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

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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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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).

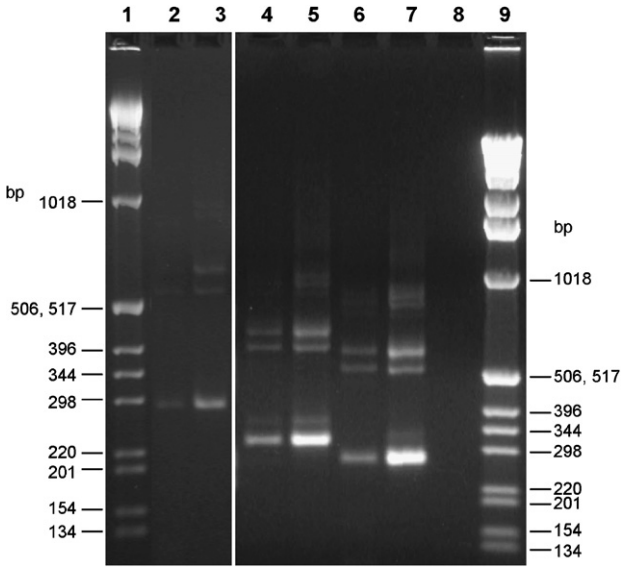


Figure 1. Comparison of PCR-amplified SL RNA gene repeats from the initial insect gut samples and the axenic cultures. Ethidium-bromide stained 2.5% agarose gel showing the SL products amplified from the gut sample 47VL (lane 2), axenic culture 47VL (= *Leptomonas tarcoles* sp. n.) (lane 3), gut sample 53CR (lane 4), axenic culture 53CR (= *Leptomonas bifurcata* sp. n.) (lane 5), gut sample 132SI (lane 6), and axenic culture 132SI (= *Leptomonas acus* sp. n.) (lane 7). Notice that the amplification products are longer than the corresponding repeat units due to utilization of overlapping primers for PCR. The smallest fragments in each lane represent monomeric repeats, while the larger fragments represent oligomeric amplified repeats. Lanes 1—3 and lanes 4—9 are from different gels, lane 8 shows the PCR negative control, and lanes 1 and 9 show the '1 kb DNA ladder' (Invitrogen).

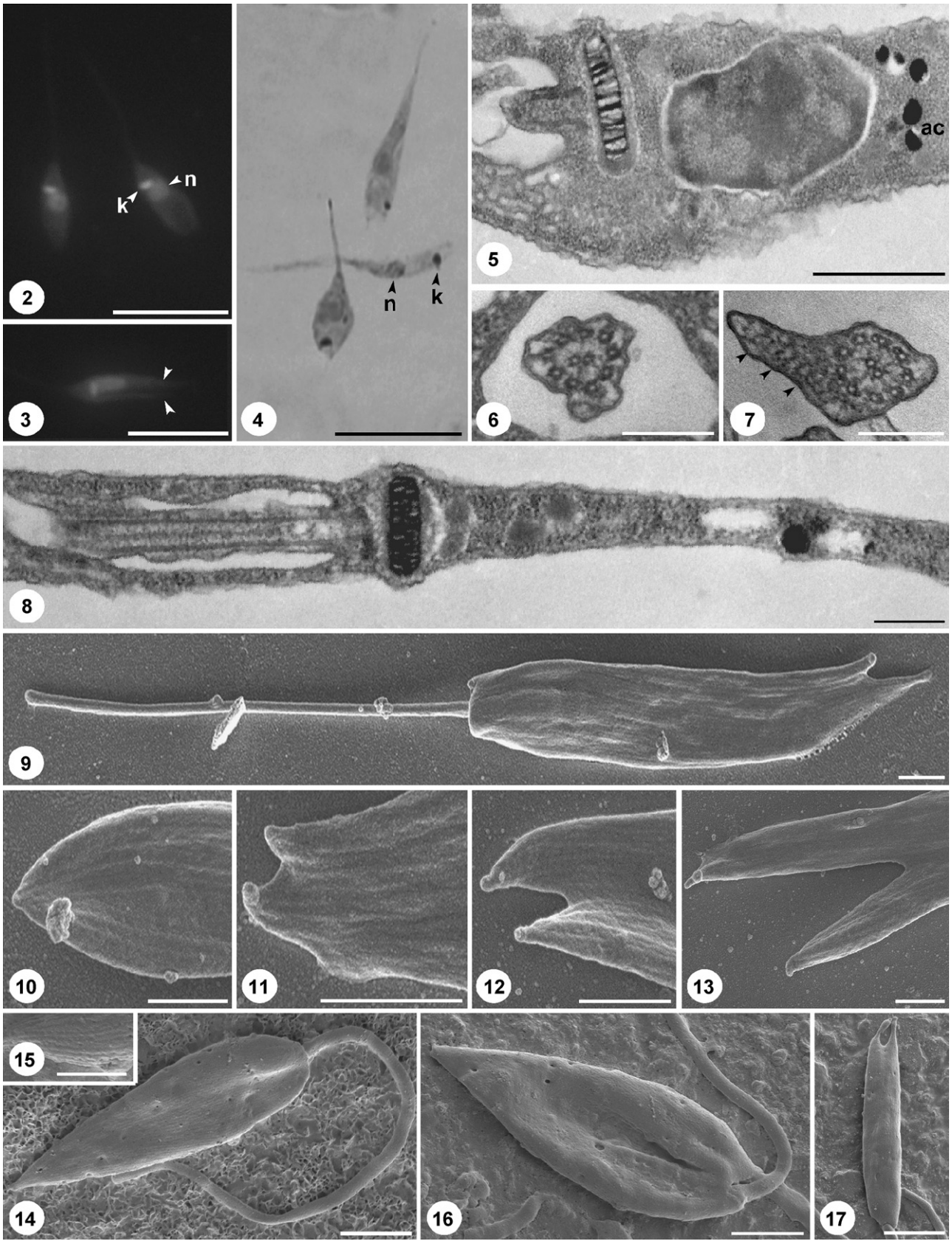
Four out of seven specimens of *Prepops* sp. (Heteroptera: Miridae) in Costa Rica (host samples 47VL, 48VL, 55VL and 56VL), all collected on vegetation along a river bank, were infected with a new trypanosomatid species labeled as TU***4*** (Maslov et al. 2007; Westenberger et al. 2004). The parasites in insects were promastigotes characterized by an elongated shape, most of them were free swimming and some were attached to the gut wall. Three sequences of SL repeats representing the insect gut DNA samples 47VL, 48VL and 55VL were 244 bp and 99.6% identical to each other (a single nt difference in 47VL). The repeat size in 56VL was exactly the same as in three other samples (see fig. 1 of Westenberger et al. 2004). In each case the parasites were readily cultivable, and axenic cultures were obtained for isolates 47VL and 55VL. The SL RNA repeats amplified from the 47VL culture cells were of the same size as the gut sample (Fig. 1). Moreover, two culture sequences shared a 99.2% identity level with three sequences from gut samples determined earlier (Maslov et al. 2007). Interestingly, an additional infected host (sample 49VL) found in the same population harbored a different trypanosomatid parasite which formed TU***18*** (Maslov et al. 2007). This organism was not cultivable, however.

Two out of seven light-trapped specimens of an unidentified species of the subtribe Eccritotarsina (Heteroptera: Miridae) from Ecuador were found to contain trypanosomatids, and one of these organisms (132SI) was denoted as TU***40*** (Maslov et al. 2007). The trypanosomatids in the host intestine represented very long promastigotes, some with a few twists along the main axis. The SL repeat sequence obtained from the respective axenic culture was 96.3—99.6% identical to the corresponding sequences from the host gut (241—243 bp long) (Fig. 1).

Morphological and Ultrastructural Characterization of the New Isolates

Light microscopy examination of cells in axenic cultures revealed the morphological differences among the three strains (Figs 2—4, 18, 19, 24, 25). The strain 53CR was represented by mostly elongated promastigotes some of which resembled a thin drop with an elongated and tapered posterior end and a wider anterior end (Figs 2, 4). Almost all cells of the strain 132SI were very long, needle-like promastigotes (Figs 24, 25). Cell of the strain 47VL varied in size from slender promastigotes to short stumpy forms, sometimes resembling choanomastigotes (Figs 18, 19).

The length of the 53CR cells varied between 8.3 and 24.8 μm (mean ± SD: 15.2 ± 3.5 μm; $n = 50$), this size range overlapping with the 132SI strain (see below). The distance between the nucleus and the kinetoplast ranged from 0.7 to 3.5 μm (1.7 ± 0.6 μm), whereas the distance from the nucleus to the posterior end varied between 5.0 and 16.8 μm (8.4 ± 2.9 μm). The flagellum was always present and its mean length was 14.5 μm (8.7—21.8 μm; 14.5 ± 3.2 μm). Many 53CR cells were slim with a bulge present around the kinetoplast (Figs 4, 8), although some cells, probably at various stages of cell cycle, were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thicker (Fig. 4). Occasionally, the 53CR cells appeared to have a split posterior end (Fig. 3).

