The Journal of
Eukaryotic Microbiology

Published by the International Society of Protistologists

J. Eukaryot. Microbiol., 59(6), 2012 pp. 537–547

DOI: 10.1111/j.1550-7408.2012.00636.x

New Species of Insect Trypanosomatids from Costa Rica and the Proposal for a New Subfamily within the Trypanosomatidae

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ABSTRACT. Several new species of trypanosomatids (Euglenozoa, Kinetoplastea, Trypanosomatidae), isolated from the intestines of Neotropical insects (Heteroptera), were genotyped on the basis of spliced leader RNA, and also defined phylogenetically using gene sequences of small subunit ribosomal RNA and glycosomal glyceraldehyde phosphate dehydrogenase. The taxonomic descriptions also included characterization using morphometry and electron microscopy. Our phylogenetic analyses placed the new species within the clade, previously designated "SE" for "Slowly Evolving" sequences of ribosomal RNA genes, a clade that also includes numerous monoxenous parasites of insects from the genera *Crithidia*, *Leptomonas*, and *Wallaceina,* as well as the dixenous genus *Leishmania*. Based on the high phylogenetic support for this clade, which is consistently recovered in all recent phylogenetic reconstructions, a proposal is put forward to recognize this natural taxon as a new subfamily, Leishmaniinae, within the family Trypanosomatidae.

Key Words. *Crithidia*, kinetoplastid, Largidae, Leishmaniinae, *Leptomonas*, Miridae, phylogeny, taxonomy.

THE insect trypanosomatids represent common parasites or commensals of the insect intestinal tract, with the prevalence levels exceeding 20% in some host populations (Maslov et al. 2007; Votýpka et al. 2010). Among the variety of insect hosts reported to be susceptible to infection with trypanosomatids, two orders, Hemiptera (Heteroptera) and Diptera, are most frequently found to harbor these parasites (Vickerman 1976; Wallace 1966). The recent spliced leader (SL) RNA-based genotyping analyses of trypanosomatids in Neotropical Heteroptera revealed a broad diversity of these organisms (Maslov et al. 2007; Westenberger et al. 2004). Phylogenetic analysis and morphological and ultrastructural characterization of the isolates available in culture allowed for a description of several new species (Maslov et al. 2010; Yurchenko et al. 2006b, 2008, 2009).

With a few notable exceptions, the newly described species of monoxenous parasites were contained within a single phylogenetic clade that is characterized by a relatively slow rate of sequence divergence of the small subunit ribosomal RNA (SSU rRNA) genes. To reflect this property, the clade was termed "SE" for "Slowly Evolving" (Merzlyak et al. 2001; Yurchenko et al. 2006a). This clade has also been consistently recovered in glycosomal glyceraldehyde dehydrogenase (gGAPDH) trees that might differ from the SSU trees in other aspects (Hamilton et al. 2004; Merzlyak et al. 2001; Podlipaev et al. 2004; Svobodová et al. 2007). Such remarkably strong phylogenetic support indicates that the SE clade represents a natural taxon within the Trypanosomatidae.

The taxonomic system of this family was established almost 50 yr ago and was based on a combination of morphology and life cycle traits (Hoare and Wallace 1966). Since then, the system was shown to be artificial with respect to some, but not all genera (Hollar et al. 1998; Merzlyak et al. 2001). This notion primarily refers to the genera *Crithidia* and *Leptomonas*, which are defined by a choanomastigote and promastigote body shape, respectively, and a monoxenous life style. Thus, some members of these genera are found within and some outside of the SE clade, testifying to the superficial nature of these genus definitions. On the other hand, the dixenous genus *Leishmania*, characterized by promastigotes in insect hosts and amastigotes in mammalian or reptilian hosts, is a monophyletic group within this clade (Hollar et al. 1998; Merzlyak et al. 2001; Simpson et al. 2006; Yurchenko et al. 2006b). The monoxenous genus *Wallaceina*, originally described under the name of *Proteomonas*, which is characterized by endomastigotes (Podlipaev 1990; Podlipaev et al. 1990), is also monophyletic within the SE clade. On the contrary, the monoxenous genus *Blastocrithidia*, excluding *Blastocrithidia miridarum*, which represents a misnamed strain (Yurchenko et al. 2009), and the dixenous genus *Trypanosoma*, both of which are characterized by more complex morphologies, having epimastigote and trypomastigote stages, respectively, are excluded from the SE clade (Hamilton et al. 2004; Maslov et al. 2010; Yurchenko et al. 2009). The monophyletic dixenous genus *Phytomonas* (promastigotes with specific adaptations to parasitism in plants), is also excluded from the SE clade (Camargo 1999; Dollet 2001; Hannaert et al. 2003; Hollar and Maslov 1997; Nawathean and Maslov 2000). Neither morphology, nor life cycle is useful for defining the natural taxon represented by the SE clade. We, therefore, propose to define this new taxon by molecular phylogenetic terms, as was performed previously for the major subdivisions within the class Kinetoplastea (Moreira et al. 2004). If morphological or ultrastructural synapomorphies are subsequently found, these still can be used for an augmented characterization of the new taxon.

MATERIALS AND METHODS

Isolation and characterization of new trypanosomatids from insect hosts. Collection and dissection of insects, preservation of intestinal samples, and establishment of primary trypanosomatid cultures were performed in the field as described previously (Westenberger et al. 2004). DNA from the intestinal samples was purified using PureLink™ Genomic DNA kit (Invitrogen, Carlsbad, CA). PCR amplification of the SL RNA gene repeats was performed as described previously (Maslov et al. 2007; Westenberger et al. 2004). The PCR products were gel-purified (Qiagen, Valencia, CA), cloned in pT7Blue system (EMD Bioscience, San Diego, CA), and sequenced as specified in the manufacturers' protocols. The axenic cultures were established at 27 °C in Brain Heart Infusion (BHI) medium (Becton Dickinson, Sparks, MD) supplemented with 10 μg/ml hemin (Podlipaev and Frolov 1987;

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Westenberger et al. 2004; Yurchenko et al. 2008). The SSU rRNA and gGAPDH genes from axenically cultivated cells were amplified as described previously (Maslov et al. 2010) and sequenced directly. The SL RNA genes from intestinal samples and cultures were cloned prior to sequencing. To distinguish SL repeat sequences derived from intestinal samples from those derived from axenic culture cells originating from the same infected insect specimen, the former are designated with the suffix "-int" and the latter with "-cult" in the following text. GenBank™ accession numbers of the new sequences determined in this study are listed in Table 1.

Light and electron microscopy. Cells were prepared for phase contrast and 4′,6-diamidino-2-phenylindole (DAPI) epifluorescence light microscopy, as well as for transmission and scanning electron microscopy as in previous studies (Yurchenko et al. 2006a,b, 2008). The kinetoplast DNA disk was measured as described elsewhere (Svobodová et al. 2007).

Phylogenetic analyses. The across-the-family neighbor-joining analysis of the most conservative regions of SL RNA repeats was performed as described elsewhere (Maslov et al. 2007; Westenberger et al. 2004). Typing units were separated using a 90% identity level threshold applied to full-size repeats (Maslov et al. 2007). The phylogenetic analysis of the concatenated SSU rRNA and gGAPDH dataset was performed using PAUP* 4.0 beta version (Swofford 1998) using maximum likelihood (ML), distance (minimum evolution, ME), and maximum parsimony (MP) criteria. The best-fitting model of the sequence evolution for ML and ME analyses was searched for using the Akaike Information Criterion (AIC) option of Modeltest 3.7 (Posada and Crandall 1998). The most optimal tree was inferred using heuristic searches. Bootstrap analyses included 100 in ML or 1,000 replicates in ME and MP. The remaining aspects of the analysis were as described in a previous study (Maslov et al. 2010).

Table **1.** GenBank™ accession numbers of the trypanosomatid sequences determined in this study.

Organism, strain/sample	SL RNA gene repeat	SSU rRNA gene	gGAPDH gene
Crithidia insperata, axenic strain 316AR	JF734885	JF717836	JF717831
Unnamed species TU***20***, sample 316AR-int	JF734884		
Unnamed species TU***12***, sample 318AR-int	JF734886		
Crithidia confusa, axenic strain 320AR	JF734887	JF717837	JF717832
Unnamed species TU***20***, sample 320AR-int	JF734888		
Leptomomas spiculata, axenic strain 331MV	JF734890		
L. spiculata, sample 331MV-int	JF734889		
L. spiculata, axenic strain 332MV	JF734891	JF717838	JF717833
L. spiculata, sample 332MV-int	JF734892		
L. spiculata, axenic strain 333MV	JF734893		
L. spiculata, axenic strain 334MV	JF734894		
Leptmononas tenua, axenic isolate 337VL	JF734895	JF717839	JF717834
	JF734896		
L. tenua, sample 337VL-int	JF734897		
L. tenua, axenic isolate 339VL	JF734900		
L. tenua, axenic isolate 341VL	JF734904		
L. tenua, sample 341VL-int	JF734903		
Unnamed species TU***13***, sample 338VL-int	JF734998		
	JF734999		
Crithidia brachyflagelli, axenic strain 340VL	JF734902	JF717839	JF7117835
C. brachyflagelli, sample 340VL-int	JF734901		
C. brachyflagelli, axenic strain 342VL	JF734906		
C. brachyflagelli, sample 342VL-int	JF734905		
C. brachyflagelli, axenic strain 343VL	JF734909		
C. brachyflagelli, sample 343VL-int	JF734907		
	JF734908		

