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Two New Species of Trypanosomatid Parasites Isolated from Heteroptera in Costa Rica

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ABSTRACT. Two new trypanosomatid species (Euglenozoa, Kinetoplastea) isolated from the intestinal tract of heteropteran insect hosts were described based on molecular phylogenetic analyses of Spliced Leader (SL) RNA gene repeats, glycosomal glyceraldehyde phosphate dehydrogenase, and small subunit ribosomal RNA genes, as well as by morphology. *Leptomonas barvae* n. sp., from a mirid host *Collaria oleosa*, was found to represent one of the closest monoxenous (one host) relatives of the dixenous (two hosts) parasitic genus *Leishmania*. This finding further supports the origin of these dixenous parasites from monoxenous progenitors in the Neotropics. *Blastocrithidia largi* n. sp., from a largid host *Largus cinctus*, is among a few members of this genus available in culture. The species is a close relative of *Blastocrithidia triatomae* and is a member of a new monophyletic phylogenetic group characterized by formation of straphanger cysts.

Key Words. *Blastocrithidia*, kinetoplastid, Largidae, *Leptomonas*, Miridae, phylogeny, taxonomy, Trypanosomatidae.

INSECT trypanosomatids represent a diverse assembly of monoxenous (single host) parasitic protists from the class Kinetoplastea that are found predominantly in Heteroptera and Diptera hosts, but are also known to parasitize other classes of insects (Vickerman 1976; Wallace 1966). The knowledge of the diversity and biogeographical distribution of insect trypanosomatids is fragmental. The emerging picture is that these organisms are distributed world-wide, reaching a high abundance and diversity in the tropics, apparently reflecting the observed species richness of their hosts in these areas (Podlipaev 2001). The parasites are found in various sections of the alimentary tracts of infected insects, and transmission is assumed to largely follow contaminative pathways, as well as coprophagy and necrophagy (Carvalho and Deane 1974; Tieszen and Molyneux 1989). To facilitate the transmission, several species are known to form flagellar cysts (i.e. straphangers) capable of long-term survival in adverse conditions. A significant impact on host fitness has been recorded for some monoxenous parasites of Diptera (Brown, Loosli, and Schmid-Hempel 2000; Brown, Schmid-Hempel, and Schmid-Hempel 2003) and Hemiptera (Schaub 1994; Schaub, Reduth, and Pudney 1990). However, individuals with a high parasite load are quite common in populations with no obvious adverse effect (D.A.M., unpubl. observ.).

While several members of the monoxenous group have been known for decades, the systematic study of insect trypanosomatids was hampered by a relatively uniform morphology observed at the light microscopy level, a requirement of axenic cultures for biochemical or ultrastructural analyses, as well as the lack of sufficient attention (Podlipaev 2000; Wallace et al. 1983). With the application of molecular phylogenetic approaches it has become clear that the morphology-based taxonomy of the group is in need of an extensive revision (Hollar, Lukeš, and Maslov 1998; Merzlyak et al. 2001; Yurchenko et al. 2008). It now appears that the genera *Leptomonas*, *Crithidia*, *Blastocrithidia*, and *Herpetomonas* are polyphyletic as their members are widely interspersed in the phylogenetic trees. However, the current taxonomy remains traditional (Hoare and Wallace 1966), mainly because of the uncertainty with respect to the criteria that would be laid in the foundation of the revised system. Another major gap is the lack of a satisfactory knowledge of trypanosomatid diversity at the level of major natural groups that might represent potential new genera. Only recently the application of molecular techniques has offered a few glimpses into the real complexity of the group (Hamilton et al. 2005; Maslov et al. 2007; Svobodová et al. 2007). An additional problem pertains to delineating species boundaries in case of genetically close entities with a clonal mode of reproduction, as exemplified by the discussion on the status of some pathogenic *Trypanosoma* species and subspecies (Brisse, Barnabé, and Tibayrenc 1998; Gibson 2003, 2007; Momen 2003).

With the aim to fill the gaps in the knowledge of the trypanosomatid diversity, we have been searching for and analyzing Neotropical trypanosomatids using a combination of traditional and culture-independent approaches. The latter approach is based on the polymerase chain reaction (PCR) amplification and sequencing of Spliced Leader (SL) RNA gene repeats. It has enabled the discovery and barcoding of nearly 50 typing units or potential new species, most of which were refractory to cultivation and, therefore, would have remained uninvestigated otherwise (Maslov et al. 2007). Meanwhile, isolation in culture is usually considered a prerequisite for a new species description because it allows for a more comprehensive analysis of the new species (Yurchenko et al. 2006a, 2006b, 2008). In this work we present a description of two new species of insect trypanosomatids. Cultivation of these organisms proved to be a challenge and highlights the difficulties with implementing the traditional approach to studying trypanosomatid biodiversity. This study also highlights the need to develop the concept of ''molecular species'' with respect to Trypanosomatidae, which would alleviate the reliance on culture for new species description.

MATERIALS AND METHODS

Collection locales and methods. Insects were collected in March 2003 and February 2004. Collection sites are described below as part of the species descriptions. In the field, trypanosomatids were detected in the gut smears of infected hosts using light microscopy, DNA samples were preserved, and the primary cultures of the parasites were established as described elsewhere (Maslov et al. 2007; Westenberger et al. 2004).

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Cultivation techniques, light and electron microscopy. Primary cultures were established in the field in the Difco brain heart infusion (BHI) medium (Becton Dickinson, Sparks, MD) supplemented with 10 μg/ml hemin and antibiotics as described previously (Westenberger et al. 2004). Subsequently, cultivation was performed in the same medium with 10% (v/v) fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA), and also in SDM79 (Brun and Schonenberger 1979) and Gibco Grace's medium (Invitrogen, Carlsbad, CA), pH 6.0, and Gibco M199 medium (Invitrogen), pH 7.0, each supplemented with 10% FBS. The cultures were kept at 26 °C. Cells were processed for light microscopy, transmission, and scanning electron microscopy as described previously (Yurchenko et al. 2006a, 2006b, 2008, 2009). The size of the kinetoplast DNA (kDNA) disk was measured as described elsewhere (Svobodová et al. 2007).

