

# **Molecular Evolution of the *period* gene and phylogeny of Neotropical Sandflies**

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## Abstract

The molecular evolution of the clock gene *period* was studied in Phlebotomine sandflies (Diptera: Psychodidae). The comparison of the synonymous and nonsynonymous substitution rates between sandflies and *Drosophila* revealed a significantly higher evolutionary rate in the latter in three out of the four regions analysed. The differences in rate were higher in the sequences flanking the Thr-Gly repetitive domain, a region that has expanded in *Drosophila* but remained stable and short in sandflies, a result consistent with the coevolutionary scenario proposed for this region of the gene.

A phylogenetic analysis including eight neotropical sandfly species and one from the Old-World was also carried out. The results showed that only the subgenus *Nyssomyia* is well supported by distance (neighbor-joining) and maximum parsimony analysis. The grouping of the other species from the subgenus *Lutzomyia* and *Migonei* group show very low bootstrap values and is not entirely consistent with classical morphological systematics of the genus *Lutzomyia*.

## Introduction

Phlebotomine sandflies (Diptera: Psychodidae) are vectors of human leishmaniasis, a disease caused by trypanosomatids of the genus *Leishmania*, that ranges from the less severe cutaneous type to fatal visceral forms (Lane 1993). Leishmaniasis is found mainly in tropical, subtropical and Mediterranean regions of the World. Thirty-two out of nearly 400 species of *Lutzomyia* sandflies, the main genus in the Americas, have been implicated as vectors in human leishmaniasis (Grimaldi and Tesh 1993), while the genus *Phlebotomus* contains the main vector species in the old world (Lane 1993). Despite their medical importance the

systematics of Phlebotominae has been controversial (Lane 1986; Dujardin et al. 1999) with very few studies of molecular evolution and phylogenetics (e.g. Aransay et al. 2000; Lins et al. submitted).

The *period* (*per*) gene controls biological rhythms in *Drosophila melanogaster* and was first identified by Konopka and Benzer (1971) who isolated three X-linked mutants that alter the fruitfly's circadian (~24h) rhythms in locomotor activity and pupae-adult emergence. Subsequently, it was shown that *per* also controls the 60s interpulse-interval rhythm in male's lovesong (Kyriacou and Hall 1980). *per* was the first behavioural gene cloned in *Drosophila* (Bargiello et al. 1984; Reddy et al. 1984) and since then, the molecular basis for the *Drosophila* clock has become a cause celebre for model of behavioural gene regulation (reviewed in Ripperger and Schibler 2001). The available evidence supports a model for the circadian pacemaker that involves the cyclical regulation of *per* gene transcription and translation via a negative feedback loop (Hall 1998; Dunlap 1999; Young 2000).

The *period* gene encodes a moderately large protein, which comprises almost 1200 amino acids (Bargiello et al. 1984; Zehring et al. 1984; Hamblen et al. 1986; Citri et al. 1987). *per* contains two regions specially important and interesting. The PAS region (Hoffman et al. 1991) is a dimerization domain located at the c2 conserved region (Colot et al. 1988). Point mutations in this region or next to it affect the interaction between PER and others proteins, including transcription factors, that is very important to pacemaker function (Huang et al. 1993). The other important but non-conserved region is the threonine-glycine (Thr-Gly) repeat (Jackson et al. 1986; Citri et al. 1987; Colot et al. 1988), that have been used in a number of population genetics and molecular evolution studies in *Drosophila* (Costa et al. 1991; 1992; Peixoto et al. 1992; 1993; Rosato et al. 1996; 1997) and appears to be involved in the temperature compensation mechanism of the biological clock (Sawyer et al. 1997; Peixoto et al. 1998). This region is also responsible for differences in the lovesong rhythms between

*Drosophila melanogaster* and *D. simulans* (Wheeler et al. 1991) that is important to the sexual isolation between these two species (Kyriacou and Hall 1982; 1986; Ritchie et al. 1999), and for that reason *per* has been considered as a speciation gene (Coyne 1992).

Homologues of *per* have also been isolated in other insects and used in molecular evolution and phylogenetics studies outside *Drosophila* (Nielsen et al. 1994; Regier et al. 1998; Gotter et al. 1999). Recently, a fragment homologous to *period* was isolated in sandflies (Peixoto et al. 2001) that extends from the end of the PAS region to the end of the Thr-Gly repetitive domain. In this article, we compare the substitution rates in this fragment within *per* in sandflies and *Drosophila*, and we use the data to carry out a phylogenetic analysis of some *Lutzomyia* species.

## **Material and Methods**

Sandflies used in this study were either from F1 progeny of wild-caught insects or from established colonies. The following species were used: *Lutzomyia dispar* (Chapada dos Guimarães, State of Mato Grosso do Sul, Brazil), *L. evandroi* (Natal, State of Rio Grande do Norte, Brazil), *L. intermedia* (Posse, State of Rio de Janeiro, Brazil), *L. longipalpis* (Lagoa Santa, State of Minas Gerais, Brazil), *L. migonei* (Posse, State of Rio de Janeiro, Brazil), *L. renei* (Lagoa Santa, State of Minas Gerais, Brazil), *L. umbratilis* (Manacapuru, State of Amazonas, Brazil), *L. whitmani* (Afonso Cláudio, State of Espírito Santo, Brazil) and *Phlebotomus duboscqi* (Keur Moussa, Senegal). Genomic DNA was extracted from sandflies according to Jowett (1998) or by using the GenomicPrep kit (Amersham Pharmacia Biotech). The extracted DNA was used as template in PCR amplifications that were carried out according to Peixoto et al. (1993) and Nielsen et al. (1994) for either 30 or 35 cycles (95° C for 30 sec, 50° C or 60° C for 30 sec or 1 min and 72° C for 1 min), with an initial

denaturation step at 95° C for 3 min. A combination of degenerated and non-degenerated primers was used and their sequences are available under request. The PCR products were electrophoresed through a 2% agarose gel using TAE Buffer (Sambrook et al. 1989), cut out from the gel and purified using the Sephaglass Bandprep Kit (Amersham Pharmacia Biotech). The purified fragments were then cloned into the pMOS *Blue* vector using the blunt-ended cloning kit (Amersham Pharmacia Biotech). Clones containing inserts were sequenced with an ABI 377XL DNA Sequencer and BigDye Terminators at Leicester University. A number of clones were sequenced from each species to verified possible PCR induced errors.