RESULTS

Isolation of new trypanosomatid species. In September 2009, a population of *Largus maculatus* near Lake Arenal in the vicinity of the town Fortuna, Costa Rica (N10°25′40″, W84° 44′47″), was sampled for the presence of trypanosomatid parasites. Out of the seven insects analyzed, trypanosomatids were found in the intestine of five specimens termed 315AR-int, 316AR-int, 318AR-int, 319AR-int, and 320AR-int. Samples 317AR-int and 321AR-int were obtained from two (out of four) predatory reduviids *Ricolla simillima* found in the same locale. Morphology of live parasites observed using light microscopy in the field resembled epimastigotes as could be inferred from their elongated slender body shape and the flexible anterior end. The organisms were heterogeneous in length, with some cells free moving and some attached to the intestinal epithelium. PCR amplification with SL RNA-specific primers yielded a 0.7 kb product with all these samples but 318AR, which resulted in a 0.5 kb product (data not shown). The obtained 754–778 bp sequences from samples 316AR, 317AR, 320AR, and 321AR were 90.8–99.6% identical to isolates 84AL, 133MD, 134MD, 150MD, and 151MD from insectivorous reduviids sampled in Ecuador. The latter were previously assigned to the typing units TU***19*** and TU***20*** (Maslov et al. 2007). However, with a continuum of identity levels observed now among these and the new sequences, these two units are merged into a single unit TU***19/20***. Using a multiple alignment of the conserved segments of the SL repeats, followed by neighbor-joining analysis to compare these sequences with the dataset previously generated for the rest of the family (Maslov et al. 2007; Westenberger et al. 2004; Yurchenko et al. 2009), it was found that these organisms are also closely related to *Blastocrithidia triatoma* (Fig. S1). The remaining analyzed intestinal sample sequence, 318AR-int from *L. maculatus*, was 476 bp long and closely matched (96.8% identity) the isolate 2VL-int from a related species *Largus cinctus* previously assigned to the typing unit TU***12*** (Maslov et al. 2007). This TU is more distantly positioned on the NJ dendrogram with respect to the other sequences from the same bug population (Fig. S1).

Cultivation was only successful for infected insect samples 316AR and 320AR, from which axenic trypanosomatid cultures were established. In both cases, the recovered organisms did not represent the trypanosomatids dominating the original

intestinal samples: the sizes of the amplified repeats in the culture 316AR-cult was 301 bp vs. 770 bp in the intestinal sample 316AR-int, whereas in the case of 320AR-cult it was 429 bp vs. 754 bp for 320AR-int (data not shown). These results indicate the presence of mixed infections in the insect hosts.

The 316AR-cult sequence was 92–93% identical with the repeat units from isolate 119YS-cult (TU***50***), a previously described species *Crithidia insperata* from a coreid host collected in the Ecuadorian Amazon region (Yurchenko et al. 2009). Analyses of several additional repeats from each strain indicated that the interstrain differences only marginally exceeded the differences among repeats from the same strain (data not shown).

The 429-bp SL RNA amplified from 320AR-cult repeat was 97.0–98.8% identical to the sequences from the organism formerly known as "*Crithidia deanei* ATCC30255" (TU***56***), and recently shown to be a misnamed isolate of an unclear origin (Yurchenko et al. 2009). This identity level falls into the range typically observed among individual repeats from a single species and even the same isolate.

Seventeen specimens of *Proba sallei* (Heteroptera, Miridae) were sampled in Monteverde, Costa Rica (N10°18′04″, W84° 48′20″), and intestinal samples 331MV-int to 334MV-int were found to be infected with needle-like promastigotes. Cultivation was successful in each of these cases. The amplified SL RNA repeats from these intestinal samples and the cultured parasites were uniformly 239-bp long and 99.6–100% identical. These sequences were > 95% similar to SL repeats of several isolates from Ecuador: 97SI, 98SI, and 110SI in the mirid hosts from the Andes, and 136YS, 137YS, 140YS, and 144YS in the coreid (and one reduviid) hosts from the Amazon (Maslov et al. 2007), constituting the same typing unit TU***32***. According to the NJ analysis (Fig. S1), this TU is a new species that is most closely related to *Leptomonas tarcoles* and *Leptomonas acus* (Yurchenko et al. 2008).

Out of the 13 dissected mirid hosts of the species *Prepops* cf. *accinctus* (Heteroptera, Miridae) found in the vicinity of Tárcoles on the costarican Central Pacific coast (N09°45′24″, W84°36′42″), an intestinal infection with trypanosomatids was observed in eight cases, labeled 337VL-int to 344VL-int. The organisms observed using light microscopy were heterogeneous in terms of size (i.e. longer and shorter forms), body shape (i.e. rigid promastigote-like and more flexible epimastigote-like), and behavior (i.e. free swimming and attached). Genotyping of the encountered parasites by amplification and sequencing of the SL RNA repeats showed that they belong to at least three groups classified as the following typing units: TU***54*** characterized by 863–864 bp repeats found in isolates 337VL-int and 341VL-int; TU***55*** with 288-289 bp repeats found in 340VL-int, 342VL-int and 343VL-int; and a mixed infection in 338VL-int composed of TU***54*** and previously identified TU***13*** with repeats of 769 bp. These three groups were not closely related to each other. Additional PCR products observed in these and the products in the remaining (339VL and 344VL) samples were not analyzed. Isolation of axenic cultures was successful for the TU***54*** and TU***55*** isolates as indicated by a match of the repeats found in cultures with those in intestinal samples. TU***13***, which represents a *Blastocrithidia*, could not be cultured, as typical for this group (Maslov et al. 2007, 2010).

A single member of the typing units, which represent new species, was chosen for the morphological and molecular phylogenetic characterization and taxonomic description: 320AR-cult (TU***56***), 332MV-cult (TU***32***), 337VL-cult (TU***54***), and 340VL-cult (TU***55***). The species boundaries were primarily drawn based on a 90% threshold rule between typing units in the SE clade. This rule may not be applicable for other groups with faster rates of sequence divergence.

Morphological characterization of the new taxa in axenic cultures. ***Strain 320AR-cult***. Light microscopy examination of DAPI- and Giemsa-stained smears revealed limited heterogeneity of oval-shaped cultured cells, most of which were choanomastigotes, with some slightly elongated ones with a more promastigote-like morphology (Fig. 1–3). The cells are equipped with a prominent flagellum, the free part of which is 4.4–8.8 μm in length (6.5 ± 1.4 μm; $n = 30$; herein and elsewhere, cell dimensions were measured after staining with Giemsa), about the same as the cell, which varies from 4.3 to 8.4 μm in length (5.9 ± 0.9 μm). The flagellum exits from a deep flagellar pocket that reaches to half of the cell (Fig. 5, 7). Perhaps as a reflection of a substantial part of the flagellum being confined to the pocket, the paraflagellar rod appears already in a region of the flagellum cross-sectioned within the flagellar pocket (Fig. 6). A noticeable feature of most 320AR-cult cells is a finger-like projection present at the exit of the flagellum from the flagellar pocket (Fig. 3–5). As is characteristic for the choanomastigote morphotype, the distance from the nucleus to the posterior end is very short, herein ranging from 0.9 to 2.5 μm (1.4 ± 0.4 μm; $n = 50$). Moreover, the nucleus is located in the vicinity of the kinetoplast disk (Fig. 5, 7), as exemplified by a very short distance of 0.7–1.8 μm (1.3 ± 0.5 μm; $n = 50$) between these two structures. The kinetoplast disk is of typical shape and size, with the width of 837 ± 160 nm and the thickness of 262 ± 85 nm (Fig. 5, 7).

Strain 332MV-cult. From all the newly described flagellates, the 332MV-cult cells are most variable in size, as their length ranges between 8.8 and 27.5 μm (17.0 ± 6.0 μm; $n = 30$). These typically very slender and slightly twisted promastigotes (Fig. 9, 11, 14) are furnished with a rather thin flagellum, also highly variable in length, which varies between 10.8 and 31.2 μm (18.3 ± 5.8 μm; $n = 50$). The kinetoplast is located at the bottom of a narrow flagellar pocket, which reaches into about one-third of the cell or less (Fig. 11). The relative position of the nucleus and the kinetoplast is also highly variable (2.3–8.2 μm; 4.5 ± 1.9 μm; $n = 50$), as both structures are close to each other in cells that are shorter and thicker (Fig. 8), but more distant in thinner elongated cells (Fig. 9, 11, 14). Another characteristic feature of the 332MV-cult promastigotes is their highly tapered posterior end (Fig. 13, 14). However, as all other followed morphological features of this strain, the distance between the nucleus and the posterior end varied between 3.0 and 9.9 μm (6.0 ± 2.5 μm; $n = 50$). Cross-section of the free region of the flagellum reveals a very prominent flagellar rod (Fig. 10). The kinetoplast has a typical straight disk shape with the width of 809 ± 165 nm and the thickness of 253 ± 62 nm (Fig. 11, 12, 14).