PCR amplification, cloning, and sequencing. The subsequent DNA purification from the gut samples followed published protocols. SL RNA gene repeats were amplified using primers M167 and M168, and the PCR products were gel-purified, cloned, and sequenced using the procedures described previously (Maslov et al. 2007; Westenberger et al. 2004). Glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) genes were amplified with primers M200 (5′-ATGGCTCC[G/A/C][G/A/C]TCAA[G/A]GT [A/T]GG [A/C]AT-3′) and M201 (5′-TA[G/T]CCCCACTCGTT[G/A]TC [G/A] TACCA-3′), using the same thermal profile as reported previously for SL amplification (Maslov et al. 2007). Small subunit ribosomal RNA (SSU rRNA) genes were amplified with primers S763 (5′-CATATGCTTGTTTCAAGGAC-3′) and S762 (5′-GACT TTTGCTTCCTCTA[A/T] TG-3′) (Maslov et al. 1996). The PCR products from isolate 67 VL were cloned with the pT7Blue system (EMD Bioscience, San Diego, CA) using the manufacturer's protocol and the clones were sequenced. The PCR products obtained from isolates 19 and 21 EC were gel-purified (Qiagen, Valencia, CA) and then sequenced directly. In each case both strands were sequenced using a combination of the amplification and internal conserved primers (Maslov et al. 1996), as necessary. The sequencing was performed by the UC Riverside IIGB Core Instrumental facility. The GenBank™ accession numbers for the new sequences determined in this work are: for *Blastocrithidia largi* n. sp.—gGAPDH gene—FJ968528, SSU rRNA gene—FJ968531, and SL RNA-5S rRNA gene repeat—FJ968530; for *Leptomonas barvae* n. sp.—gGAPDH gene—FJ968529, SSU rRNA gene—FJ968532; and for *Parabodo caudatus*—gGAPDH gene—DQ915848.

Phylogenetic analyses. The gGAPDH and SSU rRNA gene sequences were assembled and edited using Vector NTI 9.0. Alignments were generated using CLUSTAL X version 2 (Larkin et al. 2007) with gap opening penalty of 15 and gap extension penalty of 6. The alignments were manually edited using MEGA 4 (Tamura et al. 2007) by removal of the sequences representing primers, the ambiguously aligned regions, and most of the positions with gaps. The final gGAPDH alignment contained 973 characters. MODELTEST 3.06 (Posada and Crandall 1998) was used to find the best fitting model of sequence evolution by the AIC test. The model selected for the gGAPDH dataset was GTR with the proportion of invariable sites (I) as 0.2728 and the value of Γ-distribution shape parameter for variable sites as 0.6763. The across-the-family SSU rRNA gene alignment contained 1,797 characters. The respective parameters of the best-fitting model (GTR+I+Γ) selected for the SSU data set were $I = 0.5535$ and $\Gamma = 0.6570$. The second SSU rRNA gene alignment used included mainly the members of the ''SE'' clade (Merzlyak et al. 2001) and was 1,813 characters long. The selected model for this alignment was also (GTR+I+Γ) with $I = 0.8008$ and $\Gamma = 0.6520$. The maximum likelihood, distance, and parsimony phylogeny inference analyses were performed using PAUP* 4.0 beta version (Swofford 1998). Heuristic search was used in all cases. Minimum evolution was used as an optimality criterion for distance analyses. Bootstrap analyses included 100 replicates for maximum likelihood or 1,000 replicates for other methods.

RESULTS

Isolation of trypanosomatid cultures. Eight specimens of *Collaria oleosa* Distant (Hemiptera: Miridae) were collected by sweep netting in March 2003 at the 500-m elevation site (''El Ceibo'') along the Barva transect near the western boundary of the Braulio Carrillo National Park, Costa Rica (10°20′N, 84°05′W). Seven specimens were found to be heavily infected with trypanosomatids (i.e. gut samples 17–23 EC) whose morphology resembled the promastigote form. Four out of seven primary cultures, established in BHI supplemented with FBS, contained active cells 10 d after the initial inoculation. However, the subsequent transfers yielded live cultures for only 19 and 21 EC. In each case, the culture also contained yeast-like fungus that has not been investigated further. The trypanosomatid cells could be stably propagated in BHI, Grace's, and M199 media supplemented with 10% FBS. Numerous attempts were made to obtain an axenic culture by separating the actively moving trypanosomatid cells from the fungi using a device described earlier (Podlipaev and Frolov 1987). Although individual trypanosomatids were repeatedly detected in the ''clean'' part of the apparatus, subsequent attempts to propagate these cells axenically were unsuccessful. The non-axenic culture has been stably maintained in vertical 14-ml Falcon tubes filled with 10 ml of M199 medium with 10% FBS and by weekly transfers of 10% of the culture. Most of the fungal growth happens at the bottom of the tube with actively moving trypanosomatids multiplying in the entire volume of the tube. Under these conditions, the density of actively swimming trypanosomatid cells can reach $\sim 50 \times 10^6$ cell/ml after a week of growth before beginning to decline.

An operational typing unit TU***41*** had been established previously by analysis of the SL gene repeats from the insect gut samples 17, 19, 21, and 22 EC (Maslov et al. 2007) and found to represents a candidate new species. PCR amplification of the SL RNA gene repeats from 19 to 21 EC showed the same band pattern as gut samples 19–22 EC (Fig. 1) indicating that TU***41*** was recovered in culture. This new species is described below as *L. barvae* n. sp.

Four specimens of *Largus cinctus* Herrich-Schaeffer (Hemiptera: Largidae) were collected on the vegetation along a forest stream in March 2004 in the vicinity of Tárcoles, at the western boundary of the Carara National Park, Costa Rica (09°47′N, 84°37W). Abundant and actively swimming trypanosomatid cells were found in the intestines of each host specimen (i.e. samples 64, 65, 67, and 68 VL). The trypanosomatid morphotypes observed included promastigotes and epimastigotes. Initial cultivation was attempted in BHI plus 10% FBS, but only two primary cultures contained live trypanosomatids 2 wk after the initial inoculation, and a stable axenic culture was derived only from 67 VL using the same medium. Subsequently, the culture was maintained in M199 supplemented with 10% FBS. The growth was observed in 14-ml Falcon tubes almost completely filled with medium but was not observed in horizontal T25 flasks with the same amount of medium, suggesting that excess of oxygen is detrimental. This phenomenon needs to be investigated further. The culture grows slowly with an initial cell density of 0.1×10^6 cells/ml and a plateau reached at $5–6 \times 10^6$ cells/ml in 3 wk.

