Carneirinho, MG, using the AM-3 primer; **D.** Araras, SP, using the AM-4 primer. M = molecular weight marker = 100 bp (GE Healthcare).

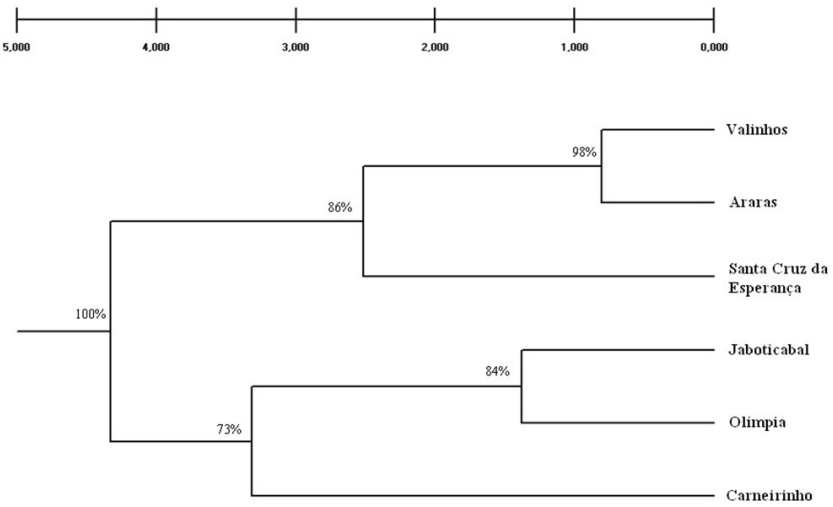

Figure 2. Genetic divergence pattern based on RAPD markers among six populations of *Zaprionus indianus*, defined by UPGMA grouping, based on Nei's (1978) genetic distances.

The genetic variability among *Z. indianus* populations resulted in a ramification pattern defined by the UPGMA method, based on Nei's genetic distance (1978) and determined by pairing populations. The genetic distance range was from 0.0804 to 0.6086 (Table 2).

Table 2. Geographic localization and genetic distances among populations of *Zaprionus indianus*, according to Nei (1978) (below the diagonal) and geographic distances in kilometers (above the diagonal).

Population	Longitude	Latitude	Altitude (m)	Distance between populations					
				Jab.	Olímp.	Carn.	SCE	Val.	Ar.
Jab.	-48°19'	-21°15'	605	*****	84.01	301	99.77	234	154
Olímp.	-48°54'	-20°44'	506	0.1379	*****	219	166	317	240
Carn.	-50°41'	-19°41'	431	0.4492	0.2140	*****	383	526	453
SCE	-47°25'	-21°17'	612	0.3137	0.4001	0.6086	*****	193	116
Val.	-46°59'	-22°58'	660	0.4618	0.3918	0.3940	0.1999	*****	79.3
Ar.	-47°23'	-22°21'	629	0.5254	0.4139	0.3836	0.3030	0.0804	*****

Jab. = Jaboticabal, SP; Olímp. = Olímpia, SP; Carn. = Carneirinho, MG; SCE = Santa Cruz da Esperança, SP; Val. = Valinhos, SP; Ar. = Araras, SP.

Genetic frequency and variability

Analysis of molecular variance (Table 3) showed that the total genetic variability between strains was 52.4%, whereas within strains it was 47.6% and the F_{st} was 0.525.

Table 3. Analysis of molecular variance for six *Zaprionus indianus* populations based on amplified DNA fragments.

Source of variation	d.f.	S.S.	S.A.S.	Components of variance	% Total variation	P	F_{st}
Between populations	5	230.36	46.07	7.79	52.37	<0.001	0.524
Within populations	24	170.20	7.09	7.09	47.63	-	-
Total	29	400.56					

d.f. = degrees of freedom; S.S. = sum of squares; S.A.A. = sum of average squares.

The descriptive statistics on the genetic variability of populations, obtained with the POPGENE software (Yeh et al., 1999) indicated less variability for the Santa Cruz da Esperança population (14.1%), followed by Carneirinho (31.0%), Olimpia (43.7%), Jaboticabal and Valinhos (47.9%), and a higher variability for Araras (52.1%).

The dendogram showed two main groupings (Figure 2), one contains populations from Carneirinho, Olimpia and Jaboticabal (Western group) and the other includes Araras, Valinhos and Santa Cruz da Esperança populations (Eastern group). In each of the two groups, two populations showed more similarity: Valinhos and Jaboticabal in the Western group and Araras and Valinhos in the Eastern group. The gene flow for Western and Eastern groups was $Nm = 0.4562$ and 0.4460; the gene flow inside the groups was higher than between them ($Nm = 0.3727$).

The correlation coefficient for genetic and geographic distances, obtained with the Mantel test, was significant ($r = 0.52$; $P < 0.05$ with 5000 permutations) suggesting isolation by distance (Figure 3).