Although a few choanomastigote-like cells were observed in the 47VL culture, only cells with promastigote morphology were measured ($n = 50$), their length varying from 6.4 to 12.4 μm (8.6 ± 1.4 μm) (Figs 18, 19). The distance between the nucleus and the posterior end and the nucleus and the kinetoplast ranged between 1.5 and 8.0 μm (3.5 ± 0.9 μm) and 0.5 and 1.9 μm (1.0 ± 0.3 μm), respectively. The length of the flagellum varied from 6.4 to 12.7 μm (9.9 ± 1.1 μm).

The 132SI promastigotes were characterized by a needle-like morphology and exceptional length that varied from 8.1 to 40.4 μm (21.7 ± 7.2 μm, $n = 50$) (Figs 24, 25). The generally short distance between the nucleus and the prominent kinetoplast (from 1.2 to 9.3 μm; 3.6 ± 1.5 μm) reflected the rather apical position of the nucleus. The distance from the nucleus to the thin posterior end of the cells ranges between 3.0 and 24.9 μm (11.5 ± 5.5 μm). All cells were flagellated, with the flagellum varying from 13.6 to 36.8 μm (25.3 ± 6.0 μm). A small and variable fraction of cells with choanomastigote-like morphology were observed in culture.

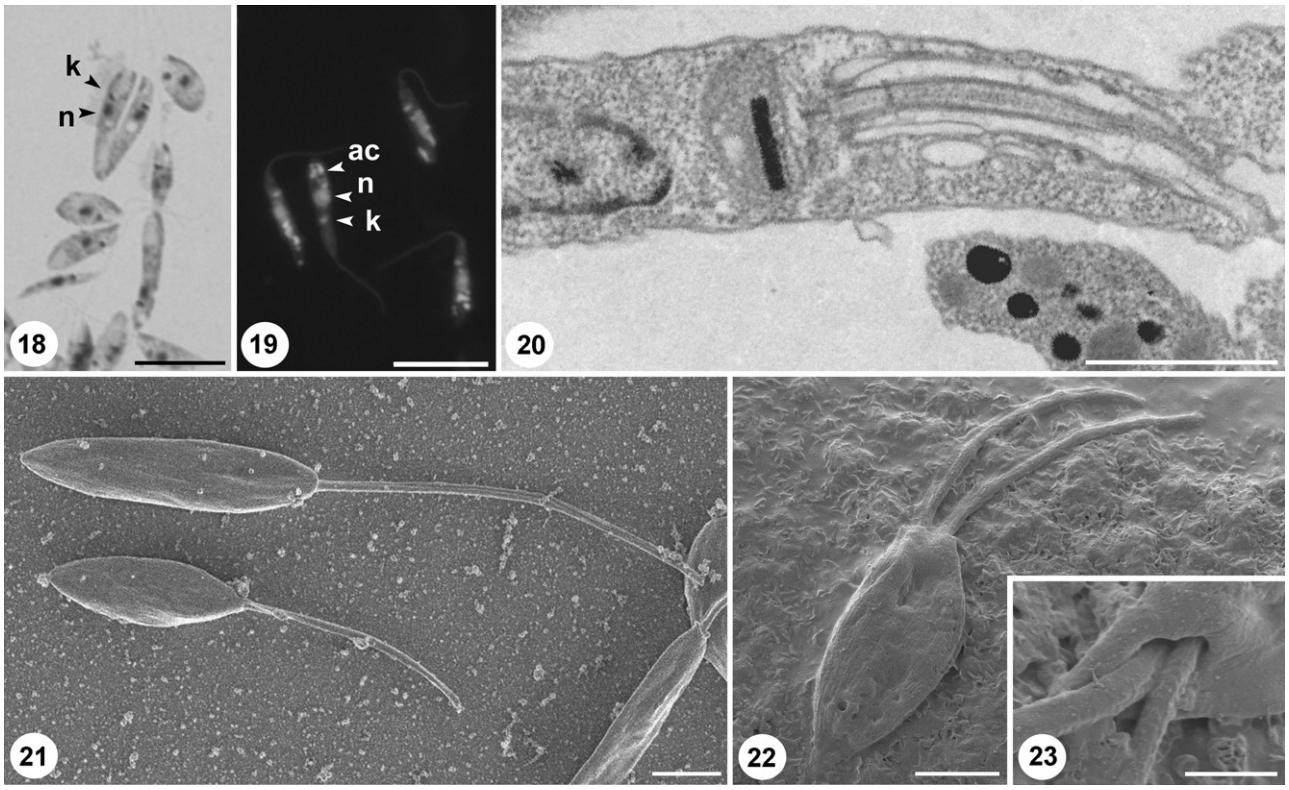
The morphology was further investigated by transmission electron microscopy (Figs 5—8, 20, 26—28). All three isolates contained characteristic features of the trypanosomatid cell, such as electron-dense kinetoplast, oval nucleus, single flagellum, a single peripherally located reticulated mitochondrion with numerous cristae, conspicuous paraxonemal rod and a complete corset of regularly spaced subpellicular microtubules. However, at the ultrastructural level, features characteristic for the individual strains were also found.

A unique ultrastructural feature of the isolate 53CR was its prominent kinetoplast disk with the DNA fibrils apparently packed into thick strands, which were separated from each other by electron-lucent zones (Figs 5, 8). A similar ultrastructure was observed in *C. fasciculata* cells in which the kinetoplast associated protein KAP1 was inactivated by a targeted gene knock-out (Lukeš et al. 2001). Numerous electron-dense acidocalcisomes are distributed throughout the anterior region of the cell (Fig. 5). The flagellum is supported by a paraxonemal rod (Fig. 7) that can be seen in a cross-sectioned flagellum only after it exits the deep flagellar pocket (Figs 6, 8). Yet the most conspicuous feature was noticed only upon the inspection of cells by scanning electron microscopy. While the majority of promastigotes have a regular spade-like to blunt posterior end (Figs 10, 14, 16), a smaller fraction of them had the end split to a varying extent (Figs 11—13, 17). The distant possibility that this is an artifact has been ruled out by analyzing the cells using cryo-scanning microscopy (Figs 14—17). In the absence of dehydration, the shape of the cells was optimally preserved with subpellicular microtubules visible under the plasmalemma (Fig. 15).

The 47VL promastigotes have a rather deep flagellar pocket (Fig. 20). The kinetoplast, located immediately next to the basal body of the flagellum, was thin, testifying of a small size of kinetoplast DNA minicircles (Lukeš and Votýpka 2000). Abundant electron-dense acidocalcisomes were well visible both in light (Fig. 19) and electron microscopy (Fig. 20). Scanning electron microscopy revealed the varying ratio between the length of the cell and its flagellum (Figs 21, 22).

For 132SI, a characteristic feature was an abundant presence of various vesicles in the cytoplasm. The elongated cells had a deep flagellar pocket (Fig. 28), adjacent to which was a prominent kinetoplast with densely packed fibrils of minicircles. The thickness of the kinetoplast (230 ± 33 nm; $n = 25$) implied the presence of large-size minicircles (Lukeš and Votýpka 2000; Yurchenko et al. 1999), and was noticeably larger than in most insect trypanosomatids. Along with acidocalcisomes and glycosomes, the cytoplasm contained numerous oval granules with

Figures 2—17. *Leptomonas bifurcata* sp. n. DAPI- (**2**, **3**) and Giemsa-stained (**4**) cultured cells with well-visible kinetoplast (k) and nucleus (n). Note the split posterior end (arrowheads) visible in some cells (**3**) Transmission electron microscopy of a thick (**5**) and thin promastigote (**8**), with a prominent bulge around the kinetoplast disk. Electron dense acidocalcisomes (ac) are located in the posterior region of the cell (**5**, **8**). In cross-sectioned flagellum a large paraxonemal rod is present (arrowheads) (**7**) but not inside the flagellar pocket (**6**). Scanning (**9—13**) and cryo-scanning (**14—17**) electron microscopy showing a variability of the posterior end, ranging from a spade-like (**14**, **16**) to blunt end (**10**), and from a slightly bifurcated (**9**, **11**, **17**) to deeply split end (**12**, **13**). Cryo-scanning electron microscopy also reveals the regular evenly-spaced corset of subpellicular microtubules (**15**) and the morphology of cells in the final stage of division (**16**). Scale bar = 10 μm (**2—4**), 2 μm (**14**, **16**, **17**), 1 μm (**5**, **6**, **9—13**, **15**), 500 nm (**7**) and 200 nm (**8**).

