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Analysis of the genetic structure of allopatric populations of *Lutzomyia umbratilis* using the *period* clock gene

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ABSTRACT

In South America, *Lutzomyia umbratilis* is the main vector of *Leishmania guyanensis*, one of the species involved in the transmission of American tegumentary leishmaniasis. In Brazil, *L. umbratilis* has been recorded in the Amazon region, and an isolated population has been identified in the state of Pernambuco, Northeastern region. This study assessed the phylogeographic structure of three allopatric Brazilian populations of *L. umbratilis*. Samples of *L. umbratilis* were collected from Rio Preto da Eva (north of the Amazon River, Amazonas), from Manacapuru (south of the Amazon River), and from the isolated population in Recife, Pernambuco state. These samples were processed to obtain sequences of the *period* gene. Phylogenetic analysis revealed the presence of two distinct monophyletic clades: one clade comprised of the Recife and Rio Preto da Eva samples, and one clade comprised of the Manacapuru samples. Comparing the Manacapuru population with the Recife and Rio Preto da Eva populations revealed high indices of interpopulational divergence. Phylogenetic analysis indicated that geographical distance and environmental differences have not modified the ancestral relationship shared by the Recife and Rio Preto da Eva populations. Genetic similarities suggest that, in evolutionary terms, these populations are more closely related to each other than to the Manacapuru population. These results confirm the existence of an *L. umbratilis* species complex composed of at least two incipient species.

1. Introduction

Cryptic species are species with discrete morphological differences that remain undetected when assessed by classical taxonomy. Therefore, several insect species have been differentiated by assessing the following factors: genetic, ecological, and behavioral characteristics, susceptibility to infection, and eating habits (Mazzoni et al., 2008). Cryptic species are a common occurrence among insect vectors of disease; instances have been identified in the Anophelinae, Culicidae, Triatominae, and Phlebotominae subfamilies (Anderson et al., 2000; Monteiro et al., 2013; Costa-Junior et al., 2015). In the Phlebotominae subfamily, low flight capacity and geographic isolation have significantly contributed to the emergence of cryptic species (Soto et al., 2001).

Lutzomyia umbratilis (Diptera: Psychodidae) is the main vector of *Leishmania guyanensis,* one of the pathogenic agents of American tegumentary leishmaniasis (ATL) (Lainson et al., 1976, 1979, 1981). This species is found in Brazil, Bolivia, Colombia, French Guyana, Guyana, Peru, Suriname, and Venezuela (Young and Duncan, 1994; Burgos and Hudson, 1994). In Brazil, *L. umbratilis* is widely distributed in the Amazon basin and there is an isolated population in remnants of the Brazilian Atlantic rain forest, in the state of Pernambuco, Northeastern Brazil (Young and Duncan, 1994; Balbino et al., 2001).

On the other side of Brazil, in the Amazon region, the presence of *L. umbratilis* naturally infected with *L. guyanensis* has been

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recorded on the east side of the Negro River and on the north side of the Amazon River, but not on the south side of the Amazon River (Lainson et al., 1994). It has been shown that bionomical (*e.g.*, longevity, fertility and larval development) differences between *L. umbratilis* populations on the north and south sides of the Amazon River have been influenced by the geographic isolation created by the geographic barrier of the river (Justiniano et al., 2004). Recently, *L. umbratilis* populations in the North and Northeast regions were evaluated using the mitochondrial gene *Cytochrome Oxidase I* (*COI*) and geometric morphometry of the wing; genetic and morphometric differences were identified, which indicates that these populations represent a species complex (De Souza Freitas et al., 2015).

