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Phylogenetic relationships of trypanosomatids parasitising true bugs (Insecta: Heteroptera) in sub-Saharan Africa

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ABSTRACT

Three hundred and eighty-six heteropteran specimens belonging to more than 90 species captured in Ghana, Kenya and Ethiopia were examined for the presence of trypanosomatid flagellates. Of those, 100 (26%) specimens were positive for trypanosomatids and the spliced leader RNA gene sequence was obtained from 81 (80%) of the infected bugs. Its sequence-based analysis placed all examined flagellates in 28 typing units. Among 19 newly described typing units, 16 are restricted to sub-Saharan Africa, three belong to previously described species and six to typing units found on other continents. This result was corroborated by the analysis of the ssrRNA gene, sequenced for at least one representative of each major spliced leader RNA-based clade. In all trees obtained, flagellates originating from sub-Saharan Africa were intermingled with those isolated from American, Asian and European hosts, revealing a lack of geographic correlation. They are dispersed throughout most of the known diversity of monoxenous trypanosomatids. However, a complex picture emerged when co-evolution with their heteropteran hosts was taken into account, since some clades are specific for a single host clade, family or even species, whereas other flagellates display a very low host specificity, with a capacity to parasitise heteropteran bugs belonging to different genera/families. The family Reduviidae contains the widest spectrum of trypanosomatids, most likely a consequence of their predatory feeding behaviour, leading to an accumulation of a variety of flagellates from their prey. The plant pathogenic genus *Phytomonas* is reported here from Africa, to our knowledge for the first time. Finding the same typing units in hosts belonging to different heteropteran families and coming from different continents strongly indicates that the global diversity of the insect trypanosomatids is most likely lower than was predicted on the basis of the "one host–one parasite" paradigm. The analysis presented significantly extends the known diversity of monoxenous insect trypanosomatids and will be instrumental in building a new taxonomy that reflects their true phylogenetic relationships.

1. Introduction

Trypanosomatid flagellates are extremely successful obligatory parasites occurring in hosts ranging from insects to all classes of vertebrates, as well as plants. Due to the nearly ubiquitous dispersal of their hosts, these protists can be found in virtually any aquatic or terrestrial environment (Simpson et al., 2006). While the medically and economically relevant members of the dixenous genera, *Trypanosoma* and *Leishmania*, have received substantial attention and thus belong to the best studied protists, all other trypanosomatids have remained largely overlooked. A common feature of nearly all monoxenous trypanosomatids is their confinement to "unimportant" insects in economic, medical or veterinary terms, with the majority of species recorded from heteropteran and dipteran hosts (Podlipaev, 1990, 2000). However, they have also been rarely, but repeatedly, encountered in warm-blooded vertebrates including humans (Morio et al., 2008; Srivastava et al., 2010).

Within the last decade, insect trypanosomatids have started to receive increasing attention, the main aim of which was to map their diversity, understand their transmission routes and lay foundations for a new taxonomy that would reflect true phylogenetic relationships (Podlipaev et al., 2004a,b; Yurchenko et al., 2006a,b; Maslov et al., 2007; Svobodová et al., 2007; Yurchenko et al., 2008, 2009; Votýpka et al., 2010; Teixeira et al., 2011). The current classification of trypanosomatids is largely based on the presence in the life cycle of one or more of the seven established

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morphologically distinct cell types (Hoare and Wallace, 1966; Wallace, 1966; Vickerman, 1976). This system was useful before the acquisition of biochemical and molecular data but it has outlived its virtue, as the data available to date show that there is almost no correlation between features observable by light and electron microscopy and molecular phylogeny (Yurchenko et al., 2009; Teixeira et al., 2011; Jirků et al., 2012). The latter method is based on sequences of the ssrRNA, spliced leader (SL) RNA, and to a lesser extent the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Regardless of the sequence used, the inclusion of newly isolated strains into phylogenetic trees has progressively rendered the monoxenous genera *Leptomonas*, *Crithidia*, *Blastocrithidia*, *Wallaceina* and *Herpetomonas* paraphyletic, whereas all three dixenous genera (*Trypanosoma*, *Leishmania* and *Phytomonas*) have retained their well-supported monophyly (Simpson et al., 2006). Moreover, detailed characterisation of cultured and host-dwelling stages has shown that at least some strains are morphologically highly variable (Zídková et al., 2010; Votýpka et al., in press), further underlining the unsuitability of morphological characters for the higher-level taxonomy of insect trypanosomatids.

Due to their extremely wide host range, insect parasites are particularly suitable to address potential correlations with hosts, environment and/or geography. In order to investigate these questions, we have assembled a large set of isolates from sub-Saharan Africa, a region from which not a single monoxenous insect trypanosomatid has been available to date. However, from this biogeographical region, information is not lacking for the dixenous genera *Trypanosoma* and *Leishmania*, of which hundreds of strains have been isolated from dipteran vectors since the beginning of the 20th century (Adams et al., 2010). Although some novel "African" clades have emerged, an extensive SL- and ssrRNA-based phylogenetic analysis convincingly showed that correlation with geography is absent for most flagellates studied. Interestingly, some well-supported clades parasitise heteropterans from a single family, while other species cross the family or even order boundaries of their hosts. Finally, although it was predicted that species richness of insect trypanosomatids may, due to their possibly high host specificity, reach millions (Stevens, 2001), making the attempt to map their diversity a challenging task, the fact that we have encountered some already known typing units (TUs) indicates much lower diversity (Votýpka et al., 2010).

