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Genetic Structure of Sympatric Populations of Female Lutzomyia Longipalpis (Diptera: Psychodidae) In Sobral and Caririaçu, Ceará State, Brazil

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Abstract

Objectives: Accurate identification of these insects is critical to avoiding mistakes in the recognition of vector and non-vector species, so the invariant phenotypic patterns displayed by the females of *L. longipalpis* require the implementation of molecular methods of identification. Our goal was to examine genetic variation in the females of *L. longipalpis* using the SNPs reported in the males of this vector species.

Methods: We trapped sand flies near houses and domestic animal shelters at two localities in the state of Ceará, Brazil, using two to five CDC miniature light traps. Genomic DNA was separately extracted from each female using 100 µl of Chelex resin, and a 525 bp fragment of the *period* gene was used to assess genetic polymorphism in the two geographically isolated populations.

Results: The results indicate the presence of three fixed polymorphisms (T124C, C171T, and C424T) within this gene. Genetic structure analysis indicated that the studied *L. longipalpis* populations can be divided into two main subgroups (1S-like and 2S-like), with the *ad hoc* quantity supporting the number K = 2.

Conclusion: Collectively, our results suggest a direct relationship between the number of spots found in the males' tergites and the genetic variation observed in the *L. longipalpis* females in the state of Ceará. The SNPs observed in the *period* gene will be useful for future studies of molecular eco-epidemiology in areas where these species occur in sympatry.

Keywords

Population genetics; Visceral leishmaniasis; sand fly; species complex; *period* gene; Northeast; *Lutzomyia longipalpis*

Introduction

The phlebotomine sand fly *Lutzomyia longipalpis sensu lato* is the principal vector of visceral leishmaniasis (VL) in the New World [1]. *L. longipalpis* males have either one pair of pale spots on the fourth tergite (the 1S morphotype) or two pairs on the third and fourth tergites (the 2S morphotype). The second pair on the third tergite may be smaller than the pair on the fourth and this is sometimes designated as an intermediate form [2]. There are reports of intermediate forms of *L. longipalpis* in some localities, especially around the northeast coast, indicating intraspecific polymorphism [2]. Several studies have shown that spot morphology cannot be used as a species-specific character [2,3]. Although these patterns of pale spots are not species-specific, they may be useful in identifying sympatric species in localities where intermediates are very rare, as occurs in Sobral [4], Jaiba, and Estrela de Alagoas [5]. The polymorphisms in the longer fragment of the *period* gene strongly suggest that this might also be the case in Bodocó and Caririaçu [6].

Molecular (microsatellite markers and speciation genes) and behavioral (sexual pheromones and courtship/mating sounds) analyses have also demonstrated the existence of differences between the 1S and 2S morphotypes and have provided further evidence of a *L. longipalpis* species complex [7]. Unlike the males, however, the females are morphologically undistinguishable: they do not present abdominal pale spots (either 1S or 2S), and this causes difficulties in studies of courtship and mating behaviors.

In Brazil, Ceará state has the third-highest number of VL cases, about 12.2% of the total reported in the country. Over the past 10 years, 4351 autochthonous cases were recorded in Ceará, and 578 of these cases (~13.3%) occurred in the municipality of Sobral [8]. In Sobral, two sympatric populations of *L. longipalpis* have exhibited differences in their copulatory courtship sounds (P3 and B), sex pheromones (9-methylgermacrene-B and cembrene), and ethological characters, as well as in their genetic variability as detected by molecular markers [6,7,9].

Multilocus analyses have also been performed on the *L. longipalpis* complex [10]. Among the studied loci, *period* (*per*) is widely known to be involved in the control of *Drosophila* circadian rhythms in eclosion and locomotor activity [11,12]. Also, *period* controls a feature of the "love song" that *Drosophila* males produce during courtship [13]. In sand flies, *per* has been utilized in population genetic studies to identify possible members of the *L. longipalpis* complex in Brazil [4,6,10].