Previously eight distinct typing units of trypanosomatids had been identified in the same locale in adults of *L. cinctus* and nymphs of *Largus* sp. by analysis of SL RNA repeats amplified from gut DNA samples (Maslov et al. 2007). These included

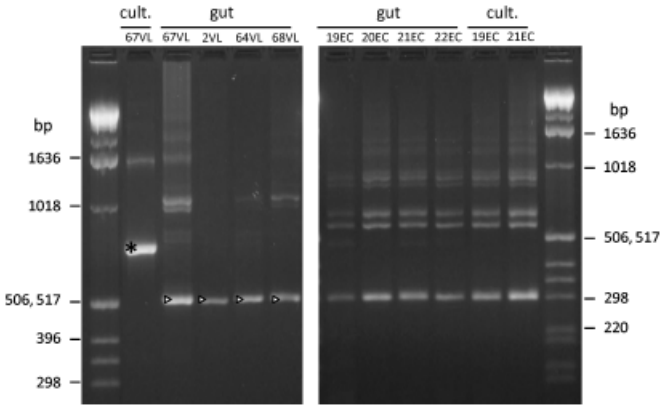


Fig. **1.** Analysis of PCR-amplified Spliced Leader (SL) RNA gene repeats from host gut samples (gut) and from cultures (cult.). Culture sample 67 VL represents axenically grown cells of *Blastocrithidia largi* n. sp., from *Largus cinctus*, with the asterisk denoting the band of monomeric SL RNA gene repeat. Host gut samples 67, 2, 64, and 68 VL correspond to trypanosomatids found in different individuals of the same host species, with arrowheads showing SL repeat monomers. Culture samples 19 and 21 EC represent two independently isolated strains of *Leptomonas barvae* n. sp., while host gut samples 19–22 EC represent trypanosomatids infecting individual specimens of *Collaria oleosa*. The fastest migrating bands represent the monomers sequenced. The gels were calibrated using 1-kb DNA ladder (Invitrogen).

TU***9***, TU***10***, TU***12–14***, TU***23***, TU***33***, and TU***34***, all of which, except TU***12***, were clustered within the *Blastocrithidia triatomae* and *Blastocrithidia leptocoridis* clade. TU***12***, which included the gut sample 67 VL(g), was somewhat more distantly related to this clade. In order to relate the culture 67 VL(c) cells with the organisms detected in these other natural hosts, the SL RNA gene repeats were compared by size (Fig. 1) and by sequence with the gut sample TU***12*** repeats and other sequences in the SL RNA gene database. The 67 VL(c) sequence obtained from cultured flagellates did not match the gut sample 67 VL(g) sequence but was closest to the sequences from TU***9***, which is formed by organisms circulating in the same host population. This result suggests that the original host was infected with at least TU***9*** and TU***12***, but only one of them was recovered in culture (Yurchenko et al. 2009). As a distinct genotype, this organism represents a new species described below as *B. largi* n. sp.

Morphological characterization of the new trypanosomatid isolates. Both 19 and 21 EC cells (i.e. *L. barvae* n. sp.) exist in culture solely as typical promastigotes (data not shown, Fig. 2), often twisted and with a pointed posterior end and oval or tapered anterior end. Because both cultures were morphologically indistinguishable, only the description of the 21 EC cells is provided below. The length of the cell (without flagellum) ranged between 10.8 and 31.0 μm (mean ± SD: 24.4 ± 10.8 μm; n = 50). The distance between the nucleus and the kinetoplast (Fig. 3) varied from 2.0 to 9.1 μm (5.6 ± 1.9 μm), whereas the distance between the nucleus and the posterior end of the cell was 4.5–14.5 μm (9.4 ± 2.9 μm). The length of the flagellum ranged from 11.0 to 35.2 μm (24.4 ± 5.7 μm).

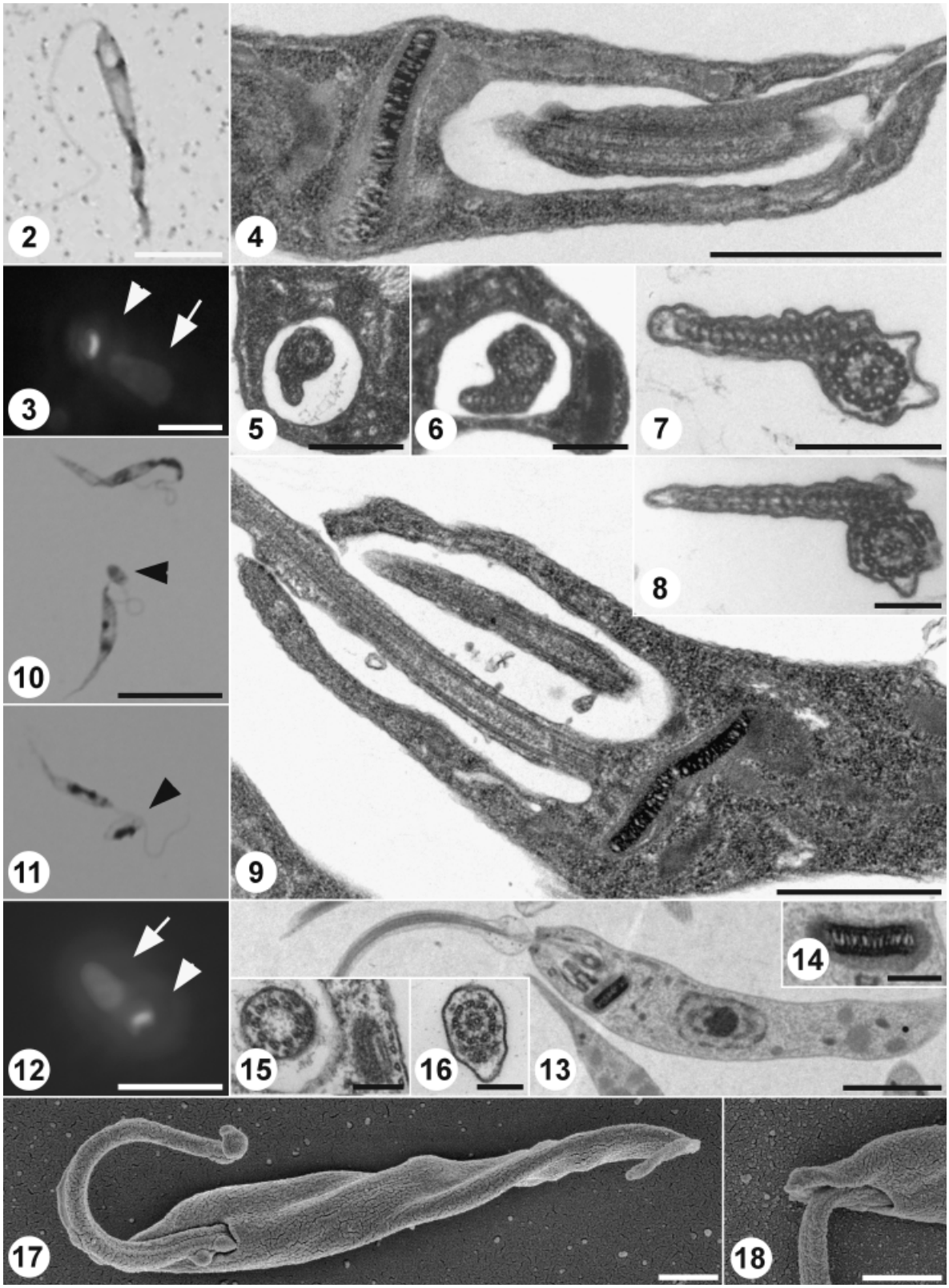
Transmission electron microscopy showed the features present in virtually every trypanosomatid species, such as an oval nucleus, a single peripherally located mitochondrion, a complete corset of subpellicular microtubules, glycosomes, and acidocalcisomes. We have noticed two aspects of 21 EC ultrastructure that are noteworthy. The thickness of the kDNA disc is 125.5 ± 39.4 nm (n = 50) and the disc is rather wide (812.0 ± 350.0 nm) (Fig. 4). The unusually thin disc shape is particularly obvious in dividing cells (Fig. 9), and testifies to the presence of small kDNA minicircles (Lukeš and Votýpka 2000). The flagellum is enforced by a conspicuous paraflagellar rod, which extends deep within the flagellar pocket (Fig. 5, 6). Moreover, cross-section of the free portion of the flagellum revealed that it is strengthened by perhaps one of the most prominent paraflagellar rods known in a kinetoplastid (Fig. 7, 8).