DNA sequence editing and alignment was carried out using GCG (Wisconsin Package Version 91, Genetics Computer Group, Madison, Wisc) and ClustalX (Thompson et al. 1997) software. The remaining analysis was done using MEGA2 (Kumar et al. 2001).

## Results

### *Molecular evolution of the period gene*

Figure 1 shows an alignment of the amino acids sequences of homologous PER proteins from four sandflies species (*L. intermedia*, *L. longipalpis*, *L. renei*, and *P. duboscqi*), from four *Drosophila* species (*D. melanogaster*, *D. pseudoobscura*, *D. virilis* and *D. yakuba*) and from the hymenopteran *Apis mellifera*.

We have divided the sequences into five regions (see Figs. 1 and 2). The first one (PAS/CLD) is very conserved and contains the majority of the second repeat of the PAS domain and all of the CLD (cytoplasmatic localization domain) (Saez and Young 1996). The second one (called here post-intron) follows an intron site in the gene and is a more variable region that precedes the highly conserved *per<sup>S</sup>* domain (third region) that includes the site of one of the original *per* mutants (Yu et al. 1987; Baylies et al. 1987). The fourth marked region

(flanking) refers to the sequences that flank the region containing repetitive sequences in *Drosophila* (Peixoto et al. 1993; Nielsen et al 1994). In the alignment, we can see the long Threonine-Glycine repetitive region in the two species of the subgroup *melanogaster* (*D. melanogaster* and *D. yakuba*), and in *D. pseudoobscura*, which has a less extensive Thr-Gly region associated with many copies of a degenerate 5 amino acid repeat (Colot et al. 1988; Peixoto et al. 1992; 1993; Nielsen et al. 1994). In contrast to *Drosophila*, this “repetitive” region is very short in sandflies. It is interesting to note that the region flanking the repeats is clearly more conserved in sandflies than in *Drosophila*.

Using the aminoacid sequences shown in figure 2 and *Apis* as the outgroup, a phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei 1987) (Fig. 3). As expected, the *Drosophila* and the sandfly lineages appear as monophyletic groups with high bootstrap values (100%). Interestingly, the separation between of the New World *Lutzomyia* and the Old World *Phlebotomus* shows a low bootstrap value (51%) perhaps reflecting the proximity between the two genera as previously suggested (Aransay et al. 2000). A higher bootstrap value (76%) was found, however, in a tree using only the synonymous changes of the PAS/CLD region (Fig. 4, see below).

One can also notice in Fig. 3 the longer branches observed for the *Drosophila* species compared to the sandfly sequences, a result that might indicate either a shorter separation time between the latter species or a higher evolutionary rate in the former. Table 1 shows a comparison of the synonymous and nonsynonymous substitution rates in *Drosophila* and in Phlebotominae for each one of the domains highlighted in Fig. 2, except the repetitive region. In the last three regions the rates of nonsynonymous over synonymous (Ka/Ks) are significantly higher in *Drosophila*. Although the synonymous rates in these three regions are somewhat lower in drosophilids, the variation in the Ka/Ks rates are mainly due to the differences in the nonsynonymous rates. In the PAS/CLD region, the rates are more similar

and not significantly different in the two lineages. Using the synonymous rates of the PAS/CLD region, a linearized Neighbor-Joining tree was constructed (Fig. 4) and used to estimate the divergence time of the Phlebotomine lineages (see Discussion).

### ***Phylogenetic analysis of Lutzomyia***

Using primers more specific for the genus *Lutzomyia*, we also amplified and sequenced fragments (~500 bp) of the *period* gene from other sandfly species to carry out a phylogenetic analysis. Figure 5 shows DNA sequence alignment of *P. duboscqi* (used as the outgroup) and eight species belonging to two subgenus and one species-group of the genus *Lutzomyia*, according to classical morphological systematics (Young and Duncan 1994): subgenus *Lutzomyia* (*L. longipalpis*, *L. renei* and *L. dispar*), subgenus *Nyssomyia* (*L. intermedia*, *L. whitmani* and *L. umbratilis*) and group *Migonei* (*L. migonei* and *L. evandroi*). The region compared includes part of the PAS/CLD domain, the intron (~60bp) that lies immediately after it and the beginning of the *per<sup>S</sup>* domain.

Figures 6a and 6b show the trees obtained using respectively the Neighbor-Joining and maximum parsimony analysis available in the MEGA2 software (Kumar et al. 2001). Both trees support with very high bootstrap values (over 98%) the subgenus *Nyssomyia* as a monophyletic clade, and place *L. intermedia* and *L. whitmani* as more closely related to each other than to *L. umbratilis*. The two trees also group together *L. dispar* and *L. renei*. However, they show low bootstrap values and disagree in the relative position of the other species and suggest that the subgenus *Lutzomyia* and the *Migonei* group might not be monophyletic.

## Discussion

The comparison of the substitution rates in four regions of the *period* gene in sandflies and *Drosophila* revealed that the PAS/CLD domain show similar evolutionary rates in these two Diptera lineages. That, however, is not necessarily a rule in insects, as the analysis of the *period* gene in *Musca domestica* revealed an unexpectedly high amino acid sequence similarity to *D. melanogaster* in a region that includes the PAS/CLD domain (Piccin et al. 2000). Another evolutionary study including a number of lepidopteran species (Regier et al. 1998), revealed a rapid evolution of the PAS/CLD domain despite the fact that this is one of the most conserved regions in *Drosophila*. Interesting enough, in one of studied species, *Antheraea pernyi*, temporal and spatial expression of *period* and *timeless* shows important differences compared to what is known in *Drosophila* (Reppert et al. 1994; Sauman and Reppert 1996).

