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. whitman*i (Afonso Cláudio, State of Espirito Santo, Brazil) and *Phlebotomus duboscqi* (Keur Moussa, Senegal). Genomic DNA was extracted from sandflies according to Jowett (1998) or by using the GenomicPrep kit (Amershan 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 (95º C for 30 sec, 50º C or 60º C for 30 sec or 1 min and 72º C for 1 min), with an initial

denaturation step at 95º 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^S 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^S 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 melifera* (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. dispa*r, 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).

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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 **Drosophila**	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 **Drosophila**	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	
per^S domain **Drosophila**	0.391 (0.068)	0.025 (0.012)	0.064	G value: 5.489 p-value: 0.01914*
per^S domain Phlebotominae	0.602 (0.063)	0.008 (0.005)	0.013	
Flanking **Drosophila**	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^S domain (ο——ο), 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

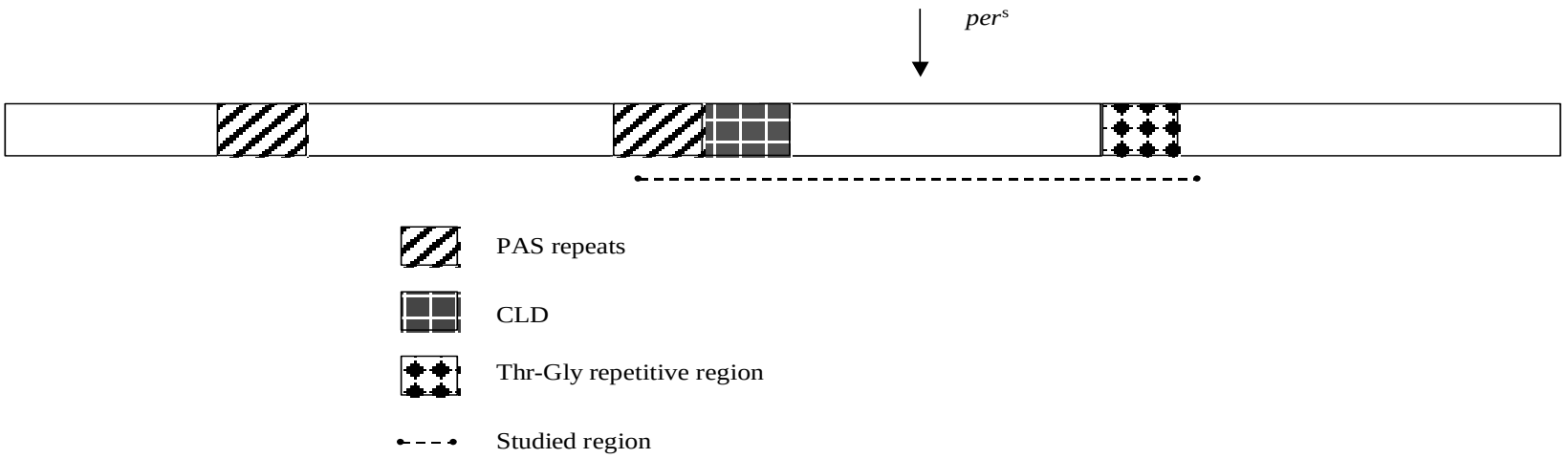
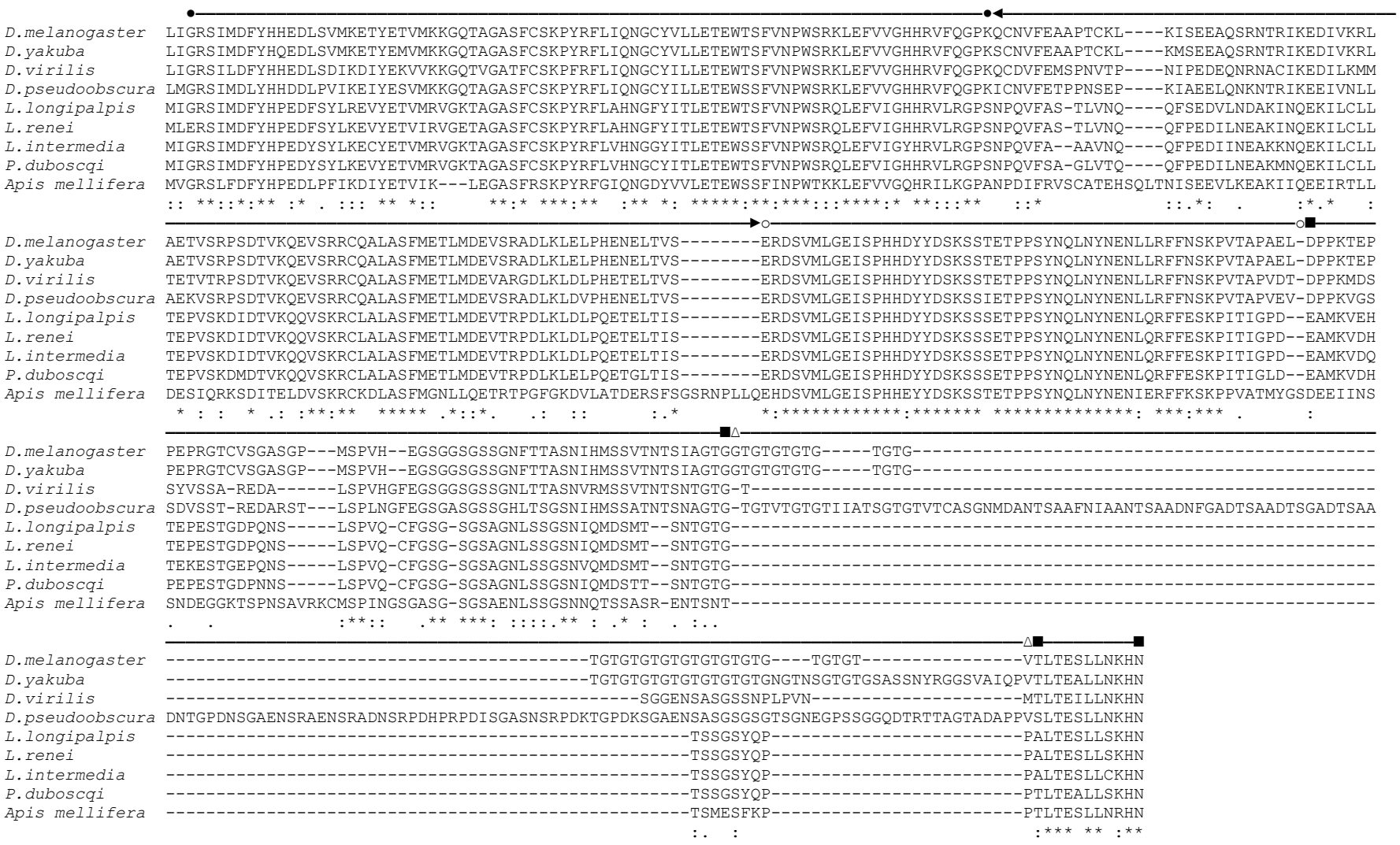

per^s
PAS repeats
CLD
Thr-Gly repetitive region
Studied region

Fig. 2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P.duboscqi PEPESTGDPNNS-----LSPVQ-CFGSG-SGSAGNLSSGSNIQMDSTT--SNTGTG----------
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Fig. 3

