New Molecular Markers for Phlebotomine Sand Flies

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Abstract

Using degenerate-primers PCR we isolated and sequenced fragments from the sand fly *Lutzomyia longipalpis* homologous to two behavioural genes in *Drosophila*, *cacophony* and *period*. In addition we identified a number of other gene fragments that show homology to genes previously cloned in *Drosophila*. A codon usage table for *L. longipalpis* based on these and other genes was calculated. These new molecular markers will be useful in population genetics and evolutionary studies in phlebotomine sand flies and in establishing a preliminary genetic map in these important leishmaniasis vectors.

Keywords: *Lutzomyia*, *cacophony*, *period*, molecular markers
Sand flies of the genus *Lutzomyia* (Diptera:Psychodidae:Phlebotominae) are vectors of leishmaniasis in the Americas (Young and Duncan, 1994; Tesh and Guzman, 1996). Despite their medical importance, there is a paucity of molecular markers in the species of this genus and related genera. So far, only a handful of genes such as those expressed in salivary glands have been characterised (Charlab et al., 1999). Behaviour is one aspect of sand fly biology where no molecular data is yet available. One area where behavioural genes are particularly important is in speciation. Studies of some of the most important vectors species, such as *Lutzomyia longipalpis*, suggest that they might represent complexes of cryptic species (Ward et al., 1988; Lanzaro et al., 1993; Lampo et al., 1999; Uribe, 1999). The occurrence of sibling species in sand flies has important epidemiological consequences (Lanzaro and Warburg, 1995) as they seem to differ in vector competence, habitat (peri-domestic x silvatic) and host preference (anthropophily x zoophily).

One of the most important forms of reproductive isolation between closely related species involves differences in courtship behaviour. In a great number of *Drosophila* species, males vibrate their wings during courtship producing a lovesong (Hall, 1994) that seems to increase the receptivity of females and has been implicated as one of the signals they used to recognise conspecific males (Kyriacou and Hall, 1982; 1986). This lovesong is therefore potentially important in the reproductive isolation between closely related species and, as a consequence, in the process of speciation. A few *Drosophila* genes controlling the courtship song have been identified and cloned (Hall, 1994).

*cacophony* is a voltage-gated calcium channel gene, also known as *Dmca1A* (Smith et al., 1996; 1998; Peixoto et al., 1997), that shows homology to mammalian calcium channel class A (Stea et al., 1995) and related channels B and E. *cacophony* is particularly interesting from an evolutionary point of view because one of its mutant alleles produces a song that resembles those of other *Drosophila* species (Peixoto and Hall, 1998). Using degenerate primer PCR we isolated a sand fly fragment homologous to the *Drosophila cacophony* gene. Fig. 1 shows the amino acid sequence encoded by a PCR fragment amplified from *L. longipalpis*, aligned to the corresponding region of the *Drosophila melanogaster cacophony* and other calcium channels. This fragment is ~270 bp and encodes the IVS6 domain of the channel. It also includes an intron (~100 bp). Fig. 2 shows a phylogenetic tree where the amino acid sequence of the *L. longipalpis* fragment is compared to other mammalian and *Drosophila* calcium channels, and clusters with the sand fly homologue of *Drosophila*’s *cac*.

Another locus that also has an effect in courtship is *period*, a gene encoding a clock protein that has been shown to control species specific courtship song rhythm differences between *D. melanogaster* and its sibling species *Drosophila simulans* (Kyriacou and Hall, 1986, Wheeler et al., 1991). *period* also controls circadian rhythms of activity in *Drosophila* (Konopka and Benzer,
1971). The behaviour of insect vector species plays an important role in the dynamics of the diseases they transmit (Klowden, 1996). The rhythmic patterns of activity and feeding presented by haematophagous insects, which are so important to disease transmission, are certainly controlled by the internal biological clock (Saunders, 1982), which is in turn under genetic control (Dunlap, 1999). Using the same strategy mentioned above we isolated a sand fly fragment homologous to the *Drosophila period* (*per*) gene. Fig. 3 shows an alignment of the putative amino acid sequences encoded by PCR fragments obtained from *Lutzomyia longipalpis* and the same region of the *per* gene of *Drosophila virilis*. The sand fly fragment is nearly 1 Kb long, includes an intron and was obtained using two pairs of degenerate primers that amplified two overlapping fragments. Its predicted amino acid sequence extends from the end of the PAS protein dimerisation domain to the Thr-Gly repeat region. These two domains are particularly important to *period* function and evolution (Costa and Kyriacou, 1998; Dunlap, 1999).

