

# Trypanosomatids are common and diverse parasites of *Drosophila*

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## SUMMARY

*Drosophila melanogaster* is an important model system of immunity and parasite resistance, yet most studies use parasites that do not naturally infect this organism. We have studied trypanosomatids in natural populations to assess the prevalence and diversity of these gut parasites. We collected several species of *Drosophila* from Europe and surveyed them for trypanosomatids using conserved primers for two genes. We have used the conserved GAPDH sequence to construct a phylogenetic tree and the highly variable spliced leader RNA to assay genetic diversity. All 5 of the species that we examined were infected, and the average prevalence ranged from 1 to 6%. There are several different groups of trypanosomatids, related to other monoxenous Trypanosomatidae. These may represent new trypanosomatid species and were found in different species of European *Drosophila* from different geographical locations. The detection of a little studied natural pathogen in *D. melanogaster* and related species provides new opportunities for research into both the *Drosophila* immune response and the evolution of hosts and parasites.

Key words: Trypanosomatidae, *Drosophila*, field populations, prevalence, diversity, phylogeny.

## INTRODUCTION

In the last 15 years, *Drosophila melanogaster* has become an important model of the genetics and evolution of innate immunity and host-parasite interactions (Hoffmann, 2003). However, much of this research relies on microbes that would never infect flies in natural populations, and we remain remarkably ignorant of the natural parasites and pathogens of *Drosophila*. For example, the immune response of *D. melanogaster* to trypanosomatids was studied using *Crithidia bombi* and *C. fasciculata*, which naturally infect bumblebees and mosquitoes respectively (Boulanger *et al.* 2001). The best-described pathogens of *Drosophila* are viruses, with numerous different families of viruses having been found infecting flies in the wild (Brun and Plus, 1980; Longdon *et al.* 2010). Recently, bacterial pathogens have also been isolated from wild flies (Corby-Harris *et al.* 2007; Juneja and Lazzaro, 2009). However, there has been very little research into the protozoan parasites of *Drosophila* for the last 40 years (see Ebbert *et al.* (2003) for exceptions).

Trypanosomatids (Kinetoplastida: Trypanosomatidae) are flagellate protozoans that infect a

wide range of invertebrates, vertebrates and plants. The dixenous (2-host) species are best known, as they include insect-vectored parasites of vertebrates. The monoxenous species, which are restricted to insects, are less well studied. The large majority of trypanosomatids that have been reported have been found in the Diptera (flies) and Hemiptera (bugs) (Podlipaev, 2001), and in these groups they can be very common. For example, a recent survey of 170 species of insects from the suborder Heteroptera found that 9% of species carried trypanosomatids, with an overall prevalence of 22% (Maslov *et al.* 2007). However, despite these recent studies the true diversity of trypanosomatids is unknown (Podlipaev, 2001). The first records of trypanosomatids in *Drosophila* were made over 100 years ago in *Drosophila confusa* (Chatton and Alilaire, 1908), and they have subsequently been discovered in a range of other species (Ebbert *et al.* 2001). In a study of 8 species of North American *Drosophila* the prevalence of infection ranged from 1 to 17% (Ebbert *et al.* 2001), although there have been reports of the prevalence reaching 40% in *D. melanogaster* (McGhee and Cosgrove, 1980). The trypanosomatids infect the gut, and transmission occurs when food is contaminated with faeces or with the cadavers of infected flies (Rowton and McGhee, 1983). Although trypanosomatids can be transmitted between distantly related species of *Drosophila* in the laboratory, rates of transmission in a new host can be much reduced suggesting that there is some degree of host specificity (McGhee *et al.* 1969).

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Trypanosomatid infections are harmful to the host, as infected *D. melanogaster* have significantly longer development times than uninfected individuals (Ebbert *et al.* 2003).

Until recently the only way to identify trypanosomatids and study their diversity was to either conduct detailed studies in culture or use microscopy to describe their morphology, but both of these approaches have their limitations. Culture-based methods tend to miss much of the diversity because many trypanosomatids cannot be cultivated (Yurchenko *et al.* 2009). Additionally, insect trypanosomatids are difficult to distinguish morphologically and phylogenies based on morphological characters have proved unreliable (Podlipaev *et al.* 2004). PCR and DNA sequencing can avoid these problems, and these approaches have now shown that insect trypanosomatids are highly diverse and have provided detailed phylogenies (Podlipaev *et al.* 2004; Yurchenko *et al.* 2006; Maslov *et al.* 2007; Votycka *et al.* 2010). The aim of this study was to use these PCR-based approaches to study trypanosomatids that infect *Drosophila*. To assess the diversity of *Drosophila* trypanosomatids, we sequenced the spliced leader RNA gene. Highly conserved exon sequences allow conserved primers to be designed that will amplify this gene from all the genera of trypanosomatids, while a hypervariable intergenic sequence allows different isolates to be discriminated (Murthy *et al.* 1992; Westenberger *et al.* 2004). Although this provides a useful barcode to identify different types of trypanosomatids, the sequences cannot be easily aligned. Therefore, we also attempted to sequence the highly conserved glycosomal *GAPDH* gene to reconstruct a phylogeny of *Drosophila* trypanosomatids, and examine how they are related to parasites from other hosts (Yurchenko *et al.* 2006). This work will give us a greater understanding of the parasites that infect this important model organism, and will identify *Drosophila* trypanosomatids that could be used to investigate host-parasite interactions.

