Molecular analysis of an odorant-binding protein gene in two sympatric species of *Lutzomyia longipalpis s.l.*

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Lutzomyia longipalpis s.l. *is the main vector of American visceral leishmaniasis (AVL) and occurs as a species complex. DNA samples from two Brazilian sympatric species that differ in pheromone and courtship song production were used to analyse molecular polymorphisms in an odorant-binding protein (*obp29*) gene. OBPs are proteins related to olfaction and are involved in activities fundamental to survival, such as foraging, mating and choice of oviposition site. In this study, the marker* obp29 *was found to be highly polymorphic in* Lu. longipalpis s.l.*, with no fixed differences observed between the two species. A pairwise fixation index test indicated a moderate level of genetic differentiation between the samples analysed.*

Key words: *Lutzomyia longipalpis s.l.* - odorant-binding proteins - leishmaniasis

The sandfly *Lutzomyia longipalpis s.l.* is the main vector of American visceral leishmaniasis (AVL) and occurs as a complex of cryptic species (Bauzer et al. 2007, Araki et al. 2009). A number of integrative studies using different approaches, such as molecular polymorphisms (Bauzer et al. 2002a, b, Bottecchia et al. 2004, Araki et al. 2009, Lins et al. 2012), microsatellites (Maingon et al. 2003), pheromones (Hamilton et al. 1999a, b, Souza et al. 2004) and courtship song analysis, have revealed the existence of at least five species in Brazil (Souza et al. 2002, Araki et al. 2009). *Lu. longipalpis s.l.* males exhibit morphological polymorphism with regard to the number of pale abdominal tergal spots and can present just one pair of pale spots on the fourth abdominal tergite (1S phenotype) or two pairs of pale spots, one on the third and the other on the fourth abdominal tergite (2S phenotype). Intermediate phenotypes (a small spot on the 3rd tergite in addition to the spot on the 4th tergite) are observed in high frequencies in some localities, indicating an intraspecific polymorphism (Ward et al. 1988). In contrast, intermediate forms are rare or nonexistent in localities where two *Lu. longipalpis s.l.* cryptic species occur in sympatry (Bauzer et al. 2002a, Araki et al. 2009). These tergal spots contain pheromone glands that are associated with *Lu. longipalpis* sexual communication (Lane et al. 1985). In the Brazilian locality of Sobral, state of Ceará, a sympatric species identified by the 2S phenotype was shown to produce the cembrene-1 (C_{20})-type pheromone, whereas a species associated with the 1S phenotype was shown to produce the 9-methylgermacrene-B (C_{16})-type pheromone (Lane et al. 1985, Hamilton et al. 1999a, b).

Proteins related to olfaction are involved in activities that are fundamental to survival, such as foraging, courtship, mating and choice of oviposition sites (Hallem & Carlson 2004). Among these molecules, odorant-binding proteins (OBPs) have been described as important components in the recognition of odours (Hekmat-Scafe et al. 2002) and several OBPs from different insect species have been cloned and sequenced (Xu et al. 2003, Zhou et al. 2004, 2008). Furthermore, an increasing number of OBPs have been identified in several non-sensorial tissues, such as salivary glands (Abdeladhim et al. 2012), head and body (Li et al. 2005, González-Caballero et al. 2013), reproductive organs (Azevedo et al. 2012), fat bodies and seminal fluid (Liu et al. 2010, Sirot et al. 2011). The wide distribution of these proteins in the organism suggests that they can perform several other physiological functions (Pelosi et al. 2006, Pelletier & Leal 2009). Phylogenetic analyses and the distribution and orientation of OBP genes in insect genomes have revealed evidence of a complex series of duplication and rearrangement events, facts that suggest that this gene family evolves rapidly (Hekmat-Scafe et al. 2002, Vieira et al. 2007) and are therefore potentially good markers for use in population genetic studies.

In this study, we analysed polymorphisms in the *obp29* gene to characterise the molecular variation of sympatric species of *Lu. longipalpis s.l.* and to hypothesise a possible role of the encoded OBPs in the reproductive isolation and adaptation of this important insect vector.