The higher nonsynonymous/synonymous rates in *Drosophila* in the other three regions suggest that the amino acid sequences closer to the repetitive domain have a higher evolutionary rate. In fact, the most significant differences in the rates between the two lineages are found in the region flanking the repeats. As mentioned above, the so-called Thr-Gly repetitive region has expanded and become highly variable in length and sequence among different *Drosophila* species (Colot et al. 1988; Peixoto et al. 1993; Nielsen et al. 1994). As observed in other insects, such as *Lucilia cuprina* (Warman et al. 2000), *Musca domestica* (Piccin et al. 2000), *Antheraea pernyi* (Reppert et al. 1994) and *Apis mellifera* (Toma et al. 2000), the Thr-Gly region of sandflies has remained short and conserved. This region's expansion and divergence in *Drosophila* compared to its stability in Phlebotominae appears therefore to be associated with the higher and lower evolutionary rates in the sequences surrounding it, respectively. In turn this further supports the model for the coevolution of the



Thr-Gly length with flanking regions proposed for *Drosophila per* (Peixoto et al. 1993; 1998; Nielsen et al. 1994).

### ***Phylogenetic analysis of Lutzomyia***

The phylogenetic tree obtained with the PAS/CLD domain (Fig. 4) suggests a similar timeframe for the evolution of the genus *Drosophila* and the subfamily Phlebotominae. Because knowledge on the phylogenetics and putative divergence times of *Drosophila* species (Powell 1997) is far greater than in sandflies, the comparison of trees based on genes with similar evolutionary rates in both lineages could provide a useful tool to date the separation of the different phlebotomine lineages, an issue still clouded with uncertainty, but relevant to different hypothesis concerning the origin of *Leishmania* (Kerr et al. 2000; Noyes et al. 2000; Momen and Cupolillo 2000). Using the divergence time proposed for the *Drosophila* species used in this work (Russo et al. 1995), the estimated divergence of the *Phlebotomus* and *Lutzomyia* lineages is between 28 to 38 million years, a time frame consistent with other proposals (Williams 1993).

Apart from the subgenus *Nyssomyia*, which is well-supported by the data presented here, the trees obtained with the *per* gene revealed some discrepancies compared with the morphological classification of the studied *Lutzomyia* species (Young and Duncan 1994), even though most bootstrap values were low and must be viewed cautiously. For example, even though *L. renei* and *L. dispar*, two species of the subgenus *Lutzomyia*, were clustered together, the position of *L. longipalpis* in both trees would make this subgenus paraphyletic. The positions of *L. migonei* and *L. evandroi* also raises doubts about the status of the *Migonei* group, a result that has some support from morphological data (Galati 1990).

There are not many molecular phylogenetic studies of the genus *Lutzomyia* so far. An analysis using *cacophony* (*cac*) (Lins et al. submitted), a calcium channel gene, also

confirmed the subgenus *Nyssomyia* as a monophyletic group. However, as with *per*, the status of the *Lutzomyia* subgenus and *Migonei* group were also not completely supported by the data.

Both *per* and *cac* are involved in courtship song production in *Drosophila*. Therefore, they are possibly implicated in the reproductive isolation between sandfly species, which also produce acoustic signals during courtship (Ward et al. 1988; Souza et al. 2001). Like other genes potentially involved in the speciation process (Ting et al. 2000), their use in phylogenetic studies of closely related sandfly species might prove to be particularly useful as demonstrated by work in *Drosophila* (Hilton and Hey 1996; Wang and Hey 1996; Gleason and Powell 1997).

## ACKNOWLEDGEMENTS

This work was supported by The Wellcome Trust and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), with additional support from Faperj, Fiocruz, CNPq and PPD/G7 (No 0847-95). We would like to thank Mr Paulo Roberto de Amoretty, Mr Robson Costa da Silva, and Mr Raimundo Nonato Lima Santos for their technical assistance.

## REFERENCES

Aransay AM, Scoulica E, Tselentis Y, Ready, PD (2000) Phylogenetic relationships of phlebotomine sandflies inferred from small subunit nuclear ribosomal DNA. *Insect Mol Biol* 9:157-168

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature* 312:752-754

Baylies MK, Bargiello TA, Jackson FR, Young MW (1987) Changes in abundance or structure of the *per* gene product can alter periodicity of the *Drosophila* clock. *Nature* 326:390-392

Citri Y, Colot HV, Jacquier AC, Yu Q, Hall JC, Baltimore D, Rosbash, M (1987) A family of unusually spliced and biologically active transcripts is encoded by a *Drosophila* clock gene. *Nature* 326:42-47

Colot HV, Hall JC, Rosbash M (1988) Interspecific comparisons of the *period* gene of *Drosophila*. *EMBO J* 7:3929-3937

Costa R, Peixoto AA, Thackeray JR, Dalglish R, Kyriacou, CP (1991) Length polymorphism in the Threonine-Glycine-encoding repeat region of the *period* gene in *Drosophila*. *J Mol Evol* 32:238-246

Costa R, Peixoto AA, Barbuji G, Kyriacou CP (1992) A latitudinal cline in a *Drosophila* clock gene. *Proc R Soc Lond [Biol]* 250:43-49

Coyne JA (1992) Genetics and speciation. *Nature* 355:511-515

Dujardin JP, Pont F Le, Martinez, E (1999) Quantitative phenetics and taxonomy of some Phlebotominae taxa. *Mem Inst Oswaldo Cruz* 94:735-741

Dunlap JC (1999) Molecular Bases for Circadian Clocks. *Cell* 96:271-290

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791

Galati EAB (1990) Sistemática dos Phlebotominae (Diptera, Psychodidae) das Américas. PhD Thesis Universidade de São Paulo (USP)

Gleason JM, Powell JR (1997) Interspecific and intraspecific comparisons of the *period* locus in the *Drosophila willistoni* sibling species. *Mol Biol Evol* 14:741-753