## MATERIALS AND METHODS

### Field samples

We collected a range of different fly species using fruit baits from several European populations. In an urban population in Athens, Greece we collected *Drosophila simulans* and *D. melanogaster* over a 5-day period in October 2007 from an area ca. 300 m in diameter. Several related species from the *obscura* group were collected from woodland locations from around the UK in the summer of 2008. In addition a small collection of *D. melanogaster* was made in Braga, Portugal, which was used only for sequencing and is not included in prevalence estimates.

### PCR and sequencing

To extract DNA, we homogenized single wild-caught flies, incubated the homogenate at 56 °C for 1 h with Chelex 100<sup>TM</sup> ion exchange resin (Bio-Rad, Hercules, CA, USA) and proteinase-K, and then boiled the supernatant which was used directly for PCR. To check that the DNA extraction process had been successful, specific primers that amplify the *ref(2)P* gene in *D. melanogaster* and *RpL32* in the *obscura* group were used. Any samples that did not amplify were discarded. Both *D. melanogaster* and *D. simulans* occur in the Greek population we sampled. Males were identified morphologically. The females were identified using PCR primers that amplify the *drosomycin* gene (dro-F 5'-CAG CCC TAA AGT ATG CCC TTC, dro-R 5'-AGC CAG GAA GAG GTA CAR GA) and yield products of 226 to 229 bp in *D. melanogaster* and 245 to 246 bp in *D. simulans*. The species in the *obscura* group were identified as *D. obscura*, *D. subobscura*, or *D. helvetica* using diagnostic PCR reactions that amplify the *cytochrome b* (*Cyt-b*) gene and the nuclear *alcohol dehydrogenase* (*Adh*) gene. A single specimen of *D. tristis* as well as 2 specimens of *D. helvetica* were identified by sequencing 852 bp of the *Cyt-b* gene, which were found to be identical to the published sequence from *D. tristis* (Accession number EF216284) and *D. helvetica* (Accession number EF216268) respectively.

To test flies for the presence of trypanosomatids, we used a diagnostic PCR that amplifies the trypanosomatid spliced leader sequence using the oligonucleotides M167, 5'-GGGAAGCTTCTGTACT (A/T)TATTGGTA, and M168, 5'-GGGAATTC-AATA(A/T)AGTACAGAAACTG, that were described by (Westenberger *et al.* 2004). We sequenced the spliced leader sequence from a subsample of 21 DNA extractions from *D. melanogaster*, *D. simulans*, *D. subobscura* and *D. helvetica* (see Table 1). We used a cloning approach where direct sequencing of PCR products showed evidence of multiple sequences being present in the same fly. For 6 DNA samples, we cloned PCR fragments using the StrataClone PCR cloning kit (Stratagene, LaJolla, CA, USA) following the manufacturer's instructions and sequenced 4 clones per sample. Before sequencing, unused PCR primers and dNTPs were digested with exonuclease 1 and shrimp alkaline phosphatase. The PCR products were then sequenced directly using the PCR primers and Big Dye reagents (ABI) on an ABI capillary sequencer. Sequences were assembled using Sequencher 4.5 (Gene Codes Corporation) and chromatograms were inspected by eye to confirm the legitimacy of any differences between sequences. In total, we analysed 34 spliced leader sequences, including 19 variable sequences from potentially multiply infected individuals. Of these sequences, 27 represent a complete repeat unit, with the caveat that they

Table 1. Spliced leader repeat sequences

(The spliced leader repeat was sequenced from 21 infected hosts; for a subset of 6 hosts, PCR fragments were cloned prior to sequencing as direct sequencing failed. Incomplete repeats include the poly-T-track, the intergenic region and the conserved Mini-exon; for these sequences, the repeat length is given in parentheses.)