In addition to these two behavioural genes, a number of other fragments were isolated which show homology to genes previously cloned in *Drosophila*. Table 1 gives a list of nine sand fly molecular markers (including *period* and *cacophony*) for which highly significant similarity to *Drosophila* genes were observed in BLASTX (Altschul et al., 1997) searches using the NCBI (National Center for Biotechnology Information) web page. Other fragments were also obtained, but not included as no clear putative *Drosophila* homologues were found. The table also gives the size of fragments, level of amino-acid identity and similarity of predicted encoded proteins and their putative functions in sand flies based on their role in *Drosophila*.

One molecular marker particularly interesting is fragment 6, which shows homology to *purity of essence*, a *Drosophila* gene coding for a calmodulin binding protein. Mutations in this locus affect behaviour and synaptic transmission at the neuromuscular junction. Mutant flies are sluggish and uncoordinated (Richards et al., 1996). In addition, mutant alleles also cause male sterility (Fabrizio et al., 1998). Of the nine markers listed, three were obtained from *Lutzomyia intermedia*, an important vector of cutaneous leishmaniasis in Brazil (Young and Duncan, 1994). One of these shares homology to *Dmca1D* (Zheng et al., 1995), a *Drosophila* voltage-gated calcium channel different from *cacophony* and more similar to mammalian channel class D (see figs. 1 and 2). Like *L. longipalpis cacophony* homologue, this fragment encodes the IVS6 domain of the channel and it also includes an intron. *Dmca1D* is highly expressed in the embryonic nervous system (Zheng et al., 1995). Some more severe mutations at this channel are lethal recessives and mutants die at the late embryonic stage, while hypomorphic alleles cause flies to have difficulty in eclosing from pupae (Eberl et al., 1998). It is not yet known whether this gene might have also an effect in behaviour.

Using the available *L. longipalpis* sequences from our work and the ones from Charlab et al.
(1999), we calculated the codon usage for this sand fly species. Table 2 shows the frequencies of the different codons used by L. longipalpis compared with the values observed in Drosophila melanogaster. Note that the majority of the amino acids, 15 out of 18, show differences in codon preference between the two species. This is consistent with the variation observed among different Drosophila species (Powell, 1997). Once more sequences are available, it will be possible to look at variation among genes as observed in other organisms.

We are currently attempting to isolate other sand fly molecular markers controlling different aspects of behaviour. These new molecular markers will be useful in population genetics and evolutionary studies. For example, based on the sequence of the L. longipalpis homologues of period and cacophony, we designed more specific primers to this species and we are now using them to study the differentiation among putative cryptic species of the L. longipalpis complex in Brazil (Oliveira et al, in press). We are also considering using the molecular markers available to initiate the construction of a preliminary genetic map in these important leishmaniasis vectors.

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Table 1.
New sand fly molecular markers.

| Fragment | Species       | Size (bp) | Putative Drosophila Homologue | Protein sequence similarity and identity | Putative function                        |
|----------|---------------|-----------|------------------------------|------------------------------------------|------------------------------------------|
| 1        | *L. longipalpis* | 921*      | period                       | 76% / 63%                                | transcriptional regulator (clock gene)    |
| 2        | *L. longipalpis* | 225*      | cacophony (Dmca1A)           | 87% / 82%                                | voltage-gated calcium channel α-1 subunit |
| 3        | *L. longipalpis* | 470*      | CG10046                      | 100% / 100%                              | RNA binding protein                      |
| 4        | *L. longipalpis* | 432       | CG15828                      | 51% / 34%                                | ligand binding or carrier                |
| 5        | *L. longipalpis* | 363       | CG9649                       | 51% / 36%                                | endopeptidase                            |
| 6        | *L. longipalpis* | 273       | purity of essence            | 94% / 84%                                | calmodulin binding protein               |
| 7        | *L. intermedia* | 291*      | CG3003                       | 60% / 43%                                | unknown                                  |
| 8        | *L. intermedia* | 249       | CG6726                       | 80% / 67%                                | peptidase                                |
| 9        | *L. intermedia* | 226*      | Dmca1D                       | 81% / 71%                                | voltage-gated calcium channel α-1 subunit |

