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Morphological Discordance of the New Trypanosomatid Species Phylogenetically Associated with the Genus *Crithidia*

Vyacheslav Y. Yurchenko[a], **Julius Lukeš**[b], **Martina Tesařová**[b], **Milan Jirků**[b], and **Dmitri A. Maslov**[c,1]

[a]Albert Einstein College of Medicine of Yeshiva University, Bronx, NY 10461, USA
[b]Biology Center, Institute of Parasitology, Czech Academy of Sciences, and Faculty of Biology, University of South Bohemia, 37005 České Budějovice (Budweis), Czech Republic
[c]Department of Biology, University of California — Riverside, 3401 Watkins Drive, Riverside, CA 92521, USA

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Three new species of monoxenous parasites from the Neotropical Heteroptera are described on the basis of the ultrastructure of cells in culture, as well as gene sequences of Spliced Leader (SL) RNA, glyceraldehyde phosphate dehydrogenase (GAPDH) and small subunit (SSU) rRNA. The results have highlighted a striking discrepancy between the morphological (dis)similarities and the phylogenetic affinities among the insect trypanosomatids. Although each of the new species is characterized by a distinct set of morphological characters, based on the predominant promastigotes observed in culture, each of them has been provisionally assigned to the genus *Leptomonas* pending the future revision of this genus. Yet, instead of the phylogenetic affinity with the other members of this polyphyletic genus, the new species are most closely related to *Crithidia* species. Thus, the extremely long promastigotes of *Leptomonas acus* sp. n. and the unique morphological features found in *Leptomonas bifurcata* sp. n. sharply contrast with their respective relatives *C. fasciculata* and *C. deanei* both of which are typical choanomastigotes. The results clearly show that the current classification at the genus level is misleading and needs to be revised. The phylogenetic clades potentially representing the candidate new genera of monoxenous trypanosomatids have started to emerge from the presented analyses.

Key words: *Crithidia*; Heteroptera; *Leptomonas*; Miridae; phylogeny; taxonomy.

Introduction

The family Trypanosomatidae, which is well known for its pathogenic dixenous members, also includes numerous organisms that are restricted to invertebrate hosts. The monoxenous trypanosomatids from insects have been traditionally placed in four large genera, *Blastocrithidia, Crithidia, Leptomonas* and *Herpetomonas*, and two smaller genera, *Rhynchoidomonas* and *Wallaceina*, which are mainly distinguished by the relative position of the nucleus and the kinetoplast (Hoare 1964; Hoare and Wallace 1966; McGhee

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[1]Corresponding author; fax +1 951 827 4286
e-mail maslov@ucr.edu (D.A. Maslov).

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and Cosgrove 1980; Podlipaev et al. 1990; Vickerman 1976; Wallace 1966). The basic morphology of a trypanosomatid cell is that of a promastigote. This is the only morphotype (excluding cysts) observed in the genus *Leptomonas*. The presence of epimastigotes is typical for the genus *Blastocrithidia,* while endomastigotes and opisthomastigotes are exclusive characters of the genera *Wallaceina* and *Herpetomonas*, respectively. Although the aforementioned morphotypes are distinct, the boundaries between these genera are often difficult to draw. The reason is that only a fraction of cells in culture or natural populations displays the characteristic shape, with the rest being promastigotes of various lengths (Fiorini et al. 2001; Podlipaev 1985; Podlipaev and Frolov 1987). The genus *Crithidia* is defined by the presence of choanomastigotes. These cells are reminiscent of shortened and inflated promastigotes, which themselves often form a continuum of shapes and sizes bordering on those of choanomastigotes (Jankevicius et al. 1993; Podlipaev 1985; Podlipaev and Frolov 1999; Romeiro et al. 2000). This lack of a clear morphological boundary makes the separation of *Crithidia* and *Leptomonas* very arbitrary.

Given the superficial nature of the morphological classification, it was not surprising that molecular phylogenies revealed that the four major genera of insect trypanosomatids were polyphyletic (Du et al. 1994; Hollar et al. 1998; Merzlyak et al. 2001; Svobodová et al. 2007; Yurchenko et al. 2006a). Several major monophyletic groups have emerged from these analyses. It is conceivable that the major groups or their subdivisions will eventually become new taxa defined by an integration of molecular and other criteria including morphology. One of the major groups found was called “slowly-evolving” or SE to reflect a relatively low rate of sequence divergence of the small subunit ribosomal RNA gene (Hollar et al. 1998; Merzlyak et al. 2001). This group has also emerged in recent analyses of the GAPDH gene which have shown that the SE group is subdivided into the clade of *Leishmania* and their monoxenous relatives on one hand, and, on the other, the large group of species assigned to the genera *Leptomonas*, *Crithidia* and *Wallaceina* (Yurchenko et al. 2006a, 2006b).

The application of molecular approaches indicated that the diversity of insect trypanosomatids is larger than might have been envisioned originally (Merzlyak et al. 2001; Simpson et al. 2006; Stevens 2001). Its broad dimensions were further revealed by the recent analyses of spliced leader (SL) RNA gene repeats amplified from the insect gut samples (Maslov et al. 2007; Westenberger et al. 2004). The trypanosomatids discovered and barcoded by this approach are classified into discrete typing units (TU) which represent candidate new species. Some of them, especially the members of the SE group, were amenable to axenic cultivation, which enables investigation of their morphology and phylogeny in detail, as described in the work presented herein. The results obtained have clearly shown that morphology, although valuable for defining species and groups of closely related species, does not always reflect the genetic or evolutionary relationships among monoxenic trypanosomatids on a larger scale. The current classification at the genus level, in particular of the genera *Leptomonas* and *Crithidia*, is highly artificial and needs to be drastically revised. These studies lay the foundation for the future taxonomic revision of these groups.

Results

Isolation and Authentication of Axenic Cultures

Twelve specimens of *Pachypoda* sp. (Heteroptera: Miridae) collected on an Araceae plant in Costa Rica were analyzed for the presence of trypanosomatids and five of them were found to be infected. The parasites found in the gut smear were present at low abundance, and most of them represented relatively small, free-swimming promastigotes, while some were aggregated. By analysis of SL RNA gene repeats amplified from the gut samples 50CR—54CR, each of them representing an individual insect host, these organisms represented a new trypanosomatid species, which was denoted as TU***3*** (Maslov et al. 2007; Westenberger et al. 2004). Cultures were obtained from three infected specimens, and one of the cultures (from the sample 53CR) was established axenically. PCR amplification of SL RNA gene repeat sequences was used to ascertain that the culture obtained faithfully represents the organism observed in the original infected specimen (Fig. 1). The 286 bp PCR product obtained from cultured cells was 99.3—99.7% identical to the respective sequences from insect gut samples (Westenberger et al. 2004). This range is well within the limits of intraspecies variability of SL RNA gene repeats (Thomas et al. 2005).