Gotter AL, Levine JD, Reppert SM (1999) Sex-linked period genes in the silkworm, *Antheraea pernyi*: implications for circadian clock regulation and the evolution of sex chromosomes. *Neuron* 24:953-65

Grimaldi G Jr, Tesh RB (1993) Leishmaniasis of the New World: current concepts and implications for future research. *Clin Microbiol Rev* 6:230-50

Hall, JC (1998) Genetics of Biological Rhythms in *Drosophila*. *Adv Genet* 33:135-184

Hamblen M, Zehring WA, Kyriacou CP, Reddy P, Yu Q, Wheeler DA, Zwiebel LJ, Konopka RJ, Rosbash M, Hall JC (1986) Germ-line transformation involving DNA from the period locus in *Drosophila melanogaster*: overlapping genomic fragments that restore circadian and ultradian rhythmicity to *per<sup>0</sup>* and *per<sup>-</sup>* mutants. *J Neurogenet* 3(5):249-291

Hilton H, Hey J (1996) DNA sequence variation at the *period* locus reveals the history of species and speciation events in the *Drosophila virilis* group. *Genetics* 144:1015-1025

Hoffman EC, Reyes H, Chu F, Sander F, Conley LH, Brooks BA, Hankinson O (1991) Cloning of a factor required for activity of the Ah (Dioxin) receptor. *Science* 252: 954-958

Huang ZJ, Edery I, Rosbash M (1993) PAS is a dimerization domain common to *Drosophila period* and several transcription factors. *Nature* 364:259-262

Jackson FR, Bargiello TA, Yun SH, Young MW (1986) Product of *per* locus of *Drosophila* shares homology with proteoglycans. *Nature* 320:185-188

Jowett T (1998) Preparation of nucleic acids In: Roberts, DB, (Ed) *Drosophila: A practical approach IRL press, Oxford* 347-371

Kerr SF, Merkelz R, Mackinnon C (2000) Further support for a Palaearctic origin of *Leishmania*. *Mem Inst Oswaldo Cruz* 95:579-81

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 68:2112-2116

Kumar S, Tamura K, Jakobsen I, and Nei, M (2001) MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics, submitted.

Kyriacou CP, Hall JC (1986) Interspecific genetic control of courtship song production and reception in *Drosophila*. *Science* 232: 494-497

Kyriacou CP, Hall JC (1982) The function of courtship song rhythms in *Drosophila*. *Anim Behav* 30: 784-801

Kyriacou CP, Hall JC (1980) Circadian rhythm mutations in *Drosophila* affect short-term fluctuations in the male's courtship song. *Proc Natl Acad Sci USA* 77:6729-6733

Lane RP (1986) Recent advances in the systematics of phlebotominae sandflies. *Insect Sci Applic* 225-230

Lane RP (1993) Sandflies. In: Lane RP, Crosskey RW (eds) *Medical Insects and Arachnids*. Chapman and Hall, London, pp 78-119

Lins R, Oliveira SG, Souza NA, Queiroz RG, Justiniano SCB, Ward RD, Kyriacou CP, Peixoto, AA Molecular evolution of the *cacophony* IVS6 region in sandflies. Submitted

Momen H, Cupolillo E (2000) Speculations on the origin and evolution of the genus *Leishmania*. *Mem Inst Oswaldo Cruz* 95:583-8

Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol* 3:418-426

Nielsen J, Peixoto AA, Piccin A, Costa R, Kyriacou CP, Chalmers D (1994) Big flies, small repeats: the "Thr-Gly" region of the *period* gene in Diptera. *Mol Biol Evol* 11:839-53

Noyes HA, Morrison DA, Chance ML, Ellis JT (2000) Evidence for a neotropical origin of *Leishmania*. *Mem Inst Oswaldo Cruz* 95:575-8

Peixoto AA, Costa R, Wheeler DA, Hall JC, Kyriacou CP (1992) Evolution of the Threonine-Glycine repeat region of the *period* gene in the *melanogaster* species subgroup of *Drosophila*. *J Mol Evol* 35:411-419

Peixoto AA, Campesan S, Costa R, Kyriacou CP (1993) Molecular evolution of a repetitive region within the *per* gene of *Drosophila*. *Mol Bio Evol* 10:127-139

Peixoto AA, Hennessy M, Townson I, Hasan G, Rosbash M, Costa R, Kyriacou CP (1998) Molecular coevolution within a clock gene in *Drosophila*. *Proc Nat Acad Sci USA* 95: 4475-4480

Peixoto AA, Gomes CA, Amoretty PR, Lins PMMA, Meireles-Filho ACA, Souza NA, Kyriacou CP (2001) New Molecular Markers for Phlebotomine Sand Flies. *Int J Parasit* 31: 635-639

Piccin A, Couchman M, Clayton JD, Chalmers D, Costa R, Kyriacou CP (2000) The clock gene *period* of the housefly, *Musca domestica*, rescues behavioral rhythmicity in *Drosophila melanogaster* Evidence for intermolecular coevolution? *Genetics* 154:747-58

Powell JR (1997) Progress and Prospects in Evolutionary Biology: The *Drosophila* Model  
*Oxford University Press, Oxford*

Reddy P, Zehring WA, Wheeler DA, Pirrota V, Hadfield C, Hall JC, Rosbash M (1984)  
Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a  
transcript involved in biological rhythms. *Cell* 38: 701-710

Regier JC, Fang QQ, Mitter C; Peigler RS, Friedlander TP, Solis MA (1998) Evolution and  
phylogenetic utility of the *period* gene in Lepidoptera. *Mol Biol Evol* 15:1172-1182

Reppert SM, Tsai T, Roca AL, Sauman I (1994) Cloning of a structural and functional  
homolog of the circadian clock gene *period* from the giant silkworm *Antheraea pernyi*.  
*Neuron* 13:1167-76

Ripperger JA, Schibler U (2001) Circadian regulation of gene expression in animals.  
*Curr Opin Cell Biol* 13:357-62