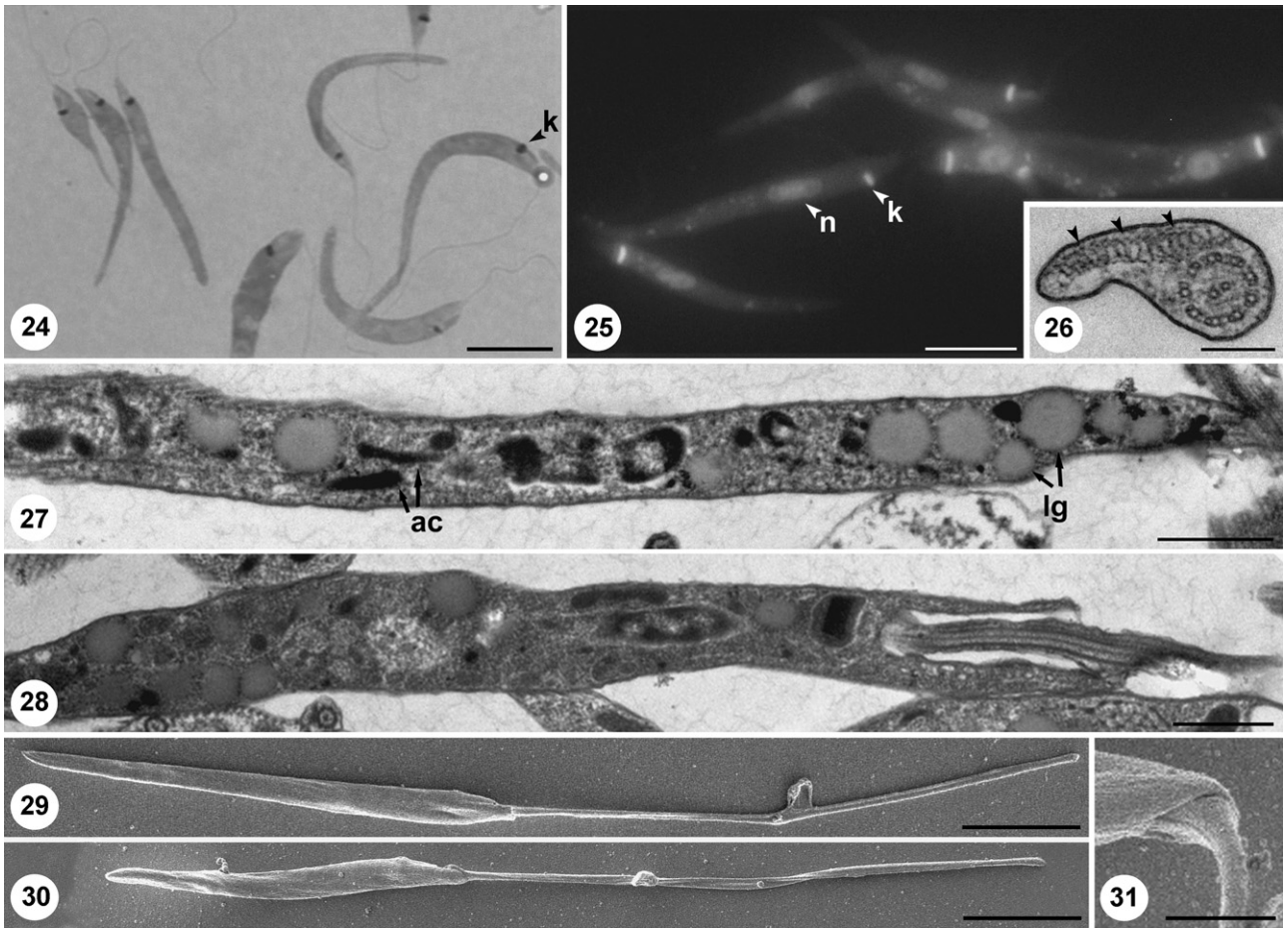


Figures 18—23. *Leptomonas tarcoles* sp. n. Giemsa- (**18**) and DAPI-stained (**19**) cultured cells with well-visible kinetoplast (k), nucleus (n) and acidocalcisomes (ac). A thin dense kinetoplast at the basis of a deep flagellar pocket is visible in Fig. 20. The varying ratio between the length of the cell and its flagellum is visualized by scanning electron microscopy (**21**). A dividing cell is seen in cryo-scan pictures (**22**, **23**). Scale bar = 10 μm (**18**, **19**), 2 μm (**21**, **22**), 1 μm (**20**, **23**).

homogeneous content, probably lipid (Figs 27, 28). The long flagellum was equipped with a prominent paraxonemal rod (Fig. 26). Examination of the needle-like promastigotes using a regular scanning electron microscope showed variability in the size of the flagellum (Figs 29, 30), yet only their processing for viewing under cryo-scan conditions revealed that the diameter of the cells is actually much closer to that of their massive and long flagellum (Fig. 31, and data not shown).

Phylogenetic Analysis

The previous SL-based genotyping of the new isolates revealed their position within the SE group (see fig. 1 of Maslov et al. 2007). The analysis had also identified the closest relatives of these organisms. Thus, the isolates 47VL, 48VL, 55VL (TU***4***) from Costa Rica were closely related to the isolate 132SI (TU***40***) and a group of isolates (TU***32***) from Ecuador. The isolates 50CR, 52CR and 53CR (TU***3***) were affiliated with *Crithidia luciliae thermophila*. However, due to the limited phylogenetic signal preserved in fast-evolving SL repeats, this marker is not appropriate for inferring the relationships beyond closely related species: the deeper branching order is generally poorly supported and unreliable unless confirmed independently. With the aim to better resolve the relationships of the new isolates within the family, we performed a phylogenetic analysis of the GAPDH and SSU rRNA sequences obtained using axenic culture cells of the isolates 47VL (TU***4***), 53CR (TU***3***) and 132SI (TU***40***). The general topology of the GAPDH tree (Fig. 32) was consistent with that described earlier (Hamilton et al. 2004, 2005; Hannaert et al. 2003; Yurchenko et al. 2006a, 2006b) and is not discussed herein. The SE clade, to which the new isolates belong, was recovered with all methods used with a high (99—98%, minimum evolution and parsimony) to



Figures 24—31. *Leptomonas acus* sp. n. Giemsa- (**24**) and DAPI-stained (**25**) cultured cells with an oval nucleus (n) and well-stained small kinetoplast (k). Transmission electron microscopy of elongated promastigotes (**27**, **28**). Note the abundant presence of electron-dense acidocalcisomes (ac) and electron-lucent lipid granules (lg) (**27**) and thick kinetoplast disc (**28**). A cross-sectioned flagellum with a prominent paraxonemal rod (arrowheads) (**26**). Ratio between size of the cell and the flagellum varies as shown by scanning electron microscopy (**29**, **30**). Cryo-scan revealed the relative thick flagellum as it emerges from the flagellar pocket (**31**). Scale bar = 10 μm (**24**, **25**), 5 μm (**29**, **30**), 1 μm (**27**, **28**, **31**), and 200 nm (**26**).

medium (63%, maximum likelihood) bootstrap support. Overall, the maximum likelihood trees had lower bootstrap support compared to the minimum evolution and parsimony trees.

In order to exclude the potential influence of the more divergent lineages on topology, in the next step the SE clade was analyzed separately (Fig. 33). The GAPDH analysis has confirmed that the isolates 47VL and 132SI represent sister taxa and that they are closely associated with the group that contains *Crithidia fasciculata*, *C. luciliae* and *Leptomonas* sp. P. The bootstrap support for these associations is high with the distance-based analysis but lower with the other methods.

In all cases, the isolate 53CR formed a highly supported group with *Crithidia deanei* ATCC30255, a relationship that was not detected by the previous SL RNA cluster analysis (Maslov et al. 2007; Westenberger et al. 2004). In order to investigate the reason for this discrepancy, two SL RNA gene repeats were amplified from *C. deanei* ATCC30255. The two nearly identical (97.2%) 429—430 bp sequences were quite dissimilar from the 665 bp sequence reported earlier for the unspecified strain of *C. deanei* (Fernandes et al. 1997), apparently reflecting the independent origin of the two strains which thereby represent different organisms. On the

other hand, the repeats from *C. deanei* ATCC30255 were 78.1—79.7% identical to the 384 bp repeat from *C. luciliae thermophila* (Fernandes et al. 1997), and both of these organisms clustered together with the 53CR SL RNA repeats (data not shown), confirming previous results (Maslov et al. 2007; Westenberger et al. 2004) and in agreement with results of the GAPDH analysis. It needs to be mentioned here that *C. luciliae* and *C. luciliae thermophila* are different organisms (Brandão et al. 2000; Roitman et al. 1977), and that the branch labeled "*C. luciliae*" in the previously published SL RNA dendrograms (Maslov et al. 2007; Westenberger et al. 2004) actually represented the sequence from *C. luciliae thermophila*.

