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Cytochrome Oxidase I as Tool to Evaluate the *Lutzomyia longipalpis* Complex: Useful Molecular Marker or Not?

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Abstract

In Latin America, *Lutzomyia longipalpis* is the principal vector of *Leishmania infantum*, and is associated with the majority of cases of visceral leishmaniasis. This species has distribution from southern Mexico to northern Argentina. Although widespread, its geographical distribution is discontinuous due to its low flight capacity associated with the large number of geographical barriers. Its geographic range includes climatic and environmental discontinuities that are associated with patterns of genetic divergence. In this study, a 487 bp fragment of the *Cytochrome Oxidase I* (*COI*) mitochondrial gene was used to evaluate two sympatric populations of *L. longipalpis* in the municipalities from Sobral (CE-1S and 2S) and Caririaçu (CE-1S and 2S) from Northeast, Brazil. A combination of probabilistic methods such as maximum likelihood and Bayesian Inference were applied to populations studied. Phylogenetic analysis revealed the presence of a single monophyletic clades composed of *L. longipalpis* morph types 1S and 2S. These results suggest that mitochondrial gene *COI* is not able to evaluate the genetic structure of the complex *L. longipalpis*.

Keywords

Lutzomyia longipalpis; Cytochrome Oxidase I; Phylogenetic analysis

Introduction

Lutzomyia longipalpis sensu lato, the principal vector of *Leishmania infantum*, the etiologic agent of American visceral leishmaniasis, has a low flight capacity and discontinued distribution throughout the Neotropical region exhibiting bionomics and genetics features compatible with a species complex [1-3]. In Brazil, *L. longipalpis* complex was first proposed by Manga Beira [4] and was based on the number of abdominal male spots (1 spot and 2 spot) on tergites. Subsequently, analysis of sex pheromones and male "lovesongs" that are produced during copulation supported the existence of a species complex in *L. longipalpis* [5-9]. These two features can have an important role in the reproductive isolation among closely related species.

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The presence of cryptic species in *Lutzomyia longipalpis* was supported initially by studies using morphological, isozyme and *Cytochrome oxidase I* (*COI*) molecular marker in populations from Central and South America leading to the identification of *L. pseudolongipalpis* [10], formally recognized as the first taxon of the *L. longipalpis* complex [11]. Genetic analyses were also realized using microsatellite markers and speciation genes (*period* gene), have provided further evidence of a species complex [3]. Thereafter, Vigoder [12] suggested that *L. cruzi* should be regarded as a cryptic species within the *L. longipalpis* complex based in copulation songs and *period* gene analysis.

Mitochondrial DNA (mt DNA) has proven useful in molecular phylogenetics of several subfamilies of vectors insects (Table 1) due to its maternal inheritance, rapid rate of divergence and lack of recombination [13-18]. Phylogenetics analysis realized on the gene *COI of L. longipalpis* revealed the existence of four clades in Latin America for this species [19]. However, little is known about the ability of this molecular marker evaluating the *L. longipalpis* complex. In light of this possibility, this study aimed to assess the phylogenetic structure of populations *L. longipalpis* from the state of Ceará using the *COI mitochondrial* gene. This mitochondrial gene (*COI)* has been implemented in many evolutionary studies of species of the subfamily Phlebotominae.

Field Collection

Field collections were done in Sobral (3°41′15″S; 40°21′5″W) and Caririaçu (07°02′31″S; 39°17′02″W), both located in the Ceará State, Northeast Region of Brazil. Sand flies were trapped in the surrounding houses and domestic animal shelters using five CDC-type miniature light traps. Sand flies were identified according to Young and Duncan [20], and *L. longipalpis sensu lato* males were separated based on the number of abdominal spots into 1S and 2S. Genomic DNA extraction was carried out using Chelex®100 (BioRad, Berkeley, California, USA), according to Costa-Junior [21]. A fragment of 487 bp of the *Cytochrome Oxidase I* (*COI)* was amplified by PCR using the universal primers described by Simon [14]. Amplification reactions were done using the Mix Go Taq Colorless kit, according to manufacturer specifications (Promega® Fitchburg, Wisconsin, USA). PCR products were visualized in 1% agarose gel through UV light and posteriorly purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega® Fitchburg, Wisconsin, USA). Sequencing was carried out in ABI 3500 automatic sequencer (Applied Biosystems, Cleveland, Ohio, USA). Only sequences with a Phred score above 30 were used in the analysis. Contigs assembly was carried out using Codon Code Aligner (Codon Code Corporation). Local alignments were done using BLAST [22]. All new sequences produced in this study have been deposited in GenBank under accession numbers: KT806399 to KT806475.