Name	Host species	Country	Clone	Complete	Repeat length	Group
1a_mel	<i>D. melanogaster</i>	Greece	1a	yes	401	A
1b_mel	<i>D. melanogaster</i>	Greece	1b	yes	419	A
1c_mel	<i>D. melanogaster</i>	Greece	1c	yes	419	A
1d_mel	<i>D. melanogaster</i>	Greece	1d	yes	419	A
2a_mel	<i>D. melanogaster</i>	Greece	2a	yes	418	A
2bd_mel	<i>D. melanogaster</i>	Greece	2b, 2d	yes	419	A
2c_mel	<i>D. melanogaster</i>	Greece	2c	yes	401	A
3a_mel	<i>D. melanogaster</i>	Greece	3a	yes	401	A
3b_mel	<i>D. melanogaster</i>	Greece	3b	yes	419	A
3c_mel	<i>D. melanogaster</i>	Greece	3c	yes	419	A
4ab_mel	<i>D. melanogaster</i>	Greece	4a, 4b	yes	401	A
4c_mel	<i>D. melanogaster</i>	Greece	4c	yes	419	A
5a_mel	<i>D. melanogaster</i>	Greece	5a	yes	419	A
5b_mel	<i>D. melanogaster</i>	Greece	5b	yes	419	A
5c_mel	<i>D. melanogaster</i>	Greece	5c	yes	419	A
5d_mel	<i>D. melanogaster</i>	Greece	5d	yes	401	A
6a_mel	<i>D. melanogaster</i>	Greece	6a	yes	419	A
6b_mel	<i>D. melanogaster</i>	Greece	6b	yes	419	A
6c_mel	<i>D. melanogaster</i>	Greece	6c	yes	419	A
7_mel	<i>D. melanogaster</i>	Greece	—	yes	419	A
8_mel	<i>D. melanogaster</i>	Greece	—	no	(369)	A
9_mel	<i>D. melanogaster</i>	Greece	—	yes	459	B
10_mel	<i>D. melanogaster</i>	Greece	—	yes	459	B
11_sim	<i>D. simulans</i>	Greece	—	no	(417)	B
12_sim	<i>D. simulans</i>	Greece	—	no	(408)	B
13_mel	<i>D. melanogaster</i>	Greece	—	no	(417)	B
14_mel	<i>D. melanogaster</i>	Greece	—	no	(417)	B
15_mel	<i>D. melanogaster</i>	Greece	—	no	(408)	B
16_mel	<i>D. melanogaster</i>	Greece	—	no	(417)	B
17_sub	<i>D. subobscura</i>	Greece	—	yes	449	C
18_hel	<i>D. helvetica</i>	UK	—	yes	449	C
19_hel	<i>D. helvetica</i>	UK	—	yes	449	C
20_sub	<i>D. subobscura</i>	UK	—	yes	425	D
21_sub	<i>D. subobscura</i>	UK	—	yes	384	E

include the overlapping primer binding sites in the conserved exon region (Table 1). We have included 7 sequences that are missing 50–51 bp of the intronic region downstream of the primer-binding site (Table 1). These sequences fully align with other complete sequences in our data set, including in the non-conserved intergenic region. The spliced leader sequences have been submitted to GenBank under the numbers HQ285325–HQ285358.

To reconstruct the parasite phylogeny, we sequenced the *GAPDH* genes from a subset of the infected flies following the protocol outlined above. To amplify the *GAPDH* gene we used the primers GAPDH\_dir, 5'-AGAGGATCCATGGCTCCG(A/C)TCAAGGTTGGC-3', and GAPDH\_rev, 5'-AGAGGATCCTTACATCTTTCGAGCTCGC-G(C/G)(C/G)GTC-3', that were described by Yurchenko *et al.* (2006). The *GAPDH* sequences have been submitted to GenBank under the numbers HQ263664–HQ263666.

#### Data analysis

We obtained most of the available sequences of both genes from GenBank, ignoring multiple accessions, and aligned them with our sequences using ClustalW and corrected the alignment by eye. As the spliced leader sequences are highly divergent, only the most similar sequences can be reliably aligned, which means that while this gene is useful for typing different isolates, it does not produce reliable phylogenies of distantly related species (Westenberger *et al.* 2004). As our spliced leader sequences did not reliably align with sequences obtained from GenBank, we reconstructed neighbour-joining trees based on Jukes-Cantor genetic distances using the program Phylip (Felsenstein, 2005) for the *Drosophila* trypanosomatid samples based on contigs produced by Sequencher 4.5.

The phylogeny of the *GAPDH* amino acid sequences was reconstructed via a Bayesian approach

Table 2. The prevalence of trypanosomatids in four species of *Drosophila*(The *obscura* group flies were not identified to species level.)

Species	Population	Infected	Uninfected	Prevalence
<i>D. melanogaster</i>	Greece	31	891	3%
<i>D. simulans</i>	Greece	2	268	1%
<i>D. obscura</i>	UK	4	161	2%
<i>D. subobscura</i>	UK	24	509	5%
<i>obscura</i> group	UK	14	225	6%

using MrBayes (v. 3.1.2) (Huelsenbeck and Ronquist, 2001). We assumed a fixed rate model of protein evolution and reconstructed the phylogeny using a model jumping method. This method allows for different models of amino acid substitution to be used in the MCMC procedure, with all models contributing to the final result weighted according to their respective posterior probability. We ran 2 runs of 4 chains for 4 000 000 MCMC generations, sampling trees every 1000 generations. All trees were drawn using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

Additionally, we reconstructed the *GAPDH* phylogeny via the DNA sequences using both a Bayesian and a maximum-likelihood approach. Bayesian posterior support values are less conservative than maximum-likelihood bootstrap support, and so both the values can be used as an upper and lower support for nodes (Douady *et al.* 2003). For the maximum likelihood trees, jModeltest (v. 0.1.1) (Guindon and Gascuel, 2003; Posada, 2008) was used to estimate the model of sequence evolution and the analysis was run in PAUP (v. 4.0b10) (Swofford, 2003). A parsimony tree created from tree bisection and reconnection with a heuristic search was used as a starting tree for the maximum likelihood analysis. A GTR+G model with a gamma distribution of rate variation and no invariable sites was used. The maximum likelihood analysis used a heuristic search with a nearest neighbour interchange algorithm. The substitution rate parameters, shape of the gamma distribution and proportion of invariable sites used were those estimated by ModelTest. Support for the nodes was calculated by 1000 non-parametric bootstraps. Bayesian trees were created using the MrBayes program (v. 3.1.2) (Huelsenbeck and Ronquist, 2001). We used a general time-reversible model, with a gamma distribution and parameters estimated from the data during the analysis. As there is likely to be a considerable amount of noise from third codon positions between these divergent sequences, a site-specific rate model was used allowing each codon position to have its own rate. Two runs of 4 chains were run for 15 000 000 MCMC generations, with trees being sampled every 5000 generations. The DNA tree is presented as Supplementary material (online version only).