Studies aiming to evaluate the genetic structure of the females of *L. longipalpis* populations have not been performed before, due to the females' monomorphic character. Using analysis of microsatellite loci and pheromone types, however, identification of the females is now possible, and so the different population genetics of the females and males in this complex can now be studied [14]. In contrast to the females, several taxonomic, evolutionary, and behavioral studies have been performed using *L. longipalpis* males [4,15-18]. Accurate identification of these insects is critical to avoiding mistakes in the recognition of vector and non-vector species [19], so the invariant phenotypic patterns displayed by the females of *L. longipalpis* require the implementation of molecular methods of identification. Our goal was to examine genetic variation in the females of *L. longipalpis* using the SNPs reported in the males of this vector species [6].

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Materials and Methods

Field collection and identification of phlebotomine sandflies

Sand fly trapping was carried out in Sobral (03°41′15″S; 40°21′5″W) and Caririaçu (07°02′31″S; 39°17′02″W) in the State of Ceará, Brazil. Both localities have a climate characterized as BSw′h′ according to the Köppen classification [20], with temperatures ranging from 23 °C to 36 °C and low annual rainfall (936 mm to 1100 mm). Both localities also are considered part of the Caatinga biome, with vegetation composed mainly of ligneous and herbaceous species with a high degree of xerophily.

Sand flies were trapped in the vicinity of houses and domestic animal shelters using two to five CDC miniature light traps positioned approximately 0.6 m from the ground. Collections were made from April 2013 to September 2014. Sand flies were identified according to Young and Duncan [21], and *L. longipalpis* females were separated for analysis. For the capture of the flies, consent from household owners was obtained before setting traps in and around their homes, and also near the shelters of domestic animals.

DNA extraction, PCR, and sequencing

Genomic DNA was separately extracted from each female using 100 µl of Chelex resin (Bio-Rad, Hercules, CA, USA) based on the protocol described by Costa Junior [6]. For each DNA sample, a 525 bp segment of the *period* gene [22] was amplified by PCR using a Master Mix Kit (Promega, Fitchburg, WI, USA). The amplicons were purified using the Genomic DNA Purification Wizard Kit (Promega, Fitchburg). Bi-directional sequencing reactions were performed on each purified PCR product using the BigDye terminator (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI3130 Sequence Analyzer (Applied Biosystems, Foster City). Each sample was sequenced in duplicate and the sequencing results evaluated with the Pregap4 program [23] based on the values of *Phred* 40 [24]. Heterozygous individuals were not observed. Consensus sequences were obtained using the Gap4 program [23] and deposited in GenBank under accession numbers KP144145 to KP144180.

Phylogenetic analysis

Of the 53 sequences of *L. longipalpis* females obtained for the *period* gene, 23 were from Sobral and 30 from Caririaçu. Sequence alignment was performed using the Muscle program present in MEGA v. 5.1 [25].

To generate a minimum evolution tree using MEGA v. 5.1 and to assess the consistency of the branches, the bootstrap test [26] with 1000 pseudo-replications was used. The sequences corresponding to the *period* gene in 1S and 2S male *L. longipalpis* from both localities (KF479047-KF479051, KF479106-KF479109, KF479075-KF479079, and KF479141-KF479145) were used as external groups.

The conserved and variables sites (parsimony-informative and singletons) were also verified using MEGA v. 5.1. For optimal viewing of polymorphisms, the parsimony-informative sites were exported into sequence logos using Weblogo v. 3.2 [27]. For the identification of SNPs, comparative analyses were done using the *period* sequences of *L. longipalpis* males with the 1S and 2S phenotypes, as described by Costa Junior [6].

Population structure

The genetic structuring of the *L. longipalpis* populations was verified using Structure v. 2.3 [28] with a model-based approach. Markov Chain Monte Carlo (MCMC) simulations were performed with 100,000 interactions of the burn-in *period* and followed by 1,000,000 steps. For each value of K (1 to 10), 10 interactions were performed to estimate the K values, and the most likely population (or cluster) number was determined by ΔK analysis, as described by Evanno [29].

Genetic diversity

Intra-population genetic diversity was assessed in terms of haplotype and nucleotide diversity, K value (number of genetic groups), number of polymorphic sites, and number of transitions and transversions; diversity was measured using DnaSP v. 4.0 [30] and Arlequin v. 3.5 [31].

Genetic differentiation was assessed with the pairwise fixation index F_{st} using Arlequin v. 3.5 [31]. The average number of substitutions per site among populations (Dxy), the total number of substitutions per site among populations (Da), the number of shared polymorphisms among populations (Ss), and the number of fixed differences among populations (Sf) were calculated using DnaSP v. 4.0 [30].

