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Genetic Differentiation in Natural Populations of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae) with Different Phenotypic Spot Patterns on Tergites in Males

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Abstract

Entomological surveys in the state of Maranhão have recorded morphologically distinct populations of *Lutzomyia longipalpis* (Lutz & Neiva). Some populations have one pair of spots (1S) on the fourth tergite, while others have two pairs (2S) on the third and fourth tergites of males. In the present study we investigated the degree of genetic polymorphism among four populations in the municipalities of Caxias, Codó and Raposa, in the state of Maranhão, Brazil, by using RAPD (Random Amplified Polymorphic DNA) markers. A total of 35 loci were identified, of which 30 were polymorphic. The highest polymorphism was observed with primer OPA 4, which produced 11 different profiles. Genetic diversity was assessed using grouping methods that produced a dendrogram in which the genotypes could be clearly separated into two main clades according to the number of spots on the male abdominal tergites. One cluster contained the populations from Caxias and Codó, and the other was formed by the populations from Raposa and Codó. The results of our RAPD analysis showed a clear separation between the populations with one and two pairs of spots. The epidemiologic significance of this genetic differentiation should be investigated in future studies.

Introduction

Lutzomyia longipalpis (Lutz & Neiva) is the principal vector of the causal agent of visceral leishmaniasis (VL) in Central and South America (Lainson & Rangel 2005). It is found in an area that extends from southern Mexico to northern Argentina (Young & Duncan 1994) and includes a variety of biomes, such as the Atlantic forest, cerrado (savanna-like grasslands) and caatinga (an area with xerophilous spiny trees and shrubs), as well as various types of climate and relief. These characteristics, whose effect is amplified by the short distance these phlebotomines can fly, can act as geographic barriers between some populations. The resulting geographic isolation favors genetic divergence and speciation, which are not only important from an evolutionary perspective, but can also lead to variations in characteristics that are of epidemiologic interest (Lanzaro *et al* 1993, Uribe 1999).

Studies supporting the hypothesis of the existence of a complex of species have been conducted using different methods to evaluate different populations of *L. longipalpis*, such as analysis of hybridization (Ward *et al* 1983), sex pheromones (Ward *et al* 1988, Hamilton *et al* 1996, 2005), isoenzyme profile analysis (Lanzaro *et al* 1993, Arrivillaga *et al* 2000), cytogenetic characters

(Yin *et al* 1999), male copulation songs (Sousa *et al* 2002, 2004, Araki *et al* 2009), analysis of mitochondrial DNA (Arrivillaga *et al* 2003) and microsatellite markers (Maingon *et al* 2003, Watts *et al* 2005).

Since the 60's males of *L. longipalpis* were observed to present a morphological differentiation in the number of abdominal spots in different geographic regions of Brazil (Mangabeira 1969). Males of *L. longipalpis* with a single pair of abdominal spots on the fourth tergite (1S) are broadly and discontinuously distribute all over South and Central America. On the other hand, males having two pairs of abdominal spots on the third and fourth abdominal tergites (2S) are more concentrated in Northeast regions of Brazil. It has been argued that the presence of abdominal spots is suggestive that *L. longipalpis* is a species complex (Ward *et al* 1985).

Other variations in populations of *L. longipalpis* from Central and South America have been reported, such as behavioral, morphological and biochemical characteristics (Dias *et al* 1998, Lampo *et al* 1999, Araki *et al* 2009), and the existence of reproductive isolation among populations from Central and South America mediated by different sex pheromones has been already suggested (Ward *et al* 1988).