The light microscopy of the 67 VL cells (i.e. *B. largi* n. sp.) suggests that they are epimastigotes (Fig. 10). This conclusion is derived from the cell's morphology characterized by a lateral position of the kinetoplast and also from the flexible and gradually thinning anterior end of the cells, which is due to the flagellar attachment to the cell body. Giemsa staining revealed the often twisted and elongated shape of cells equipped with a long flagellum. Importantly, about 1% of epimastigotes carried a typical oval straphanger cyst, 2–3 μm in diameter, attached to the flagellum (Fig. 10, 11). Both in the slender epimastigote and the straphanger, the kinetoplast and the nucleus stained as prominent dots. In most cases, the straphanger was attached at or close to the base of the flagellum (Fig. 10, 11). Cells of this isolate were 12.4–19.5 μm in length (15.4 ± 2.1 μm; n = 50). The distance between the kinetoplast and the nucleus (Fig. 12) was 1.6–4.7 μm (2.8 ± 0.8 μm), whereas the distance from the nucleus to the tip of the posterior end was 4.7–12.1 μm (7.8 ± 1.7 μm). The flagellum size was typically 16.3 ± 2.9 μm.

Measurement of kinetoplast disks in transmission electron micrographs of the 67 VL cells revealed that this structure is thicker than its homologue in the 21 EC cells (312.9 ± 77.7 nm, n = 50), yet it is at the same time quite narrow in diameter (744.0 ± 155.9 nm) (Fig. 13). Moreover, the upper and lower face of the disk is characteristically dense, while the density of the stretched taut minicircle DNA fibers is lower than in a typical kDNA disk (Fig. 13, 14). Within the flagellar pocket, the flagellum is not supported with a paraflagellar rod (Fig. 15), which in the free part of the flagellum is almost inconspicuous (Fig. 16). Scanning electron microscopy (Fig. 17, 18) showed that a flagellar pocket opens laterally with the emergent flagellum positioned adjacent to the cell body, as expected for epimastigotes, before becoming free. The cell's anterior end is usually tapered, as is the posterior end, and the flagellum closely adheres to the cell body within a shallow groove that extends from the flagellar pocket to the anterior end. At least in fixed cells, no undulating membrane is seen. In a few cases the flagellum is detached from the cell's body (Fig. 18). However, observations of live cells indicate that flagellum is inseparable from cell body in most cases. The cell's entire anterior end represents a highly flexible structure that actively participates in cell movements along with the free part of the flagellum.

Phylogenetic affinities of the new species. The gGAPDH and SSU rRNA gene sequences of isolates 21 EC and 67 VL were PCR amplified from the respective cultures and infected gut samples. For each isolate and each gene the same sequence was produced from both sources, confirming authenticity of the cultures. Only the most conserved sequence positions were subjectively selected and used for phylogenetic analyses, even though this inevitably leads to the loss of resolution. Moreover, the fast evolving sequences of the salivarian trypanosomes were excluded from the SSU analysis (see Hamilton et al. 2004, for a discussion of this problem). The phylogenetic trees were rooted with the sequences of *Bodo saltans* and *P. caudatus*, the first of these being closest known kinetoplastid relatives of Trypanosomatidae (Doležel et al. 2000; Simpson et al. 2000).

The gGAPDH maximum likelihood tree (Fig. 19) showed that isolate 21 EC (i.e. *L. barvae* n. sp.) belongs to the ''SE'' clade that includes a variety of monoxenous insect parasites along with the clade of dixenous *Leishmania* species (Yurchenko et al. 2006b).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The *L. barvae* n. sp. lineage is found in a sister-group relationship with the previously established monophyletic group that brings together *Leishmania* and *Leptomonas costaricensis* (Yurchenko et al. 2006b). This relationship is consistent with the position of the corresponding TU***41*** in the SL RNA gene cladogram amidst the *Leishmania* lineages (Maslov et al. 2007).

The across-the-family SSU rRNA analysis has strongly favored the inclusion of *L. barvae* n. sp. in the SE clade (Fig. 20). An alternative alignment that includes only the members of the ''SE'' clade along with several representative *Leishmania* species was used to investigate an unrooted tree topology. The best maximum likelihood tree displays the branch of *L. barvae* n. sp. as the closest sister lineage to the *Leishmania* clade (Fig. 21).