Strain 337VL-cult. All 337VL-cult cells were typical promastigotes (Fig. 15–17). The cells were slender and small, with their cell length without flagellum varying between 5.2 and 10.3 μm (7.3 ± 1.7 μm; $n = 30$). The distance between kinetoplast and nucleus ranged from 0.5 to 2.5 μm (1.5 ± 0.5 μm; $n = 50$), whereas the thin posterior region of the cell from nucleus to the posterior end varied in length from 1.1 to 3.4 μm (2.0 ± 1.1 μm; $n = 50$) (Fig. 16, 19). DAPI staining of the cultured cells revealed that quite frequently, the oval nucleus was juxtaposed to the concave sausage-shaped kinetoplast (Fig. 15, 19–21). The length of the flagellum was virtually identical with that of the cell, ranging from 4.4 to 10.8 μm (7.2 ± 2.0 μm; $n = 50$) (Fig. 17), and the flagellum was equipped with an inconspicuous paraflagellar rod (Fig. 18). Cells fixed for TEM were usually widest in the

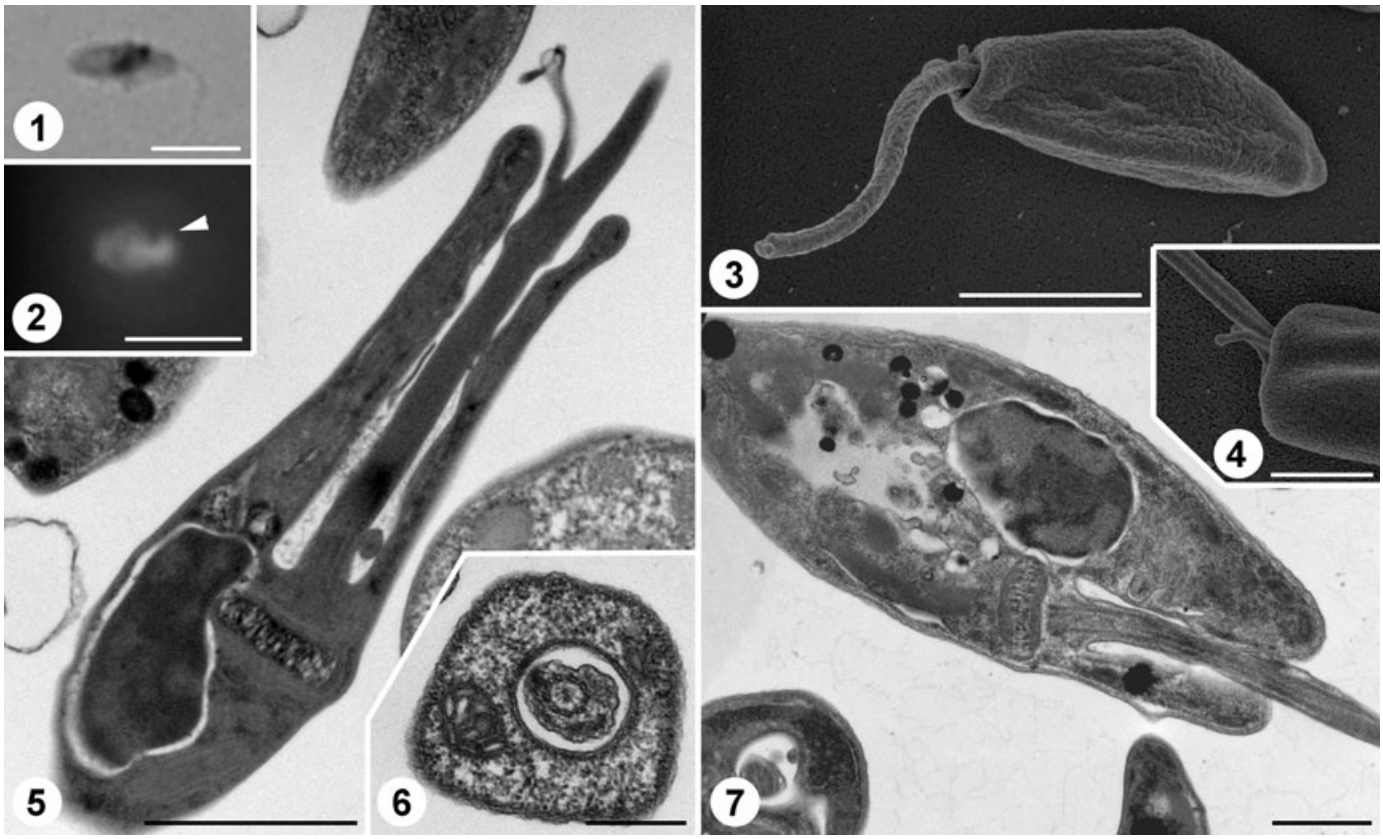


Fig. **1–7.** Light (1, 2), scanning (3, 4), and transmission (5–7) electron microscopy of *Crithidia confusa* n. sp. (isolate 320AR-cult). **1**. Giemsa-stained (arrowhead points to the kinetoplast) and **2**. DAPI-stained cultured cells of slightly elongated shape. **3**. Scanning electron microscopy of the predominant cell type in the culture. **4**. Finger-like projection invariably present at the basis of the flagellum. **5, 7**. Transmission electron microscopy of longitudinally sectioned choanomastigotes with the nucleus juxtaposed to the kinetoplast. **6**. Cross-sectioned flagellum within the flagellar pocket bearing inconspicuous paraflagellar rod. Scale bar = 5 μm (1, 2), 2 μm (3), 1 μm (4, 5, 7), and 500 nm (6).

region of the kinetoplast (Fig. 19, 20). A characteristic feature of the 337VL-cult promastigotes was a relatively deep flagellar pocket (Fig. 19, 21). As measured in TEM micrographs, the dimensions of the usually slightly bent kinetoplast disks (n = 30) were 689 ± 142 nm in width and 231 ± 69 nm in thickness (Fig. 19–21).

Strain 340VL-cult. Morphologically, the 340VL-cult cells are rather uniform, with the cells falling in a quite narrow range of 6.5–9.6 μm in length (8.3 ± 1.1 μm; n = 20), most of them being oval-shaped choanomastigotes with a short and stout flagellum, the length of which varied from 5.3 to 10.4 μm (8.8 ± 1.6 μm; n = 50) (Fig. 22–24). Most cells had a bulge on the flagellum at the exit of the flagellar pocket (Fig. 23). As shown by DAPI staining and in TEM micrographs, the nucleus in most cells was located next or close to the kinetoplast: the distance ranged from 1.0 to 2.3 μm (1.6 ± 0.5 μm; n = 50) (Fig. 22, 25). The deep and wide flagellar pocket runs through more than a half of the length of the cell (Fig. 25, 26). Moreover, the cell's posterior region was very short (1.7–3.9 μm; 2.4 ± 1.0 μm; n = 50), as is typical for the choanomastigote cell type. The cytoplasm of the blunt-ended posterior region contained numerous glycosomes and acidocalcisomes. The mitochondrion contained a prominent kinetoplast that was 822 ± 123 nm wide and 290 ± 51 nm thick (n = 20) (Fig. 25).

Phylogenetic position of the new isolates within the family. The genes for glycosomal glycerophosphate dehydrogenase (gGAPDH) and SSU rRNA were amplified and sequenced for each strain under investigation. The concatenated gene sequences were aligned with a representative set of species from the known trypanosomatid clades and used to infer the most optimal maximum likelihood tree (Fig. 27). Only the most conserved positions of the multiple sequence alignment were used for molecular phylogenetic analysis after manual inspection and removal of the fast evolving regions that could not be unambiguously aligned. The downside of this cautious approach is the apparent loss of resolution (basal polytomy) in the consensus tree (data not shown). This is usually compensated, however, by reliability of the following clades that still could be recovered on such a tree (marked with open circles at the nodes in Fig. 27): (1) the clade herein proposed to be recognized as a new subfamily Leishmaniinae, formerly SE clade; this clade includes the new species described in this study; (2) clade E of endosymbiont-bearing monoxenous trypanosomatids; (3) clade H, a group including several opisthomastigote-shaped species; (4) clade P of *bona fide* dixenous plant parasites; and (5) clade T of various *Trypanosoma* species. Relationships among these clades remain uncertain, as can be seen from the low (< 50%) bootstrap support of the particular branching order in the tree shown (Fig. 27). The basal polytomy of the consensus tree includes several lineages (also marked with open circles in the shown most optimal tree) represented herein by individual organisms with either known (i.e. *Blastocrithidia largi*) or anticipated (i.e. *Sergeia podlipaevi*, *Leptomonas collosoma*) numerous relatives.