The relationship between the group including the isolates 47VL, 132SI and related *Crithidia* species on one hand and the clade of 53CR-*C. deanei* on the other was not confidently resolved with the GAPDH dataset. Although the best likelihood (Fig. 33) and parsimony (data not shown) trees favored their inclusion in a single monophyletic clade, this was not the case with the best distance tree (data not shown), and all three methods showed a polytomy in the majority consensus trees derived from the respective bootstrap trees (data not shown).

The preliminary SSU rRNA analyses confirmed that the new isolates are associated with monoxenous trypanosomatids of the SE group (data not shown). An across-the-family SSU analysis is prone to artifacts due to highly unequal substitution rates in many lineages and the presence of poorly alignable hypervariable regions (Philippe 1998); therefore, the subsequent analysis was limited to the SE group with less divergence and more uniform rates. The downside of a high level of conservation across the entire alignment was that the overall bootstrap support for the SSU trees inferred was generally low with polytomies in the majority consensus trees (data not shown). Nonetheless, the association of the isolates 132SI and 47VL with *C. fasciculata* had an exceptionally strong support with all the methods used (Fig. 34). As could be expected based on the GAPDH analyses, the isolate 53CR was separated from the other two isolates, but a verification of its specific association with *C. deanei* ATCC30255 awaits the availability of the SSU sequence from the latter.

Overall, the phylogenetic analyses indicated that the new isolates represent unique and distinct phylogenetic lineages, which merit a separate species status. The organisms are phylogenetically associated with several named *Crithidia* species, yet in no case were choanomastigotes a predominant morphotype in culture or in nature, nor were they even detected with certainty. The promastigotes of different length invariably represented the predominant cell type, although short-length promastigotes, such as those observed in 47VL, could not be reliably distinguished from choanomastigotes. Thus, based on morphology, which remains the current basis of the genus-level trypanosomatid taxonomy (Hoare and Wallace 1966; Wallace 1966), each organism should be classified as a member of the genus *Leptomonas* Kent, 1880.

Diagnosis

Phylum Euglenozoa Cavalier-Smith, 1981
Class Kinetoplastea Honigberg, 1963
Subclass Metakinetoplastida Vickerman, 2004
Order Trypanosomatida (Kent, 1880) Hollande, 1952
Family Trypanosomatidae Doflein, 1951.

***Leptomonas bifurcata* sp. n.** (Figs 2—17)

Specific characters: Most cells are medium-sized (up to 15.2 μm) or short promastigotes, with an elongated posterior end and a variable-length flagellum. The most characteristic for this species is the split posterior end observed in some cells in culture. Another unique feature is a fine structure of the kinetoplast with thick DNA fibers separated by translucent zones. In addition, *L. bifurcata* sp. n. is distinguished by its unique 285—286 bp SL RNA gene repeats (GenBank™ accession numbers EF152330, DQ860230, DQ860231) which are markedly different in size and sequence from repeats of the two closest known relatives, *C. deanei* ATCC30255 (429—430 bp, EF546790, EF546791) and *C. luciliae thermophila* (384 bp, U96171).

Type host: Intestine of *Pachypoda* sp. (Heteroptera, Miridae). The xenotype (post-dissection remains of the host) is stored in the UCR Entomology Museum (UCRC ENT 140279).

Type locality: In the vicinity of Tárcoles, at the western boundary of the Carara National Park (09°47′N, 84°37′W), Province Puntarenas, Costa Rica.

Type material: The hapantotype represents the axenic culture of the isolate 53CR deposited in the American Type Culture Collection (ATCC PRA-214).

Etymology: The name was given after the uniquely split posterior end of some cells.

***Leptomonas tarcoles* sp. n.** (Figs 18—23)

Specific characters: Relatively small promastigotes are variable in shape from slender to stumpy forms. The flagellum of varying size exits from a rather deep flagellar pocket. Prominent acidocalcisomes are visible in Giemsa and DAPI-stained cells. The species is identified by its 244—245 bp SL RNA gene repeat sequences (GenBank™ accession numbers EF546788, EF546789, DQ860227, DQ860228, DQ860232) which are only 77—80% identical to repeats from its two closest known relatives, *L. acus* sp. n. (241—243 bp, EF152331, DQ864270) and an undescribed species represented by TU*32* (Maslov et al. 2007) (240—241 bp, DQ864274, DQ864275, DQ864301, DQ864302, DQ864306).

Type host: Intestine of *Prepops* sp. (Heteroptera: Miridae). The xenotype is stored in the UCR Entomology Museum (UCRC ENT 138206).

Type locality: In the vicinity of Tárcoles, at the western boundary of the Carara National Park (09°47′N, 84°37′W), Province Puntarenas, Costa Rica.

Type material: The hapantotype represents the axenic culture of the isolate 47VL deposited in the American Type Culture Collection (ATCC PRA-241).

Etymology: The name was given after the collection locale.

***Leptomonas acus* sp. n.** (Figs 24—31)

Specific characters: Most cells are long (up to 40.4 μm) needle-like promastigotes with a lower frequency of various shorter forms. The kinetoplast has a diameter of ~850 nm and is exceptionally thick (230 nm). Numerous vesicles are present in the cytoplasm. The species is distinguished by its unique 241—243 bp SL RNA gene repeat sequences (EF152331, DQ864270).

Type host: Intestine of a species from the subtribe Eccritotarsina (Heteroptera: Miridae). The xenotype is deposited in the UCR Entomology Research Museum (UCRC ENT 140278).

Type locality: In the San Isidro forest reserve, the vicinity of Cosanga (00°35′S, 77°53′W), Province Napo, Ecuador.

Type material: The hapantotype represents the axenic culture of the isolate 132SI deposited in the American Type Culture Collection (ATCC PRA-213).

Etymology: The species name reflects a needle-like shape of most cells observed in culture and the original infection.

Discussion

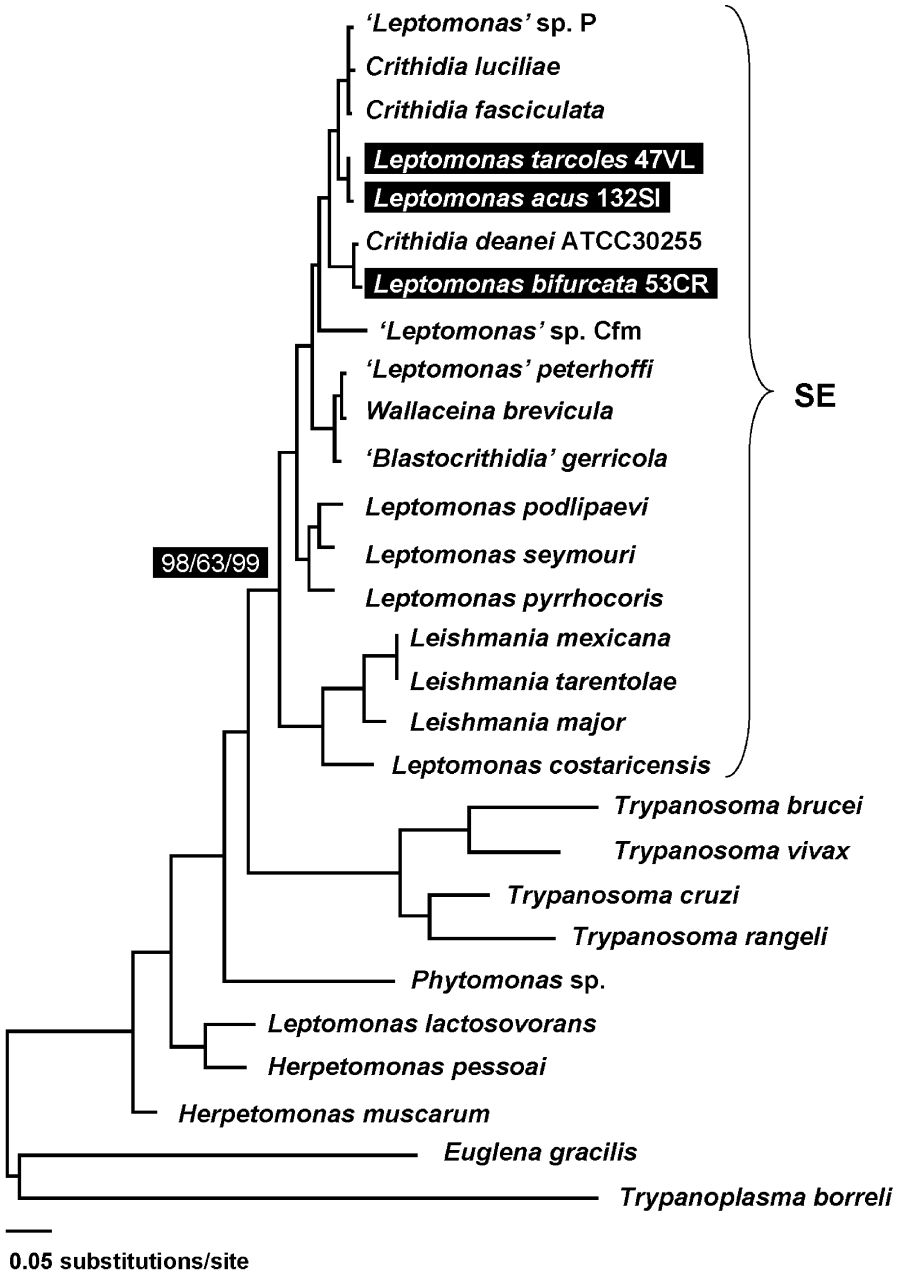
Three new species of Trypanosomatidae were isolated from heteropteran hosts in Costa Rica and Ecuador. The parasites were found to be related to monoxenous trypanosomatids from the SE group (Hollar and Wallace 1998; Merzlyak et al. 2001). Based on morphology and in accordance with the existing classification of Trypanosomatidae (Hoare et al. 1966; Wallace 1966), the new species were assigned to the genus *Leptomonas* Kent, 1880. Given that this genus is artificial and needs a revision, this assignment is provisional.