Differences in the proportion of infected flies were analysed in contingency tables using a Fisher Exact Test. In tables of more than 2 rows and 2 columns, significance was assessed by generating 100 000 random contingency tables with the same marginal values using a Monte Carlo procedure and taking the proportion with more extreme deviations as the probability (Lewontin and Felsenstein, 1965).

## RESULTS

### Prevalence

We tested 2129 wild-caught *Drosophila* for infection with trypanosomatids using a diagnostic PCR reaction that amplifies the spliced leader RNA gene. All 5 identified species that we collected – *D. melanogaster*, *D. simulans*, *D. obscura*, *D. subobscura* and *D. helvetica* – were infected (Table 2). Note that we did not further determine the species of uninfected flies from the *obscura* group by sequencing. The prevalence of infection varied significantly among these species (Fisher Exact Test:  $P=0.02$ ), but was always below 5%. This variation was not entirely caused by samples coming from different geographical regions, as the prevalence differed between the 2 species from Greece (*D. melanogaster* and *D. simulans*; Fisher Exact Test:  $P=0.02$ ) but not the 2 from the UK (*D. obscura* and *D. subobscura*; Fisher Exact Test:  $P=0.36$ ). Multiple spliced leader sequences from a single fly were detected in 28.5% of samples, suggesting that these flies may have been infected by 2 trypanosomatids with distinct spliced leader RNA genotypes. Alternatively, this may indicate microheterogeneity of spliced leader repeat units, which has been reported in both *Leishmania major* and *Trypanosoma cruzi* (Thomas *et al.* 2005).

*Drosophila obscura* and *D. subobscura* were collected from 14 sites scattered across the UK. In *D. obscura*, there was significant geographical variation in prevalence (Table 3; Fisher Exact Test:  $P=0.03$ ). For example, in the 2 populations with the largest sample size the prevalence ranged from 0.7% to 7%. In *D. subobscura*, where we had smaller samples, there was no significant geographical variation in prevalence (data not shown; Fisher Exact Test:  $P=0.08$ ).

Table 3. The prevalence of trypanosomatids in different populations of *Drosophila obscura* from across the UK

Population	Infected	Uninfected	Prevalence
Bristol A	1	14	7%
Bristol C	2	12	14%
Derbyshire A	2	52	4%
Derbyshire B	2	29	6%
Essex	1	144	0.7%
Falmouth A	2	67	3%
Falmouth AC	5	25	17%
Kent	8	103	7%
Sussex	1	41	2%

#### Barcoding trypanosomatid samples

To distinguish different trypanosomatids we sequenced the spliced leader sequence. The spliced leader RNA is encoded in the genome in many copies arranged in a tandem array. The sequence of each repeat consists of a highly conserved exon, a variable intron and a hypervariable intergenic sequence (Murthy *et al.* 1992). It has been found that a 20 bp sequence of the exon can be used to define groups of related species, and it is therefore a useful 'barcode' that can be used to identify trypanosomatids to a level akin to a genus (Westenberger *et al.* 2004; Maslov *et al.* 2007).

We sequenced this 20 bp region and found that it was identical in 33 of 34 individual sequences (AACTAACGCTATTATTGTTA), suggesting that most of these trypanosomatids are closely related. The hosts consisted of 14 Greek *D. melanogaster* and 2 Greek *D. simulans*, 2 UK *D. subobscura* and 2 UK *D. helvetica*. Barcoding assigns these trypanosomatid samples to a group consisting of *Sergeia podlipaevi*, several *Herpetomonas* species, one species of *Leptomonas* and several unidentified Trypanosomatidae isolated from Heteroptera in Egypt and Southwest China. The only other sequence that was identified was from a single *D. subobscura* from the UK, which was identical to that found in the 'SE clade' (AACTAACGCTATATAAGTAT, Maslov *et al.* 2007). This highly specious group contains numerous isolates from many species of insects, and includes the genera *Crithidia* and *Leptomonas*, which contain a number of insect parasites.