Nucleotide sequences were aligned using Muscle [23] incorporated in MEGA v. 5.0 [24]. Phylogenetic analysis was carried out with the Maximum Likelihood criterion using PhyML [25]. The consistency of the branches was assessed using 1000 bootstrap replicates. We use also the Bayesian inference (BI) analysis, which was implemented with Mr Bayes [26]. BI analysis included two simultaneous independent

Table 1: Evolutionary studies of sandflies using mitochondrial marker *Cytochrome Oxidase I*.

Genus	Species	Journal	Methods	Status
Lutzomyia	*Lutzomyia anduzei*	Parasit Vectors	Neighbor Joining	Population genetic
-	*Lutzomyia umbratilis*	Parasit Vectors	Maximum Likelihood	Population genetic
-	*Lutzomyia shannoni*	J Med Entomol	Maximum Likelihood	Population genetic
-	*Lutzomyia ayacuchensis*	Acta trop	Maximum Likelihood	Population genetic
-	*Lutzomyia longipalpis*	Infect Genet Evol	Neighbor-joining	Phylogeography
-	*Lutzomyia andina*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia nevesi*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia serrana*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia robusta*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia longiflocosa*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia quasitownsendi*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia sauroida*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia spinicrassa*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia torvida*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia youngi*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia walkeri*	Am J Trop Med Hyg	Maximum Likelihood	Phylogeny
-	*Lutzomyia shannoni*	J Med Entomol	Neighbor-joining	Molecular identification
-	*Lutzomyia trapidoi*	Parasit Vectors	Neighbor-joining	Population genetic
-	*Lutzomyia trapidoi*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia vespertilionis*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia panamensis*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia camposi*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia carpeteri*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia gomezi*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia olmeca*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia ovallesi*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia carrerai thula*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia sanguinaria*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia aclydifera*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia dysponeta*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia triramula*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia guyanensis*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia runoides*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia trinidadensis*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia galindoi*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
-	*Lutzomyia hamata*	PLoS Negl Trop Dis	Neighbor-joining	Phylogeny
Sergentomyia	*Sergentomyia minuta*	Acta trop	Bayesian inference	Molecular identification
Euphlebotomus	*Phlebotomus perniciosus*	Acta trop	Bayesian inference	Molecular identification
Euphlebotomus	*Phlebotomus sergenti*	Acta trop	Bayesian inference	Molecular identification
Euphlebotomus	*Phlebotomus ariasi*	Acta trop	Bayesian inference	Molecular identification
Euphlebotomus	*Phlebotomus barguesae*	Parasit Vectors	Neighbor-joining	Molecular identification
Euphlebotomus	*Phlebotomus argentipes*	Parasit Vectors	Maximum likelihood	Molecular identification

runs of the Markov Chain Monte Carlo (MCMC) for 100 million generations, sampling every 1,000 generations with a burn-in of 25%. *Phlebotomus sergenti* was used as the out group for both phylogenetic methods.

Conclusion

We obtained 77 sequences of the gene *COI from L. longipalpis* male, being 59 (29 1S and 30 2S) from Caririaçu and 18 (101S and 82S) from Sobral. The Maximum Likelihood analysis (Figure 1) and Bayesian Inference (Figure 2) suggested a single genetic group associated with 1S and 2S morph types, and was unable to indicate the ancestral relationship and geographic origin of populations. In a previous study using the *period* speciation gene was detected two subgroups (1S and 2S) linked with abdominal spots in *L. longipalpis* from Sobral, Caririaçu and Bodocó municipalities [27]. According with Costa-Junior [21], the *per* gene can be used as a barcode to *L. longipalpis* since it could also indicate the ancestral relationship between individuals with 1S and 2S based on polymorphic sites fixed in the two morpho types [21]. In our analysis were not observed