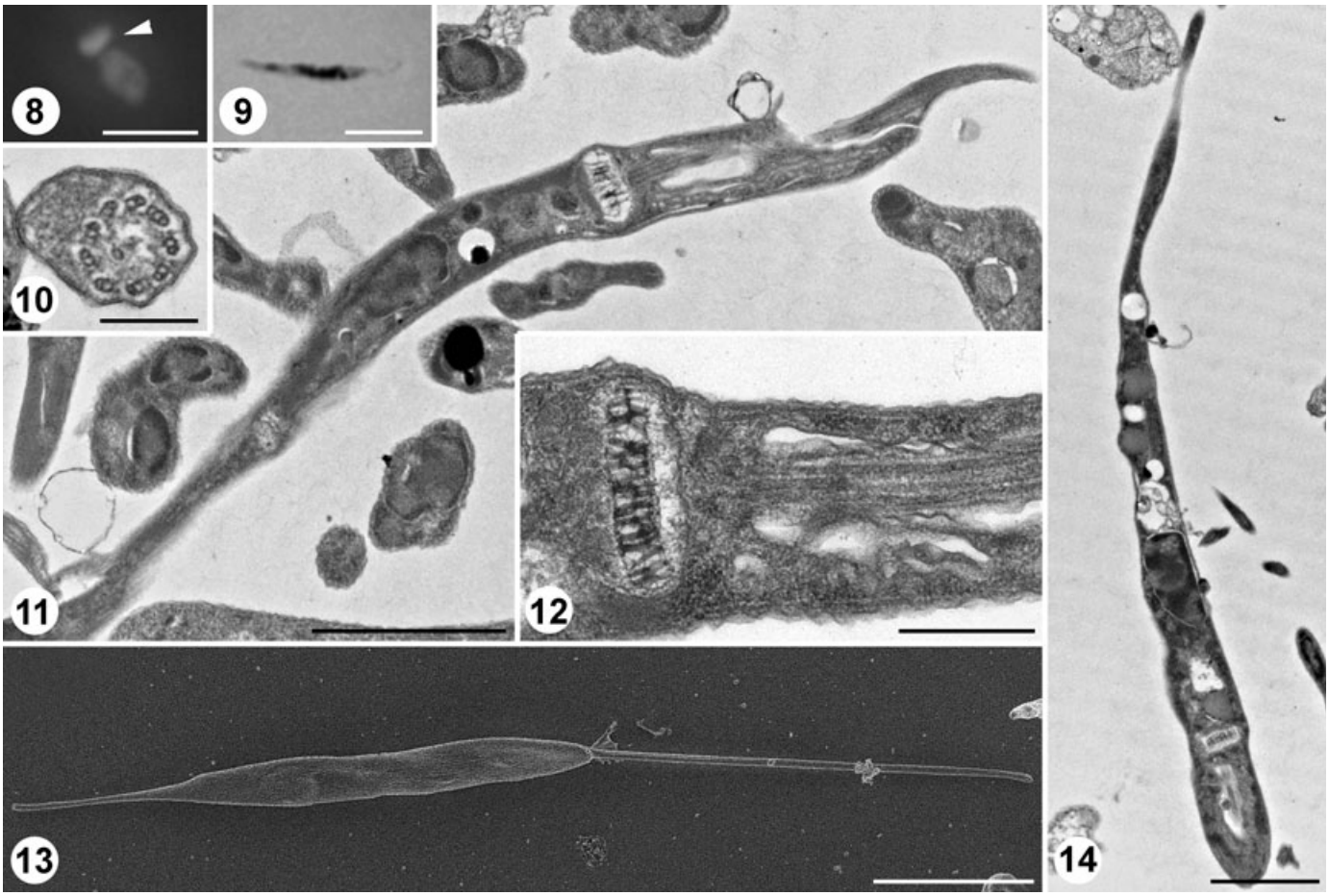


Fig. **8–14.** Light (8, 9), scanning (13), and transmission (10–12,14) electron microscopy of *Leptomonas spiculata* n. sp. (isolate 332MV-cult). **8**. DAPI-stained (arrowhead points to the kinetoplast) and **9**. Giemsa-stained cells in culture. **10**. Transmission electron microscopy of a paraflagellar rod-bearing flagellum cross-sectioned outside of the cell. **11, 14**. Longitudinal sections of highly elongated and thin promastigotes. **12**. A typical disk-shaped kinetoplast with one twist of the minicircle fibers. **13**. Scanning electron microscopy of a typical slender and twisted promastigote. Scale bar = 10 μm (8, 9), 5 μm (10), 2 μm (12, 14), 500 nm (13), and 200 nm (11).

DISCUSSION

Justification for proposed new species. Each of the new isolates was found within the large clade of monoxenous and dixenous trypanosomatids that was previously termed "SE" (Merzlyak et al. 2001). This clade is characterized by relatively conserved sequences of the rRNA genes, and is consistently recovered with a high bootstrap support in the SSU rRNA- and gGAPDH-based phylogenetic reconstructions. This is also the case in the current study: 85% bootstrap support was observed with ML, and 100% with distance and MP. There are several subgroups within the SE clade recoverable with a high bootstrap support (i.e. Cf, Wa, Cc, Lp, Le) as well as individual lineages (marked with filled circles at the nodes in Fig. 27) that are recoverable on the consensus majority tree. These groups or lineages in turn represent potential subdivisions, perhaps tribes and genera, within the proposed Leishmaniinae. The same set of major lineages was obtained in an unrooted tree exclusively composed of the SE trypanosomatids (data not shown).

The rules to delineate species boundaries applied herein to insect trypanosomatids of the SE clades are consistent with interspecies divergence levels observed in *Leishmania*, the type genus for the new subfamily. As typical for the entire SE clade, *Leishmania* species are characterized by a very slow divergence rate of their SSU rRNA genes, with closely related species being practically indistinguishable by this criterion, necessitating application of faster evolving phylogenetic markers (Croan et al. 1997; Luyo-Acero et al. 2004; Noyes et al. 2002; Yurchenko et al. 2006b). Mini-exon genes were also proved useful to separate species; however, the divergence within the subgenus *L.* (*Viannia*) (i.e. among *L. braziliensis*, *L. guyanensis*, and *L. panamensis*) was only 92% and between *L. mexicana* and *L. amazonensis* (often considered a subspecies of *L. mexicana*) was only 94% (Fernandes et al. 1994). The threshold level of 90% was, therefore, chosen for species boundaries for other members of the SE group (Maslov et al. 2007; Westenberger et al. 2004).

The positions occupied by the five cultured trypanosomatids are consistent with the associations revealed by the analysis of the SL RNA repeats. These include the relationship of isolate 320AR-cult (TU***56***) and the strain *C. deanei* ATCC30255, which deserves a special attention. As discussed previously, the strain ATCC30255 does not represent the original species *C. deanei*, and its actual origin is obscure (Yurchenko et al. 2009). The previous SL RNA analysis also showed that the misnamed strain is actually more close (but not identical) to the isolate named *Crithidia luciliae thermophila* with 77%

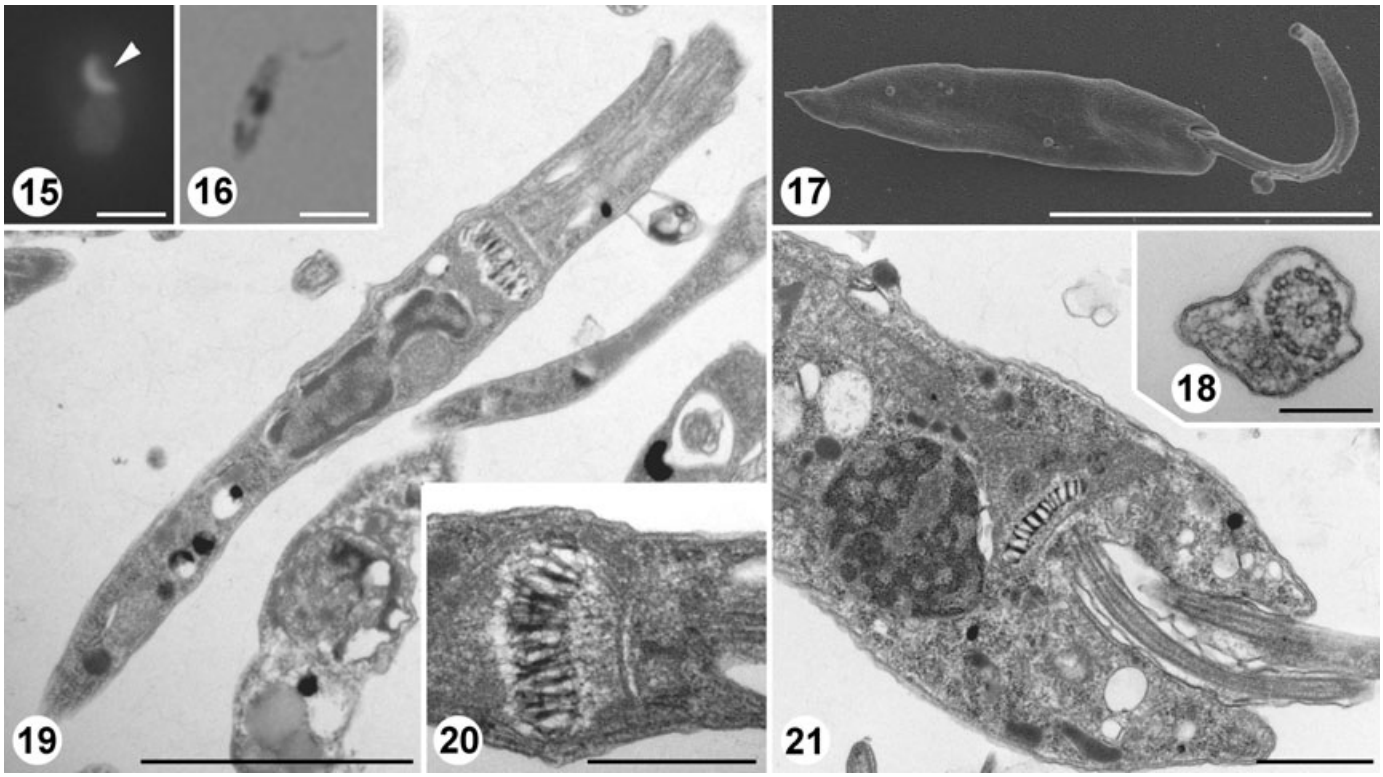


Fig. **15–21.** Light (15, 16), scanning (17), and transmission (18–21) electron microscopy of *Leptomonas tenua* n. sp. (isolate 337VL-cult). **15**. DAPI-stained (arrowhead points to the kinetoplast) and **16**. Giemsa-stained culture cells. **17**. Scanning electron microscopy of a typical promastigote. **18**. Cross-sectioned flagellum with a prominent paraflagellar rod. **19**.Transmission electron microscopy of a slender promastigote with oval-shaped nucleus. **20, 21**. A cross-section of the slightly bend kinetoplast revealing the presence (20) and the absence (21) of minicircle twist. Scale bar = 5 μm (15–17), 2 μm (19), 1 μm (21), 500 nm (20), and 200 nm (18).