2. Materials and methods

2.1. Collection of insects

In July and August 2009, two of the authors (Votýpka and Lukeš) intensely sampled the following localities in Ghana for heteropteran insects: villages Abutia-Kloe (6°28′54″N 0°25′19″E; 60 m above sea level (a.s.l.)), Dzolo (6°40′36″N 0°24′28″E; 60 m a.s.l.) and Maste (6°39′22″N 0°30′53″E; 60 m a.s.l.) near Ho (6°34′48″N 0°29′2″E; 60 m a.s.l.), Kokrobite Beach (5°29′42″N 0°22′8″W; 10 m a.s.l.) near Accra, Cape Coast (Fort Victoria) (5°6′24″N 1°14′57″W; 20 m a.s.l.), outskirts of the Kakum National Park close to Abrafo (5°20′29″N 1°22′58″W; 30 m a.s.l.), around the village of Beyin (5°0′0″N 2°39′6″W; 20 m a.s.l.) and in the vicinity of Elubo (5°14′40″N 2°43′44″W; 150 m a.s.l.). In May 2010, insects were captured at South Horr (2°5′52″N 36°55′16″E; 1020 m a.s.l.) in northern Kenya. Finally in March 2011, heteropterans were sampled at Shiraro (14°24′17″N 37°47′16″E; 1030 m a.s.l.) in north-western Ethiopia. For more information on the DNA isolates studied, their insect hosts and localisation and intensity of infection see Table 1.

Heteropterans were collected on vegetation by sweep-netting during the day, and less frequently using light traps during the night. Within 12 h after capture, insects were killed with 96% ethanol, washed and dissected in 0.9% sterile saline solution, and checked for parasite infection under a portable microscope as described elsewhere (Votýpka et al., 2010). Heteropteran specimens captured in Ghana were dissected by carefully pulling the intact intestinal tract from its body by removing the last abdominal segments. From most specimens, midgut and hindgut with Malpighian tubes were squeezed separately with a cover slip and examined for the presence of motile flagellates. During microscopic examination, the parasite stages were ranked by morphology into one of the three morphotypes described for trypanosomatid flagellates: (medium) promastigotes, (long slender) leptomonads and (short) choanomastigotes. The intensity (four categories) and location (midgut occasionally quoted as abdominal or thoracic midgut, hindgut and Malpighian tubes) of the infection in the intestinal tract were established.

Upon detection of trypanosomatids, part of the infected tissue was smeared upon a microscopic slide, fixed with methanol, air dried and stored until further use. Using sterile tools, the rest of the tissue sample was carefully transferred in 100 µl of 2% SDS and 100 mM of EDTA solution and kept at the ambient temperature until transfer to the laboratory (1–2 weeks), where it was kept at −20 °C until further use. Whereas all heteropteran bugs captured in Ghana were dissected as described above, the specimens obtained in Kenya and Ethiopia, after collection in the field, were stored in alcohol and individually inspected for trypanosomatid infection by PCR.

The dissection was performed so that the insect hosts were preserved for subsequent determination and they have been deposited in the collection of The National Museum, Prague, Czech Republic. The host species were identified according to available taxonomic revisions and by comparison with collections of particular specialists and The Natural History Museum, London, UK. The following specialists are responsible for identifications: F. Chérot (Miridae; Université Libre de Bruxelles, Belgium), D. Chłond (Reduviidae from Ethiopia and Kenya; University of Silesia, Katowice, Poland), É. Guilbert (Tingidae; Muséum National d'Histoire Naturelle, Paris, France), E. Kondorosy (Lygaeoidea from Kenya; Pannon University, Keszthely, Hungary), N. Nieser (Nepomorpha and Gerromorpha; Tiel, the Netherlands), D. Rédei (Reduviidae from Ghana; Hungarian Natural History Museum, Budapest, Hungary), J.L. Stehlík (Pyrrhocoroidea; Moravian Museum, Brno, Czech Republic), and P. Kment (remaining groups).

2.2. DNA isolation, PCR amplification and sequencing

Total DNA was isolated from the infected samples using a High Pure PCR Template Preparation Kit (Roche, Germany) and 10–50 ng were used for PCR amplification of the target genes. The SL RNA and ssrRNA genes were amplified using the kinetoplastid-specific primer pairs M167 and M186, and S762 and S763, respectively, as described elsewhere (Maslov et al., 1996; Westenberger et al., 2004). Upon gel-purification using a Gel Extraction Kit (Roche), both strands of the PCR-amplified ssrRNA genes were directly sequenced using the above primers, as well as internal primers 577F (5′-GCC AGC ACC CGC GGT-3′), 577R (5′-ACC GCG GGT GCT GGC-3′), 1510F (5′-CAG GTC TGT GAY GCT G-3′) and 1510R (5′-CAG CRT CAC AGA CCT G-3′). GenBank™ accession numbers of small subunit genes are listed in Table 1. Due to persistent difficulties with direct cloning of PCR products, the SL RNA gene amplicons were cloned into the pCR-TOPO vector (Invitrogen, USA).

2.3. Phylogenetic analysis

SL RNA alignment was constructed after trimming of the sequences as described earlier (Votýpka et al., 2010, in press). Briefly, for species comparisons, only the most conserved section of the SL