Molecular markers are considered useful tools for differentiating between cryptic species and identifying genetic variation among populations (Bauzer et al., 2002; Waycott et al., 2002; Uchimura et al., 2006). In *Drosophila melanogaster*, genes that control reproductive behavior were isolated and cloned; these molecular markers have enormous potential for intra and inter population analysis. Among these, the *period* gene is particularly interesting because it controls the rhythms of locomotor activity (Konopka and Benzer, 1971), and it controls a feature of the "lovesongs" that males produce during courtship (Kyriacou and Hall, 1980). Both of these behavioral characteristics have been implicated in maintaining sexual isolation between species (Kyriacou and Hall, 1980, 1982, 1986; Ritchie et al., 1999; Sakai and Ishida, 2001). Therefore, the *period* gene has been called a "speciation gene," because it controls differences in lovesongs rhythms that may contribute to reproductive isolation (Coyne, 1992). In sand flies, the *period* gene has also been utilized in studies of population genetics in order to identify possible members of the *Lutzomyia longipalpis* complex in Brazil (Bauzer et al., 2002, 2007). In a recent study, the *period* gene revealed moderate geographical structuring between the *L. longipalpis* populations of Ceará State, and revealed significant variability among the 1S and 2S phenotypes (Costa-Junior et al., 2015).

In the current study, we have analyzed the presence of polymorphism in the *period* gene of three Brazilian populations of *L. umbratilis*. Our goal was to investigate genetic differentiation and the speciation process in this putative species complex.

2. Material and methods

2.1. Field collection and identification of phlebotomine sandflies

Field collections were done in Rio Preto da Eva (2°50′50″S/59°56′28″W) and Manacapuru (3°12′41″S/60°26′20) municipalities, located in the Amazonas State, North Region of Brazil, and in the Atlantic Forest Ecological Reserve of Dois Irmãos (8°03′14″S/34°52′52″W) in Pernambuco State, Recife Municipality, Northeast Region of Brazil.

Adult specimens were collected from tree trunks by suction using CDC light traps. The samples were conserved in 95% alcohol, at −20 °C. Sandflies were identified using the keys of Young and Duncan (1994).

2.2. DNA extraction, PCR, and sequencing

For molecular analysis of the *period* nuclear marker, 73 specimens of *L. umbratilis* were used: 36 from Manacapuru; 23 from Rio Preto da Eva; and 14 from Recife. Genomic DNA extraction was carried out using Chelex®100 (BioRad, Berkeley, California, USA), according to Costa-Junior et al. (2015).

For each DNA sample isolated, a segment of 489 bp of *per* (Mazzoni et al., 2002) was amplified by PCR using the Master Mix kit (Promega, Fitchburg, WI). PCR products were visualized in 1% agarose gel under UV light, and they were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega® Fitchburg,Wisconsin, USA). Sequencing was carried out in an ABI 3500 automatic sequencer (Applied Biosystems, Cleveland, Ohio, USA).

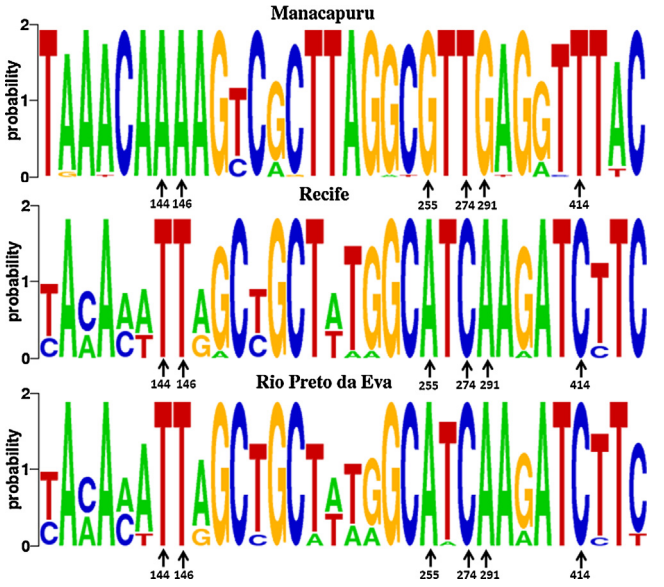

Fig. 1. Schematic representation of polymorphisms in a fragment of 489 bp from the *Period* gene using Weblog.
The sequences shown were obtained from *L. umbratilis* collected in Recife, State of Pernambuco, Rio Preto da Eva and Manacapuru, State of Amazonas, Brazil. Font size is indicative of the frequency of a nucleotide at any given site. Fixed (black arrows).