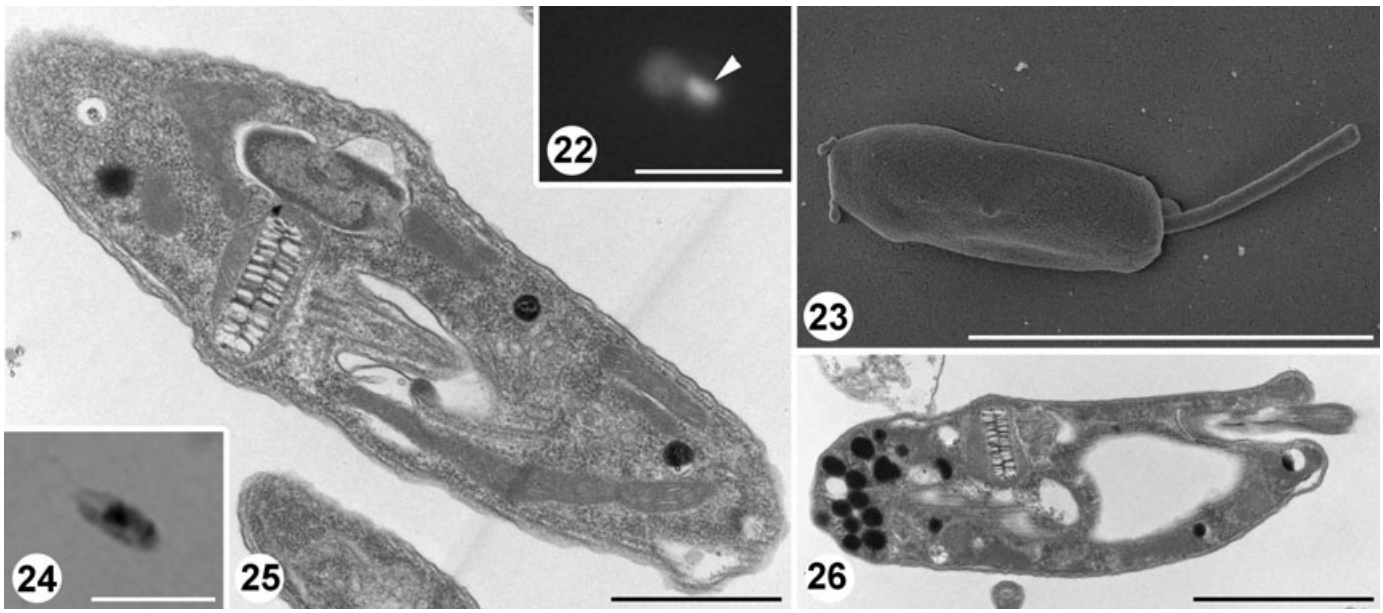


Fig. **22–26.** Light (22, 24), scanning (23), and transmission (25, 26) electron microscopy of *Crithidia brachyflagelli* n. sp. (isolate 340VL-cult). **22**. DAPI-stained (arrowhead points to the kinetoplast) and **24**. Giemsa-stained cultured oval-shaped cells. **23**. Scanning electron microscopy of a choanomastigote with a characteristically short flagellum. **25, 26**. Transmission electron microscopy of choanomastigotes with a deep flagellar pocket. Note that the minicircles make one twist in the kinetoplast disk. Scale bar = 10 μm (22, 24), 5 μm (23), 1 μm (25), and 200 nm (26).

identity of SL RNA repeats (Fernandes et al. 1997; Yurchenko et al. 2009). Based on the comparison of SL RNA repeats, gGAPDH, and SSU rRNA sequences between 320AR-cult and ATCC30255, and consistent with the typical choanomastigote body shape observed in both strains, it is clear that these two strains represent the same genetic entity, which we, therefore, propose to recognize as a new species described below under the name *Crithidia confusa* n. sp. Another known relative of this species is *Leptomonas bifurcata*, which is, nonetheless, clearly dissimilar in its gene sequence, cell size, and occurrence of a split posterior end (Yurchenko et al. 2008), features that are not seen in *C. confusa* n. sp.

The close relationship between costarican isolate 316AR-cult from a largid bug and isolate 199YS of *C. insperata* from a coreid host collected in the Ecuadorian Amazon was confirmed using our phylogenetic analysis, as well as morphological similarities. These organisms were nearly indistinguishable by the SSU rRNA sequences (i.e. 99.8% identity within the overlapping sequenced region of 2,113 bp) and gGAPDH sequences (i.e. 99.2% identity within the 969 bp long sequences), but as shown above, still could be distinguished by SL RNA repeat sequences, albeit marginally at 92–93% identity. Nonetheless, we realize that the difference between 316AR-cult and 119YS is not commensurate with a separate species level with both isolates belonging to the same typing unit TU***50***. These isolates represent the same species, *C. insperata*, with a seemingly broad Neotropical distribution and a relaxed host specificity.

It is worth mentioning herein that isolates 316AR-cult and 320AR-cult did not match the predominant infection detected using PCR in intestinal samples that were barcoded as 316AR-int and 320AR-int, both belonging to TU***19/20***. According to the analysis of SL repeats by NJ (Fig. S1), this typing unit represents an unnamed species of *Blastocrithidia* closely related to *B. triatoma*. This mismatch was a clear evidence for a mixed infection in each host, in which the cultivable trypanosomatid species were only the minor components (Yurchenko et al. 2009).

The phylogenetic tree also showed a pair of closely related organisms represented by the costarican isolate 332MV-cult (TU***32***) and *L. acus* 132SI from Ecuador (TU***40***) (Yurchenko et al. 2008). However, herein, in spite of the high similarities of 99.8% for the SSU rRNA and 99.6% for the gGAPDH gene sequences, the distinction is clearly possible on the basis of the SL RNA gene repeats with identity level just under 80%. Nonetheless, the similarity is also traced on the morphological level with both organisms being highly elongated promastigotes, which is reflected in the proposed new species name, *Leptomonas spiculata* n. sp. (see below) for 332MV-cult, although the latter cells on average are smaller than *L. acus*. The new species includes the remaining TU***32*** organisms from Costa Rica (samples 331MV-334MV, both culture and intestinal) and Ecuador (intestine samples 97SI, 98SI, and 110SI from the Andes and 136YS, 137YS, 140YS, and 144YS from the Amazon) (Maslov et al. 2007; this study). These isolates have been found predominantly in mirids and coreids. One of these specimens (140YS) came from an insectivorous reduviid indicating a prey-to-predator transmission of trypanosomatids.

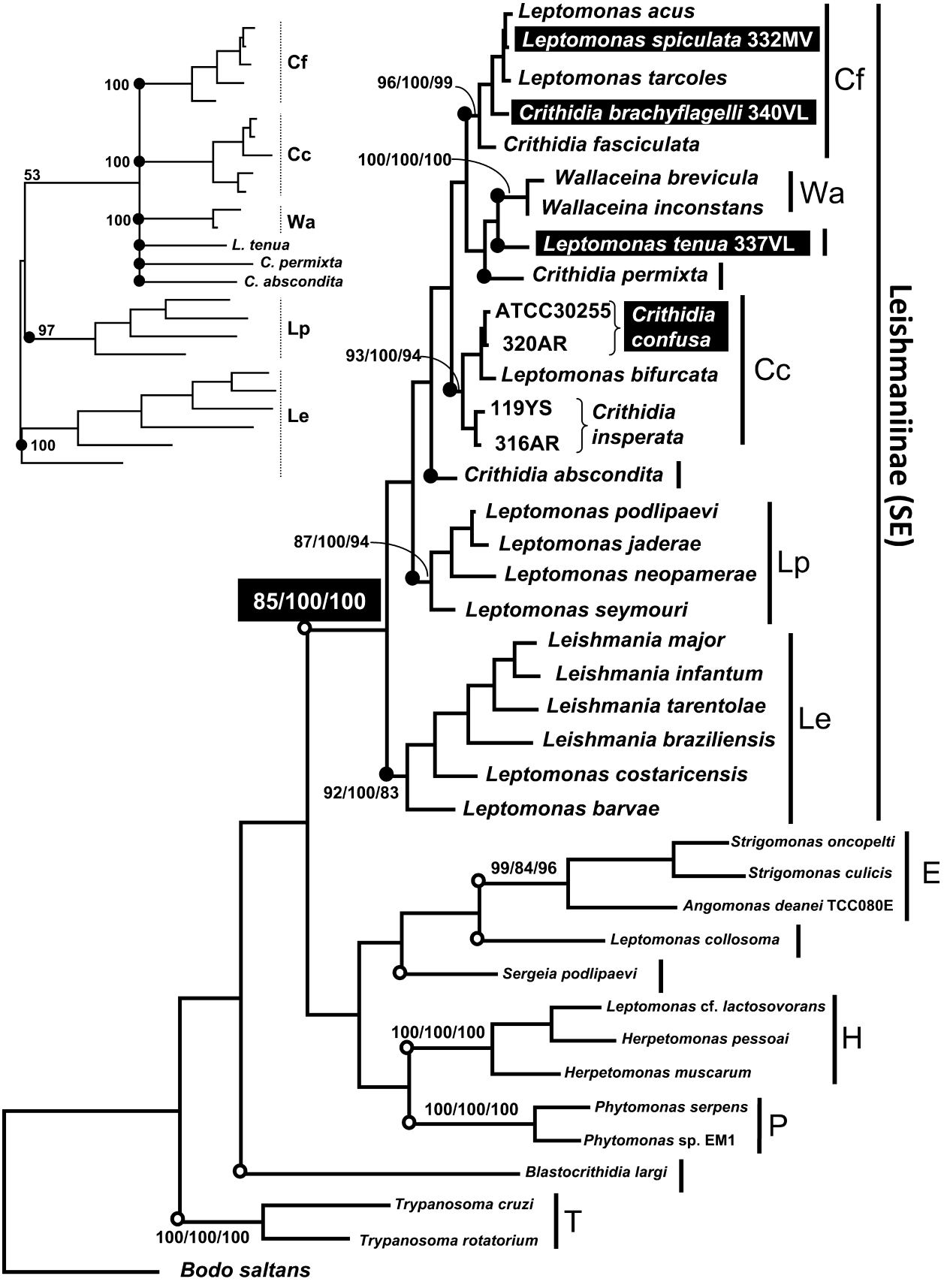
The isolates 340VL-cult (TU***55***) and in particular 337VL-cult (TU***54***) occupy well-separated positions within the SE clade on the phylogenetic tree. Isolate 340VL-cult is phylogenetically related to *C. fasciculata*, sharing the same choanomastigote morphotype (Wallace 1966), but it is also close to the group of three typical *Leptomonas* species. Of these, *Leptomonas tarcoles* is somewhat similar morphologically, with occasional choanomastigotes observed in cultures (Yurchenko et al. 2008). Although the predominant promastigotes of this species also match the 340VL-cult cell dimensions, the noticeable difference is the rounded posterior end and the shorter flagellum in 340VL-cult, henceforth named *Crithidia brachyflagelli* n. sp. So far, this species is known only from mirid hosts in Costa Rica.