The genomic DNA from samples collected in the Brazilian locality of Sobral (3º41'S 40º20'W) was the same as that used by Bauzer et al. (2002a.) Genes that were isolated from *Lu. longipalpis s.l.* and searched

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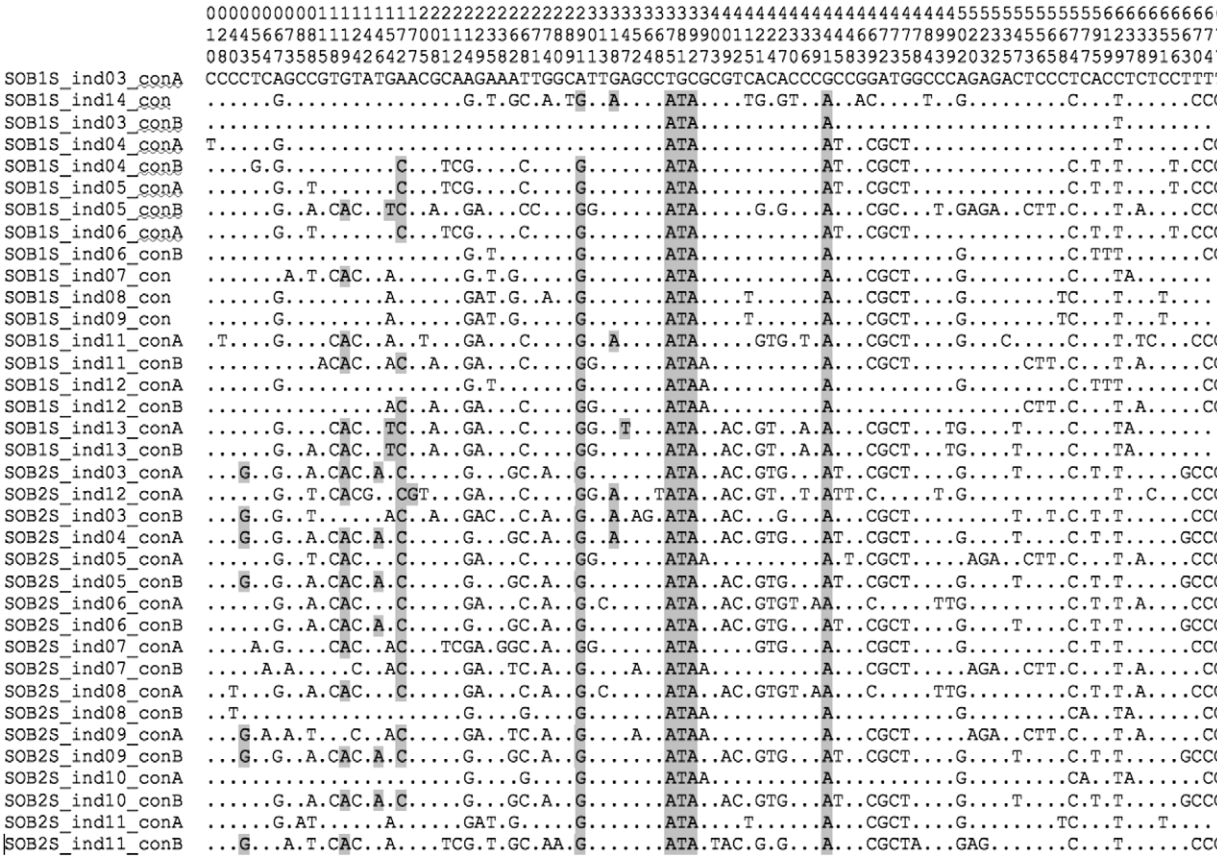
for using the Antennae EST database developed in our laboratory (Dias 2008) directed the choice of specific oligonucleotide primers for the *obp29* gene. Polymerase chain reaction (PCR) reactions were performed according to the Go Taq DNA Polymerase instructions manual (Promega) using a thermocycler (GeneAmp PCR System 9700/Applied Biosystems) with forward (5'-GGGAGTGAGAAAAATGAGATCAAA-3') and reverse (5'-ACTTGACATTGCTTTTCTGTGCAGG-3') OBP29 primers. The following programme was used: 30 cycles of 95ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min. The obtained fragments were purified using the QIAquick PCR Purification kit (QIAGEN) and cloned using the pGEM–T Vector kit (Promega). Plasmid DNA was isolated using the mini-preparation alkaline lysis method (Sambrook & Russel 2001) in 96-well MicroWell Plates and the DNA was cleaned using filter plates (Multiscreen-Millipore). Sequencing of the cloned fragments was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction v.3.1 kit (Applied Biosystems) and the ABI 3730 Sequencer by the Program on Technological Development of Health (Fiocruz, Rio de Janeiro, Brazil) sequencing service (Otto et al. 2008). The obtained sequences were edited using the Bioedit v.7.0.5 programme (Hall 1999) and an alignment was performed using the CLUSTALW full multiple alignment option within the BioEdit software. Different measures of genetic polymorphism were calculated using DnaSP v.5 (Librado & Rozas 2009), including the number of segregating sites (S), the nucleotide diversity pi (π) which is the average number of nucleotide substitutions per site between any two sequences and the parameter theta (θ), which is estimated from the number of segregating sites. The estimated π and θ parameters were used to perform Tajima's D test of neutrality (Tajima 1989). The Hudson, Kreitman & Aguadé neutrality test (HKA) was performed using the *period* gene (Bauzer et al. 2002a) as the second *locus*. Both neutrality tests were executed using the DnaSP v.5 software (Librado & Rozas 2009). A population structure analysis was conducted by separating two groups of sequences based on the abdominal spot phenotype (1S or 2S). The population subdivision was calculated using the programme ProSeq3.0 (Filatov 2002) and the significance of the pairwise fixation index test was estimated by a permutation approach. The sequences were submitted to GenBank (accessions KF669570-KF669605).