Only sequences with a PHRED score above 30 were used in the analysis. Contig assembly was carried out using CodonCode Aligner (CodonCode Corporation). Local alignments were done using BLAST (Altschul et al., 1990). All new sequences produced in this study have been deposited in GenBank under accession numbers: KT722623–KT722695.

2.3. Phylogenetic analysis

Nucleotide sequences were aligned using Muscle (Edgar, 2004), incorporated with MEGA v. 5.0 (Tamura et al., 2011). Phylogenetic relationships among the sequences were inferred by Bayesian Inference (BI) analysis, which was implemented with MR. BAYES (Ronquist and Huelsenbeck, 2003) using the evolutionary model (TPM2uf) that best fit the period data sets; the best fit model was determined using the jMODELTEST (Posada, 2008). BI analysis included two simultaneous independent runs of the Markov Chain Monte Carlo (MCMC) for 100 million generations, sampling every 1000 generations with a burn-in of 25%. *Lutzomyia whitmani* and Phlebotomus dubosqui were selected as outgroups in the phylogenetic analysis.

2.4. 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 (Rozas et al., 2003) and Arlequin v. 3.5 (Excoffier and Lischer, 2010). The frequencies of polymorphic sites were also assessed using the WebLogo tool (http://weblogo.berkeley.edu/logo.cgi).

Tajima's *D* neutrality test was performed using Arlequin v. 3.5 (Excoffier and Lischer, 2010). Genetic differentiation was assessed