The isolate 337VL-cult, herein named *Leptomonas tenua* n. sp., forms a monophyletic group with the two *Wallaceina* species in the best ML tree (Fig. 27), but this relationship is not supported using bootstrap analysis (inset in Fig. 27). Nor is there morphological resemblance between the small and slender promastigotes of this organism and the endomastigotes typical for *Wallaceina*. The known occurrence of the new species is also limited to the costarican Miridae.

Justification for proposal of the new subfamily. The well-supported monophyly of the SE clade testifies to this group being a natural taxon within the family Trypanosomatidae. The rank of this taxon deserves further discussion. In the current taxonomic system, members of several trypanosomatid genera are included in this clade. Among those, only the genus *Leishmani*a with exclusion of the genus *Endotrypanum*, which has a dubious status (Cupolillo et al. 2000), and possibly the genus *Wallaceina*, represent natural taxa. Members of the remaining genera, *Crithidia* and *Leptomonas,* are interspersed within the SE clade, and some *Leptomonas* species are also found elsewhere in the phylogenetic tree. These two genera are certainly in need of revision, but the natural status of the genus *Leishmania* does not cause any concerns. The rank of the SE clade, therefore, lies below the family and above the genus, with the subfamily being the logical choice.

Using a character-based approach, new taxa are defined by a sum of traits thought to be exclusively found in their members (i.e. shared derived characters). The usefulness and limitations of this approach have been debated (see e.g. Benton 2000; De Queiroz and Gauthier 1990, 1992), but it remains the predominant approach in systematics including that of kinetoplastids. More recently, the source of evidence supporting new taxa began to shift toward results of molecular phylogenies with morphological and other nonsequence-based characters playing an auxiliary role (Maslov et al. 2010; Moreira et al. 2004; Teixeira et al. 2011; Yurchenko et al. 2006a,b, 2008, 2009). Moreover, a strongly supported clade might be encountered with no unifying set of nonmolecular traits currently known. This seems to be the case for the SE clade and our proposed new subfamily: at this time, the only synapomorphies that could be used to define this taxon represent those sequence alignment positions that were used by the process of phylogeny inference. None of the basic trypanosomatid morphotypes has been exclusively ascribed to this clade to help with its characterization, and so far no ultrastructural features have been found to serve a similar purpose. The new taxon thus remains to be defined strictly phylogenetically as outlined by De Queiroz and Gauthier (1990) and Sereno (1999). The node-based definition ("statement specifying a clade composed of the most recent ancestor of two or more reference taxa and all descendants"; Sereno 1999) is particularly appropriate, as it excludes potential earlier branches that might not be strongly associated with the SE clade. On the contrary, if they are, only an emendation of the reference taxa list would be required.

The bootstrap consensus tree also shows that the SE clade is further subdivided into several well-supported subclades and a few individual lineages. All, but one of these represents monoxenous parasites of insects. The subclade Le, recovered with the high bootstrap support (i.e. 92% with ML, 100%


Cf
Cc
Wa
Lp
Le
100
100
53
100
L. tenua
C. permixta
C. abscondita
97
100
Leptomonas acus
Leptomonas spiculata 332MV
Leptomonas tarcoles
Crithidia brachyflagelli 340VL
Crithidia fasciculata
96/100/99
100/100/100
Wallaceina brevicula
Wallaceina inconstans
Leptomonas tenua 337VL
Crithidia permixta
ATCC30255
320AR
Crithidia confusa
93/100/94
Leptomonas bifurcata
119YS
316AR
Crithidia insperata
Crithidia abscondita
Leptomonas podlipaevi
Leptomonas jaderae
Leptomonas neopamerae
Leptomonas seymouri
87/100/94
85/100/100
Leishmania major
Leishmania infantum
Leishmania tarentolae
Leishmania braziliensis
Leptomonas costaricensis
Leptomonas barvae
92/100/83
Strigomonas oncopelti
Strigomonas culicis
Angomonas deanei TCC080E
99/84/96
Leptomonas collosoma
Sergeia podlipaevi
Leptomonas cf. lactosovorans
Herpetomonas pessoai
Herpetomonas muscarum
100/100/100
100/100/100
Phytomonas serpens
Phytomonas sp. EM1
Blastocrithidia largi
Trypanosoma cruzi
Trypanosoma rotatorium
100/100/100
Bodo saltans
Cf
Wa
Cc
Lp
Le
E
H
P
T
Leishmaniinae (SE)

with distance, and 83% with MP), includes two insect parasites: *Leptomonas barvae* and *Leptomonas costaricensis*, branching off relatively early, and the monophyletic group of dixenous *Leishmania*. Although only a few *Leishmania* lineages were included in this analysis, the earlier studies convincingly demonstrated the monophyly of this genus (Cupolillo et al. 2000; Noyes et al. 2002; Yurchenko et al. 2006b). The topological position of the dixenous parasites as the terminal clade in subclade Le represents additional evidence for their origin from the monoxenous parasites.

The rest of the tree contains several other well-supported clades as discussed above. Each of these topological elements (additionally marked with bars in Fig. 27) represent a natural subdivision within the Trypanosomatidae and some of them (e.g. *Phytomonas* and *Trypanosoma*) match the existing taxonomic units. An important taxonomic revision of the members of clade E (endosymbiont-bearing monoxenous trypanosomatids) has been carried out recently (Teixeira et al. 2011). The remaining clades still await their detailed taxonomic scrutiny.

TAXONOMIC SUMMARY

Class Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida Kent, 1880 stat. nov. Hollande, 1952

Family Trypanosomatidae Doflein, 1951

Subfamily Leishmaniinae, n. subfamily Maslov and Lukeš in Jirků et al. 2012. Diagnosis. A well-supported monophyletic group of monoxenous parasites of insects currently assigned to the genera *Crithidia*, *Leptomonas*, and *Wallaceina,* and the dixenous parasites of insects and vertebrates included in the genera *Leishmania* and *Endotrypanum*, previously termed SE, for relatively "slow evolution" (low rate of sequence divergence) of ribosomal RNA genes. Emerges late in the best tree reconstructions, but may appear as part of the basal polytomy in bootstrap consensus trees. Composed of the most recent common ancestor of *Crithidia fasciculata* (clade Cf, Fig. 27), *Wallaceina brevicula* (clade Wa), *Crithidia permixta*, *Crithidia confusa* (clade Cc), *Leptomonas tenua*, *Crithidia abscondita*, *Leptomonas podlipaevi* (clade Lp), *Leishmania major* (clade Le), and all its descendants. The type genus is *Leishmania* Ross (1903).

The authorship of the new taxa (the subfamily and the species) proposed in this study is attributed to Maslov and Lukeš and the formal way of citation should be Maslov and Lukeš in Jirků et al. 2012.

***Crithidia confusa* n. sp. Maslov and Lukeš in Jirků et al. 2012. Generic assignment.** Based on the typical choanomastigote morphology displayed by the cells and in accordance with the existing taxonomic system (Hoare and Wallace 1966), the new species belongs to the genus *Crithidia* Lèger 1902. This genus was later found to be polyphyletic. However, as *Crithidia* species include several names deeply entrenched in the parasitological literature, including, in particular, the type species *Crithidia fasciculata* Lèger, 1902, the complete disbanding of this genus is deemed to produce much confusion. We, therefore, suggest to continue ascribing new species of monoxenous endosymbiont-free choanomastigotes to this genus, provided that such organisms are also members of the SE clade.