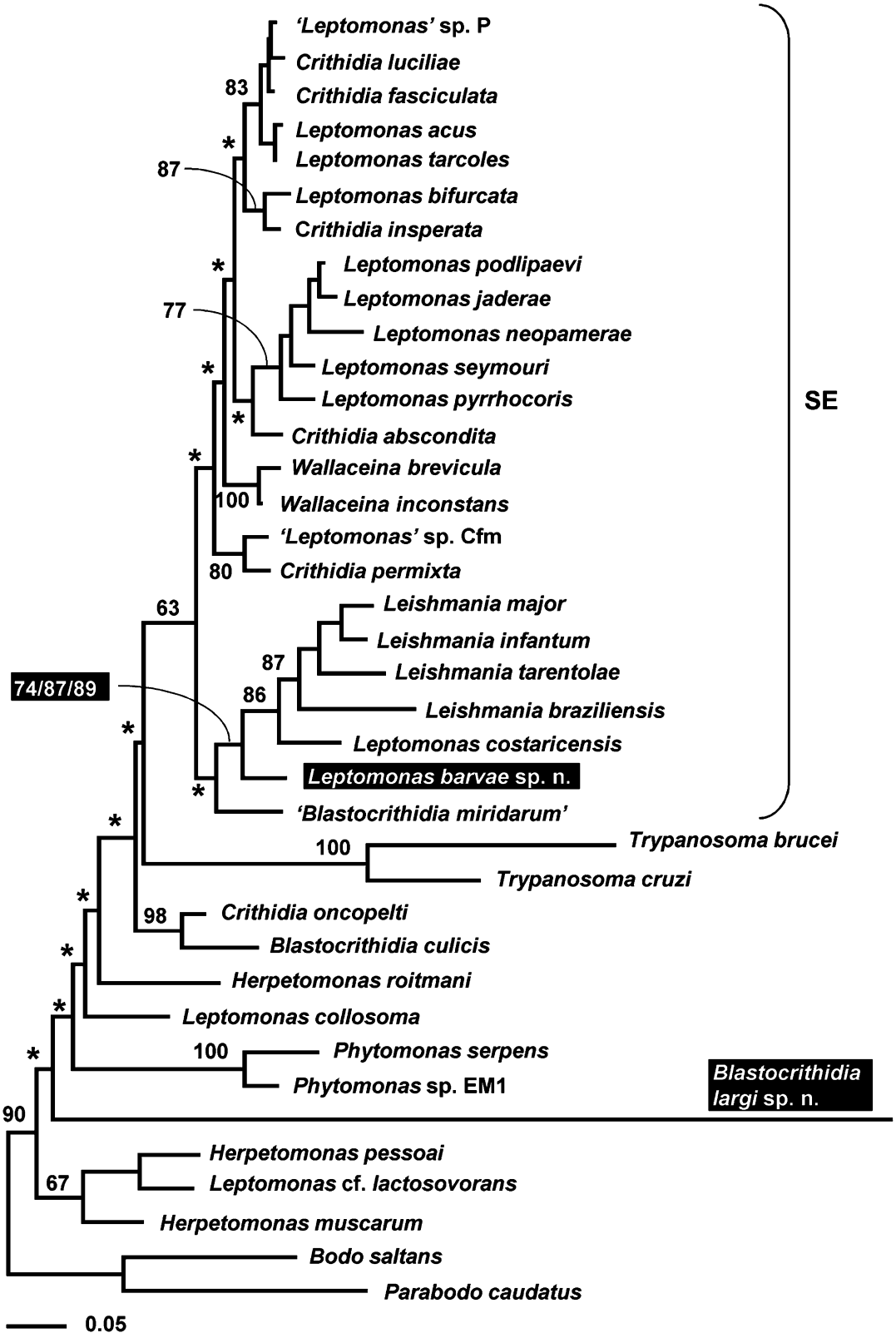
The trypanosomatids represented by the second culture analyzed in this study, *B. largi* n. sp., isolate 67 VL, formed a well-separated long branch in the gGAPDH tree (Fig. 19). The SSU rRNA analysis (Fig. 20) has revealed that this species is most closely related to *B. triatomae*. This association is fully consistent with the position of isolate 67 VL, which is a member of SL RNA TU***9*** in the SL RNA cladogram within a large clade that also includes *B. triatomae*, *B. leptocoridis*, and a score of unnamed species (Maslov et al. 2007). While the SSU rRNA sequences of *B. largi* and *B. triatomae* are 99.5% similar, their SL RNA gene repeats are only 69.6% similar. Besides *B. triatomae* and *B. largi* n. sp., no gGAPDH and SSU rRNA sequences have been published from this group, which has proved refractory to cultivation (Maslov et al. 2007). The two blastocrithidias are found in a highly supported sister-group relationship with *Leptomonas jaculum* (Fig. 20). The position of the *B. largi–B. triatomae–L. jaculum* clade in the gGAPDH and SSU rRNA trees was not well resolved with any methods used.

DISCUSSION

Establishment of new species. We have described two new species of insect trypanosomatids from Costa Rica. We have followed the integrative approach (Yurchenko et al. 2006a, 2006b, 2008, 2009) that combines morphology with molecular data to allow for a comparison of the new species with the rest of the family, as well as for identification when isolates of these species are encountered in nature. This approach, in particular a detailed morphological characterization, is greatly facilitated by availability of the organisms in culture, although, as shown by the case of *L. barvae* n. sp., not necessarily axenic cultures. However, without disputing the importance of cultures for a comprehensive analysis of the physiology, biochemistry, and ultrastructure of trypanosomatids under study (Wallace et al. 1983), it is clear that reliance on cultures is becoming an obstacle for future development of trypanosomatid systematics. To some extent the availability of infected insects can substitute cultures for ultrastructure analysis in species description (Frolov and Malysheva 1993; Frolov and Scarlato 1989), but these are not always available. Our data show that a significant segment of trypanosomatid diversity is represented by organisms that are refractory to cultivation with the methods and media currently utilized (Maslov et al. 2007; Westenberger et al. 2004; Yurchenko et al. 2009). The situation is thus similar to prokaryotic microorganisms, many or even most of them uncultivable or yet uncultured (Fraser et al. 2009; Handelsman 2004). Fortunately, just as bacterial microbiologists are circumventing this obstacle by the further development of metagenomics approaches, biodiversity studies of trypanosomatids have reached the level when sufficient information can be extracted directly from insect gut samples (Adams et al. 2008; Ferreira et al. 2008; Hamilton et al. 2008). Moreover, morphological data for insect trypanosomatids are often misleading or uninformative, especially at the genus level, as shown by numerous cases of discrepancy between the morphological and phylogenetic affinities of the studied organisms (Hollar et al. 1998; Merzlyak et al. 2001; Yurchenko et al. 2008). With morphology playing an auxiliary role, the basic taxonomic framework is now defined by molecular phylogenetic analyses. The big unresolved issue, however, is that of the trypanosomatid species concept, which is not clearly defined and which cannot be solved by declaring a certain sequence similarity cut-off value to delineate species boundaries. Instead, it seems that only a combination of genomic data with ecological, physiological, and morphological information should be used to identify peaks on the adaptive landscape that can be called separate species. The question of how much weight to assign to the different criteria should be decided based on the biological context of each particular case. For example, the presence of a single gene that allows *Trypanosoma brucei rhodesiense* to infect humans is sufficient for considering this organism as a separate taxon (species or subspecies) within the *T. brucei* group despite the absence of a large overall genetic divergence (Gibson 2007).