Ritchie MG, Halsey EJ, Gleason JM (1999) *Drosophila* song as a species-specific mating  
signal and the behavioural importance of Kyriacou and Hall cycles in *D. melanogaster* song.  
*Anim Behav* 58:649-657

Rosato E, Peixoto AA, Gallippi A, Kyriacou CP, Costa R (1996) Mutational mechanisms,  
phylogeny, and evolution of a repetitive region within a clock gene of *Drosophila*  
*melanogaster*. *J Mol Evol* 42:392-408



Rosato E, Peixoto AA, Costa R, Kyriacou CP (1997) Linkage disequilibrium, mutational analysis and natural selection in the repetitive region of the *clock* gene, *period*, in *Drosophila melanogaster*. *Genet Res* 69:89-99

Russo CA, Takezaki N, Nei M (1995) Molecular phylogeny and divergence times of drosophilid species. *Mol Biol Evol* 12:391-404

Saez, L, Young, MW (1996) Regulation of nuclear entry of the *Drosophila* clock proteins *period* and *timeless*. *Neuron* 17:911-920

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory manual *Cold Spring Harbor Laboratory Press, Cold Spring Harbor*

Sauman I, Reppert SM (1996) Circadian clock neurons in the silkworm *Antheraea pernyi*: novel mechanisms of Period protein regulation. *Neuron* 17:889-900

Sawyer L, Hennessy M, Peixoto AA, Rosato E, Parkinson H, Costa R, Kyriacou CP (1997) Natural variation in a *Drosophila* clock gene and temperature compensation. *Science* 278, 2117-2120

Souza NA, Ward RD, Hamilton JGC, Kyriacou CP, Peixoto AA. Copulation songs in three siblings of *Lutzomyia longipalpis* (Diptera:Psychodidae). Trans R Soc Trop Med Hyg. In press.

Thackeray JR, Kyriacou CP (1990) Molecular evolution in the *Drosophila yakuba period* locus. *J Mol Evol* 31:389-401

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Ac Res* 24, 4876-4882

Ting CT, Tsaur SC, Wu CI (2000) The phylogeny of closely related species as revealed by the genealogy of a speciation gene, *Odysseus*. *Proc Natl Acad Sci USA* 97:5313-5316

Toma DP, Bloch G, Moore D, Robinson GE (2000) Changes in period RNAm levels in the brain and division of labor in honey bee colonies. *Proc Natl Acad Sci USA* 97:6914-6919

Wang RL, Hey J (1996) The speciation history of *Drosophila pseudoobscura* and close relatives: inferences from DNA sequence variation at the *period* locus. *Genetics* 144:1113-1126

Ward RD, Phillips A, Burnet B, Marcondes CB (1988) The *Lutzomyia longipalpis* complex: reproduction and distribution. In: Service MW (ed) *Biosystematics of Haematophagous Insects*. Oxford University Press, Oxford, pp. 258-269.

Warman GR, Newcomb RD, Lewis RD, Evans CW (2000) Analysis of the circadian clock gene *period* in the sheep blow fly *Lucilia cuprina*. *Genet Res* 75:257-67

Wheeler DA, Kyriacou CP, Greenacre ML, Yu Q, Rutilia JE, Rosbash M, Hall JC (1991) Molecular transfer of a species-specific behavior from *Drosophila simulans* to *Drosophila melanogaster*. *Science* 251:1082–1085

Williams P (1993) Relationships of phlebotominae sand flies (Diptera). *Mem Inst Oswaldo Cruz* 88:177-183

Young DG, Duncan MA (1994) Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera:Psychodidae). *Mem Amer Ent Inst* 54:1-881

Young MW (2000) Life's 24-hour clock : molecular control of circadian rhythms in animal cells. *Trends Biochem Sci* 25: 601-605

Yu Q, Jacquier AC, Citri Y, Hamblen M, Hall JC, Rosbash M (1987) Molecular mapping of point mutations in the *period* gene that stop or speed up biological clocks in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 84:784-788

Zehring WA, Wheeler DA, Reddy P, Konopka RJ, Kyriacou CP, Rosbash M, Hall JC (1984) P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell* 39:369-376

**Table 1:** Synonymous and nonnynonymous substitution rates in *Drosophila* and Phlebotominae.

Regions	Synonymous substitution rate Ks	Nonsynonymous substitution rate Ka	Ka/Ks	G-test (Synonymous X Nonsynonymous)
PAS/CLD <b>Drosophila</b>	0.601 (0.044)	0.063 (0.015)	0.105	G value: 1.928 p-value: 0.16502 (not significant)
PAS/CLD Phlebotominae	0.590 (0.046)	0.040 (0.011)	0.068	
Post-intron <b>Drosophila</b>	0.520 (0.042)	0.114 (0.019)	0.219	G value: 9.297 p-value: 0.00229**
Post-intron Phlebotominae	0.711 (0.044)	0.040 (0.011)	0.056	
<i>per<sup>S</sup></i> domain <b>Drosophila</b>	0.391 (0.068)	0.025 (0.012)	0.064	G value: 5.489 p-value: 0.01914*
<i>per<sup>S</sup></i> domain Phlebotominae	0.602 (0.063)	0.008 (0.005)	0.013	
Flanking <b>Drosophila</b>	0.487 (0.057)	0.154 (0.026)	0.316	G value: 11.080 p-value: 0.00087***
Flanking Phlebotominae	0.636 (0.060)	0.049 (0.013)	0.077	

Standard errors based on bootstrapping (Kumar et al. 2001) are shown in brackets.

The G-tests were calculated on the mean number of synonymous and nonsynonymous changes in the two lineages.

## Legends to figures

Fig. 1: Schematic representation of the PER protein with its domains and the region studied in this paper.

Fig. 2: Alignment of protein sequences encoded by a segment of the *period* genes from different sandfly species (*L. intermedia*, *L. longipalpis*, *L. renei*, and *P. duboscqi*), *D. melanogaster* (Citri et al. 1987), *D. yakuba* (Thackeray and Kyriacou 1990), *D. pseudoobscura* and *D. virilis* (Colot et al. 1988), and *A. mellifera* (Toma et al. 2000). The sequences are divided in five regions: PAS/CLD (●—●); Post-intron (◀—▶); *per<sup>S</sup>* domain (○—○), Flanking (■—■), Repetitive (Δ—Δ) (see text for more details). Only part of this last region is shown in the case of *D. pseudoobscura*.