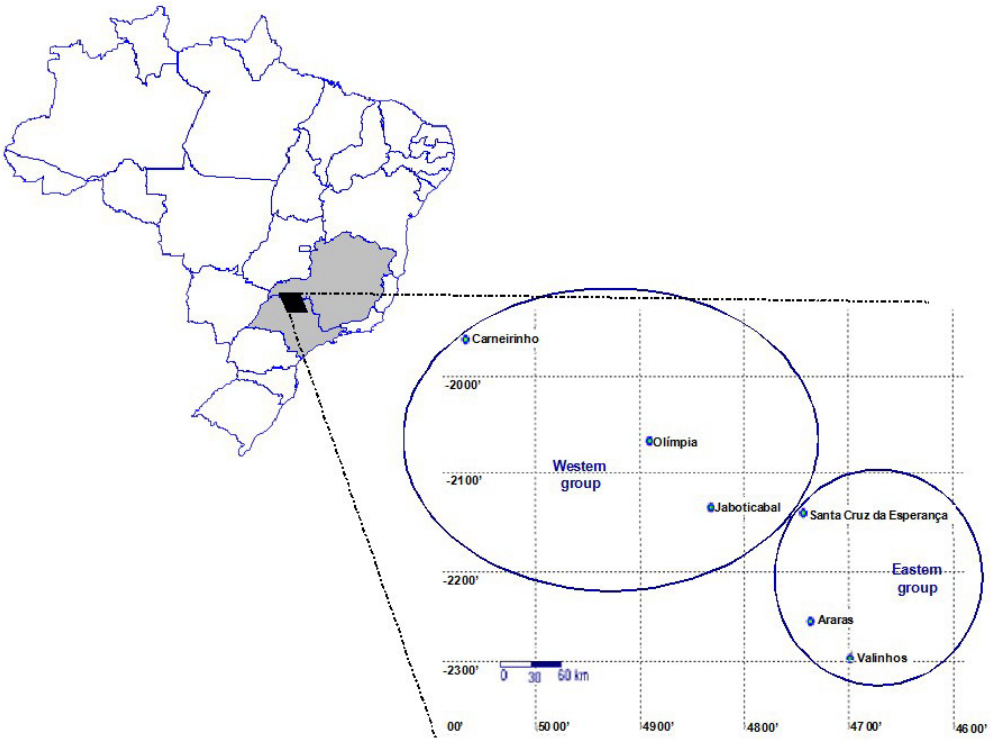

Figure 3. Map showing the distribution of the *Zaprionus indianus* populations. Ellipses indicate the groups formed by UPGMA clustering, based on Nei's (1978) genetic distances.

DISCUSSION

Studies of the dynamics of the colonization of flies in fruit orchards are of relevance, not only because of economic considerations, as a result of damage to fruit, but also because of biological aspects. It is possible that crop infestation by *Z. indianus* and the increasing range of this introduced species will threaten the stability of pre-established *Drosophila* communities, which are very rich in number of species.

Investigations have been made of colonization dynamics and genetic variability of *Z. indianus* populations in Brazil, including several studies using genetic markers such as allozymes (Mattos-Machado et al., 2005), chromosomal inversion polymorphisms (Ananina et al., 2007), and quantitative traits (David et al., 2006; Loh et al., 2008). Ours is, to the best of our knowledge, the first study of genetic variability of *Z. indianus* Brazilian populations based only on molecular markers.

We found higher genetic variability among than within the fly populations. In general, low variability has been detected in American populations of *Z. indianus* samples in comparisons of isofemale lines from African and American populations (David et al., 2006) and in studies of mitochondrial DNA (Yassin et al., 2009) from Egyptian samples. Mattos-Machado et al. (2005) reported low variability in Brazilian populations compared to African and Asiatic samples, based on allozyme analyses. However, studies of chromosomal inversion carried out by Ananina et al. (2007) also revealed five new inversions in Brazilian populations, which were therefore found to

be more polymorphic than Indian populations. High variability was also observed in wing size and shape in relation to growth temperature in two natural populations, Rio de Janeiro, Brazil, and São Tomé Island, near Africa (Loh et al., 2008). Low variability was only found in Santa Cruz da Esperança, SP. It is possible that the population in Santa Cruz, SP, has been subjected to particular selective forces or adaptive differentiation caused by anthropomorphic factors.

When we examined the geographic distribution of the populations, we found two groups, based on genetic distance, including cities at different latitudes (Figure 3). Our results indicate that the six populations of *Z. indianus* are genetically related to their geographical distributions; the closer the two populations, the more genetically similar they were. An exception to this rule was observed when we compared populations of Santa Cruz da Esperança and Jaboticabal, SP; despite being at the same latitude, they had different variabilities of 14.1 and 47.9%, respectively. Although Santa Cruz da Esperança and Jaboticabal are only 100 km from each other, they are separated by a large sugarcane monoculture, which could explain the difference.

Latitudinal differentiation for *Z. indianus* has not been found in Brazilian populations, based on quantitative traits, such as body size, thoracic bristles (David et al., 2006) and chromosomal polymorphism (Ananina et al., 2007). However, it is possible that the *Z. indianus* flies, recently introduced in Brazil (Vilela et al., 1999), have been undergoing local differentiation processes, due to selective pressures of natural selection. Based on our data, the two groups of flies demonstrate signs of an incipient selection process, which may subsequently lead to a cline, as Alves and Bélo (2002) described for a nearby region, when they examined *Musca domestica* populations. Similar results had been observed in Egypt populations, based on mitochondrial DNA and chromosomal inversion polymorphism, despite the low genetic variability that was found (Yassin et al., 2009).

We found that the populations could be classified into two clearly different groups, which we called western and eastern patterns, based on geographic distribution (Figure 3). The higher value for gene flow between the two groups (western and eastern) compared to the value for all populations is also indicative of population structure. Genetic and geographic correlation data support the distance isolation model, although the F_{st} value determined for our study was high (0.524). It is known that F_{st} above 0.15 is an indication of differentiation of fragmented populations (Frankham et al., 2006). Similar results were reported previously (Mattos-Machado et al., 2005), in a study of allozyme variability in populations of *Z. indianus* collected from Poços de Caldas, MG, and Brasília, DF, with an observed F_{st} of 0.105, although there was no correlation with geographic distance. Behavioral and ecological factors, such as temperature, latitude and longitude, may be responsible for the low gene flow values (0.3727) and high F_{st} between the two groups, which may be responsible for the population structure that we observed.

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