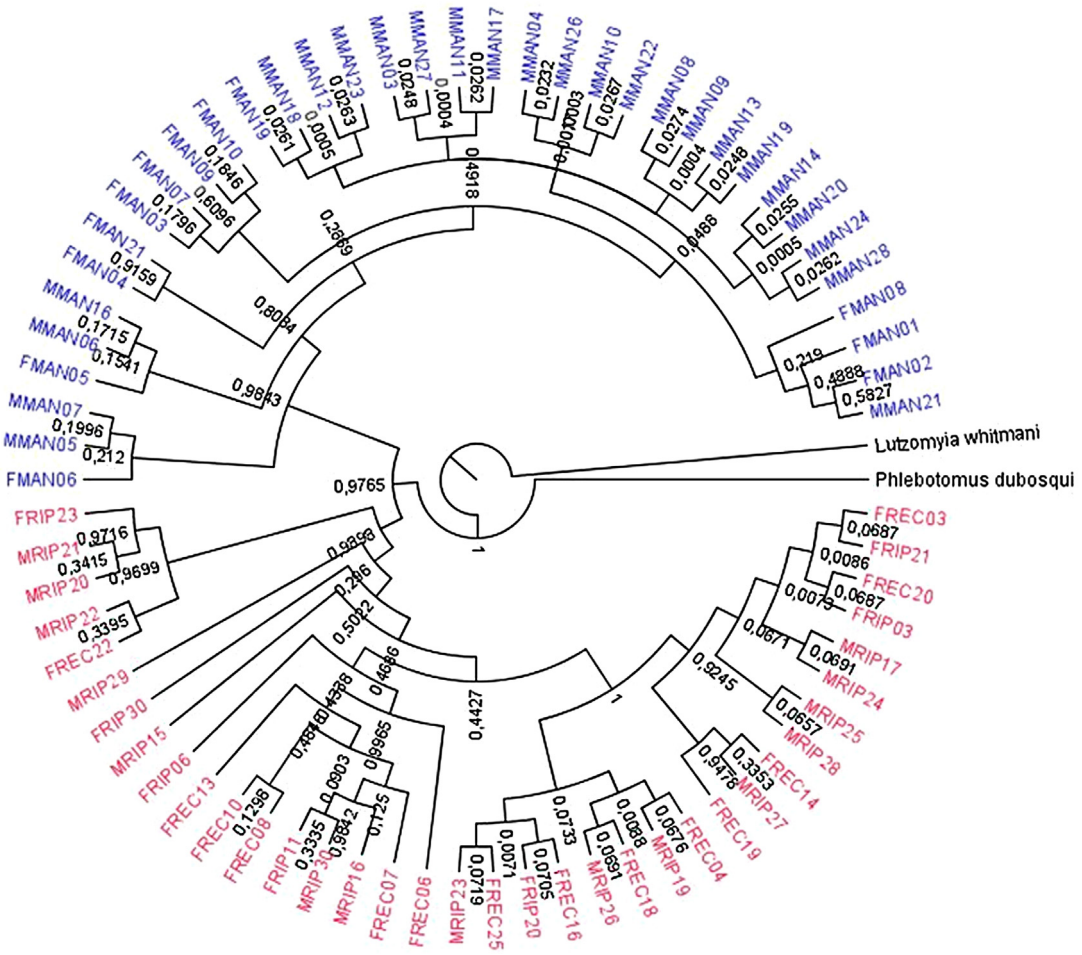

Fig. 2. Bayesian Inference (BI) topology tree of the 73 sequences of *Lutzomyia umbratilis* inferred under the TIM1 + I model.
Numbers on each branch (above branch) represent posterior probabilities obtained in the BI. *Lutzomyia whitmani* and *Phlebotomus dubosqui* were used as outgroups.

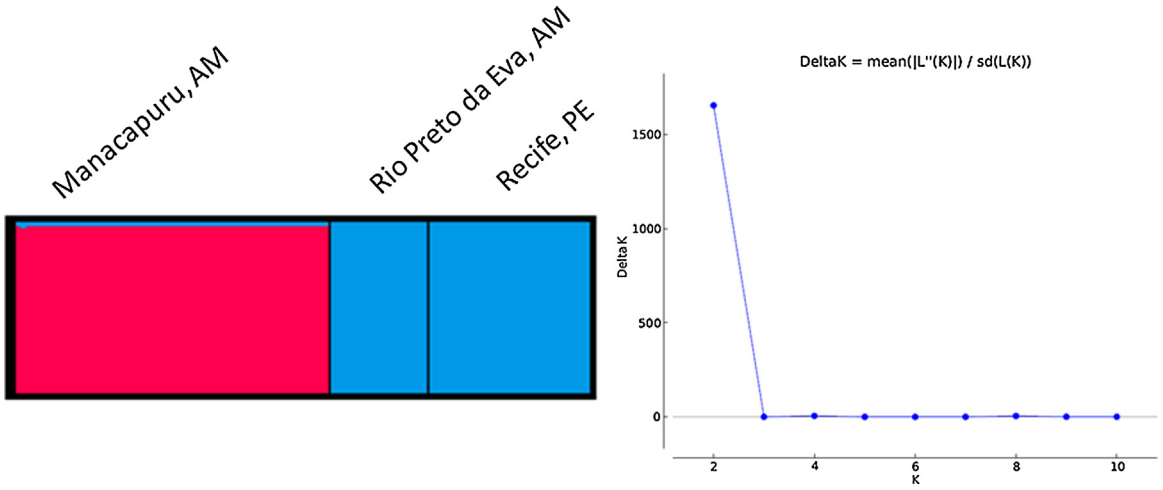

Fig. 3. Bar plots and ΔK values ranging from 1 to 10 by the STRUCTURE software, inferring the genetic structure of *L. umbratilis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The Manacapuru specimens were assigned to the red group, and the Rio Preto da Eva and Recife specimens were assigned to the blue group. The Evanno method predicted that the most likely number of populations was two.

with the pairwise fixation index F_{st} using Arlequin v. 3.5 (Excoffier and Lischer, 2010).

The average number of substitutions per site among populations (D_{xy}), the total number of substitutions per site among populations (D_a), the number of shared polymorphisms among populations (S_s), and the number of fixed differences among populations (S_f) were calculated using DnaSP v. 4.0 (Rozas et al., 2003).