With respect to the trypanosomatid isolates described herein, the phylogenetic analyses clearly indicate that the respective organisms represent new taxa. One of them, *L. barvae* n. sp., along with *L. costaricensis* from the same locale (Yurchenko et al. 2006b), represents a close relative of the dixenous *Leishmania* parasites. It needs to be mentioned that *L. costaricensis* had been isolated from a predatory reduviid host species *Ricolla simillima*, which might have acquired this trypanosomatid from an infected prey of unknown identity, not excluding a sandfly. While there is a formal possibility of *L. costaricensis* with the related trypanosomatid G755 (from a sandfly) (Noyes et al. 1997) representing highly divergent dixenous parasites of sandflies and some vertebrates, this possibility is ruled out for *L. barvae* n. sp. The latter species has only been found and with high prevalence in a population of the phytophagous bug *C. oleosa* (Heteroptera: Miridae). This finding indicates that *L. barvae* n. sp. is a specific parasite of this host species. Whether or not it may also parasitize the plant on which its host feeds is unknown, but in any case this trypanosomatid certainly is not a divergent species of *Leishmania*, which are defined as dixenous parasites of sand flies. This finding further strengthens the theory that postulates the origin of *Leishmania* from monoxenous parasites of insects (Baker 1963; Lainson and Shaw 1987). The findings are also consistent with the proposed Neotropical origin of the genus *Leishmania* (Noyes 1998).

Fig. **2–18.** Light (2, 3, 10–12), transmission (4–9, 14–16), and scanning (17, 18) electron microscopy of *Leptomonas barvae* n. sp. (2–9) and *Blastocrithidia largi* n. sp. (10–18). **2.** Giemsa- and **3.** DAPI-stained cultured cells of *L. barvae* n. sp. with an oval nucleus and large intensely stained kinetoplast. **4.** Transmission electron microscopy of the anterior part of a promastigote, with a deep flagellar pocket and a thin kDNA disk. **5, 6.** Cross-sections of a flagellum in the flagellar pocket revealing the presence of a paraflagellar rod inside the pocket. **7, 8.** The free portion of the flagellum supported with an extremely developed paraflagellar rod. **9.** Longitudinal section through a dividing promastigote. **10, 11.** Giemsa-stained cells of *B. largi* n. sp. revealing straphanger cysts attached to the basal part of the epimastigote flagellum. A frequently occurring slender cell lacking straphangers (**10**; upper cell). **12.** A DAPI-stained cell. **13.** A cell with a shallow flagellar pocket. **14.** Kinetoplast with characteristic dense zones at its upper and lower faces. **15.** Cross-sectioned flagellum lacking the paraflagellar rod within the flagellar pocket. **16.** Cross-sectioned free flagellum with inconspicuous paraflagellar rod. **17.** Epimastigote cell with flagellum closely adjacent to cell body. **18**. A cell with short anterior end and partially detached flagellum reminiscent of promastigotes. Scale bar = 10 ím (2, 3, 10, 11), 5 ím (12), 2 ím (13), 1 ím (4, 9**,** 17**,** 18), 500 nm (14), and 200 nm (5–8, 15, 16).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Crithidia luciliae
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Leptomonas neopamerae
Leptomonas seymouri
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Wallaceina inconstans
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Leishmania major
Leishmania infantum
Leishmania tarentolae
Leishmania braziliensis
Leptomonas costaricensis
Leptomonas barvae sp. n.
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Trypanosoma brucei
Trypanosoma cruzi
Crithidia oncopelti
Blastocrithidia culicis
Herpetomonas roitmani
Leptomonas collosoma
Phytomonas serpens
Phytomonas sp. EM1
Blastocrithidia largi sp. n.
Herpetomonas pessoai
Leptomonas cf. lactosovorans
Herpetomonas muscarum
Bodo saltans
Parabodo caudatus
SE
74/87/89
0.05

Three previously described Neotropical *Leptomonas* species isolated from mirid hosts, *Leptomonas bifurcata*, *Leptomonas tarcoles*, and *Leptomonas acus* (Yurchenko et al. 2008), are phylogenetically separated from *L. barvae* n. sp. It is worth mentioning here that *L. acus* cells are also equipped with a prominent paraflagellar rod (Yurchenko et al. 2008), indicating characteristic expansion of this classical character of the kinetoplastid cell in this lineage. Two additional trypanosomatid species described from Miridae are *Blastocrithidia miridarum* (Podlipaev and Frolov 1987) and *Leptomonas mycophilus* (Frolov and Skarlato 1991) isolated in North-Western Russia. The phylogenetic position of the first of these species is unknown due to a replacement of the organism originally described by morphology with a faster growing species (A. Kostygov, pers. commun.) that is represented by the published sequences of ''*B. miridarum*'' (Merzlyak et al. 2001). The original *B. miridarum* possessed endomastigotes and flagellar cysts, clearly distinguishing it from *L. barvae* n. sp. The distinction between *L. mycophilus* and *L. barvae* n. sp., besides a large geographic distance and different host species, is less clear, and no molecular data are available for *L. mycophilus*. Both species appeared to require fungus for growth in culture, however, the taxonomic significance of this feature is not clear. Culture promastigotes of *L. mycophilus* are significantly shorter compared with those of *L. barvae* n. sp. Formation of ''rosettes'' and differentiation of motile cells into cyst-like immotile cells described for *L. mycophilus* (Frolov and Skarlato 1990; Frolov et al. 1991) have not been observed in *L. barvae* n. sp. Unfortunately, the culture of *L. mycophilus* or suitable material for DNA extraction is not available (A. Kostygov, pers. commun.), currently precluding a morphological or DNA-based comparison of the two species. Nevertheless, on the basis of these distinct differences we established the new species, *L. barvae* n. sp.