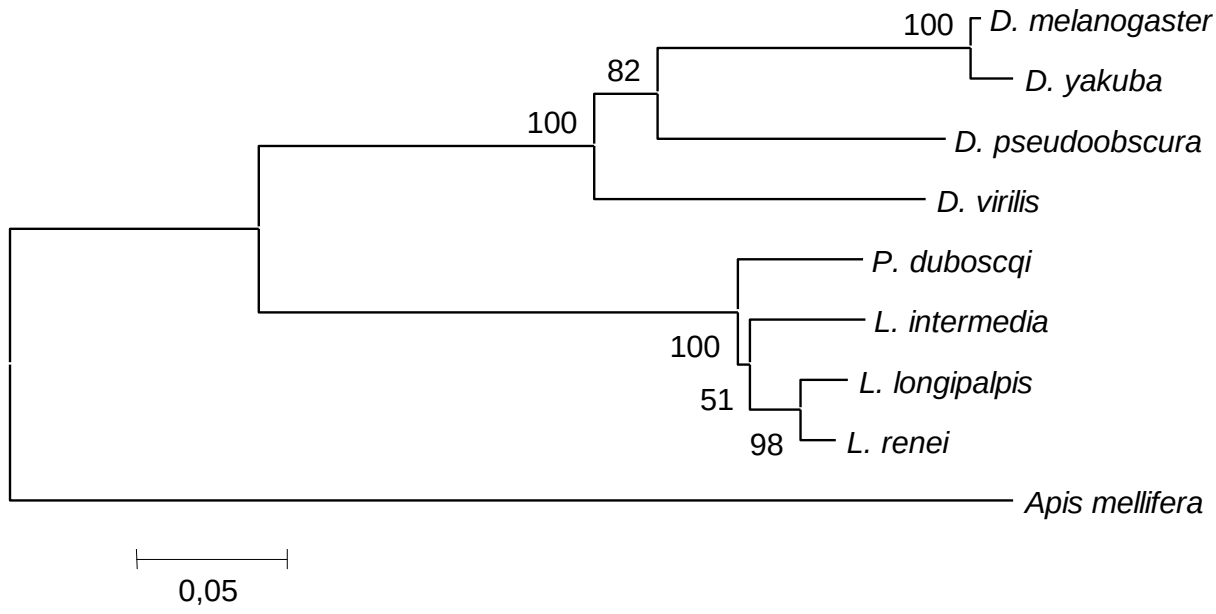
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Fig. 4

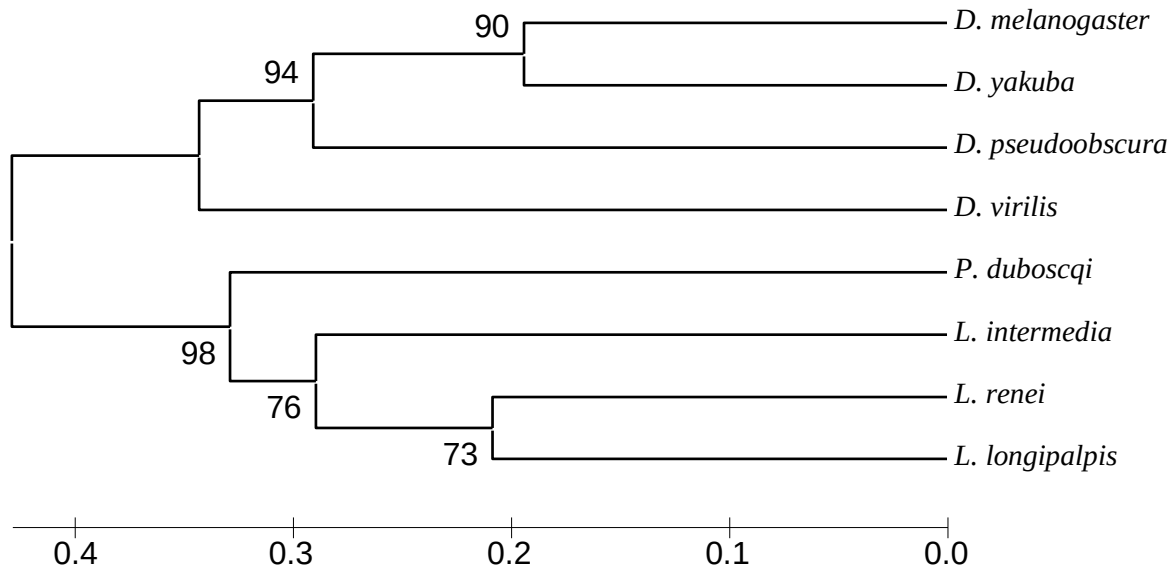
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Fig. 5