The haplotype network was created with NETWORK v. 4.6 (www.fluxus-engineering.com) using the median-joining method

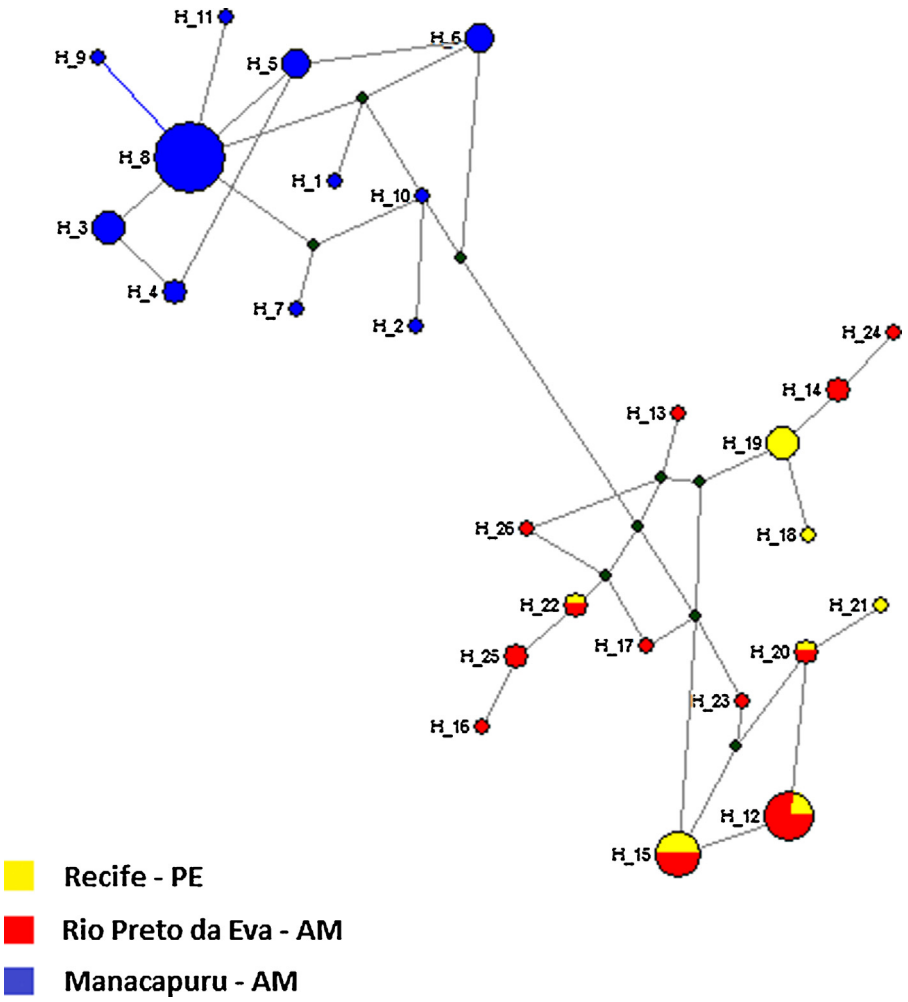

Fig. 4. Haplotype network of *L. umbratilis* showing 51 interconnected haplotypes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The size of the circles are proportional to the number of individuals observed for each haplotype. The small circles (green) represent mutational events lost during the evolutionary process.

(Bandelt et al., 1999) to verify the level of haplotype sharing and the frequency distribution among populations.

2.5. Population structure

Genetic structure analysis was performed using Structure v. 2.3 (Pritchard et al., 2000). Simulations were carried out with 20,000 interactions of burn-in, followed by 200,000 generations of Markov Chain Monte Carlo, adjusted 1–10 for each "*K*" population. Ad hoc quantity ΔK (Evanno et al., 2005) was used to determine the most accurate number of "*K*" groups.