Results

Altogether, 53 *L. longipalpis* females were analyzed. The analyzed sequence region (525 bp) exhibited 63 (12%) polymorphic sites, including 39 (~61.3%) parsimony-informative sites and 24 (~38.7%) singletons. Among the polymorphic sites, 73% of the nucleotide substitutions were transitions and 27% were transversions (Table 1).

Three single nucleotide polymorphisms (SNPs) were identified within the 525 bp fragment of the *period* gene used in our analyses. Based on the SNPs, we found 22 1S-like specimens (12 FCAR and 10 FSOB) and 31 2S-like specimens (18 FCAR and 13 FSOB). All SNPs can be used to separate the phenotypes (1S and 2S). The SNPs identified at nucleotide positions T124C and C171T, the first and second nucleotides in the 1S and 2S phenotypes, respectively, are located within exon 1 and are fixed in *L. longipalpis* females from both localities in Ceará (Figure 1). The SNP identified at position C424T (within exon 2) is fixed in the *L. longipalpis* females from Sobral and Caririaçu, just as it is in *L. longipalpis* males.

In the genetic structure analysis of *L. longipalpis* females, with each population assessed separately, two distinct genetic groups associated with abdominal spot patterns were observed, with each sequence possessing a probability (Q) greater than 80% of belonging to each genetic group (Figure 2). When sequences from both localities were combined, the genetic assignment test indicated the presence of two genetic groups associated with the 1S and 2S morphotypes, as suggested by the *Δk* peak that indicated the presence of two genetically distinct populations.

The minimum evolution analysis revealed two distinct and well-supported clades with bootstrap values of 80%. This result indicates that the separation is associated with the abdominal spots (1S and 2S) of *L. longipalpis* males (Figures 3 and 4).

Similarly, when F_{st} was used to verify the genetic structuring at the two localities (Caririaçu and Sobral), the presence of two morphotypes related to 1S and 2S was again observed. The *L. longipalpis* female 1S-like populations of Caririaçu and Sobral display low F_{st} values when compared to each other, forming a genetically similar group (Group 1S-like). A similar pattern was observed when comparing the 2S-like populations amongst themselves. However, the F_{st} values were the highest when comparing the two different phenotypes, even from

Table 1: Intra-population genetic diversity measures for each sample.

Samples	N	Hd	π	NS	H	K	TS\TV	S	Pi
Sobral 1S	10	1.0000	0.01636	27	10	8.55556	21**6**	13	15
Sobral 2S	13	1.0000	0.01206	20	13	6.30769	16**5**	7	14
Caririaçu 1S	12	1.0000	0.01721	28	12	9.0000	**19\11**	12	17
Caririaçu 2S	18	1.0000	0.01395	34	17	7.29412	**26\11**	19	16
1S-like full˙	22	1.0000	0.01692	37	22	8.84848	**28\11**	15	23
2S-like full˙	31	0.99785	0.01317	38	30	6.88602	**29\12**	20	19
Total	53	0.99927	0.02091	63	52	10.93759	**46\17**	24	39

N, sample size ; Hd, haplotypic diversity.
π, nucleotide diversity ; NS, number of polymorphic sites.
h, haplotype ; K, average number of nucleotide difference ; TS\TV, number of transitions\number of transversions.
S, singletons ; Pi, parsimony-informative sites
˙All females 1S-like and 2S-like.

Table 2: Genetic differentiation among *L. longipalpis* populations.

Populations		F_{st}	Dxy	Da	Ss	Sf
Sobral 1S	Sobral 2S	0.41906	0.02446	0.01025	8	1
Caririaçu 1S	Caririaçu 2S	0.48151	0.03004	0.01447	10	2
Sobral 1S	Caririaçu 1S	0.01095	0.01697	0.00019	18	0
Sobral 1S	Caririaçu 2S	0.40014	0.02526	0.01011	11	1
Sobral 2S	Caririaçu 1S	0.50063	0.02931	0.01467	8	3
Sobral 2S	Caririaçu 2S	0.00162	0.01302	0.00002	17	0
1S-like full*	2S-like full*	0.45395	0.02755	0.01251	14	1

Fst : pair-wise genetic differentiation.
Dxy : average number of nucleotide substitutions per site between populations.
Da : number of net nucleotide substitutions per site between populations.
Ss : number of shared polymorphisms between population pairs.
Sf : number of fixed differences between population pairs.
*All females 1S-like and 2S-like.