Species diagnosis. Cells are typical choanomastigotes with no discernible diagnostic features at the morphology level. The species is identified by the unique sequences of the SSU rRNA, gGAPDH, and especially SL RNA genes.

Type host. Intestine of *Largus maculatus* Schmidt 1931 (Heteroptera: Largidae). The xenotype is deposited in the UCR Entomology Museum.

Type locality. In vicinity of Fortuna near Lake Arenal (N10°25′40″, W84°44′47″), province Alajuela, Costa Rica.

Type material. The designated hapantotype is the Giemsa-stained slide of axenic culture of isolate 320AR-cult deposited the parasitological collection in the Institute of Parasitology (České Budějovice). The culture is deposited in the American Type Culture Collection (ATCC PRA-346).

Etymology. The Latin word *confusa* meaning *confusable* or *confusing* used for the species name reflects the previous confusing situation regarding the strain ATCC30255. That strain was earlier mistakenly believed to represent *C. deanei*, an endosymbiont-bearing trypanosomatid with a strong phylogenetic affinity to other endosymbiont-containing species. Although the origin of the ATCC30255 strain remains obscure, we have found herein that it belongs to the new species.

Gene sequence. The GenBank™ accession numbers of the *C. confusa* n. sp. (isolate 320AR) sequences determined in this study are JF717837 (SSU rRNA), JF717832 (gGAPDH), and JF734887 (SL RNA gene repeats).

***Leptomonas spiculata* n. sp. Maslov and Lukeš in Jirků et al. 2012. Generic assignment.** The only morphotype observed is a typical highly elongated promastigote. According to the existing taxonomic nomenclature of Hoare and Wallace (1966), monoxenous promastigotes are ascribed to the genus *Leptomonas* Kent, 1880. This is an artificial taxon with the dubious type species, and, however, it is preferable to preserve this name rather than wreak havoc by a massive renaming of the numerous species placed in this genus over the long time. Consequently, we suggest to continue using the genus name *Leptomonas*, but limiting this practice only to monoxenous trypanosomatids of promastigote body shape that are monophyletic with the other members of the SE clade.

Fig. **27.** The phylogenetic tree of Trypanosomatidae with emphasis on the position of the new species assigned to the new subfamily Leishmaniinae, formerly the "slowly evolving" clade. The tree was inferred from the dataset of concatenated SSU rRNA and gGAPDH genes of selected species (39 taxa) aligned with CLUSTAL X, ver. 2. The ambiguously aligned positions were manually removed, the length of the final alignment was 2,890 characters. The tree shown is the best maximum likelihood (ML) tree (*Ln*-likelihood = –20656.58288) inferred using heuristic search – PAUP 4.0 and the best-fitting model (GTR + I + Γ) determined by the AIC option of Modeltest (propotion of invariable sites I = 0.4413, and the value of gamma-distribution shape parameter Γ = 0.5489). Major clades within trypanosomatids recovered on the majority-rule consensus tree (not shown) are marked with open circles at the ancestral nodes. The percentage values of bootstrap support are shown for the nodes of the relevant clades only: the first number representing ML (100 replicates), the second value representing minimum evolution and the third – parsimony (1,000 replicates in each case). The bootstrap support values for more basal nodes were below 50% and are not shown. Bootstrap values within the terminal clades are omitted for clarity and due the lack of relevance. Scale bar represents 0.05 substitutions per site. The tree was rooted using the sequences of *Bodo saltans*. GenBank™ accession numbers of the retrieved SSU rRNA and gGAPDH sequences are given in Table S1. The inset shows topology of the SE clade shown in the majority-rule consensus of the ML bootstrap trees (alignment of 25 taxa and 2,991 characters, 100 replicates, *Ln*-likelihood = –9958.04241, GTR + I + Γ, I = 0.6776, Γ = 0.6544). The clades Cf, Cc, Wa, Lp, and Le, and the individual lineages of *Leptomonas tenua*, *Crithidia permixta*, and *Crithidia abscondita* recoverable on the SE bootstrap tree (inset) are also marked with filled circles on the entire-family best ML tree. Bootstrap values are shown for the relevant clades only.

Species diagnosis. The new species is identifiable by unique sequences of SL RNA gene repeats. The SSU rRNA and gGAPDH gene sequences are > 99% identical to *L. acus* and *Leptomonas tarcoles* not allowing for separation of the three species. Cells display the elongated promastigote body shape shared by several other species which, therefore, is not diagnostic.

Type host. Intestine of *Proba sallei* Stahl, 1862 (Heteroptera: Miridae). The xenotype is deposited in the UCR Entomology Museum.

Type locality. In the town of Monteverde (N10°18′04″, W84°48′20″), province Puntarenas, Costa Rica.

Type material. The designated hapantotype is the Giemsa-stained slide of axenic culture of isolate 332MV-cult deposited the parasitological collection in the Institute of Parasitology (České Budějovice). The culture of isolate 332MV-cult is deposited in the American Type Culture Collection (ATCC PRA-348).

Etymology. The Latin word *spiculata* meaning *spear-* or *dart-like* reflects the sharp-pointed appearance of the cells.

Gene sequence. The GenBank™ accession numbers of the sequences are JF717838 (SSU rRNA), JF717833 (gGAPDH), and JF734889–JF734894 (SL RNA gene repeats).

***Leptomonas tenua* n. sp. Maslov and Lukeš in Jirků et al. 2012. Generic assignment**. Slender promastigotes represent the only observed morphotype in this species, which is thereby assigned to the polyphyletic genus *Leptomonas* Kent, 1880.

Species diagnosis. Although the new species cannot be reliably distinguished from the closely related *Wallaceina* spp. and *Crithidia permixta* by SSU rRNA sequences (~ 99% idenity) or gGAPDH sequences (~ 93–95% idenity), this species is clearly identifiable by SL RNA gene repeats, which are only ~ 54% identical to *C. permixta* and ~ 61% to *W. inconstans*. Cells morphology (slender promastigotes) is shared by many species and is not diagnostic.

Type host. Intestine of *Prepos* cf. *accinctus*. (Heteroptera: Miridae). The xenotype is deposited in the UCR Entomology Museum.

Type locality. In the vicinity of Tárcoles (N09°45′24″, W84° 36′42″), province Puntarenas, Costa Rica.

Type material. The designated hapantotype is the Giemsa-stained slide of axenic culture of isolate 337VL-cult deposited the parasitological collection in the Institute of Parasitology (České Budějovice). The culture of isolate 337VL-cult is deposited in the American Type Culture Collection (ATCC PRA-349).

Etymology. The species name is derived from the Latin word *tenua* indicating a slim or slender appearance typical for cells of this species.

Gene sequence. The GenBank™ accession numbers of the sequences are JF717839 (SSU rRNA), JF717834 (gGAPDH) and JF734895–JF734897, JF734900, JF734903, JF734904 (SL RNA gene repeats).

***Crithidia brachyflagelli* n. sp. Maslov and Lukeš in Jirků et al. 2012. Generic assignment.** The new species is ascribed to the polyphyletic genus *Crithidia* Lèger, 1902 based on the predominance of choanomastigotes.

Species diagnosis. The new species is identified by the sequences of its SL RNA gene repeats and the SSU rRNA and gGAPDH gene sequences. Cells' morphology (choanomastigotes) does not reveal distinct diagnostic characters.

Type host. Intestine of *Prepos* cf. *accinctus* (Heteroptera: Miridae). The xenotype (postdissection remains of the host of the trypanosomatid isolate 340VL) is deposited in the UCR Entomology Museum.

Type locality. In the vicinity of Tárcoles (N 09°45′24″, W84° 36′42″), province Puntarenas, Costa Rica.

Type material. The designated hapantotype is the Giemsa-stained slide of axenic culture of isolate 340VL-cult deposited the parasitological collection in the Institute of Parasitology (České Budějovice). The culture of isolate 340VL-cult is deposited in the American Type Culture Collection (ATCC PRA-345).

Etymology. The species name represents a combination of a Greek and Latin words characterizing a short (*brachys,* βραχύς, Greek for short) flagellum observed in cells from this species.

Gene sequence. The GenBank™ accession numbers of the sequences are JF717840 (SSU rRNA), JF717835 (gGAPDH) and JF734901, JF734902, JF734905–JF734909 (SL RNA gene repeats).

ACKNOWLEDGMENTS

We thank T. Henry (Smithsonian Institution, Washington, DC), J. L. Stehlík (Moravian Museum, Brno), and P. Kment (National Museum, Prague) for host species identification, and R. Blanco, F. Campos, R. Vargas and the Organization for Tropical Studies for help with permits in Costa Rica. This study was supported by a UCR Academic Senate grant and a Fulbright Foundation International Scholarship award to D. A. M., and grants 2B06129, LC07032, and 6007665801 from the Ministry of Education of the Czech Republic, and the Premium Academiae award to J. L.

LITERATURE CITED

Benton, M. J. 2000. Stems, nodes, crown clades, and rank-free lists: Is Linnaeus dead? *Biol. Rev.*, **75**:633–648.

Camargo, E. P. 1999. *Phytomonas* and other trypanosomatid parasites of plants and fruit. *Adv. Parasitol.*, **42**:29–112.