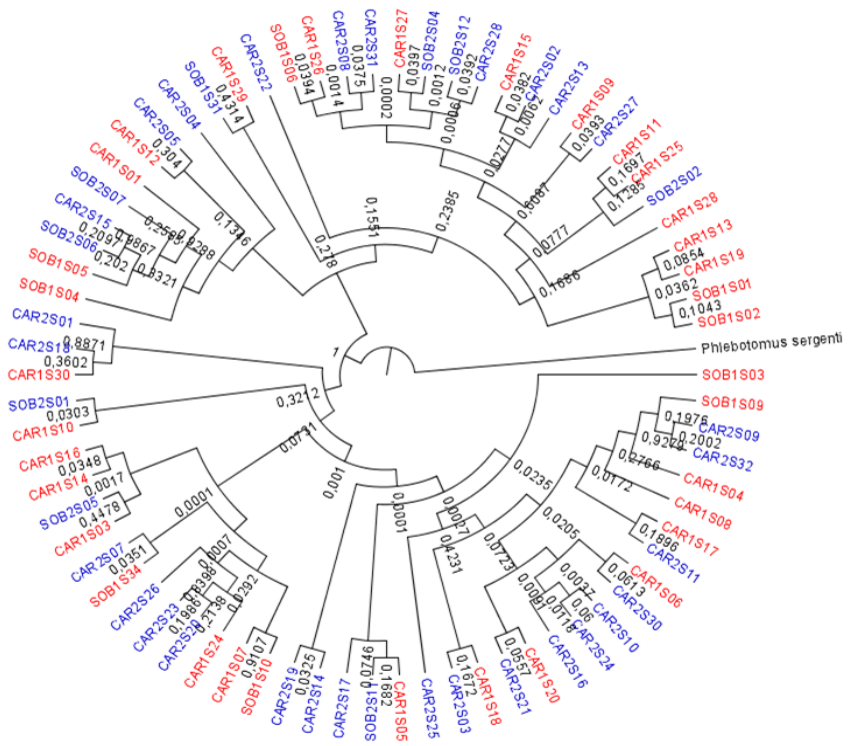

Figure 1: Maximum likelihood tree obtained of the TVM+ I+G model has shown the results of using 487 bp from *Lutzomyia longipalpis COI* marker. The localities of Caririaçu (CAR) and Sobral (SOB), State of Ceará, Brazil.

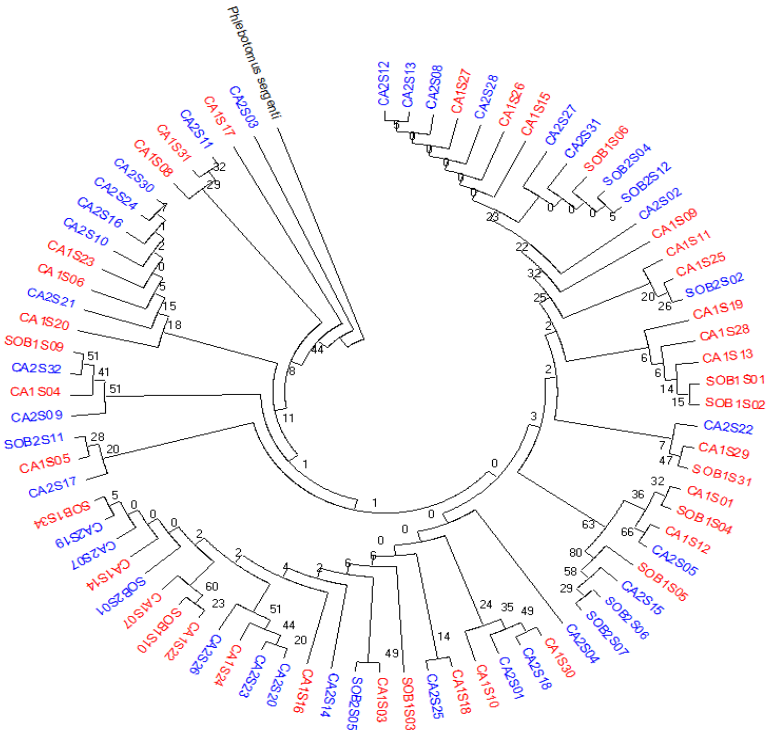

Figure 2: Bayesian Inference (BI) topology tree obtained of the HKY + I model has shown the results of using 487 bp from *Lutzomyia longipalpis COI* marker. The localities of Caririaçu (CAR) and Sobral (SOB), State of Ceará, Brazil.

similar results to previous studies when used the speciation gene *period* suggesting that the mitochondrial gene *COI* do not have appropriate features to analyze the complex *Lutzomyia longipalpis*. The lack of sub structuring observed between sympatric populations of *L. longipalpis* using mitochondrial gene *COI* is probably related to evolutionary factors e.g. speciation incipient, natural selection, genetic drift and genetic introgression. Despite this molecular marker *(COI)* has proven effective in the evolutionary approaches of various species of sand flies (Table 1), in our study, the results indicate a lower sensitivity of this *COI* marker to assess the sympatric populations of the municipalities of Sobral e Caririaçu, Ceará State, Northeast Region of Brazil. Thus, we concluded that the use of other molecular markers (e.g. markers *period* and *paralytic*) is necessary to evaluate the sub structuring of sympatric populations of the complex *L. longipalpis* [21,28].

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