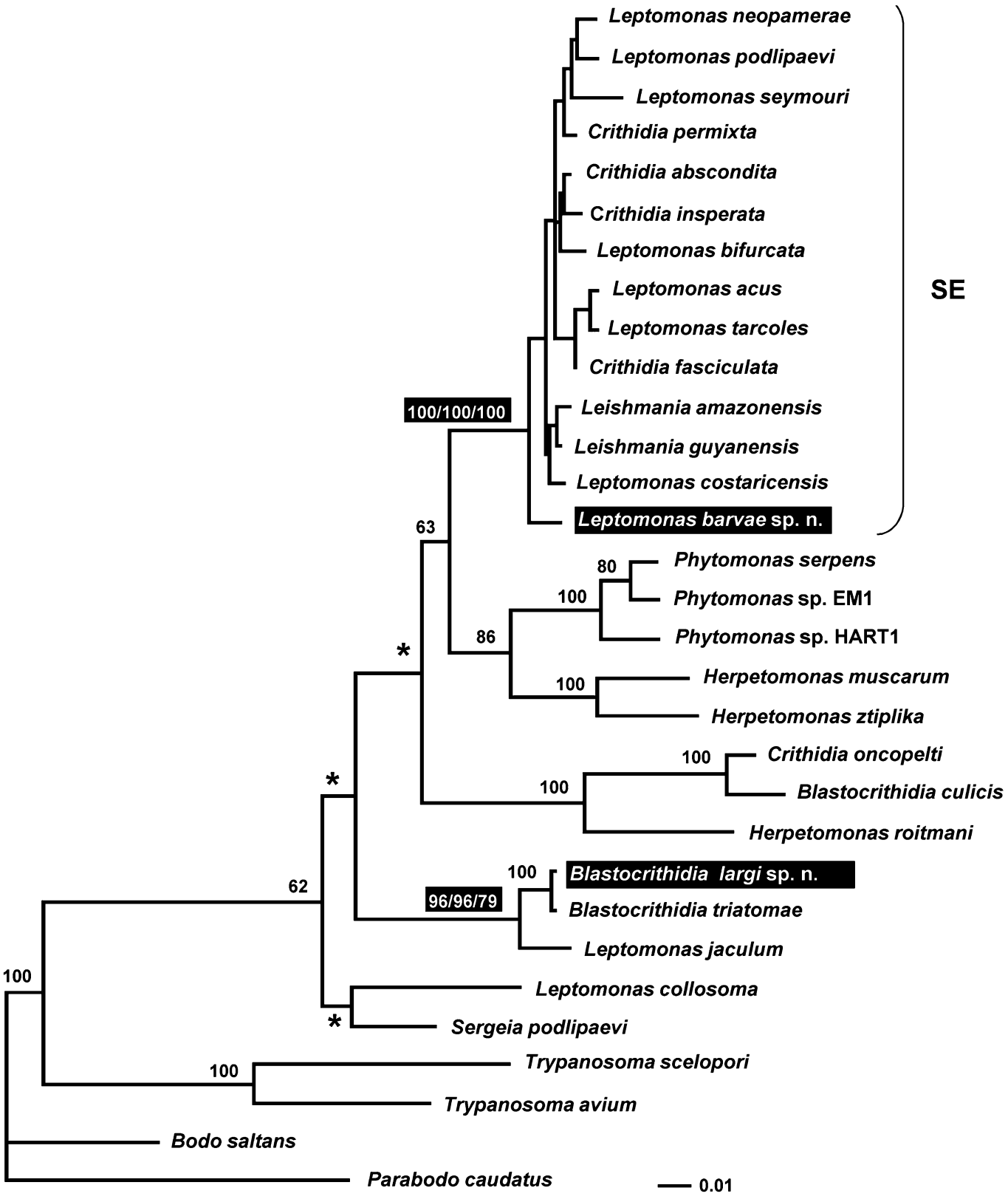
As the phylogenies show, the second species described herein, *B. largi* n. sp., is related to *B. triatomae*. The separate status of the new species is supported by a high level of SL RNA repeat divergence that exceeds the interspecies divergence levels observed in some other well established species (e.g. the species of *Leishmania*), although it falls within the range of the intraspecific variability of *Trypanosoma cruzi* (Thomas et al. 2005; Tibayrenc 1998). However, the differences in vectors and geographic origin also separate *B. largi* n. sp. and *B. triatomae*.

The morphological data are fully consistent with the close association of *B. largi* n. sp. and *B. triatomae*. First, epimastigote body shape is the predominant morphotype of *B. largi* n. sp. cells in culture. Second, the flagellar cyst-like cells (straphangers) and free cysts are observed in this species, as they are in *B. triatomae*, *B. leptocoridis*, and several other species of *Blastocrithidia* (Cerisola et al. 1971; Schaub et al. 1990; Wallace 1966). These cysts represent the products of unequal cell division characterized by condensed cell content, the lack of a flagellum, and formation of a dense cortical complex under the cell membrane (Peng and Wallace 1982; Reduth and Schaub 1988; Tieszen, Molyneux, and Abdel-Hafez 1985). In *B. triatomae*, such cysts were reported to be resistant to adverse environmental conditions (Schaub et al. 1990) and serve for more efficient dissemination of the parasite. Several *Leptomonas* species with various modes of cyst formation have also been described, including *Leptomonas oncopelti*, *Leptomonas wallacei*, and *L. jaculum* (Frolov, Skarlato, and Shaglina 1991; Malysheva, Frolov, and Skarlato 2006; McGhee and Hanson 1962; Romeiro et al. 2000). These species do not form typical epimastigotes, which distinguishes these species and the blastocrithidias, including *B. largi* n. sp. So far, molecular characterization of the cyst-forming leptomonads has been performed only for *L. jaculum* (Kostygov and Frolov 2007). This species has a sister-group relationship with *B. largi* n. sp. and *B. triatomae*. It will be interesting to verify if other cyst-forming *Blastocrithidia* and *Leptomonas* are members of the same monophyletic clade, which might then represent a new natural taxon.

Nearly 40 named species of *Blastocrithidia* have been described over the last century (Podlipaev 1990; Wallace 1966) with a few findings in Largidae. Validity of most of these species and their relationship to *B. largi* n. sp. or *B. triatomae* are uncertain. Besides those mentioned above, only *Blastocrithidia culicis* has been investigated by molecular methods. This organism is only distantly related to *B. largi* n. sp. and belongs to a separate clade that includes endosymbiont-bearing trypanosomatids, testifying to the polyphyly of the genus (Hollar at al. 1998; Merzlyak et al. 2001). Cysts are not observed in *B. culicis*. In conclusion, on the basis of SL RNA repeat divergence, host and geographic origin, we therefore propose the new species *B. largi* n. sp.

Phylogeny of trypanosomatids. The phylogenetic position of the *B. largi* n. sp. and *B. triatomae* lineage is uncertain, although this question is potentially important for understanding evolution of the Trypanosomatidae. Based on morphology, a hypothesis had been put forward regarding monoxenous blastocrithidias as precursors of the advanced dixenous parasites, such as trypanosomes (Baker 1973; Hoare 1972). Indeed, the presence of cysts may be viewed as a plesiomorphy because it is also observed in bodonids, such as *P. caudatus* (Brooker and Ogden 1972) and *Dimastigella trypaniformis* (Breunig et al. 1993), which are regarded to be close to the bodonid progenitor of trypanosomatids (Simpson et al. 2000; Vickerman 1994). Moreover, the epimastigote body shape