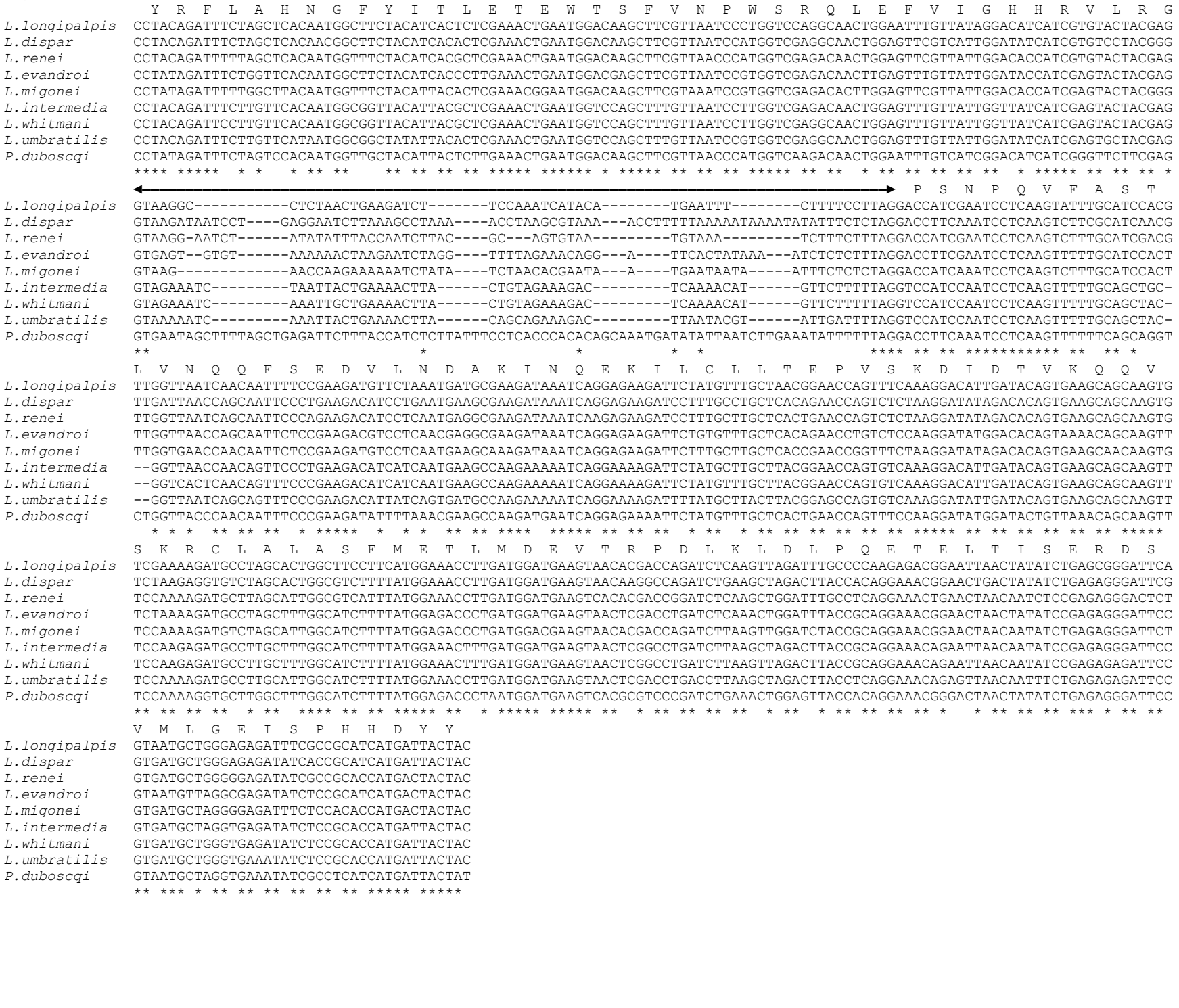
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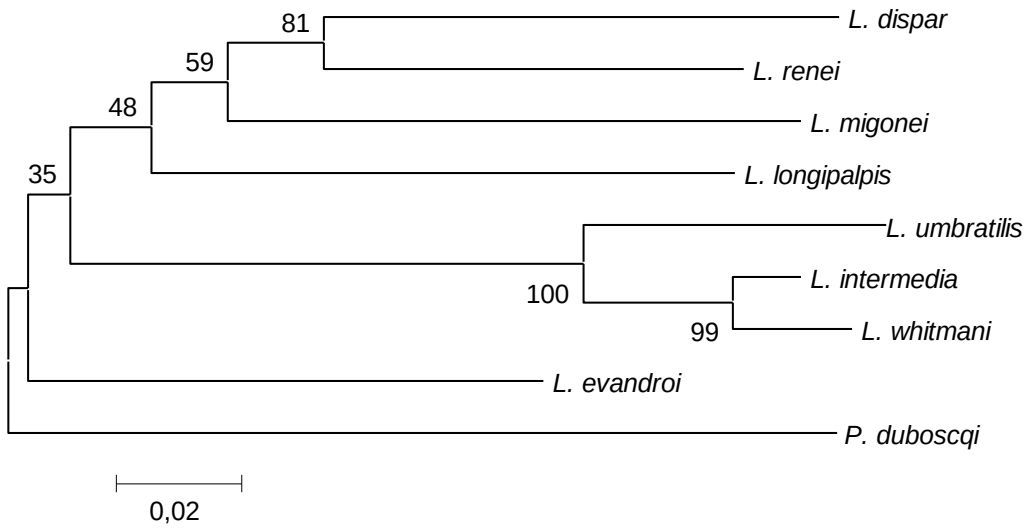
Fig. 6

