

Courtship Song Genes and Speciation in Sand Flies

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Lutzomyia longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) is a vector of visceral leishmaniasis in the Americas and it might represent a complex of sibling species. Reproductive isolation between closely related species often involves differences in courtship behaviour. *cacophony* (*cac*) and *period* (*per*) are two *Drosophila* genes that control features of the "lovesong" males produce during courtship that has been implicated in the sexual isolation between closely related species. We are using gene fragments from *L. longipalpis*' homologues of these two genes to study the speciation process in this putative species complex.

Key words: *Lutzomyia longipalpis* - *cacophony* - *period* - polymorphism

Genes controlling aspects of reproductive behaviour are among the best molecular markers in the study of speciation in insect vectors. In *Drosophila*, males vibrate their wings during courtship producing a kind of "lovesong". This acoustic signal is potentially involved in the reproductive isolation between closely related species (Kyriacou & Hall 1982, 1986, Ritchie et al. 1999). Among the genes controlling this "lovesong" (Hall 1994), *cacophony* (*cac*) and *period* (*per*) are particularly interesting as they control features of the song that are known to vary between species (see below).

cac codes for a voltage-gated calcium channel α -1 subunit, a large protein that forms the pore of the channel. *cac* is a very large (over 45kb) and complex gene that presents alternative splicing and RNA editing of its transcript (Smith et al. 1996, 1998a, b, Peixoto et al. 1997). It is also the site of song (*cac*^S), visual (*night-blind-A*) and lethal (*L13*) mutations. In vertebrates, voltage-gated calcium channels are divided in many different classes according to their electrophysiological characteristics, pharmacology, sequence similarities and tissue distribution (Stea et al. 1995). *cac*, also known as *Dmca1A*, shows homology to vertebrate calcium

channel classes A, B and E. The song produced by its mutant allele *cac*^S is characterized by longer interpulse-intervals (IPIs) and pulses that contain more cycles than normal (Schilcher 1977, Kulkarni & Hall 1987, Peixoto & Hall 1998). These are two features that commonly show differences among various *Drosophila* species (e.g. Hoikkala & Lumme 1987, Ritchie & Gleason 1995).

The clock gene *per* controls a different feature of the "lovesong" of *Drosophila*. *D. melanogaster* and related species (Kyriacou & Hall 1980, Demetriades et al. 1999) present cyclic variation in their mean IPI. These song rhythms are species-specific and they have been implicated as one of the signals females used to recognise conspecific males (Kyriacou & Hall 1982, 1986, Ritchie et al. 1999). Because *per* controls the differences in song rhythms that seem to contribute to the reproductive isolation between *D. melanogaster* and its sibling species *D. simulans* (Wheeler et al. 1991), it has been called a "speciation gene" (Coyne 1992).

Acoustic communication is also a feature of the courtship behaviour of *Lutzomyia longipalpis* (Ward et al. 1988), a vector of visceral leishmaniasis in the Americas and a putative complex of sibling species (Ward et al. 1988, Lanzaro et al. 1993, Alexander et al. 1998, Uribe 1999). Using PCR with degenerated primers, we cloned and sequenced gene fragments from *L. longipalpis*' putative homologues of *per* and *cac*. In the case of *per*, the fragment is nearly 1 Kb long and includes a small intron (~50 bp). It encodes the end of the PAS protein dimerisation domain and the Thr-Gly repeat region. The *cac* fragment is ~270 bp and

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encodes the IVS6 domain of the channel. It also includes an intron (~100 bp). Based on these first sequences we designed more specific oligos for this species and we are now using them to study the molecular variation and differentiation among populations of the *L. longipalpis* complex in Brazil.