The new species of parasites from Miridae are clearly different from all previously described trypanosomatid species for which the molecular data are available. However, for most of the nearly 70 named *Leptomonas* species and uncertain number of unnamed species (Podlipaev 1990), there was no molecular or detailed morphological characterization. There are only two records of *Leptomonas* in mirid bugs. One of those is *Leptomonas* sp. found in an unidentified host species in Uganda (Robertson 1912). This organism lacks a description but based on its biogeographical origin it is most likely different from the species described herein. In addition, a well-described species, *Leptomonas mycophilus,* was isolated from *Phytocoris* sp. in North-Western Russia (Frolov and Skarlato 1991). This trypanosomatid is well distinguished from *L. bifurcata* sp. n. by densely packed kinetoplast DNA fibers and by the lack of the characteristic posterior end protrusions. The species from Russia differs from *L. acus* sp. n. by cell dimensions including a much smaller cell size in culture (7—8 μm) and a shorter flagellum (~2.5 μm). The cell size of *L. mycophilus* is close to *L. tarcole*s sp. n., yet the new species has a longer flagellum and the kinetoplast that is positioned anterior with respect to the nucleus instead of by its side, as in *L. mycophilus*. The diameter of the kinetoplast DNA disk in *L. mycophilus* appears to be smaller compared to *L. tarcole*s sp. n. In addition, *L. mycophilus* required a specific fungus or its extract for growth in culture, while no similar dependence was observed for any of the new species.

To the best of our knowledge, the only additional described trypanosomatid from Miridae is *Blastocrithidia miridarum* (Podlipaev and Frolov 1987), and that organism stands apart from the *Leptomonas* species described herein due to the presence of epimastigotes and flagellar cysts (straphangers) in the former.

According to the GAPDH and SSU phylogenetic analyses (Figs 32—34) *L. acus* sp. n. and *L. tarcoles* sp. n. have a strong affinity with *C. fasciculata*, the type species for the genus *Crithidia* Léger, 1902 (Wallace 1966). This association seems to be at

odds with the peculiar long promastigote morphology observed in most cells of *L. acus*. On the other hand, *L. tarcoles* displays variable length cells, the shortest of which can possibly be regarded as choanomastigotes. In any case, in spite of the drastic morphological differences, *L. acus* and *L. tarcoles* are indisputably each other's closest relatives. The third new species, *L. bifurcata* sp. n., is phylogenetically associated with *C. deanei* Carvalho, 1973 (strain ATCC30255) and *C. luciliae thermophila* Roitman et al. 1977 which were assigned to the genus *Crithidia* based on the choanomastigote morphology. It should be mentioned that at least two different organisms have been associated with the name *C. deanei*, while *C. luciliae thermophila* is too distant from *C. luciliae* Wallace and Clark, 1959 to be regarded its subspecies (Brandão et al. 2000; Clark 1997; Fernandes et al. 1997; Hollar et al. 1998; this work). In any case, no morphological features resembling the posterior end protrusions observed in *L. bifurcata* have been reported for any of these



trypanosomatids. Thus, the morphological properties of the three new species would be uninformative or even misleading in an attempt of revealing relationships of these organisms within the family.

If the classification system of monoxenous trypanosomatids is to reflect their genetic and evolutionary relationships, development of a new set of criteria for the genus-level taxonomy has become imperative. The analysis performed here facilitates building a phylogenetic framework necessary for a meaningful revision of the Trypanosomatidae. Several clades have emerged within the SE group in this (Fig. 33) and previous analyses (Hollar et al. 1998; Merzlyak et al. 2001; Yurchenko et al. 2006a, 2006b). One of the clades (LE, Figs 33, 34) corresponds to the well-established genus *Leishmania*. As shown by several works, this clade, represented in our analyses by a few sequences only, includes all studied *Leishmania* and *Endotrypanum* species (the latter presumably being some mis-named *Paraleishmania*) (Cupolillo et al. 2000; Noyes et al. 1997; Yurchenko et al. 2006b). Considering the genus-level status of the LE clade, it is advisable to propose that the remaining major subgroups of the SE clade also merit a separate genus rank. Due to the uninformative or misleading nature of morphological characters observed among their members, these new genera should be defined mainly by the means of genosystematics. Additional characters, including morphology, should be auxiliary. A precedent was set recently when a well-supported clade was used as the ground for establishing a new genus of trypanosomatids (Svobodová et al. 2007).

The pronounced heterogeneity of the genus *Crithidia* observed by genotyping (Brandão et al. 2000; Clark 1997; Du and Chang 1994; Fernandes et al. 1997) reflects the polyphyly of this genus (Du et al. 1994; Hollar et al. 1998; Merzlyak et al. 2001). If the name *Crithidia* is to be retained, it should stay with the group that includes its type species *C. fasciculata*. The clade C (Fig. 33) comprising this species and *C. luciliae* is therefore the primary candidate for the revised, monophyletic genus *Crithidia*. The clade also includes an organism previously referred to as *Leptomonas* sp. P (Bulat et al. 1999; Podlipaev et al. 2004) with cells closely resembling choanomastigotes (A. Kostygov, pers. commun.). The association of this organism with the two *Crithidia* species is very strong. It remains to be seen if this clade includes the remaining 'canonical' *Crithidia* species, such as *C. acanthocephali*. However, the endosymbiont-containing crithidias, such as *C. oncopelti*, *C. desouzai* and *C. deanei*, should definitely belong to a different genus or genera [e.g. *Strigomonas* or *Angomonas* (Brandão et al. 2000; de Souza and Corte-Real 1991)], as indicated by molecular phylogenies (Du et al. 1994; Hollar et al. 1998; Merzlyak et al. 2001). The same applies to another organism with a frequent occurrence of choanomastigotes in culture, *Crithidia* sp. Cfm (= *Leptomonas* sp. Cfm) (Merzlyak et al. 2001; A. Kostygov, pers. commun.). Its position is clearly separate from the named *Crithidia* or other species. Thus, choanomastigote species should be divided among several genera.

The NR clade contains a few geographically restricted species that were classified as members of the three separate genera based on

Figure 32. Glyceraldehyde phosphate dehydrogenase (GAPDH) gene phylogenetic tree of the Trypanosomatidae with the emphasis on position of the new trypanosomatid species. The GAPDH sequences were aligned along the entire length using CLUSTALX with gap opening weight = 12 and gap extension weight = 5. After exclusion of the primers the alignment was 1050 nt long. The analyses were performed by a heuristic search under the GTR+Γ+*I* model (gamma-distribution shape parameter was 0.7562, proportion of invariable sites was 0.2662). *Ln*-likelihood of the best maximum likelihood tree shown was −9161.69443. The bootstrap values for the support of the SE clade were derived with the minimum evolution (first value, 1000 replicates), maximum likelihood (100 replicates, second value), and parsimony analyses (1000 replicates, third value). GenBank™ accession numbers of the reference sequences were: *Blastocrithidia gerricola* (AF322391), *Crithidia fasciculata* (AF047493), *Crithidia luciliae* (AF053740), *Euglena gracilis* (L39772), *Herpetomonas muscarum* (DQ092548), *Herpetomonas pessoai* (AF047494), *Leishmania major* (AF047497), *Leishmania mexicana* (X65226), *Leishmania tarentolae* (DQ092549), *Leptomonas costaricensis* (DQ383650), *Leptomonas lactosovorans* (AF053741), *Leptomonas peterhoffi* (AF322390), *Leptomonas podlipaevi* (DQ019000), *Leptomonas pyrrhocoris* (AY029072), *Leptomonas seymouri* (AF047495), *Leptomonas* sp. Cfm (AF320820), *Phytomonas* sp. (AF047496), *Trypanoplasma borreli* (X74535), *Trypanosoma brucei brucei* (X59955), *Trypanosoma cruzi* (X52898), *Trypanosoma rangeli* (AF053742), *Trypanosoma vivax* (AF047500), and *Wallaceina brevicula* (AF316620). The genus names given in quotation marks indicate that the taxonomic status of organisms in culture does not correspond to the original species description (see Discussion).