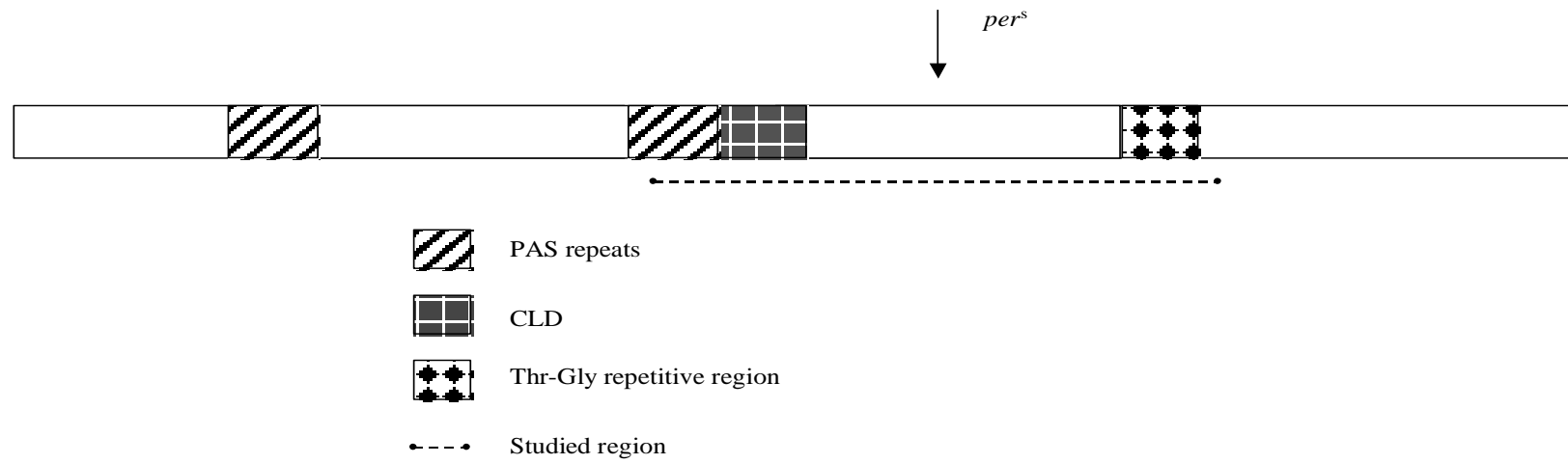
Fig. 3: Neighbor-joining tree using PERIOD amino acid sequences shown in Fig. 2. Numbers on the nodes represent the percentage bootstrap values based on 500 replicates (Felsenstein, 1985).

Fig. 4: Linearized Neighbor-Joining tree using only the synonymous changes in the PAS/CLD region (Nei and Gojobori 1986). Numbers on the nodes represent the percentage bootstrap values based on 500 replicates.

Fig. 5: Alignment of the DNA sequences from a region of the *period* gene from different sandfly species. The translated amino-acid sequence (of *L. longipalpis*) and the intron position are shown above the DNA sequences.


Fig. 6: Neighbor-Joining (A) and Maximum Parsimony (B) trees using the DNA sequences shown in Fig. 4. Numbers on the nodes represent the bootstrap percentage values based on 500 replicates Kimura 2-parameter distances were used in “A” and Close-Neighbor-Interchange (search level 3) with random addition trees (10 replicates) was used in “B”.

Fig. 1




*D.melanogaster* LIGRSIMDFYHHEDLSVMKETYETVMKKGQTAGASFCSKPYRFLIQNGCYVLLETETWTSFVNPNWSRKLEFVVGHRVFQGPQCNVFEEAPTCKL----KISEEAAQSRNTRIKEDIIVKRL  
*D.yakuba* LIGRSIMDFYHQEDLSVMKETIEMVMKKGQTAGASFCSKPYRFLIQNGCYVLLETETWTSFVNPNWSRKLEFVVGHRVFQGPQCNVFEEAPTCKL----KMSEEAAQSRNTRIKEDIIVKRL  
*D.virilis* LIGRSILDFYHHEDLSYDKIDIEYKVVKMGQTATGATFCSPKPRFLIQNGCYILLTETWTSFVNPNWSRKLEFVVGHRVFQGPQCDVFEMSPNVTP---NIPEDEQNRRACIKEDIILKMM  
*D.pseudoobscura* LMGRSIMDLYHDDLPVIKEIYESVMKKGQTAGASFCSKPYRFLIQNGCYILLETEWSSFVNPNWSRKLEFVVGHRVFQGPQICNVFETPPNSEP---KIAEELQNKNTRIKEEIVNL  
*L.longipalpis* MIGRSIMDFYHPEDFSYLREYVETVMRVGKTAGASFCSKPYRFLAHNGFYITLETETWTSFVNPNWSRQLEFVIGHHRVLRGPSNPQVFAS--TLVNQ---QFSEDLNDAKINQEIKLCLL  
*L.renei* MLERSIMDFYHPEDFSYLKEYVETVIRVGETAGASFCSKPYRFLAHNGFYITLETETWTSFVNPNWSRQLEFVIGHHRVLRGPSNPQVFAS--TLVNQ---QFPEDILNEAKINQEIKLCLL  
*L.intermedia* MIGRSIMDFYHPEDYSYLKEYVETVMRVGKTAGASFCSKPYRFLVHNGGYITLETETWSSFVNPNWSRQLEFVIGHHRVLRGPSNPQVFA--AAVNQ---QFPEDIINEAKKNQEIKLCLL  
*P.duboscqi* MIGRSIMDFYHPEDYSYLKEYVETVMRVGKTAGASFCSKPYRFLVHNGCYITLETETWTSFVNPNWSRQLEFVIGHHRVLRGPSNPQVFA--GLVTQ---QFPEDILNEAKMNQEIKLCLL  
*Apis mellifera* MVGRSLDFYHPEDLPFIKDIIYETVIK---LEGASFRSKPYRFGIQNGDYVLETEWSSFIPNPTWKLEFVVGQHRLIKGPANPDIFRVSCATEHSQLTNISEEVLKEAKIIQEEIRTLL  
:: \*\*:\*.\*\* :\* . ::: \*\* \*\*: \*:\* \*\*\*:\*\* :\*\* \*: \*\*\*\*\*:\*\*:\*\*\*::\*\*\*\*\*: \* \*\*::\*\* : :\* :.:\* . :.\* :