Figure shows the first results we obtained with the analysis of the IVS6 region of *cac* in the population of Natal (State of Rio Grande do Norte). Length variation was observed among PCR fragments amplified from different individuals. Sequencing of these fragments revealed a number of differences due to insertion/deletion events and point mutations within the intron in this region of *L. longipalpis' cac* (Figure). Preliminary analysis of a number of other sand flies from Natal and from the population of Lapinha (Lagoa Santa, State of Minas Gerais) confirms that this intron of *cac* is highly polymorphic and indicates that the two populations are quite differentiated. For example, the Table shows the frequencies of haplotypes associated with two major deletions found in this intron. Significant differences are observed between the populations of Natal and Lapinha ($X^2 = 10.26$; d.f. = 2; $p = 0.0059$). The haplotype associated with deletion 1 has a frequency of 36.4% (8/22) in Natal but has yet to be observed in Lapinha, while in this latter population the haplotype associated with deletion 2 is fairly common (30%; 6/20), although it was not found in Natal.

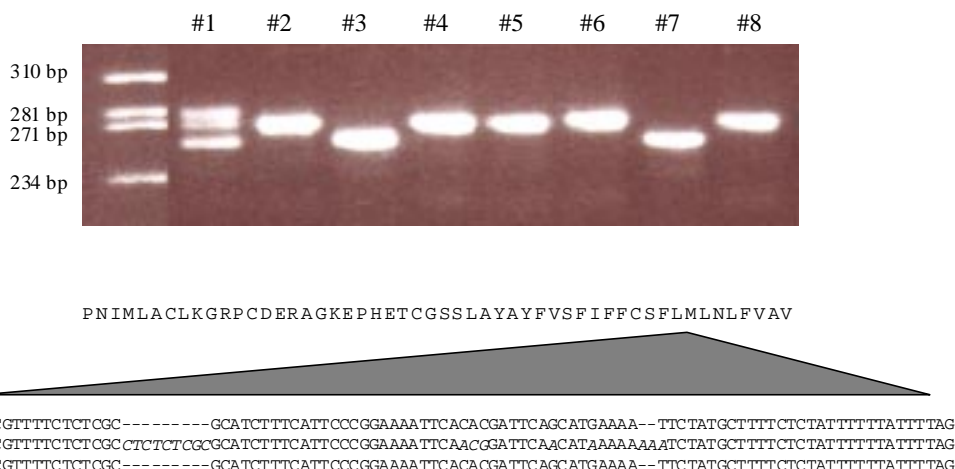
We also started the analysis of the molecular variation in a region of the *per* gene fragment of *L. longipalpis* in the populations of Lapinha and Jacobina (State of Bahia). The first results also in-

TABLE
cacophony haplotype frequencies in two populations of *Lutzomyia longipalpis*

Haplotypes	Natal	Lapinha
del I	36.3%	-
del II	-	30%
others	63.7%	70%
Total	22	20

dicating that these two populations are quite differentiated. We sequenced a 266 bp fragment in 35 individuals (17 of Lapinha and 18 of Jacobina). Of a total of 33 polymorphic sites found so far, only six are shared between the two populations. The calculated *Fst* for the data is 0.3911 (Hudson et al. 1992, Rozas & Rozas 1999). This figure predicts only 0.39 migrants per generation (*Nm*), a value nearly ten times smaller than the ones predicted by isoenzyme loci (Mukhopadhyay et al. 1998, Mutebi et al. 1999, Azevedo et al. 2000).

The differentiation between the Natal, Lapinha and Jacobina populations at the molecular level is supported by the first results obtained with the analysis of the “lovesongs” produced by *L. longipalpis* males from these very same populations (Souza & Peixoto, unpublished), and raises the question of whether they represent different sibling species. Cryptic speciation is one of the most interesting phenomena in evolution and its occurrence in phlebotomine sand flies has important epidemiological consequences (Lanzaro & Warburg 1995). The results obtained so far with



Length polymorphism in the IVS6 intron of *cacophony* in *Lutzomyia longipalpis*. Top: agarose gel (NuSieve) showing the length variation among PCR amplified fragments from different *L. longipalpis* males. Note that sand fly #1 is heterozygous for two different length variants. Bottom: amino acid sequence of the IVS6 region and intron sequences of some of the PCR fragments showed on top.

the analysis of the molecular variation in *cac* and *per* suggest that courtship song genes will be very useful to enhance our knowledge of the speciation process in the *L. longipalpis* complex.

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