Four types of pheromones are known to be produced by *L. longipalpis* males (Hamilton *et al* 1999, 2004), which are released by secretory glands located near the abdominal spots (Lane & Ward 1984, Spiegel *et al* 2002, Hamilton 2008). Hamilton *et al* (2005) reported no correlation between morphology of the abdominal spots and the type of pheromone produced. On the other hand, recent molecular data associated with the analysis of male copulation songs and pheromones of *L. longipalpis* from different regions of Brazil suggest the existence of two main groups of populations (Araki *et al* 2009). The first group represents a single species with males producing Burst-type copulation songs and cembrene-1 as pheromone, while the second group is more heterogeneous and probably represents a number of incipient species producing different combinations of songs and pheromones, demonstrating a high level of divergence and gene-flow complexity among populations of *L. longipalpis* in Brazil (Araki *et al* 2009).

Other studies supporting or refuting the hypothesis of a species complex in Latin America have been carried out in an attempt to determine the taxonomic status of *L. longipalpis*. However, although *L. longipalpis* is considered to be a species complex, there is no consensus on the delimitation among members of the complex, differences among the species or their distribution (Bauzer *et al* 2007). Currently, only *Lutzomyia pseudolongipalpis* (Arrivillaga & Feliciangeli), from Venezuela, is recognized as a distinct species of *L. longipalpis* (Arrivillaga & Feliciangeli 2001).

Entomological surveys in the state of Maranhão (Rebêlo *et al* 1999ab, 2010) detected that *L. longipalpis* is widely distributed in the state and is associated with phytogeographic regions and endemic areas of visceral leishmaniasis. In light of the growing number of cases of VL recorded in urban areas and the presence of morphologically distinct populations (one and two abdominal spots), the aim of this study was to evaluate the genetic basis of morphological differences between males of these populations of sandflies in endemic areas of visceral leishmaniasis.

Material and Methods

Area studied

The study was carried out in three endemic areas for VL in the state of Maranhão: the municipalities of Raposa, Caxias and Codó where *L. longipalpis* with different phenotypic patterns can be found.

Caxias and Codó, which are located east in the state, are characterized by mixed cerrado and babassu palms (*Orbignya phalerata*) and a climate that is a transition from semi-humid to semi-arid. The municipality of Raposa is located in São Luís Island and presents a semi-humid climate and secondary vegetation (IBGE 2009). The town of Raposa is approximately 290 km away from Codó and 100 km away from Caxias.

Phlebotomines were collected during 2007 with battery-operated CDC (Centers for Disease Control) light traps installed in peridomiciles in the study areas for 12 h (6 pm to 6 am). The insects captured were stored in freezer, identified under a stereomicroscope according to Young & Duncan (1994) and registered by collection area and morphological pattern of spots on the tergites.

DNA extraction and RAPD-PCR analysis

DNA was extracted from 40 *L. longipalpis* males (10 from each population), according to Mukhopadhyay *et al* (1998). Ten *Melipona fasciculata* (Smith) bees were used as outgroups. In all amplification reactions, ultrapure water was used as a negative control. DNA was extracted by adding 20 µl of STE buffer (0.1 M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA) to each specimen, and heating at 95°C for 10 min. Specimens were then macerated and the same buffer added to a final volume of 50 µl. Samples were heated again at 95°C for 10 min and centrifuged at 348 *g* for 2 min. The supernatant containing the DNA (40 µl) was collected and used for further analysis.

The DNA was amplified by RAPD-PCR in a Model MJ96+ Peltier Thermal Cycler, according to Lima *et al* (2000). The amplification reactions were prepared in a final volume of 30 µl containing 1 x buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM dNTP mix, 1.3 µM primer, 2.0U Taq DNA polymerase and 2 µl of DNA. The

PCR amplification conditions were as follows: an initial denaturation step of 3 min at 94°C followed by five cycles of 1 min at 93°C, 40s at 35°C and 2 min at 72°C; 40 cycles of 1 min at 93°C, 40 s at 42°C and 2 min at 72°C; and a final extension of 5 min at 72°C. The amplification products (2 µl) were subjected to electrophoresis in a 1.5% agarose gel in TBE buffer (90mM Tris-borate, 1mM EDTA) at 90 V for 2h. Gels were stained with ethidium bromide (0.1 mg/µl) and recorded in a UV photodocumentation system.