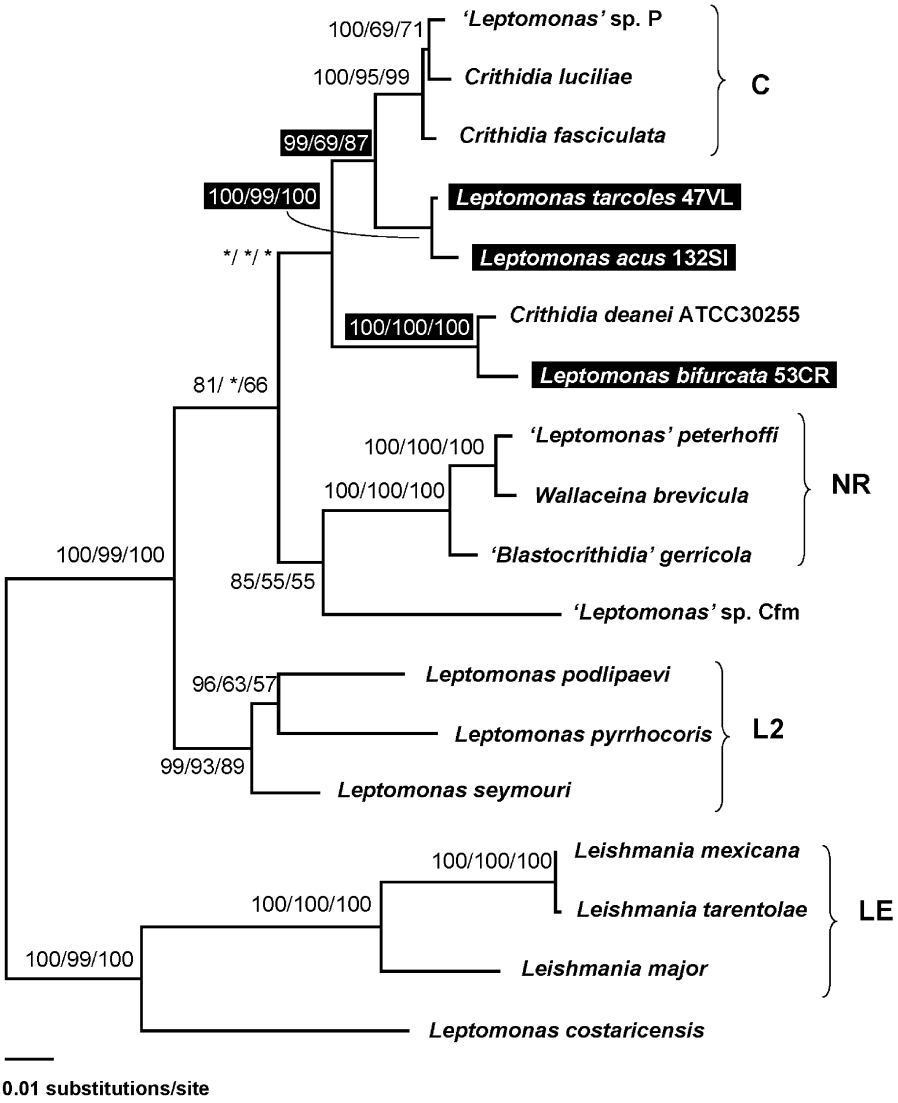


Figure 33. Topology of the SE clade derived by the maximum likelihood analysis with heuristic search of the GAPDH dataset after deletion of non-SE clade taxa. *Ln*-likelihood of the best maximum likelihood tree shown was −3968.13067. Bootstrap values shown represent minimum evolution (the first value), maximum likelihood (the second value), and parsimony (the third value) analyses. Asterisks show that the clade was recovered with frequency less than 50%. Clade designations are as in the previous studies (Merzlyak et al. 2000; Yurchenko et al. 2006a, 2006b).

morphology observed in the original infected hosts (Podlipaev 1985; Podlipaev et al. 1990). Nonetheless, by the molecular criteria applied to cells in the established cultures (Bulat et al. 1999; Podlipaev et al. 2004; Yurchenko et al. 2006a), these organisms are all very closely related to each other and to *W. brevicula*. Podlipaev (2003) suggested that this discrepancy is due to the presence of *Wallaceina*-like organisms in the original mixed infections followed by the recovery of only that component in culture. This is confirmed by the presence of endomastigotes in cultures of *B. gerricola*, *L. peterhoffi*, and several others from this region (A. Kostygov, pers. commun.), all of which thus represent various isolates of *Wallaceina*. The type species, *W. inconstans* (Frolov and Malysheva 1989; Podlipaev et al. 1990), is also a member of the NR clade (Fig. 34) (Bulat et al. 1999; Merzlyak et al. 2001). Therefore, the NR clade represents the genus *Wallaceina*.