Although the exon is useful to identify major groups of trypanosomatids, it evolves too slowly to distinguish more closely related species or strains. To do this we examined 1 spliced leader RNA repeat unit. Examining the sequences, it was clear that they fell into 5 major sequence groups (Fig. 1). Groups A–D all share the exon sequence AACTAACGCTATTATTGTTA and group E has the sequence AACTAACGCTATATAAGTAT. The sequence groups show variation in repeat length (see Table 1),

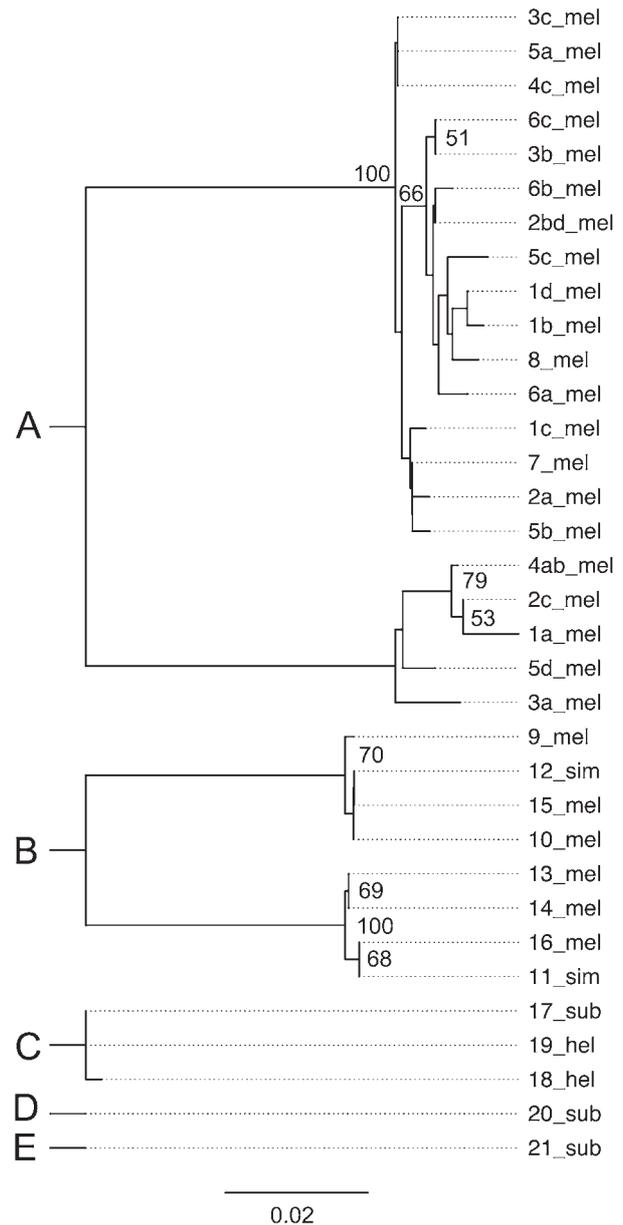


Fig. 1. Neighbour-joining trees of the spliced leader sequences illustrating genetic diversity within sequence groups. The taxa are labelled according to the species of *Drosophila* the trypanosomatids were found in, following the nomenclature in Table 1. The sequences form 5 groups (A–E), within which the genetic distance between sequences is 13% or less. The sequences in the different groups are too divergent to reliably align (except for the conserved region).

which is caused by indels in the highly variable intergenic region. Additionally, groups D and E show small indels in the intronic region (a 1 bp insertion and a 5 bp deletion respectively). The pair-wise genetic similarity between sequences within each group is very high, with an average of 95.4% in group A, 94.7% in group B and 99.8% in group C, and both the intergenic and intronic regions could be aligned. The maximum pair-wise genetic distance in these groups is 12.6%, 7.7% and 0.2% respectively.

However, between the groups there is a very high level of divergence, and, although some stretches of homologous sequence are clearly visible in the intronic region, reliable sequence alignments across the repeat unit were impossible. Therefore there are distinct species or strains of trypanosomatids that infect *Drosophila*. We refrained from further phylogenetic analyses of the spliced leader sequence because these methods rely on perfect alignments (Ogden and Rosenberg, 2006), and reliable alignments were not possible.

To examine how the strains are distributed across our different fly species, we made separate sequence alignments of each sequence group and reconstructed neighbour-joining trees of these sequences (Fig. 1). It is clear that there are strong effects of host species in which genotypes of trypanosomatids are found. However, there are no significant differences between the groups that are found in closely related species collected from the same location and habitat – Greek *D. simulans* and *D. melanogaster*, or members of the *obscura* group from the UK – suggesting that there is transmission of parasites between these species.

#### Phylogeny

The rapid evolution of the spliced leader RNA sequence means that it cannot be used to infer the phylogeny of distantly related strains of trypanosomatids, so instead we attempted to amplify the *GAPDH* gene (glyceraldehyde-3-phosphate dehydrogenase), which is more conserved (Yurchenko *et al.* 2006). Unfortunately, this proved to be difficult, and we only obtained sequences from 3 trypanosomatid samples found in *D. tristis* and *D. obscura* from the UK, and *D. melanogaster* from Portugal, from which, in turn, we failed to obtain the spliced leader sequence. Similar difficulties in sequencing both the spliced leader and *GAPDH* gene have been reported before (i.e. Votypka *et al.* 2010; Maslov *et al.* 2010) and Maslov *et al.* (2010) have pointed out that additional phylogenetic markers will need to be analysed in order to resolve the trypanosomatid phylogeny. As the sequences were highly divergent, we reconstructed phylogenies using protein sequences as well as DNA sequences. The *D. tristis* and *D. obscura* trypanosomatid samples form a monophyletic group that is distant from the sample found in *D. melanogaster* (Fig. 2 and S1-Online version only). The sequences are most closely allied to other monoxenous species that infect insects. The closest relatives of the *D. melanogaster* trypanosomatid sample are *Herpetomonas muscarum*, *H. megaseliae* and ‘isolate 37EC’, which naturally infect house flies, scuttle flies and a heteropteran bug (Yurchenko *et al.* 2009). There is only weak support for the relationships of the *D. tristis* and *D. obscura* trypanosomatid samples to other species, but the most likely

relative is a parasite of mosquitoes, *Blastocrithidia culicis*.