RAPD-PCR reactions were performed individually for each of the twenty primers tested. There was no intrapopulation variation in any of the analyses carried out. We then performed 40 amplifications using pools of 10 males from each population for interpopulation analysis. Primers OPA-3 (5'AGT CAG CCA C3'), OPA-4 (5'AAT CGG GCT G3'), OPA-9 (5'GGG TAA CGC C3') and OPA-15 (5'TTC CGA ACC C3') were then chosen according to the quality of the bands generated and the reproducibility of the experiments.

Statistical analysis

Only the most visible bands were chosen as molecular markers and scored as present (1) or absent (0) in the populations studied to produce a binary matrix for each locus selected. To calculate the genetic similarity indices, the binary matrix was subjected to the unweighted pair-group average (UPGA) method to construct a dendrogram using the STATISTIC version 7.1 software. The binary matrix was also used to calculate the percentage of polymorphism obtained with each primer according to the following formula: $P = nbp/nbt$, where P = percentage polymorphism (or polymorphism rate), nbp = number of polymorphic bands, and nbt = number of total bands.

Results

Of the twenty primers that could potentially be used to analyze populations of *L. longipalpis*, only four (OPA-3, OPA-4, OPA-9 and OPA-15) (Table 1) were selected from our preliminary RAPD-PCR tests, as they produced informative, reproducible and clear information on the genetic variation for the specimens and populations studied (Fig 1).

All primers selected produced different RAPD fragment patterns. The number of clear and reproducible fragments generated by these primers varied from five to 12, and the size of the amplification products varied from 400 to 2072 bp. The average number of amplicons produced per primer was 8.7.

The four primers used yielded the amplification of 35 loci. Of all the amplicons analyzed, 30 (85.7%) were polymorphic (Table 1). Primer OPA-4 produced the largest number of total and polymorphic loci, and OPA-15 the smallest number of total loci (five). The percentage of polymorphic loci varied between 70% (OPA-03) and 100% (OPA-15). The fact that polymorphism was detected with all of the primers used showed that there is a high degree of genetic variability among the populations studied.

Table 1 Polymorphism pattern detected by RAPD-PCR in four populations of *Lutzomyia longipalpis*.

Primers	Number of loci obtained	Number of polymorphic loci	Polymorphism (%)
OPA-3	10	7	70.0
OPA-4	12	11	92.0
OPA-9	8	7	87.5
OPA-15	5	5	100.0
Total	35	30	-

The genetic distance varied between 0.15 and 0.50 (Table 2). The shortest genetic distance (0.15) was observed between populations of males with a single pair of spots collected in Caxias and Codó (neighboring municipalities 100 km apart), while the greatest (0.50) was observed between populations in the same municipalities but with different phenotypes (one or two pairs of spots), yielding two very distinct clades. One clade included the Caxias 1S and Codó 1S populations (a single tergite spot), and the other, the Raposa 2S and Codó 2S populations (2 tergite spots) (Table 2, Fig 2).

Discussion

The degree of polymorphism observed in this study agrees with the findings of Balbino *et al* (2006), who investigated the genetic structure of seven natural populations of *L. longipalpis* in the Northeast of Brazil (São Luís, Teresina, Patos, João Pessoa, Itamaracá, Calumbi), using RAPD molecular markers. They detected a high degree of genetic variation among these populations when they analyzed the 24 amplified fragments obtained using three primers. Despite the usual problems associated with the reproducibility of RAPD markers, the high degree of polymorphism observed with *L. longipalpis* indicates these markers are a viable alternative tool for wide range studies on the genetic makeup and differentiation of populations of *L. longipalpis*.