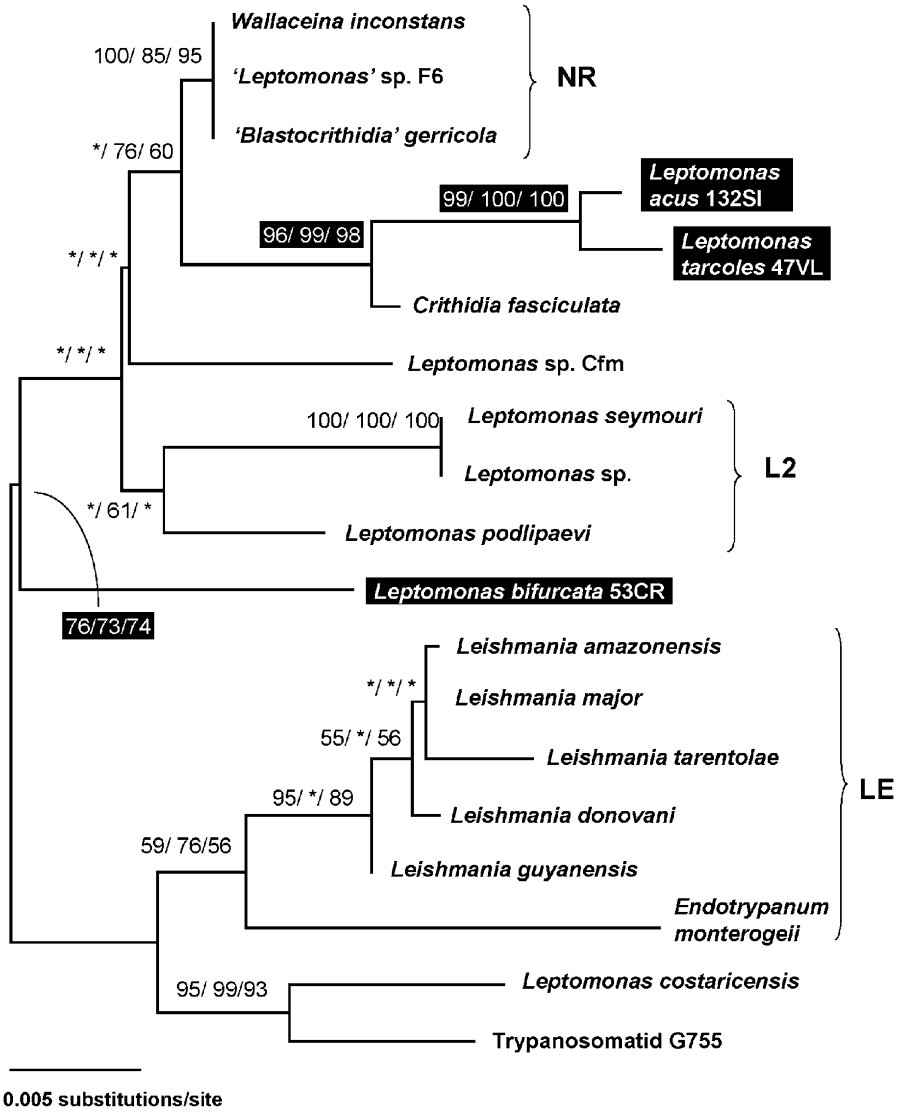


Figure 34. Small subunit ribosomal RNA (SSU rRNA) maximum likelihood tree of the SE clade. The SSU sequences were aligned using CLUSTALX with gap opening weight = 5 and gap extension weight = 2. After a manual removal of several ambiguously aligned positions and exclusion of the primers, the alignment was 2020 nt long. The analyses were performed by heuristic search with $I = 0.8421$ and $\Gamma = 0.8177$. *Ln*-likelihood of the best maximum likelihood tree shown was −4141.93162. The bootstrap values were obtained with the minimum evolution (first value, 1000 replicates), maximum likelihood (second value, 100 replicates), and parsimony analyses (third value, 1000 replicates). The following sequences were retrieved from GenBank™: *Blastocrithidia gerricola* (AF153036), *Crithidia fasciculata* (Y00055), *Endotrypanum monterogeii* (X53911), *Leishmania amazonensis* (X53912), *Leishmania donovani* (X07773), *Leishmania guyanensis* (X53913), *Leishmania major* (X53915), *Leishmania tarentolae* (M84225), *Leptomonas costaricensis* (DQ383648), *Leptomonas podlipaevi* (DQ383649), *Leptomonas seymouri* (AF153040), *Leptomonas* sp. (X53914), *Leptomonas* sp. F6 (AF153042), *Leptomonas* sp. Cfm (AF153041), *Wallaceina inconstans* (AF153044), and undescribed trypanosomatid species G755 (U59491).

It remains to be seen, however, if the members of this clade merit the status of separate species.

The genus *Leptomonas* Kent, 1880 is invalid not only because of its polyphyly (Hollar et al. 1998; Merzlyak et al. 2001; Yurchenko et al. 2006a) but also due to the uncertain identity or even the very existence of its type species, *Leptomonas bütschlii* Kent, 1880 (Wallace 1966). The organisms

fitting the associated generic diagnosis turned out to be particularly diverse phylogenetically, as illustrated by this and other studies. In the absence of the type species, it is uncertain which clade should bear the traditional name, so it might be best to abandon this name in the future revision. The clade L2 (Figs 33, 34) including *L. pyrrhocoris*, *L. seymouri* and *L. podlipaevi* is an obvious candidate for a new genus primarily defined by the monophyly of this group.

Phylogenetically as well as morphologically, the new *Leptomonas* species described herein should belong to genera different from those represented by the members of the L2 or C clades. The question is still open whether each of these species merits the status of a new genus or they can be grouped together with their respective closest relatives. Although shared morphology loses its value as a unifying taxonomic character, the differences still can be important, and a caution is needed before uniting morphologically diverse organisms into the same taxon. In the absence of a broader genomic comparison between *L. acus* and *L. tarcoles* or between *L. bifurcata* and *C. deanei* it is uncertain how indicative the apparent closeness inferred from a few genetic markers really is. A phylogenomic analysis, based on inclusion of multiple gene sequence data, is a prerequisite for the classification revision necessary.

Methods

Isolation of trypanosomatid cultures: Field stages (insect dissection, microscopic examination of the intestinal contents and establishment of the primary parasite cultures) were performed as described earlier (Maslov et al. 2007; Westenberger et al. 2004). Bacterial growth in cultures was prevented with antibiotics (chloramphenicol at 100 μg/ml, tetracycline at 50 μg/ml, and ampicillin at 100 μg/ml). Axenic cultures were established as described previously (Podlipaev et al. 1987).

Microscopy: Light microscopy and scanning and transmission electron microscopy were performed as described previously (Yurchenko et al. 2006a, 2006b). Samples for cryoelectron microscopy were prepared as follows. Cells (10 μl aliquots) fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, were dropped on a grid (1000 mesh size, 3 mm) glued onto an aluminum target, which was mounted on a holder for 10 mm stubs. The sample was flash frozen ($<10^{-3}$ K/s) in slushy nitrogen. After freezing, the sample was transferred to a high vacuum preparation chamber (ALTO 2500, Gatan). The surface of the sample was then sublimated by $-95\,^{\circ}$C for 7 min. Sublimation was followed by coating the sample with 3 nm layer of a mixture of platinum and palladium at $-135\,^{\circ}$C. Coated sample was inserted into the chamber of the Field Emission Scanning Electron Microscope JEOL JSM-7401F and images were obtained by the secondary electron signal at an accelerating voltage of 1 kV using the GB high mode.

Isolation of DNA, PCR amplification, cloning and sequencing: Total cell DNA was isolated from axenic cultures by lysis with sarcosyl-pronase and deproteinization using phenol-chloroform (Maslov et al. 1996). PCR amplification of the spliced leader (SL) RNA, glyceraldehyde phosphate dehydrogenase (GAPDH) and small subunit (SSU) rRNA genes, gel-purification, cloning and sequencing were performed as described previously (Maslov et al. 2007; Westenberger et al. 2004; Yurchenko et al. 2006a, 2006b).

The following gene sequences determined in this work have been deposited in GenBank™ under the following accession numbers: *Leptomonas acus* 132SI GAPDH (DQ910926), *L. acus* 132SI SSU rRNA (DQ910923), *L. acus* 132SI SL RNA gene repeat (EF152331), *Leptomonas bifurcata* 53CR GAPDH (DQ910928), *L. bifurcata* 53CR SSU rRNA (DQ910925), *L. bifurcata* 53CR SL RNA gene repeat (EF152330), *Leptomonas tarcoles* 47VL GAPDH (EF546787), *L. tarcoles* 47VL SSU rRNA (EF546786), *L. tarcoles* 47VL SL RNA gene repeats (EF546788, EF546789), *Leptomonas* sp. P GAPDH (EF546793), *Crithidia deanei* ATCC30255 GAPDH (EF546792), *C. deanei* ATCC30255 SL RNA gene repeats (EF546790, EF546791).

Phylogenetic analysis: The GAPDH and SSU rRNA datasets and phylogenetic procedures used were mainly the same as in the previous analysis (Yurchenko et al. 2006b). After primer removal, the sequences were aligned using CLUSTALX, version 1.81 (Thompson et al. 1997). The GAPDH alignment was 1050 nt long. The sequences were unambiguously aligned over the entire length. The SSU alignment after removal of several ambiguously aligned positions was 2020 nt long. A general time-reversible model (GTR+I+Γ) of sequence evolution was the best-fitting model for the GAPDH dataset by the AIC test of MODELTEST, version 3.06 (Posada and Crandall 1998). The proportion of invariable sites (I) was 0.2662, and the Γ-distribution shape parameter for variable sites (Γ) was 0.7562. The best-fitting MODELTEST-derived model for the SSU rRNA dataset was TrN+I+Γ, with $I = 0.8421$ and $\Gamma = 0.8177$. Maximum likelihood (ML), minimum evolution (ME) and parsimony tree inferences were made using PAUP* 4.0 beta version (Swofford 1998). Bootstrap analyses were done using 100 replicates (ML) or 1000 replicates (ME and parsimony).

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References

Brandão AA, Miranda A, Degrave WM, Sousa MA (2000) The heterogeneity of choanomastigote-shaped trypanosomatids as analyzed by their kDNA minicircle size: taxonomic implications. Parasitol Res **86**: 809—812

Bulat SA, Mokrousov IV, Podlipaev SA (1999) Classification of trypanosomatids from insects and plants by the UP-PCR (universally-primed PCR) technique and cross dot blot hybridization of PCR products. Europ J Protistol **35**: 319—326

Clark CG (1997) Riboprinting: a tool for the study of genetic diversity in microorganisms. J Eukaryot Microbiol **44**: 277—283

Cupolillo E, Medina-Acosta E, Noyes H, Momen H, Grimaldi Jr. G (2000) A revised classification for *Leishmania* and *Endotrypanum*. Parasitol Today **16**: 142—144

de Souza MA, Corte-Real S (1991) Postnuclear kinetoplast in choanomastigotes of *Crithidia deanei* Carvalho, 1973. Proposal of a new genus. Rev Inst Med Trop S Paulo **33**: S8

Du Y, Chang K-P (1994) Phylogenetic heterogeneity of three *Crithidia* spp vs. *Crithidia fasciculata*. Mol Biochem Parasitol **66**: 171—174

Du Y, Maslov DA, Chang K-P (1994) Monophyletic origin of β-division proteobacterial endosymbionts and their coevolution with insect trypanosomatid protozoa *Blastocrithidia culicis* and *Crithidia* spp. Proc Natl Acad Sci USA **91**: 8437—8441

