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Field and experimental evidence of a new caiman trypanosome species closely phylogenetically related to fish trypanosomes and transmitted by leeches

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ABSTRACT

Trypanosoma terena and *Trypanosoma ralphi* are known species of the South American crocodilians *Caiman crocodilus, Caiman yacare* and *Melanosuchus niger* and are phylogenetically related to the tsetse-transmitted *Trypanosoma grayi* of the African *Crocodylus niloticus.* These trypanosomes form the Crocodilian clade of the terrestrial clade of the genus *Trypanosoma.* A PCR-survey for trypanosomes in caiman blood samples and in leeches taken from caimans revealed unknown trypanosome diversity and frequent mixed infections. Phylogenies based on SSU (small subunit) of rRNA and gGAPDH (glycosomal Glyceraldehyde Phosphate Dehydrogenase) gene sequences revealed a new trypanosome species clustering with *T. terena* and *T. ralphi* in the crocodilian clade and an additional new species nesting in the distant Aquatic clade of trypanosomes, which is herein named *Trypanosoma clandestinus* n. sp. This new species was found in *Caiman yacare, Caiman crocodilus* and *M. niger* from the Pantanal and Amazonian biomes in Brazil. Large numbers of dividing epimastigotes and unique thin and long trypomastigotes were found in the guts of leeches (*Haementeria* sp.) removed from the mouths of caimans. The trypanosomes recovered from the leeches had sequences identical to those of *T. clandestinus* of caiman blood samples. Experimental infestation of young caimans (*Caiman yacare*) with infected leeches resulted in long-lasting *T. clandestinus* infections that permitted us to delineate its life cycle. In contrast to *T. terena, T. ralphi* and *T. grayi,* which are detectable by hemoculturing, microscopy and standard PCR of caiman blood, *T. clandestinus* passes undetected by these methods due to very low parasitemia and could be detected solely by the more sensitive nested PCR method. *T. clandestinus* n. sp. is the first crocodilian trypanosome known to be transmitted by leeches and positioned in the aquatic clade closest to fish trypanosomes. Our data show that caimans can host trypanosomes of the aquatic or terrestrial clade, sometimes simultaneously.

1. Introduction

Flagellates of the genus *Trypanosoma* (Euglenozoa, Kinetoplastea, Trypanosomatidae) are obligate parasites of all vertebrate classes and are distributed into two major phylogenetic lineages: the Terrestrial clade composed of trypanosomes of mammals, snakes, lizards, crocodilians and birds and the Aquatic clade containing trypanosomes of aquatic leeches and aquatic (fishes) or semi-aquatic (chelonians, anurans and platypus) hosts and, oddly, a trypanosome of chameleon (Stevens et al., 2001; Hamilton et al., 2005, 2007).

Trypanosomes infect Neotropical and Afrotropical crocodilians (Telford, 1995, 2009; Viola et al., 2008a; Fermino et al., 2013). Crocodilians of the family Alligatoridae predominate in the New World and are distributed in the genera *Caiman*, *Paleosuchus* and *Melanosuchus,* generically called caimans. The Afrotropical Crocodylidae originated in Australasia and after transoceanic dispersal

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(Oaks, 2011) flourished in South America (Scheyer et al., 2013). In the American continent, Alligatoridae and Crocodylidae encountered plenty of opportunities of trypanosome switching in the wide connected and disconnected wetlands shaping the present day hydrographic basins (Fermino et al., 2013).

Although there are many reports of the occurrence of trypanosomes in reptilians (revised by Telford, 1995, 2009), only three species from crocodilians were molecularly characterized: *Trypanosoma grayi* (Hoare, 1929, 1931), and *Trypanosoma ralphi* and *Trypanosoma terena* (Viola et al., 2008a; Fermino et al., 2013). The reported hosts of these trypanosomes are *Crocodylus niloticus* and *O. tetraspis* in Africa and the caimans *Caiman crocodilus, Caiman yacare* and *Melanosuchus niger* in South America. Available phylogenetic data show very close phylogenetic relationships between the African *T. grayi* from *Crocodylus niloticus* and the Brazilian *T. ralphi* from caimans, as well as between the Brazilian *T. terena* from caimans and an unnamed African trypanosome from *O. tetraspis.* These South American and African crocodilian trypanosomes form the major Crocodilian clade comprising the clades Terena, Ralphi and Grayi; each clade harbors a number of closely related genotypes (Fermino et al., 2013). The phylogenetic relationships between the crocodilian trypanosomes concur with the worldwide transoceanic dispersal of *Crocodylus* during the Miocene that ended in either South America or Africa (Oaks, 2011).