Our results revealed two groups that coincided with the morphological characteristics observed in males. First, the populations with the shortest genetic distances were those with one pair of spots, which came from the municipalities that were geographically closest (Caxias and Codó), showing that the species examined may belong to the same population and that the short genetic distance detected may just be

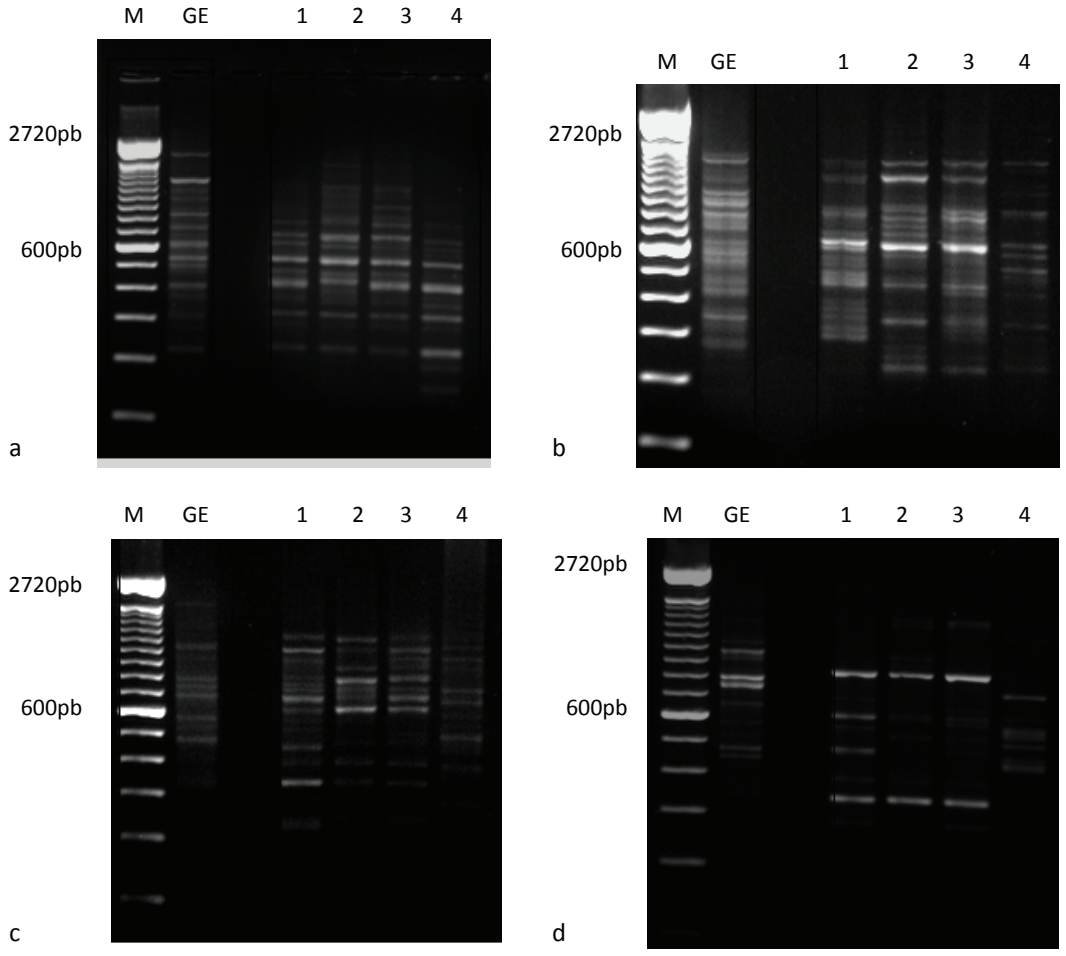

Fig 1 RAPD profile generated by the primers OPA-03 (a), OPA-04 (b), OPA-09 (c) and OPA-15 (d) in samples from males with one and two pairs of abdominal spots from *Lutzomyia longipalpis* populations captured in three different regions of Maranhão endemic for VL. M = marker; OG = outgroup (bee); 1 = population with two pairs of spots collected in Raposa; 2 = population with one pair of spots collected in Caxias; 3 = population with one pair of spots collected in Codó; 4 = population with two pairs of spots collected in Codó.

the result of intrapopulation variability. Secondly, the populations with the greatest genetic distances belong to the two distinct phenotypes (one and two pairs of spots) from the neighboring municipalities mentioned

Table 2 Genetic distance matrix among the *Lutzomyia longipalpis* populations obtained using STATISTIC version 7.0 and the binary matrices constructed based on the presence or absence of RAPD markers.