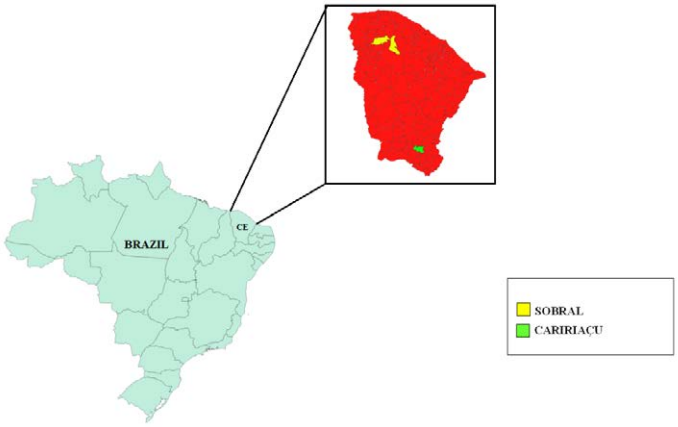

Figure 1: Map of Ceará showing the localities of Sobral and Caririaçu, where the females of *Lutzomyia longipalpis* were collected.

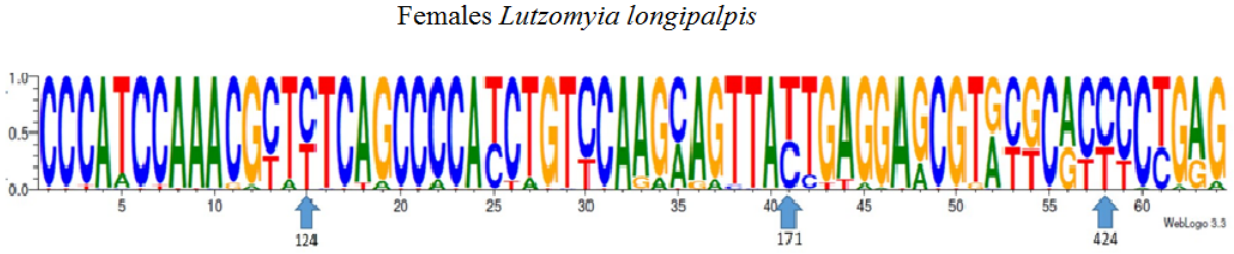

Figure 2: Schematic representation of polymorphisms in a 525 bp fragment of the *period* gene using Weblog. Shown are the sequences obtained from *L. longipalpis* females collected in Sobral and Caririaçu, Ceará, Brazil. Font size is indicative of the frequency of a nucleotide at any given site. Fixed (blue arrows).