#### DISCUSSION

We have found that trypanosomatids are common parasites of *Drosophila*, and have for the first time characterized the diversity of these parasites in natural populations. The sequences from the spliced leader sequence show that the large majority of trypanosomatids circulating in European *Drosophila* populations are genetically distinct from those found in other large surveys of trypanosomatids as it was impossible to align the sequences. Within this group there are several distinct genotypes that can be identified from the more variable regions of the spliced leader sequence, and it is possible that these are different species of the parasites. How these different strains relate to other trypanosomatids is still uncertain, as we had difficulty in sequencing more conserved genes from the parasites, failing to obtain any *GAPDH* sequences from the samples for which we sequenced the spliced leader sequence and vice versa. However, the sequences that we did obtain show that they are most closely related to other monoxenous species that only infect insects (as opposed to the dixenous species of the two genera *Trypanosoma* and *Leishmania* that infect vertebrates), and belong to at least 2 different lineages on the trypanosomatid phylogeny.

The PCR approach that we have used may detect very low numbers of parasites. It is possible that some of the trypanosomatids detected may have been picked up during feeding in the wild, without having replicated significantly in the fly. However, this is unlikely as all the flies were maintained on agar or fly medium for several days before the PCR assay. Additionally, it is known that naturally occurring trypanosomatid infections do establish in *Drosophila* (e.g. Ebbert *et al.* 2001, 2003). Furthermore, the infection in the Portuguese specimen was detected by microscopy (Ferreira, unpublished data); this sample showed morphological similarity to *Leptomonas drosophilae* as described by Chatton and Leger (1911). Further work is required to establish whether all the organisms that we have detected are true parasites of these species of *Drosophila*.

*Drosophila* is widely used as a model species to study both invertebrate immune systems and the evolution of hosts and parasites. Trypanosomatids are a potential new system for these studies, as they represent one of the few natural parasites of *Drosophila* that we know of and many species can be cultured *in vitro*. They may be naturally co-evolving with *Drosophila*, potentially leading to flies evolving specific immune responses to counter these infections, and in turn parasites having been selected to evade *Drosophila* immune responses. Much of the research into the *Drosophila* immune systems has