A 685-bp *opb29* fragment encompassing the coding region and part of the 3' UTR was obtained. A total of 18 consensus sequences were analysed from the Sobral 1S and 2S populations. Of the 685 analysed nucleotides sites, 93 (13.6%) were variable and the nucleotide diversi-



Alignment of the variable sites in the *Lutzomyia longipalpis s.l. odorant-binding protein 29* haplotypic sequences. Dots indicate nucleotides shared with the first sequence. Positions of the polymorphic sites are indicated on the top of the figure. Non synonymous sites are shown in gray.

ty estimation was similar for the two species analysed (π = 0.035 for Sobral 1S and π = 0.0331 for Sobral 2S). Similarly, the parameter θ was found to be essentially the same for the two species (0.0310 and 0.0309 for Sobral 1S and Sobral 2S, respectively). Figure shows an alignment of all the polymorphic sites observed in the analysed *opb29* fragment. Twenty three polymorphic sites were exclusive to Sobral 1S, 24 were exclusive to Sobral 2S and 54 were shared between the species; non-synonymous mutations were found at 12 sites (6 exclusive to S1S, 3 exclusive to S2S and 3 shared). A moderate and significant genetic differentiation (Fst = 0.1098; $p < 0.001$; 1,000 permutations) was computed between the two species. The genetic divergence and polymorphism data were used to test departures from neutrality, with both Tajima's D statistics (Tajima 1989) and the HKA test (Hudson et al. 1987) indicating no departure from neutrality.

The performed molecular analysis revealed no fixed differences between the two analysed populations. Although some exclusive synonymous and non-synonymous mutations were found, a clearer pattern of differentiation was not obtained. Therefore, we cannot infer a possible role of the *obp29* gene in responses to specific pheromones or in the process of reproductive isolation. The level of genetic differentiation was not as high as that observed for other nuclear markers (Bauzer et al. 2002a, b, Bottecchia et al. 2004, Araki et al. 2009, Lins et al. 2012). This finding can be explained by the recent origin of these species within the *Lu. longipalpis* complex and the consequent retention of ancestral polymorphisms. Alternatively, one might also consider introgression events if reproductive barriers allowed rare events of hybridisation. Future studies expanding this analysis to other *obp* genes might help in the elucidation of a putative functional role of OBPs in the speciation process occurring in *Lu. longipalpis s.l.*

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