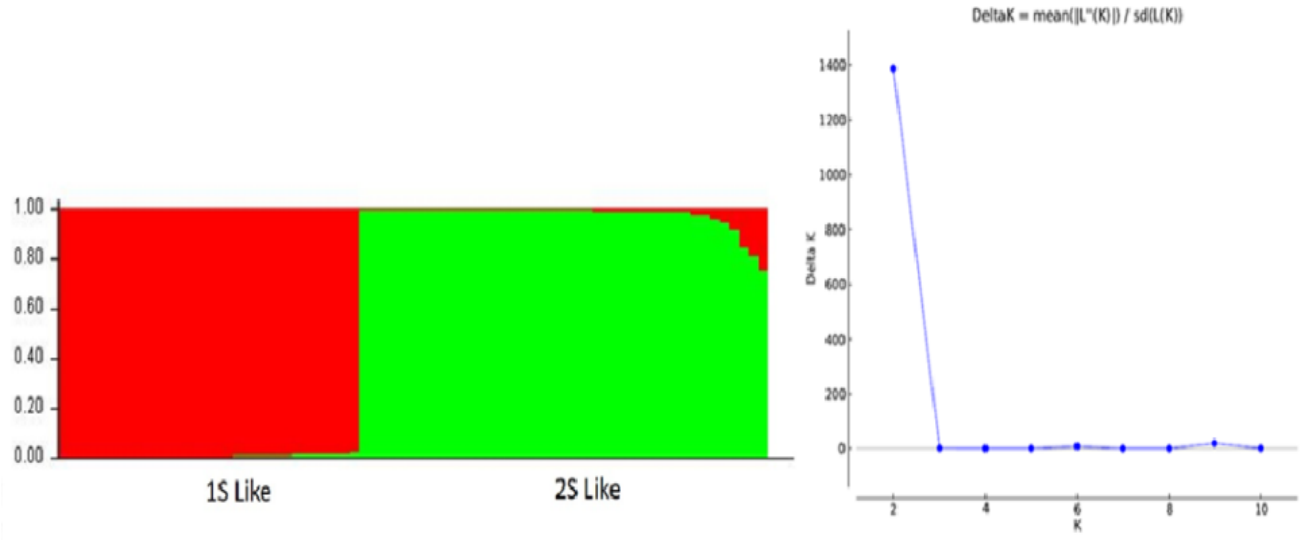

Figure 3: Bar plots and Δ*K* values ranging from 1 to 10 as drawn by STRUCTURE software, representing the genetic structure of *L. longipalpis* females. The 1S-like specimens were assigned to the red group and 2S-like specimens were assigned to the green group. The Evanno method predicted that the most likely number of populations was two.

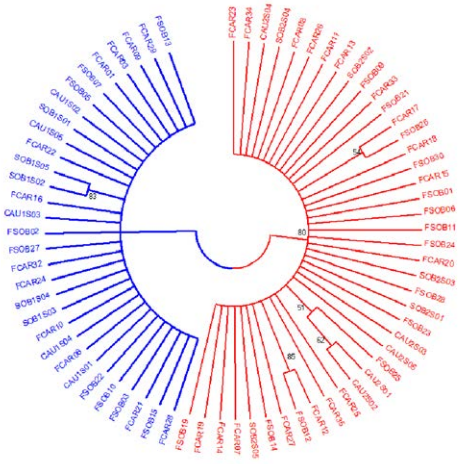

Figure 4: Minimum evolution topology tree of the 53 sequences of *Lutzomyia longipalpis* inferred under the K2P model. Numbers on the interior branches represent posterior probabilities obtained in the ME analysis.

the same locality (Table 2). Identical values of F_{st} were observed when populations of *L. longipalpis* males from the municipalities of Sobral and Caririaçu were examined [6].

Discussion

Analysis of the parsimony-informative sites indicated the presence of two group of female *Lutzomyia longipalpis* (1S-like and 2S-like). In a previous study, *L. longipalpis* males have been assessed for gene polymorphisms in the *period* gene with the aim of distinguishing the 1S and 2S morphotypes based on four SNPs in the populations of Sobral and Caririaçu [6]. These results indicate that there may be prezygotic barriers between the members of the *L. longipalpis* species complex, as suggested by Maingon [14] using microsatellite data and sex pheromones.

The fixed polymorphisms (T124C, C171T, and C424T) observed in the *period* gene will be particularly useful for the differentiation of 1S and 2S females in localities where they occur as sympatric species, just as they are in male *L. longipalpis* [6]. We also identified two sites with high frequencies that can be used in combination as markers for cryptic species occurring sympatrically in Sobral and Caririaçu. In male *L. longipalpis*, a fixed SNP causing a missense mutation in the amino acid sequence was previously identified in the gene *paralytic*, and it was able to separate the two main groups of *L. longipalpis* that produce different copulation songs [15,18]. The *period* gene is an effective tool to identify members of a species complex, as proved in *Lutzomyia umbratilis* [32].

The minimum evolution approach for *L. longipalpis* females collected in Caririaçu and Sobral indicated the presence of two genetically separated populations, and each collected sample belonged to either the 1S or the 2S morphotype. Our analyses also indicated that these two genetically distinct populations are correlated with abdominal spot patterns, as previously suggested for sympatric populations of *L. longipalpis* [6,10].

Conclusion

The SNPs observed in the *period* gene were useful to relate *L. longipalpis* females to the 1S and 2S phenotypes of the males, and they may be useful in the design of probes for future studies of molecular eco-epidemiology in areas where these species occur in sympatry.

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