3. Results

Altogether, 73 specimens of *L. umbratilis* were analyzed. The analyzed sequence region (489 bp) exhibited 35 (7.1%) polymorphic sites; these were comprised of 27 (~77.1%) parsimony-informative sites and 8 (~22.9%) singletons. Among the polymorphic sites, 54.2% of the nucleotide substitutions were transitions and 45.8% were transversions. Analysis of the parsimony informational sites identified six fixed single nucleotide polymorphisms (SNP) within the 489 bp fragment of *per* that was used in our analysis (Fig. 1). No non-synonymous nucleotide substitutions were found within the exonic region. Five indels were observed in the intronic region.

The Bayesian Inference analysis indicated difference, revealing two distinct clades; both clades were well-supported with probability values of 0.98 or 98% (Fig. 2). This result indicates that the Recife and Rio Preto da Eva populations are more closely related evolutionarily, which reinforces the possibility that Recife individuals are ancestrally linked to individuals from Rio Preto da Eva.

Genetic-structure analysis indicated that the populations studied divide into two main subgroups, with the *ad hoc* quantity supporting the number $K = 2$. Cluster analysis corroborated the separation of the samples into two clades (Fig. 3).

Twenty-six alleles were observed across the three populations. The most frequent haplotypes were: H8, shared by 18 Manacapuru individuals; and H12, shared by 9 Recife and Rio Preto da Eva individuals (Fig. 4). The greatest number of haplotypes (15) was observed in the Recife and Rio Preto da Eva populations, which shows that these populations possess a higher level of genetic diversity than the Manacapuru population.

Intrapopulational analysis of *L. umbratilis* specimens revealed a higher level of nucleotide and haplotype diversity in specimens from Rio Preto da Eva and Recife, than in specimens from Manacapuru (Table 1). In addition, the Rio Preto da Eva and Recife populations exhibited a higher level of differentiation than the

Table 1
Neutrality tests and intra-population genetic diversity measures for each sample.

Samples	Tajima's *D*	*N*	Hd	$\pi \pm$ SE	NS	*H*	*K*
Manacapuru	−1.31653	36	0.737	0.00315 ± 0.00058	11	11	1.525
Rio Preto da Eva	0.79751	23	0.885	0.01392 ± 0.00121	18	12	6.735
Recife	0.56387	14	0.857	0.01326 ± 0.00157	16	7	6.418
Total	0.01495	73	0.909	0.01984 ± 0.00071	35	26	9.908

Tajima's *D* (*p < 0.05); *N*: sample size; HD: haplotypic diversity; $\pi \pm$ SE: nucleotide diversity and standard errors (SE); NS: number of polymorphic sites; *H*: haplotype; *K*: average number of nucleotide difference.

Table 2
Genetic differentiation among samples.

Populations		F_{st}	D_{xy}	D_a	S_s	S_f
Rio Preto da Eva	Recife	0.00000	0.01335	−0.00024	15	0
Rio Preto da Eva	Manacapuru	0.76062	0.03076	0.02223	1	6
Recife	Manacapuru	0.80522	0.03105	0.02284	1	6

F_{st}: pair-wise genetic differentiation; D_{xy}: average number of nucleotide substitutions per site between populations; D_a: number of net nucleotide substitutions per site between populations; S_s: number of shared polymorphisms between pairs of population; S_f: number of fixed differences between pairs of populations.

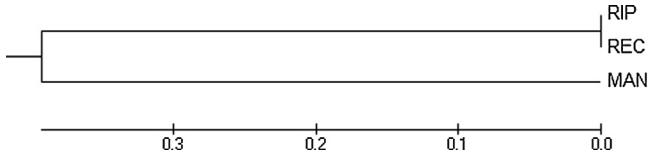

Fig. 5. AMOVA UPGMA tree for populations of *L. umbratilis*.
UPGMA tree constructed from the F_{st} values for each phenotype and their respective localities. The populations of Rio Preto da Eva (RIP) and Recife (REC) are separate from the Manacapuru (MAN) population.

Manacapuru population; this reflects the high level of genetic divergence that exists between the two clades (Table 2).

Tajima's *D* test was not significant ($P > 0.05$) for the populations studied. The haplotype fixation index (F_{st}) was significant (0.76062–0.80522) when comparing the Rio Preto da Eva and Recife populations with the Manacapuru population (Table 2)—this reflects the high level of genetic divergence between these two groups (Fig. 5).