* Genomic DNA of *Lutzomyia longipalpis* and *L. intermedia* was prepared according to Jowett (1998) or using the GenomicPrep from Amersham Pharmacia Biotech. From 50 ng to 100 ng of genomic DNA were used in the PCR. The reactions were carried out in a Perkin-Elmer GeneAmp PCR system 9600 for 35 cycles (95°C for 0.5 min; 37°C to 55°C for 1.0 min; 72°C to 1.0 min). In some cases reamplifications were also carried out. Different combinations of degenerate primers were used. PCR products were electrophoresed in 2% TAE agarose gels, bands were purified using the Sephaglas BandPrep kit Band (Amersham Pharmacia Biotech) and cloned into pMOSBlue vector. Sequencing was done automatically at Fundação Oswaldo Cruz (Rio de Janeiro, Brazil) and at University of Leicester (UK). The GCG software (Version 7, 1991) was used for DNA sequence editing and analysis. These fragments include putative intron sequences. Protein sequence similarity and identity values were obtained from BLASTX (Altschul et al., 1997) searches and they are based on comparisons to *Drosophila melanogaster* in all cases, except for period where the sequence of *D. virilis* was used.
The frequencies are calculated from a total of 3683 codons. Values in brackets are from *D. melanogaster* (Powell, 1997) and they are shown here for comparison. The most frequent used codons in both species are in italics.

**Table 2**
Codon usage in *L. longipalpis*

| Amino Acid | Codon | Frequency* | Amino Acid | Codon | Frequency |
|------------|-------|------------|------------|-------|-----------|
| Gly        | GGG   | 0.21 (0.06) | Ser        | AGU   | 0.18 (0.12) |
| Gly        | GGA   | 0.43 (0.28) | Ser        | AGC   | 0.18 (0.24) |
| Gly        | GGU   | 0.26 (0.23) | Ser        | UCG   | 0.08 (0.22) |
| Gly        | GGC   | 0.10 (0.43) | Ser        | UCA   | 0.22 (0.09) |
|            |       |            |            |       | 0.14 (0.08) |
| Glu        | GAG   | 0.35 (0.71) | Ser        | UCC   | 0.19 (0.25) |
| Glu        | GAA   | 0.65 (0.29) | Arg        | AGG   | 0.17 (0.10) |
| Asp        | GAU   | 0.66 (0.52) | Arg        | AGA   | 0.25 (0.08) |
| Asp        | GAC   | 0.34 (0.48) | Arg        | CGG   | 0.04 (0.14) |
|            |       |            |            |       | 0.15 (0.14) |
| Val        | GUG   | 0.22 (0.47) | Arg        | CGU   | 0.22 (0.18) |
| Val        | GUA   | 0.16 (0.10) | Arg        | CGC   | 0.16 (0.35) |
| Val        | GUU   | 0.48 (0.18) |            |       |            |
| Val        | GUC   | 0.14 (0.25) | Gln        | CAG   | 0.47 (0.73) |
|            |       |            |            |       | 0.53 (0.27) |
| Ala        | GCG   | 0.09 (0.18) |            |       |            |
| Ala        | GCA   | 0.36 (0.16) | His        | CAU   | 0.71 (0.34) |
| Ala        | GCU   | 0.34 (0.19) | His        | CAC   | 0.29 (0.66) |
| Ala        | GCC   | 0.21 (0.47) |            |       |            |
| Lys        | AAG   | 0.47 (0.75) | Leu        | UUG   | 0.21 (0.18) |
| Lys        | AAA   | 0.53 (0.25) | Leu        | UUA   | 0.11 (0.04) |
|            |       |            |            |       | 0.13 (0.08) |
| Asn        | AAU   | 0.74 (0.42) | Leu        | CUU   | 0.20 (0.09) |
| Asn        | AAC   | 0.26 (0.58) | Leu        | CUC   | 0.20 (0.16) |
| Ile        | AUA   | 0.17 (0.16) | Pro        | CCG   | 0.15 (0.30) |
| Ile        | AUU   | 0.54 (0.32) | Pro        | CCA   | 0.44 (0.24) |
| Ile        | AUC   | 0.30 (0.52) | Pro        | CCU   | 0.24 (0.11) |
|            |       |            |            |       | 0.17 (0.35) |
| Thr        | ACG   | 0.25 (0.24) | Phe        | UUU   | 0.52 (0.33) |
| Thr        | ACA   | 0.35 (0.19) | Tyr        | UAU   | 0.45 (0.34) |
| Thr        | ACU   | 0.23 (0.15) | Tyr        | UAC   | 0.55 (0.66) |
| Thr        | ACC   | 0.17 (0.42) |            |       |            |
| Cys        | UGU   | 0.49 (0.27) | Phe        | UUC   | 0.48 (0.67) |
| Cys        | UGC   | 0.51 (0.73) | End        | UAG   | 0.10 (0.28) |
| Met        | AUG   | 1.00 (1.00) | End        | UAA   | 0.60 (0.53) |
|            |       |            |            |       | 0.30 (0.18) |
| Trp        | UGG   | 1.00 (1.00) |            |       |            |