Croan, D. G., Morrison, D. A. & Ellis, J. T. 1997. Evolution of the genus *Leishmania* revealed by comparison of DNA and RNA polymerase gene sequences. *Mol. Biochem. Parasitol.*, **89**:149–159.

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

De Queiroz, K. & Gauthier, J. 1990. Phylogeny as a central principle in taxonomy: phylogenetic definitions of taxon names. *Syst. Zool.*, **39**:307–322.

De Queiroz, K. & Gauthier, J. 1992. Phylogenetic taxonomy. *Annu. Rev. Ecol. Syst.*, **23**:449–480.

Dollet, M. 2001. Phloem-restricted trypanosomatids form a clearly characterized monophyletic group among trypanosomatids isolated from plants. *Int. J. Parasitol.*, **31**:459–467.

Fernandes, O., Murphy, V. K., Kurath, U., Degrave, W. M. & Campbell, D. A. 1994. Mini-exon gene variation in human pathogenic *Leishmania* species. *Mol. Biochem. Parasitol.*, **66**:261–271.

Fernandes, O., Teixeira, M. M. G., Sturm, N. R., Sousa, M. A., Camargo, E. P., Degrave, W. M. & Campbell, D. A. 1997. Mini-exon gene sequences define six groups within the genus *Crithidia*. *J. Eukaryot. Microbiol.*, **44**:535–539.

Hamilton, P. B., Stevens, J. R., Gaunt, M. W., Gidley, J. & Gibson, W. C. 2004. Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. *Int. J. Parasitol.*, **34**:1393–1404.

Hannaert, V., Bringaud, F., Opperdoes, F. R. & Michels, P. 2003. Evolution of energy metabolism and its compartmentation in Kinetoplastida. *Kinetoplastid Biol. Dis.*, **2**:11.

Hoare, C. A. & Wallace, F. G. 1966. Developmental stages of trypanosomatid flagellates: a new terminology. *Nature*, **212**:1385–1386.

Hollar, L. & Maslov, D. A. 1997. A phylogenetic view on the genus *Phytomonas*. *Mol. Biochem. Parasitol.*, **89**:295–299.

Hollar, L., Lukeš, J. & Maslov, D. A. 1998. Monophyly of endosymbiont containing trypanosomatids: phylogeny versus taxonomy. *J. Eukaryot. Microbiol.*, **45**:293–297.

Luyo-Acero, G. E., Uezato, H., Oshiro, M., Takei, K., Kariya, K., Katakura, K., Gomez-Landires, E., Hashiguchi, Y. & Nonaka, S. 2004. Sequence variation of the cytochrome *b* gene of various human infectiong members of the genus *Leishmania* and their phylogeny. *Protist*, **128**:483–491.

Maslov, D. A., Yurchenko, V. Y., Jirků, M. & Lukeš, J. 2010. Two new species of trypanosomatid parasites isolated from Heteroptera in Costa Rica. *J. Eukaryot. Microbiol.*, **57**:177–188.

Maslov, D. A., Westenberger, S. J., Xu, X., Campbell, D. A. & 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. *J. Eukaryot. Microbiol.*, **54**:57–65.

Merzlyak, E., Yurchenko, V., Kolesnikov, A. A., Alexandrov, K., Podlipaev, S. A. & Maslov, D. A. 2001. Diversity and phylogeny of insect trypanosomatids based on small subunit rRNA genes: polyphyly of *Leptomonas* and *Blastocrithidia*. *J. Eukaryot. Microbiol.*, **48**:161–169.

Moreira, D., Lopéz-Garcia, P. & Vickerman, K. 2004. An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. *Int. J. Syst. Evol. Microbiol.*, **54**:1861–1875.

Nawathean, P. & Maslov, D. A. 2000. The absence of genes for cytochrome *c* oxidase and reductase subunits in maxicircle kinetoplast DNA of the respiration-deficient plant trypanosomatid *Phytomonas serpens*. *Curr. Genet.*, **38**:95–103.

Noyes, H., Pratlong, F., Chance, M., Ellis, J., Lanotte, G. & Dedet, J. P. 2002. A previously unclassified trypanosomatid responsible for human cutaneous lesions in Martinique (French West Indies) is the most divergent member of the genus *Leishmania* ss. *Parasitology*, **124**:17–24.

Podlipaev, S. A. 1990. Catalogue of the world fauna of Trypanosomatidae (Protozoa). *Proc. Zool. Inst. Leningrad*, **140**:1–178.

Podlipaev, S. A. & Frolov, A. O. 1987. Description and laboratory cultivation of *Blastocrithidia miridarum* sp. n. (Mastigophora, Trypanosomatidae). *Parasitologia*, **21**:545–552. (in Russian)

Podlipaev, S. A., Frolov, A. O. & Kolesnikov, A. A. 1990. *Proteomonas inconstans* n. gen., n. sp. (Kinetoplastida: Trypanosomatidae)—a parasite of the hemipteran bug *Calocoris sexguttatus* (Hemiptera: Miridae). *Parasitologia*, **24**:339–345. (in Russian)

Podlipaev, S., Votýpka, J., Jirků, M., Svobodová, M. & Lukeš, J. 2004. *Herpetomonas ztiplika* n. sp. (Kinetoplastida: Trypanosomatidae): a parasite of the blood-sucking biting midge *Culicoides kibunensis* Tokunaga, 1937 (Diptera: Ceratopogonidae). *J. Parasitol.*, **90**:342–347.

Posada, D. & Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**:817–818.

Ross, R. 1903. Further notes on Leishman's bodies. *Br. Med. J.*, **ii**:1401.

Sereno, P. 1999. Definitions in phylogenetic taxonomy: critique and rationale. *Syst. Biol.*, **48**:329–351.

Simpson, A. G. B., Stevens, J. R. & Lukeš, J. 2006. The evolution and diversity of kinetoplastid flagellates. *Trends Parasitol.*, **22**:168–174.

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

Swofford, D. L. 1998. PAUP* 4.0: Phylogenetic Analysis Using Parsimony (and Other Methods), Beta Version. Sinauer Associates, Inc., Sunderland.

Teixeira, M. M. G., Borghesan, T. C., Ferreira, R. C., Santos, M. A., Takata, C. S. A., Campaner, M., Nunes, V. L. B., Milder, R. V., de Souza, W. & Camargo, E. P. 2011. Phylogenetic validation of the genera *Angomonas* and *Strigomonas* of trypanosomatids harboring bacterial endosymbionts with the description of new species of trypanosomatids and of proteobacterial symbionts. *Protist*, **162**:503–524.

Vickerman, K. 1976. The diversity of the kinetoplastid flagellates. *In*: Lumsden, W. H. R. & Evans, D. A. (ed.), Biology of the Kinetoplastida. Academic Press, London. p. 1–34.

Votýpka, J., Maslov, D. A., Yurchenko, V., Jirků, M., Kment, P., Lun, Z. R. & Lukeš, J. 2010. Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in South-West China reveals both endemism and global dispersal. *Mol. Phylogenet. Evol.*, **54**:243–253.

Wallace, F. G. 1966. The trypanosomatid parasites of insects and arachnids. *Exp. Parasitol.*, **18**:124–193.

Westenberger, S. J., Sturm, N. R., Yanega, D., Podlipaev, S. A., Zeledón, R., Campbell, D. A. & Maslov, D. A. 2004. Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene. *Parasitology*, **129**:537–547.

Yurchenko, V. Y., Lukeš, J., Jirků, M. & 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. *Int. J. Syst. Evol. Microbiol.*, **59**:893–909.

Yurchenko, V., Lukeš, J., Xu, X. & Maslov, D. A. 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, V. Y., Lukeš, J., Jirků, M., Zeledón, R. & Maslov, D. A. 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.

Yurchenko, V. Y., Lukeš, J., Tesařová, M., Jirků, M. & Maslov, D. A. 2008. Morphological discordance of the new trypanosomatid species phylogenetically associated with the genus *Crithidia*. *Protist*, **159**:99–114.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Neighbor-joining (NJ) analysis of Spliced Leader RNA repeats of Trypanosomatidae with emphasis on the new species. To enable a meaningful alignment at least for closely related taxa, full-size repeats were truncated by eliminating the most rapidly evolving part of the intergenic region. The remaining part included the sequence from −100 position upstream of the exon to the beginning of the exon, followed by the exon itself (excluding the primer annealing sites) and the intron (ending just upstream of the T-block, which marks the transcription termination site). The sequences were aligned using Clustal X; the alignment contained 149 taxa and 225 characters. The NJ analysis was performed using PAUP* 4.0 beta version using Kimura two-parameter distances. Species of *Trypanosoma* were used to root the tree. Taxa included representatives of typing units (and/or species, when appropriate) of insect trypanosomatids discovered and bar-coded up to date, as well as most " older" species for which SL sequences are known. Typing unit or species name is followed by isolate (in parentheses) used for the analysis. As typing units are defined by the 90% threshold rule applied to full-size repeats, although the presented tree is based on the most conserved segments of the sequences, some typing units appear more closely related to each other than they really are (e.g. TU***56*** = *Crithidia confusa* and *Crithidia luciliae thermophila*). New species described in this study are highlighted with red. The isolates characterized in this study for the first time are shown in bold. When a block of several isolates is given in parentheses, the listing order reflects their branching order on the NJ tree.

Table S1. GenBank™ accession numbers of the retrieved SSU rRNA and gGAPDH sequences

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Received: 03/31/11; 01/29/12, 04/20/12; accepted: 04/23/12