4. Discussion

In this study, we compared individuals from Recife and Rio Preto da Eva with individuals from Manacapuru, and we observed the presence of five indels in the intron of the *period* gene. Mutations found in the intron can be neutral or they can alter gene regulations (Hoy, 2013). According to Chong et al. (2013) in several species of *Drosophila sp.* the emergence of deletions may relate primarily to fitness by creating greater adaptability among individuals. This highlights the need for studies that seek to determine the relationship between these indels and the regulation of the *period* gene, and thus to determine the role that the *period* gene has played in generating bionomic differences between populations on the north and south margins of the Amazon River.

The fixed polymorphisms observed in the *period* gene will be particularly useful for the differentiation of these three populations, as was the case for *L. longipalpis* (Costa-Junior et al., 2015; Araki et al., 2009; Bauzer et al., 2002) (Fig. 1). De Souza Freitas et al., (2015) used the mitochondrial gene *Cytochrome oxidase I* to identify 13 fixed polymorphisms in individuals from Rio Preto da Eva and Recife, thereby indicating that these population are ancestrally related.

Analysis indicated the presence of two distinct clades in *L. umbratilis*: Manacapuru (Clade I), and Rio Preto da Eva and Recife (Clade II). De Souza Freitas et al., (2015) observed similar results when they evaluated these same populations using the mitochondrial marker *Cytochrome Oxidase I* and geometric morphometrics of the wing. The presence of a clade formed by Rio Preto da Eva and Recife individuals suggests that there was a continuum of intercrossing between the North and Northeast populations, as suggested by Ready et al. (1998). However, ecological vicariance could have contributed to the segregation of these populations, primarily via geological and climatic changes—a phenomena that has been observed among other organisms in the Amazon region (Haffer, 2008).

Highly significant F_{st} values (0.76062–0.80522) were observed when comparing the Rio Preto da Eva and Recife populations with the Manacapuru population (Table 2)—this reflects a high level of genetic divergence between these two groups. In the species *Lutzomyia intermedia* and *L. whitmani* significant F_{st} values were not observed in analyses using the *period* gene (Mazzoni et al., 2006). The high genetic divergence observed between Amazonian populations of *L. umbratilis* suggests that a long period of isolation along opposite margins of the river has contributed to the fact that the Rio Preto da Eva and Recife populations have a more closely related evolutionary history. According to Ting et al. (2000); genes involved in reproductive isolation, such as the *period* gene, may record the phylogenetic history of closely related groups more consistently.

In the taxonomic analysis of the *L. umbratilis* complex, phylogenetic analysis showed that the utilized *period* marker had a high discriminatory capacity; thereby demonstrating the presence of two clades within the populations from the Central Amazon and the Northeast Region. Genes controlling the production of acoustic signals during courtship are useful molecular markers for studying the speciation process, because these "lovesongs" are an important indicator of sexual behavior and reproductive isolation in many insect species (Lins et al., 2008). Genes controlling acoustic signals were used previously to study *L. longipalpis*, *L. whitmani*, *L. intermedia* with considerable efficiency (Costa-Junior et al., 2015; Mazzoni et al., 2006). The applicability of this nuclear marker was bolstered by statistical support from the maximum likelihood tree, which detected the presence of two clades for *N. umbratilis*.

5. Conclusions

Our results indicate the presence of two distinct clades in *L. umbratilis*: Manacapuru (Clade I), and Rio Preto da Eva and Recife (Clade II). Genetic analysis suggests that the Recife and Rio Preto da Eva populations are ancestrally linked. The genetic differentiation detected in this study using the *period* gene was also observed in studies using morphological, genetic and biologic analysis (De Souza Freitas et al., 2015; Justiniano et al., 2004). These results contribute to knowledge of the *L. umbratilis* complex in Brazil, and they further the understanding of the phylogenetic relationships that exist between populations in the Northern and Northeastern regions.

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