Fernandes O, Teixeira MM, Sturm NR, Sousa MA, Camargo EP, Degrave WM, Campbell DA (1997) Mini-exon gene sequences define six groups within the genus *Crithidia*. J Eukaryot Microbiol **44**: 535—539

Fiorini JE, Takata CSA, Teofilo VM, Nascimento LC, Faria-E-Silva PM, Soares MJ, Teixeira MMG, de Souza W (2001) Morphological, biochemical and molecular characterization of *Herpetomonas samuelpessoai camargoi* n. subsp., a trypanosomatid isolated from the flower of the squash *Cucurbita moschata*. J Eukaryot Microbiol **48**: 62—69

Frolov AO, Malysheva MN (1989) The developmental cycle of *Crithidia brevicula* (Trypanosomatidae) in the laboratory culture. Tsitologya **31**: 971—975

Frolov AO, Skarlato SO (1991) Description of *Leptomonas mycophilus* sp. n. (Trypanosomatidae) from the bug *Phytocoris* sp. (Miridae). Parasitologia **25**: 99—103

Hamilton PB, Stevens JR, Gaunt MW, Gidley J, Gibson WC (2004) Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. Int J Parasitol **34**: 1393—1404

Hamilton PB, Stevens JR, Gidley J, Holz P, Gibson WC (2005) A new lineage of trypanosomes from Australian vertebrates and terrestrial bloodsucking leeches (Haemadipsidae). Int J Parasitol **35**: 431—443

Hannaert V, Bringaud F, Opperdoes FR, Michels PA (2003) Evolution of energy metabolism and its compartmentation in Kinetoplastida. Kinetoplastid Biol Dis **2**: 11

Hoare CA (1964) Morphological and taxonomical studies on mammalian trypanosomes. X. Revision of the systematics. J Protozool **11**: 200—210

Hoare CA, Wallace FG (1966) Developmental stages of trypanosomatid flagellates: a new terminology. Nature **212**: 1385—1386

Hollar L, Lukeš J, Maslov DA (1998) Monophyly of endosymbiont containing trypanosomatids: Phylogeny versus taxonomy. J Eukaryot Microbiol **45**: 293—297

Jankevicius SI, de Almeida ML, Jankevicius JV, Cavazzana M Jr., Attias M, de Souza W (1993) Axenic cultivation of trypanosomatids found in corn (*Zea mays*) and in phytophagous hemipterans (*Leptoglossus zonatus* Coreidae) and their experimental transmission. J Eukaryot Microbiol **40**: 576—581

Lukeš J, Votýpka J (2000) *Trypanosoma avium*: novel features of the kinetoplast structure. Exp Parasitol **96**: 178—181

Lukeš J, Hines JC, Evans CJ, Avliyakulov NK, Prabhu VP, Chen J, Ray DS (2001) Disruption of the *Crithidia fasciculata* KAP1 gene results in extensive structural rearrangement of the kinetoplast disc. Mol Biochem Parasitol **117**: 179—186

Maslov DA, Lukeš J, Jirků M, Simpson L (1996) Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol Biochem Parasitol **75**: 197—205

Maslov DA, Westenberger SJ, Xu X, Campbell DA, Sturm NR (2007) Discovery and barcoding by analysis of Spliced Leader RNA gene sequences of new isolates of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador. J Eukaryot Microbiol **54**: 57—65

McGhee B, Cosgrove B (1980) Biology and physiology of the lower Trypanosomatidae. Microbiol Rev **44**: 140—165

Merzlyak E, Yurchenko V, Kolesnikov AA, Alexandrov K, Podlipaev SA, Maslov DA (2001) Diversity and phylogeny of insect trypanosomatids based on small subunit rRNA genes: polyphyly of *Leptomonas* and *Blastocrithidia*. J Eukaryot Microbiol **48**: 161—169

Noyes HA, Arana BA, Chance ML, Maingon R (1997) The *Leishmania hertigi* (Kinetoplastida; Trypanosomatidae) complex and the lizard *Leishmania*: their classification and evidence for a neotropical origin of the *Leishmania-Endotrypanum* clade. J Eukaryot Microbiol **44**: 511—517

Philippe H (1998) Molecular Phylogeny of Kinetoplastids. In Coombs GH, Vickerman K, Sleigh MA, Warren A (eds) Evolutionary Relationships among Protozoa. Kluwer Academic Publishers, Dordrecht, pp 25—56

Podlipaev SA (1985) New species of lower trypanosomatids from insects (Hemiptera: Heteroptera) belonging to the families Gerridae and Nabidae: their life cycle stages in nature and in culture. Proc Zool Inst USSR Acad Sci **129**: 35—47

Podlipaev SA (1990) Catalogue of world fauna of Trypanosomatidae (Protozoa). Proc Zool Inst USSR Acad Sci **217**: 1—177

Podlipaev SA (2003) Host specificity of homoxenic trypanosomatids. Parasitologia **37**: 3—17

Podlipaev SA, Frolov AO (1987) Description and laboratory cultivation of *Blastocrithidia miridarum* sp. n. (Mastigophora, Trypanosomatidae). Parasitologia **21**: 545—552

Podlipaev SA, Frolov AO (1999) *Crithidia borea* sp. n. — a parasite of *Dixa* sp. (Diptera: Dixidae). Parasitologia **33**: 152—156

Podlipaev SA, Frolov AO, Kolesnikov AA (1990) *Proteomonas inconstans* n. gen., n. sp. (Kinetoplastida: Trypanosomatidae) — a parasite of the hemipteran bug *Calocoris sexguttatus* (Hemiptera: Miridae). Parasitologia **24**: 339—345

Podlipaev SA, Sturm NR, Fiala I, Fernandes O, Westenberger SJ, Dollet M, Campbell DA, Lukeš J (2004) Diversity of insect trypanosomatids assessed from the spliced leader RNA and 5S rRNA genes and intergenic regions. J Eukaryot Microbiol **51**: 283—290

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics **14**: 817—818

Robertson M (1912) Notes on some flagellate infections found in certain Hemiptera in Uganda. Proc Royal Soc Sect B **85**: 234—240

Roitman I, Mundim MH, De Azevedo HP, Kitajima EW (1977) Growth of *Crithidia* at high temperature: *Crithidia hutneri* sp. n. and *Crithidia luciliae thermophila* s. sp. n. J Protozool **24**: 553—556

Romeiro A, Solé-Cava A, Sousa MA, de Souza W, Attias M (2000) Ultrastructural and biochemical characterization of promastigote and cystic forms of *Leptomonas wallacei* n. sp isolated from the intestine of its natural host *Oncopeltus fasciatus* (Hemiptera: Lygaeidae). J Eukaryot Microbiol **47**: 208—220

Simpson AGB, Stevens JR, Lukeš J (2006) The evolution and diversity of kinetoplastid flagellates. Trends Parasitol **22**: 168—174

Stevens JR (2001) One million insects — a lot of parasites? Trends Parasitol **17**: 119—120

Svobodová M, Zídková L, Čepička I, Oborník M, Lukeš J, Votýpka J (2007) *Sergeia podlipaevi* gen. nov., sp. nov. (Trypanosomatidae, Kinetoplastida): a new parasite of the biting midges (Ceratopogonidae, Diptera). Int J Syst Evol Microbiol **57**: 423—432

Swofford DL (1998) PAUP* 4.0: Phylogenetic Analysis Using Parsimony (and Other Methods), beta version. Sinauer Associates, Inc., Sunderland, MA

Thomas S, Westenberger SJ, Campbell DA, Sturm NR (2005) Intragenomic spliced leader RNA array analysis of kinetoplastids reveals unexpected transcribed region diversity in *Trypanosoma cruzi*. Gene **352**: 100—108

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25**: 4876—4882

Vickerman K (1976) The Diversity of the Kinetoplastid Flagellates. In Lumsden WHR, Evans DA (eds) Biology of the Kinetoplastida. Academic Press, London, New York, San Francisco, pp 1—34

Wallace FG (1966) The trypanosomatid parasites of insects and arachnids. Exp Parasitol **18**: 124—193

Westenberger SJ, Sturm NR, Yanega D, Podlipaev SA, Zeledón R, Campbell DA, Maslov DA (2004) Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the Spliced Leader RNA gene. Parasitology **129**: 537—547

Yurchenko V, Hobza R, Benada O, Lukeš J (1999) *Trypanosoma avium*: large minicircles in the kinetoplast DNA. Exp Parasitol **92**: 215—218

Yurchenko V, Lukeš J, Xu X, Maslov DA (2006a) 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). J Eukaryot Microbiol **53**: 103—111

Yurchenko VY, Lukeš J, Jirků M, Zeledón R, Maslov DA (2006b) *Leptomonas costaricensis* sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus *Leishmania*. Parasitology **133**: 537—546