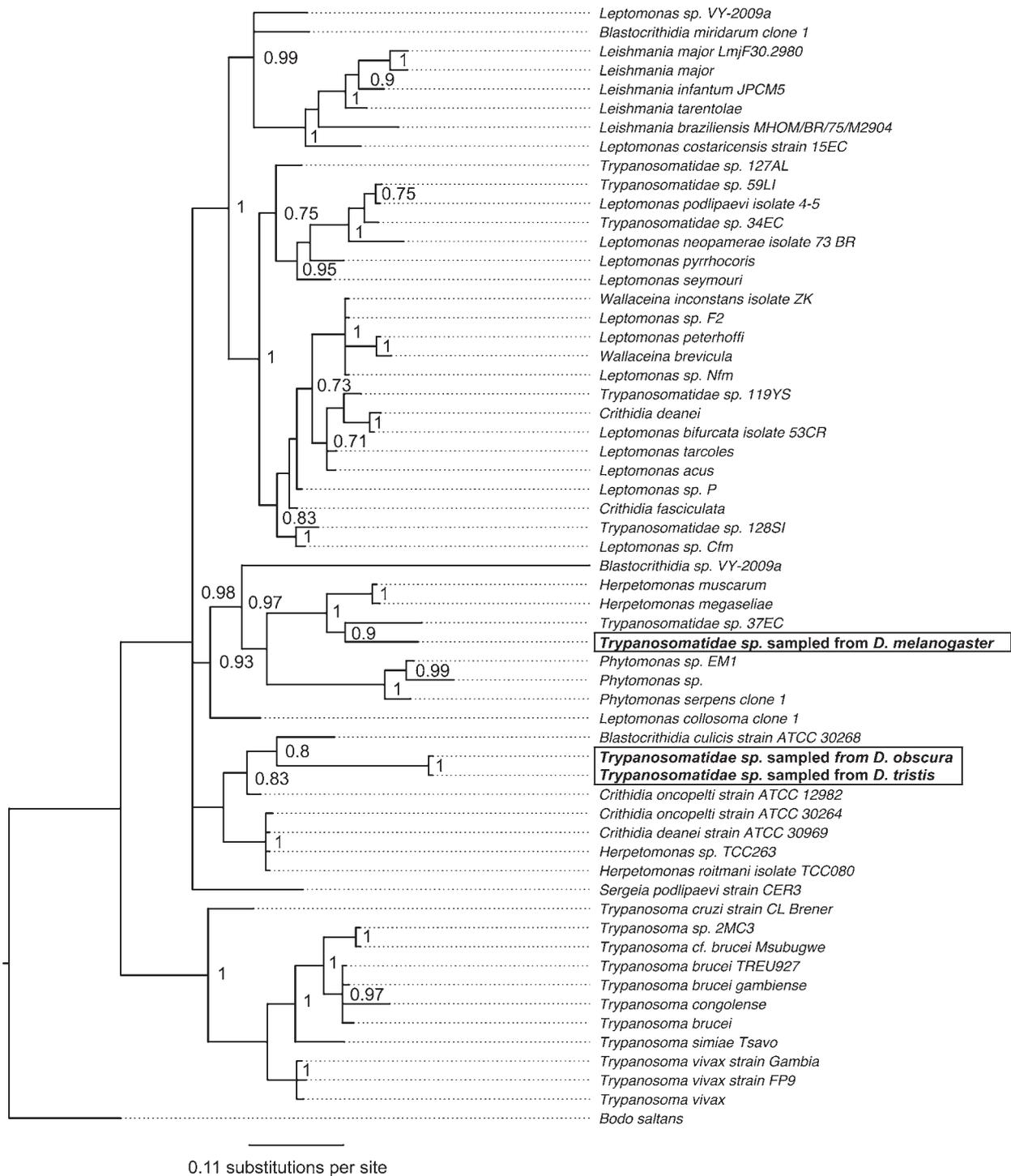


Fig. 2. Phylogeny of the GAPDH gene reconstructed from protein sequences. The tree was reconstructed using the Bayesian method and node labels show the posterior support for the clade.

focussed on systemic immune responses to bacteria and fungi. It is therefore also interesting to find that protists that infect the gut are common in fly populations, and studying the immune response to these parasites promises to reveal new aspects of the invertebrate immune response. As *Drosophila* is a model system particularly amenable to molecular and immunological studies, this avenue of research also has the potential to yield new methods of fighting monoxenous trypanosomatid infections in economically

important insects such as honeybees and bumblebees as well as in dioxenous infections in which insects vector vertebrate parasites. Important ecological questions also remain unanswered. We found that different genotypes of the parasites are found in different host species. It is currently unclear whether this host specificity is the result of parasites being physiologically adapted to certain host species, or if it instead emerges simply because related species share related habitats or were collected from the same sites.

In conclusion, trypanosomatids represent an exciting new opportunity for evolutionary and immunological research in *Drosophila*.