Trypanosomes of the three species discovered in crocodilians to date were detected by culturing, standard PCR and microscopic examination of peripheral blood. However, these methods may fail to detect tissue-dwelling or scarce blood flagellates and culturing could select against trypanosomes that are fastidious or refractory to cultivation. The alligatorid trypanosomes present varied morphology in the blood and tissues of their hosts that could be the result of the inherent polymorphism of trypanosomes or concomitant infections with more than one species (Viola et al., 2008a; Fermino et al., 2013). The alleged polymorphisms of blood trypanosomes may also result from the occurrence of mixed infections that have been disclosed by molecular analyses of parasites directly from blood samples of anurans, fishes and mammals (Ferreira et al., 2007; Gu et al., 2007; Grybchuk-Ieremenko et al., 2014; Thompson et al., 2013; Lemos et al., 2015). In addition, molecular studies revealed a number of trypanosomes from wildlife displaying blood forms that are morphologically indistinguishable (Ferreira et al., 2007, 2008; Viola et al., 2008a,b; 2009; Cavazzana et al., 2010; Fermino et al., 2013; Lemos et al., 2015). These facts have prevented the correct appraisal of the whole assemblage of trypanosomes and the extent of their diversity and host and geographical ranges.

Nothing is known about the vectors of trypanosomes of South American caimans. Their close relative *T. grayi* is transmitted to African crocodiles by tsetse flies. A range of hematophagous flies serve as vectors of trypanosomes of reptiles, including tsetse flies for crocodiles (Hoare, 1929, 1931) and sand flies for lizards (Ayala, 1971; Ayala and McKay, 1971; Cristensen and Telford, 1972; Viola et al., 2008b). Leeches are known to transmit trypanosomes of aquatic and semi-aquatic animals including fishes (Letch, 1977; Karlsbaak et al., 2005; Khan, 1976; Hayes et al., 2014; Lemos et al., 2015), anurans (Martin and Desser, 1991; Siddall and Desser, 1992), snakes (Pessoa and Fleury, 1969; Chia and Miller, 1984), turtles (Woo, 1969; Siddall and Desser, 1992) and platypus (Paparini et al., 2014).

Trypanosomes of anurans (Ayala, 1971; Martin and Desser, 1991; Siddall and Desser, 1992; Ferreira et al., 2008) and serpents (Chia and Miller, 1984; Viola et al., 2008b) are transmitted by leeches in aquatic environments and by insects such as sand flies and culicids in more terrestrial niches (Desser et al., 1975). Moreover, a survey performed using nested PCR suggested that terrestrial leeches could transmit trypanosomes of toads and marsupials in Australia (Hamilton et al., 2005). Therefore, flies and leeches could transmit the trypanosomes of the semiaquatic caimans, although no vectors have been identified to date.

In the present study, we surveyed for trypanosomes in blood and tissues samples from 122 specimens of South American caimans and the guts of 208 leeches collected from the mouths of caimans. Field surveys, morphological and experimental infections of caimans and phylogenetic analyses were employed to investigate life cycles, species diversity and phylogenetic relationships of crocodilians trypanosomes.

2. Materials and methods

2.1. Collection sites, caiman handling and blood sampling

Caimans were captured in the Amazonian (AM), Araguaia-Tocantins (TO), Paraguay-Paraná (PP) and Orinoco (OR) river basins. The collection sites are shown in Fig. 1 and Table 1. The capture of animals and all ensuing procedures were conducted as described previously (Fermino et al., 2013) according to the recommendations of IBAMA (the Brazilian Institute for the Environment and Renewable Natural Resources), permit Number 10080-2 and the protocol (108/2013) approved by the Committee on the Ethics of Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo.

Caiman blood samples and liver tissue preserved in ethanol (v/v) were stored in the Blood Sample Collection (BSC) and Tissue Sample Collection (TSC) of the Trypanosomatid Culture Collection of the University of São Paulo (TCC – USP). Total DNA was extracted from blood and tissue samples as previously described using the traditional phenol-chloroform method. DNA samples from all trypanosome-positive crocodilian samples were also preserved in the TCC.

2.2. Collection and identification of leeches and survey for trypanosomes

Leeches were collected from the mouths of the caimans, and identified as *Haementeria* sp. by COI barcodes (Folmer et al., 1994). Leeches were immediately preserved in ethanol (v/v) or brought to the laboratory and kept for 15–20 days in plain water until they completely digested the ingested blood meal and became almost transparent. Then, the guts of the leeches were examined by microscopy. Leeches positive for trypanosomes were used for smears on glass slides, inoculation into culture tubes and DNA preparations using the phenol-chloroform method.

2.3. PCR amplification and phylogenetic analyses of SSU rRNA and gGAPDH gene sequences

The nested-PCR of SSU rRNA sequences (~987 bp) of trypanosomes from crocodilian blood samples was performed as described previously (Noyes et al., 1999). PCR amplification of the V7V8 region of the SSU rRNA and gGAPDH sequences from leech trypanosomes was performed as previously described (Borghesan et al., 2013). The nested PCR amplification of gGAPDH sequences was performed using the first round primers (GAPDH SF) 5′ GTG GCG GTK GTY GAC ATG AAC A3′ and (GAPDH SR) 5′ TTG GAG TCR TAG ATR GAG CT3′, and the second round internal primers (GAP 3F) 5′ GTG AAG GCG CAG CGC AAC 3′ and (GAP 5R) 5′ CCG AGG ATG YCC TTC ATG 3′. The two rounds of amplification (30 cycles each) were performed using PCR reaction mixtures and conditions described previously (Borghesan et al., 2013).

Amplified DNA were cloned, and 10–20 clones were sequenced for each gene from each trypanosome sample. Sequencing of cloned amplified DNA allowed for the detection of mixed