* The frequencies are calculated from a total of 3683 codons. Values in brackets are from *D. melanogaster* (Powell, 1997) and they are shown here for comparison. The most frequent used codons in both species are in italics.
Legends to Figures

Fig. 1. Alignment of the amino acid sequence encoded by a fragment obtained from *L. longipalpis* (called here Llca1A) compared to the IVS6 domain of mammalian (rat alpha-1A, human alpha-1D and rat alpha-1G) and *Drosophila* (Dmca1A and Dmca1D) voltage-gated calcium channels. The amino acid sequence encoded by a fragment obtained from *L. intermedia* (called here Lica1D) is also included (see also Table 1). The software ClustalX (Thompson et al., 1997) was used for the alignment. Accession numbers of protein sequences used: P54282 (rat alpha-1A), Q01668 (human alpha-1D), O54898 (rat alpha-1G), P91645 (Dmca1A or cacophony), Q24270 (Dmca1D).

Fig. 2. Phylogenetic tree using sequences of Fig. 1. The “neighbour-joining” method available at ClustalX software (Thompson et al., 1997) was used to construct the tree. The numbers on nodes represent the “bootstrap” percentage values based on a 1000 replicates.

Fig. 3. Alignment of the amino acid sequence encoded by a fragment obtained from *L. longipalpis* compared to the protein sequence encoded by the *period* gene of *Drosophila virilis* (accession number S02035).
Llca1A          PNIMLACLKGR---PCDERAGKEPHE--TC-GSSLAYAYFVSIFFCFLMLNLFVAV
Dmca1A          PNIMLACLKGR---ACDDDAEKAPGE--YC-GSTLAYAYFVSIFFCFLMLNLFVAV
alpha-1A        HNIMLSCLSGK---PCDKNSGIQKPE---C-GNEFAYFYVSIFFLCSFLMLNLFVAV
alpha-1D        QEIMMCNPGK---LCPEDSYNPGEHTC-GSNFAIVYFISFYMLCFLINLFLVAV
Dmca1D          QEIMMCAQP-DVKNMNSTPG---EPC-GSIIIAYFYISFYVLCFLINLFLVAV
Llca1D          QDIMMDCSRAQP-DVKCDMDSTPG---EPC-GSIIIAYFYISFYVLCFLINLFLVAV
alpha-1G        NGIMKDTLR-----DCDOEST------CYNTVISPYFVSFLTAQFLNVNVVIAV

**           **  :         * .. ::  **:** . . *:::*:.:**
D. virilis  L. longipalpis

LIGRSILDYHEDLSIKDIYEKVKQTVGATFCSKPFRFILQNCYILLETEWTSF

L. longipalpis

MIGRSIMDFYHPQILREVYETVMRGKTAGASFCSKPYPFHLANGFYITLETEWTSF

D. virilis

L. longipalpis

*:*****:*** **:* :**::*:**::*:*::* **:******:

D. virilis

VNPWSQLEFVIGHHRLGLPSNPQVFASV-LVNQQFEDVLNADAKINGKLCLILEPVM

L. longipalpis

VNPWSRKLFEVGVHHRVQGPKQCDVFEMSPNTPNPEDEQNRNNACIKEDILKMMETV

*:******:****:*****::**:*:** :**  :  *. ::.**  *     :*.** ::**.*

D. virilis

TRPSDTVQVEVRSQHALASFMETLMDEVARGDLKDLPHETELTVSERSVMLGEISPH

L. longipalpis

SKDIDTVKQVSKALASFMETLMDEVTRFPLDLIPQETELTISERDSVMLGEISPH

*: * **:*******:*************:* ****:****:**:*****:**:**

D. virilis

HDYYDSKSTETTPSYQLYNENLLRFNSKFPVTAPVTDPPKMDSSYVSSAR-EDALS

L. longipalpis

HDYYDSKSSSETTPSYQLYNENLQRFESKPHETPESTGPQNSLS

*:******:*******:*******:* **:*:* * .: * *:. :;::*

D. virilis

PVHGFEGSGGSNSNLLTASNVMSSVTNNTSNTGTSGGENSAGSNPLPVNMTLTE

L. longipalpis

PVQCF-GSG-SGSSANGNLSGNSIQMDSTM--SNTGTS-----SGSYQP-P---ALTE

*: * **:*******:*************:* ****:****:**:*****:**:**

D. virilis

ILLN

L. longipalpis

SLLS

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