A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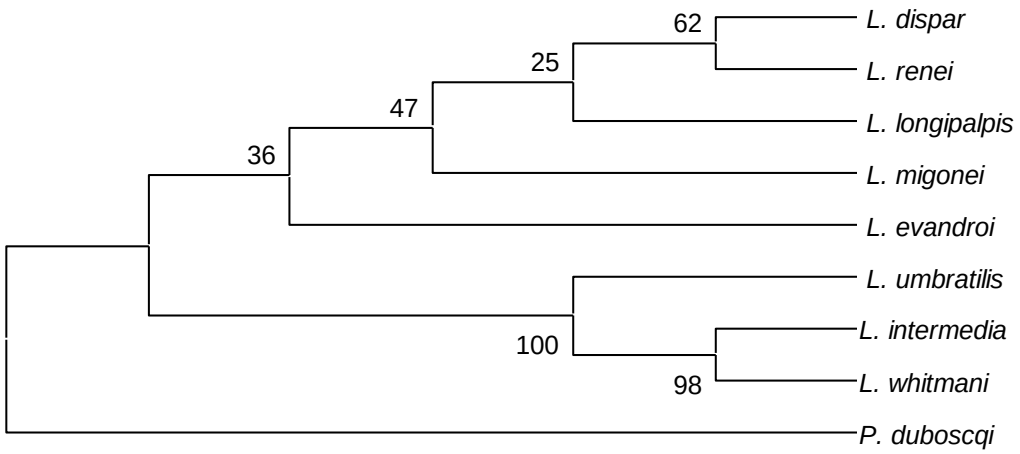
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