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*D.melanogaster* AETVSRPSDTVKQEVSRRCQALASFMETLMDEVSRADLKLELPHENELTVS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLLRFFNSKPVTAPAE--DPKTEP  
*D.yakuba* AETVSRPSDTVKQEVSRRCQALASFMETLMDEVSRADLKLELPHENELTVS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLLRFFNSKPVTAPAE--DPKTEP  
*D.virilis* TETVTRPSDTVKQEVSRRCQALASFMETLMDEVARGDLKLDPHELTETVS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLLRFFNSKPVTAPVD--DPKMD  
*D.pseudoobscura* AEKVSRPSTVKQEVSRRCQALASFMETLMDEVSRADLKLDVPHENELTVS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLLRFFNSKPVTAPVEV--DPKVG  
*L.longipalpis* TEPVSKDIDTVKQQVSKRCLALASFMETLMDEVTRPDLKLDLPQETELTIS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLQRFFESKPITIGPD--EAMKVEH  
*L.renei* TEPVSKDIDTVKQQVSKRCLALASFMETLMDEVTRPDLKLDLPQETELTIS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLQRFFESKPITIGPD--EAMKVDH  
*L.intermedia* TEPVSKDIDTVKQQVSKRCLALASFMETLMDEVTRPDLKLDLPQETELTIS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLQRFFESKPITIGPD--EAMKVDQ  
*P.duboscqi* TEPVSKDMDTVKQQVSKRCLALASFMETLMDEVTRPDLKLELPQETGLTIS-----ERDSVMLGEISP HHYYDSKSSTETPPSYNQLNYNENLQRFFESKPITIGD--EAMKVDH  
*Apis mellifera* DESIQRKSDITELDVSKRCKDLASFMGNLLQETRTPGFQKDVLATDERSFSGSRNPLQEHDSVMLGEISP HHEYDSKSSTETPPSYNQLNYNENIERFFSKPPVATMYGSDEEIINS  
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*D.melanogaster* PEPRGTCVSGASGP---MSPVH--EGSGGGSGSGNFTTASN IHMS SVTNTSIAGTG GTGTGTGTG---TGTG-----  
*D.yakuba* PEPRGTCVSGASGP---MSPVH--EGSGGGSGSGNFTTASN IHMS SVTNTSIAGTG GTGTGTGTGTG---TGTG-----  
*D.virilis* SYVSSA-REDA----LSPVHGFE GSGSGSGSNLTASNVRMSSVTNTSNTGTG-T-----  
*D.pseudoobscura* SDVSST-REDARST---LSPLNFGFE GSGSGSGHLTSGSNIHMSSATNTSNAGTG-TGTVTGTGTIIATSGTGTVTCASGNMDANTSAAFNIAANTSAADNFGADTSAADTSGADTSA  
*L.longipalpis* TEPESTGDPQNS---LSPVQ-CFGSG-SGSAGNLSSGSNIQMDSMT--SNTGTG-----  
*L.renei* TEPESTGDPQNS---LSPVQ-CFGSG-SGSAGNLSSGSNIQMDSMT--SNTGTG-----  
*L.intermedia* TEKESTGEPQNS---LSPVQ-CFGSG-SGSAGNLSSGSNVQMDSMT--SNTGTG-----  
*P.duboscqi* PEPESTGDPNNS---LSPVQ-CFGSG-SGSAGNLSSGSNIQMDSMT--SNTGTG-----  
*Apis mellifera* SNDEGGTKSPNSAVRKC MSPINGSGASG-SGAENLSSGSNNQTSSASR-ENTSN-----  
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*D.melanogaster* -----TGTGTGTGTGTGTGTGTGTG---TGTGT-----VTLTESLLNKHN  
*D.yakuba* -----TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGSASSNYRGGSVAIQVTLTEALLNKHN  
*D.virilis* -----SGGENSASGSSNPLPVN-----MTLTEILLNKHN  
*D.pseudoobscura* DNTGPDNSGAENSRAENSRADNRPRPDISGASNSRPDGTGPKSGAENSASGSGSGTSGNEGPFSSGGQDTRTTAGTADAPPVSLTESLLNKHN  
*L.longipalpis* -----TSSGSYQP-----PALTESLLSKHN  
*L.renei* -----TSSGSYQP-----PALTESLLSKHN  
*L.intermedia* -----TSSGSYQP-----PALTESLLCKHN  
*P.duboscqi* -----TSSGSYQP-----PTLTEALLSKHN  
*Apis mellifera* -----TSMESFKP-----PTLTESLLNRHN  
: : : \*\* \*