Variable	Bee	Raposa (2S)	Caxias (1S)	Codó (1S)	Codó (2S)
Bee	0.00				
Raposa (2S)	0.69	0.00			
Caxias (1S)	0.69	0.42	0.00		
Codó (1S)	0.71	0.31	0.15	0.00	
Codó (2S)	0.48	0.33	0.50	0.48	0.00

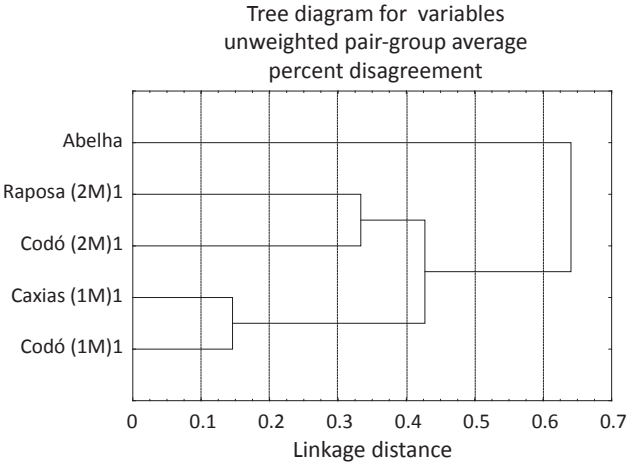

Fig 2 Dendrogram of *Lutzomyia longipalpis* populations with one and two pairs of spots on the abdominal tergites produced using STATISTIC version 7.0 and binary matrices constructed based on the presence or absence of RAPD markers.

above. Therefore, our data suggest that although species belong to the same ecological region, they may belong to different populations/species that segregated as a result of selective pressures, such as different pheromones and pre-mating behavior, therefore, reducing gene flow between these populations. The two populations in which males have two pairs of spots showed moderate genetic distance between them. This can be explained by the geographic distance between the cities where they were collected (Raposa and Codó).

The suggestion of the existence of a complex of cryptic species in the Brazilian populations is not new. Maingon *et al* (2008) described different types of behavior among these males in terms of the sounds they make during precopulatory courting and the type of sex pheromone they produce, and correlate these with the two phenotypes (one or two pairs of spots on the abdominal tergites). These authors also discussed the reproductive isolation in the different populations and stressed the importance of population substructures in the epidemiology of VL, which is also supported by our data.

Our results also support the findings of a study by Lins *et al* (2008), who identified a clear difference between two populations of males with one and two pairs of spots collected in Sobral (CE), using the *paralytic* (*para*) gene. This gene encodes for a sodium channel associated with resistance to pyrethroid insecticides and production of courtship songs in *Drosophila melanogaster* (Meigen), as a molecular marker. Lins *et al* (2008) were the first authors to demonstrate the association of the *para* gene with fixed differences between these two populations, raising the possibility that they may be associated with an incipient process of resistance to insecticides in these populations of *L*. *longipalpis*.

A better understanding of the genetic, evolutionary and epidemiologic significance of the one-pair-of-spots and two-pairs-of-spots male phenotypes of *L*. *longipalpis* can be gained from a comparative analysis of sympatric, such as those in the municipality of Codó, and allopatric populations, such as those in the municipalities of Raposa and Caxias. Our RAPD data showed a clear separation between populations with one and two pairs of spots, supporting recent studies that suggest that these distinct phenotypes may reflect the existence of subspecies or cryptic species. Given the importance of *L*. *longipalpis* as a vector of leishmaniasis the epidemiologic significance of each phenotype should be investigated in more detail in future studies.

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