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## REFERENCES

- Boulanger, N., Ehret-Sabatier, L., Brun, R., Zachary, D., Bulet, P. and Imler, J.L.** (2001). Immune response of *Drosophila melanogaster* to infection with the flagellate parasite *Crithidia* spp. *Insect Biochemistry and Molecular Biology* **31**, 129–137.
- Brun, P. and Plus, N.** (1980). The viruses of *Drosophila*. In *The Genetics and Biology of Drosophila* (ed. by Ashburner, M. and Wright, T. R. F.), pp. 625–702. Academic Press, London, UK.
- Chatton, E. and Alilaire, E.** (1908). Coexistence d'un *Leptomonas* (*Herpetomonas*) et d'un *Trypanosoma* chez un muscicide non vulnérant, *Drosophila confusa* Staeger. *Comptes rendus des Seances de la Societe de Biologie, Paris* **64**, 1004–1006.
- Chatton, E. and Leger, A.** (1911). *Eutrypanosomes, Leptomonas* et *Leptotrypanosomes* chez *Drosophila confusa* Staeger (Muscide). *Comptes rendus des Seances de la Societe de Biologie, Paris* **70**, 34–36.
- Corby-Harris, V., Pontaroli, A. C., Shimkets, L. J., Bennetzen, J. L., Habel, K. E. and Promislow, D. E. L.** (2007). Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Applied and Environmental Microbiology* **73**, 3470–3479. doi:10.1128/aem.02120-06.
- Douady, C. J., Delsuc, F., Boucher, Y., Doolittle, W. F. and Douzery, E. J. P.** (2003). Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. *Molecular Biology and Evolution* **20**, 248–254. doi:10.1093/molbev/msg042.
- Ebbert, M. A., Burkholder, J. J. and Marlowe, J. L.** (2001). Trypanosomatid prevalence and host habitat choice in woodland *Drosophila*. *Journal of Invertebrate Pathology* **77**, 27–32. doi:10.1006/jjipa.2000.4989.
- Ebbert, M. A., Marlowe, J. L. and Burkholder, J. J.** (2003). Protozoan and intracellular fungal gut endosymbionts in *Drosophila*: prevalence and fitness effects of single and dual infections. *Journal of Invertebrate Pathology* **83**, 37–45. doi:10.1016/s0022-2011(03)00033-8.
- Felsenstein, J.** (2005). *PHYLIP (Phylogeny Inference Package) Version 3.69*. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- Guindon, S. and Gascuel, O.** (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704. doi:10.1080/10635150390235520.
- Hoffmann, J. A.** (2003). The immune response of *Drosophila*. *Nature* **426**, 33–38. doi:10.1038/nature02021.
- Huelsenbeck, J. P. and Ronquist, F.** (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755. doi:10.1093/bioinformatics/17.8.754.
- Juneja, P. and Lazzaro, B. P.** (2009). *Providencia sneebia* sp. nov. and *Providencia burhodogranariae* sp. nov., isolated from wild *Drosophila melanogaster*. *International Journal of Systematic and Evolutionary Microbiology* **59**, 1108–1111. doi:10.1099/ijs.0.000117-0.
- Lewontin, R. C. and Felsenstein, J.** (1965). The robustness of homogeneity tests in 2 X n tables. *Biometrics* **21**, 19–33.
- Longdon, B., Obbard, D. J. and Jiggins, F. M.** (2010). Sigma viruses from three species of *Drosophila* form a major new clade in the rhabdovirus phylogeny. *Proceedings of the Royal Society of London, B* **277**, 35–44. doi:10.1098/rspb.2009.1472.
- Maslov, D. A., Westenberger, S. J., Xu, X., Campbell, D. A. and Sturm, N. R.** (2007). Discovery and barcoding by analysis of spliced leader RNA gene sequences of new isolates of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador. *Journal of Eukaryotic Microbiology* **54**, 57–65. doi:10.1111/j.1550-7408.2006.00150.x.
- Maslov, D. A., Yurchenko, V. Y., Jirku, M. and Lukes, J.** (2010). Two new species of trypanosomatid parasites isolated from Heteroptera in Costa Rica. *Journal of Eukaryotic Microbiology* **57**, 177–188. doi:10.1111/j.1550-7408.2009.00464.x.
- McGhee, R. B. and Cosgrove, W. B.** (1980). Biology and physiology of the lower Trypanosomatidae. *Microbiological Reviews* **44**, 140–173.
- McGhee, R. B., Hanson, W. L. and Schmittner, S. M.** (1969). Isolation, cloning and determination of biologic characteristics of five new species of *Crithidia*. *Journal of Eukaryotic Microbiology* **16**, 514–520. doi:10.1111/j.1550-7408.1969.tb02310.x.
- Murthy, V. K., Dibbern, K. M. and Campbell, D. A.** (1992). PCR amplification of mini-exon genes differentiates *Trypanosoma cruzi* from *Trypanosoma rangeli*. *Molecular and Cellular Probes* **6**, 237–243. doi:10.1016/0890-8508(92)90022-P.
- Ogden, T. H. and Rosenberg, M. S.** (2006). Multiple sequence alignment accuracy and phylogenetic inference. *Systematic Biology* **55**, 314–328. doi:10.1080/10635150500541730.
- Podlipaev, S.** (2001). The more insect trypanosomatids under study—the more diverse Trypanosomatidae appears. *International Journal for Parasitology* **31**, 647–651. doi:10.1016/S0020-7519(01)00139-4.
- Podlipaev, S. A., Sturm, N. R., Fiala, I., Fernanades, O., Westenberger, S. J., Dollet, M., Campbell, D. A. and Lukes, J.** (2004). Diversity of insect trypanosomatids assessed from the spliced leader RNA and 5S rRNA genes and intergenic regions. *Journal of Eukaryotic Microbiology* **51**, 283–290.
- Posada, D.** (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**, 1253–1256. doi:10.1093/molbev/msn083.
- Rowton, E. D. and McGhee, R. B.** (1983). Transmission of *Herpetomonas* in Laboratory Populations of *Drosophila melanogaster*. *Journal of Eukaryotic Microbiology* **30**, 669–671. doi:10.1111/j.1550-7408.1983.tb05341.x.
- Swofford, D. L.** (2003). *PAUP\*. Phylogenetic Analysis using Parsimony (\*and other Methods)* Sinauer Associates, Sunderland, MA, USA.
- Thomas, S., Westenberger, S. J., Campbell, D. A. and Sturm, N. R.** (2005). Intragenomic spliced leader RNA array analysis of kinetoplasts reveals unexpected transcribed region diversity in *Trypanosoma cruzi*. *Gene* **352**, 100–108. doi:10.1016/j.gene.2005.04.002.
- Westenberger, S. J., Sturm, N. R., Yanega, D., Podlipaev, S. A., Zeledon, N. R., Campbell, D. A. and Maslov, D. A.** (2004). Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene. *Parasitology* **129**, 537–547. doi:10.1017/S003118200400592X.
- Votycka, J., Maslov, D. A., Yurchenko, V., Jirku, M., Kment, P., Lun, Z. R. and Lukes, J.** (2010). Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in South-West China reveals both endemism and global dispersal. *Molecular Phylogenetics and Evolution* **54**, 243–253. doi:10.1016/j.ympev.2009.10.014.
- Yurchenko, V. Y., Lukes, J., Jirku, M. and Maslov, D. A.** (2009). Selective recovery of the cultivation-prone components from mixed trypanosomatid infections: a case of several novel species isolated from neotropical Heteroptera. *International Journal of Systematic and Evolutionary Microbiology* **59**, 893–909. doi:10.1099/ijs.0.001149-0.
- Yurchenko, V., Lukes, J., Xu, X. and Maslov, D. A.** (2006). An integrated morphological and molecular approach to a new species description in the trypanosomatidae: The case of *Leptomonas podlipaevi* n. sp., a parasite of *Boisea rubrolineata* (Hemiptera: Rhopalidae). *Journal of Eukaryotic Microbiology* **53**, 103–111. doi:10.1111/j.1550-7408.2005.00078.x.