Fig. 3

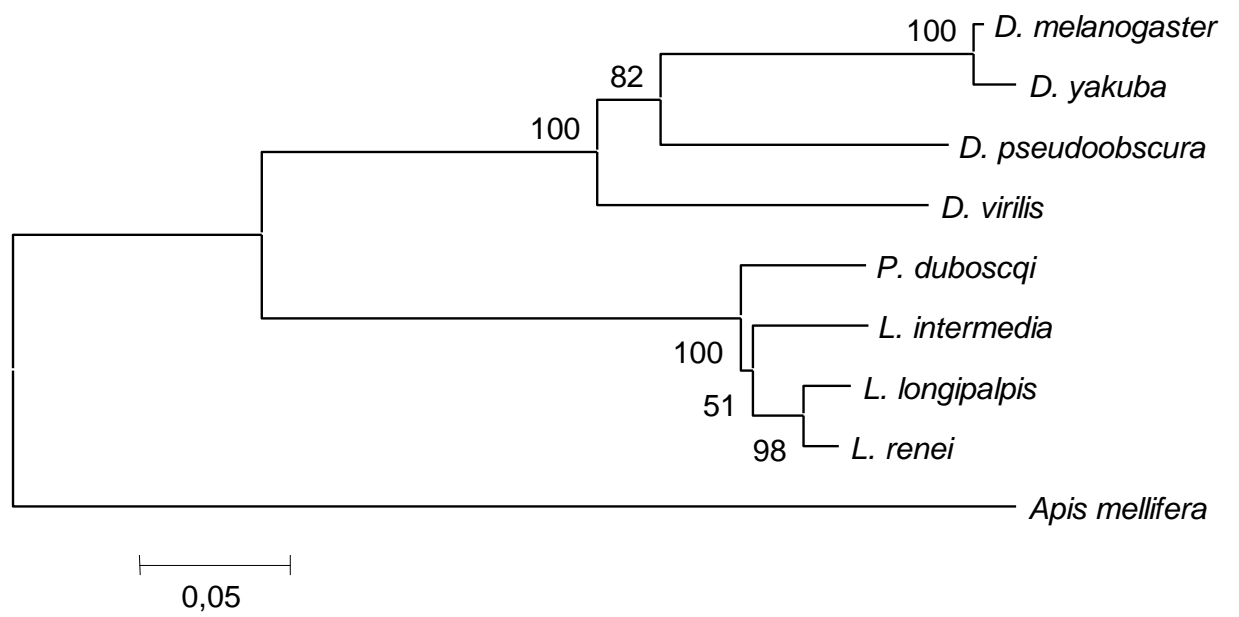
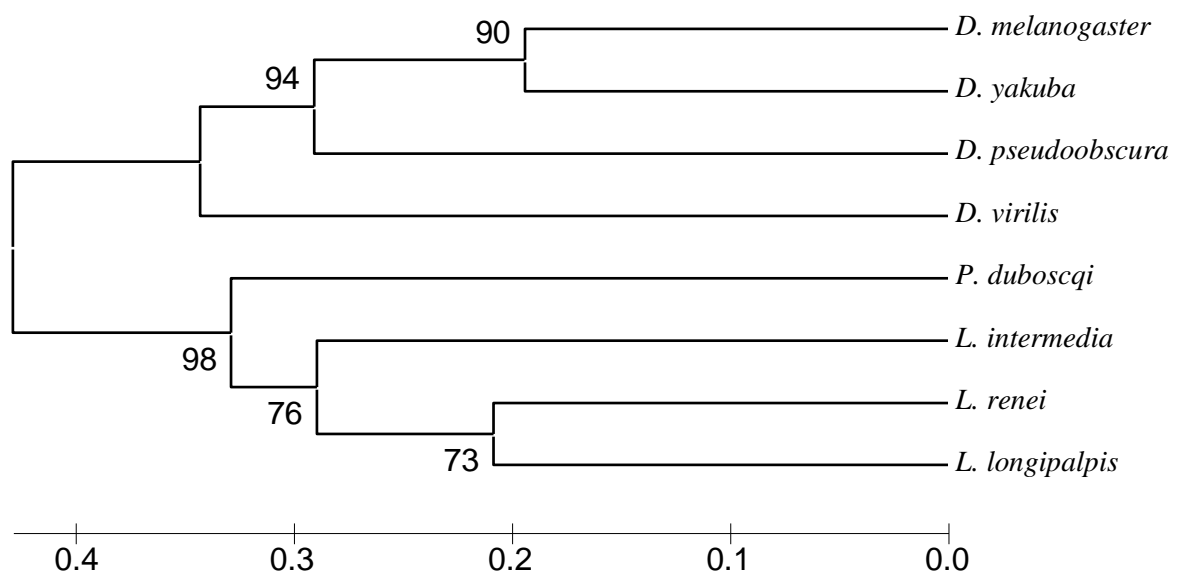




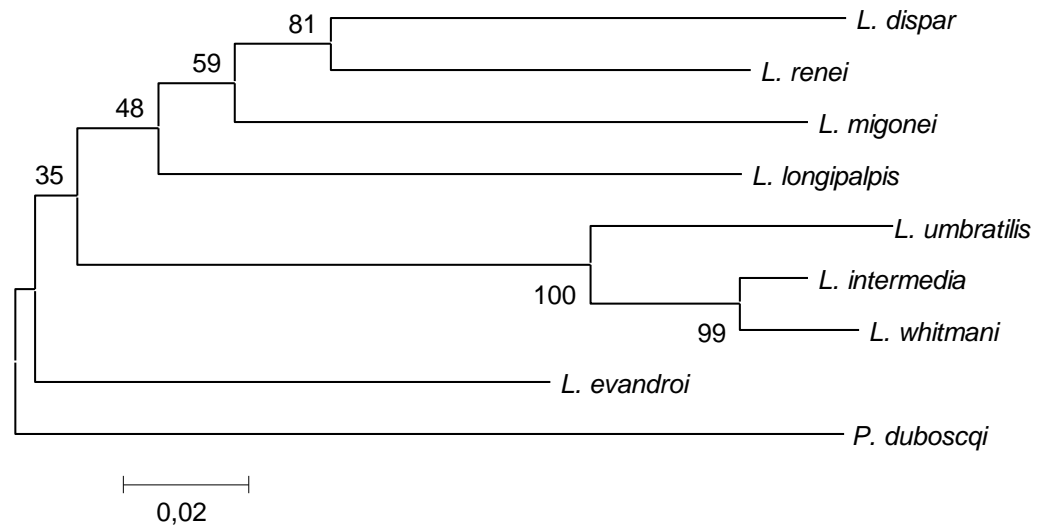
Fig. 4



[illegible]

Fig. 6

**A**



**B**