Fig. **19.** A maximum likelihood tree ($-Ln$-likelihood = $-11{,}274.9783$) derived from glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene sequences of selected Trypanosomatidae lineages and showing the position of the two new species, *Leptomonas barvae* n. sp. and *Blastocrithidia largi* n. sp. The alignment and other tree parameters are described in ''Materials and Methods''. The tree was rooted using bodonids the *Parabodo caudatus* and *Bodo saltans*. The designation ''SE'' indicates a major trypanosomatid clade that includes mainly monoxenous parasites of insects and also includes the clade of dixenous *Leishmania*. Triple bootstrap values shown for the group of *Leishmania* with the closest relatives represent maximum likelihood (the first value), followed by minimum evolution and parsimony, respectively. Other bootstrap values shown represent only maximum likelihood. Bootstrap values for terminal and most subterminal clades are not shown. Asterisk indicates that the respective clade was not recovered in the bootstrap majority consensus tree. The scale bar corresponds to 0.05 substitutions per site. GenBank™ accession numbers of the retrieved gGAPDH sequences are: *Blastocrithidia culicis* (EU079136), ''*Blastocrithidia miridarum*'' (EU084896), *Bodo saltans* (EU084900), *Crithidia abscondita* (EU076606), *Crithidia fasciculata* (AF047493), *Crithidia insperata* (EU076605), *Crithidia luciliae* (AF053740), *Crithidia oncopelti* (EU079134), *Crithidia permixta* (EU076607), *Herpetomonas muscarum* (DQ092548), *Herpetomonas pessoai* (AF047494), *Herpetomonas roitmani* (EU079130), *Leishmania major* (AF047497), *Leishmania tarentolae* (DQ092549), *Leptomonas acus* (DQ910926), *Leptomonas bifurcata* (DQ910928), *Leptomonas* cf. *lactosovorans* (EU076602), *Leptomonas collosoma* (EU084898), *Leptomonas costaricensis* (DQ383650), *Leptomonas jaderae* (EU076603), *Leptomonas neopamerae* (DQ910927), *Leptomonas podlipaevi* (DQ019000), *Leptomonas pyrrhocoris* (AY029072), *Leptomonas seymouri* (AF047495), ''*Leptomonas*'' sp. Cfm (AF320820), ''*Leptomonas*'' sp. P (EF546793), *Leptomonas tarcoles* (EF546787), *Phytomonas serpens* (EU084892), *Phytomonas* sp. EM1 (EU084895),*Trypanosoma brucei brucei* (X59955), *Trypanosoma cruzi* (X52898), *Wallaceina brevicula* (AF316620), and *Wallaceina inconstans* (EU076608). The gGAPDH sequences of *Leishmania braziliensis* and *Leishmania infantum* were retrieved from the respective genome databases (http://www.genedb.org/genedb). Names of taxa given in quotation marks indicate that sequences obtained from cultures are likely to misrepresent the species originally described under the respective names as discussed previously (Yurchenko et al. 2009).


Leptomonas neopamerae
Leptomonas podlipaevi
Leptomonas seymouri
Crithidia permixta
Crithidia abscondita
Crithidia insperata
Leptomonas bifurcata
Leptomonas acus
Leptomonas tarcoles
Crithidia fasciculata
Leishmania amazonensis
Leishmania guyanensis
Leptomonas costaricensis
Leptomonas barvae sp. n.
SE
100/100/100
63
80
100
86
*
Phytomonas serpens
Phytomonas sp. EM1
Phytomonas sp. HART1
100
Herpetomonas muscarum
Herpetomonas ztiplika
100
Crithidia oncopelti
*
100
Blastocrithidia culicis
Herpetomonas roitmani
100
Blastocrithidia largi sp. n.
62
96/96/79
Blastocrithidia triatomae
Leptomonas jaculum
100
Leptomonas collosoma
*
Sergeia podlipaevi
100
Trypanosoma scelopori
Trypanosoma avium
Bodo saltans
Parabodo caudatus
0.01

with a relatively short undulating membrane may be regarded as an intermediate step toward a fully developed trypomastigote morphotype. The correct phylogenetic placement of these organisms in the family tree can support or negate this scenario, proving important for our understanding of the origin and evolution of the Trypanosomatidae. The work that still needs to be done includes the following. First, it will be necessary to find additional members of this clade to minimize a potential long branch attraction artifact (Philippe et al. 2005), to which this relatively fast-evolving lineage may be prone. Second, combined gene datasets need to be developed, which might result in a robust tree topology derived from a larger amount of data (Delsuc, Brinkmann, and Philippe 2005; Driskell et al. 2004).

Molecular foundations of trypanosomatid phylogeny. The question of which gene(s) should be used for the development of a taxonomic framework for the Trypanosomatidae deserves separate attention. In addition to the SL RNA-based genotyping, which mainly serves for identification and barcoding of new trypanosomatid isolates, phylogenetic analyses of the family were mainly based on gGAPDH and SSU rRNA genes (Hamilton et al. 2004; Hollar et al. 1998; Merzlyak et al. 2001; Yurchenko et al. 2006a, 2008). Although useful to determine the relationship of a new species with the previously established major clades (Hollar et al. 1998; Merzlyak et al. 2001), neither gene is suitable for inferring a robust phylogeny of the entire family, which still remains an unsolved problem. The gGAPDH sequences can be relatively well aligned across the family with a minimal number of gaps, and were instrumental in verifying the monophyly of trypanosomes (Hamilton et al. 2004). However, this dataset may contain some bias as demonstrated by the position of the *Herpetomonas* clade in the gGAPDH trees, which is incongruent with analyses based on SSU rRNA genes (Hamilton et al. 2004; Yurchenko et al. 2006a). Moreover, for unknown reasons, gGAPDH sequences are difficult to amplify from members of the *Blastocrithidia* group, including *B. triatomae* (D.A.M. and V.Y.Y., unpubl. observ.). With respect to SSU rRNA genes, the main problem has been related to several large fast evolving regions in these genes, which, if not removed, may lead to tree reconstruction artifacts (Philippe 1998; Philippe and Adoutte 1998), and if removed, render alignments with little phylogenetic signal. The need to analyze additional phylogenetic markers for Trypanosomatidae, such as DNA and RNA polymerase genes (Croan et al. 1997), has become imperative.

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

***Leptomonas barvae* n. sp. Maslov and Lukeš**

Generic assignment. The only morphotype of the organism in culture is promastigote. Based on the existing taxonomic system (Hoare and Wallace 1966; Wallace 1966), the new species is assigned to the genus *Leptomonas* Kent, 1880. This is done with the realization that the genus is polyphyletic and, probably, even invalid, and therefore, should be revised in the future.

Species diagnosis. The cells are typical promastigotes, often twisted, with a pointed posterior end and a flagellum size close to that of a body. The distinguishing morphological features represent the thin (125.5 ± 39.4 nm) and wide (812.0 ± 350.0 nm) kDNA disk and a noticeable paraflagellar rod extending deep within the flagellar pocket. The species is mainly defined by phylogenetically using SL RNA repeat, SSU rRNA and gGAPDH gene sequences, which show that this organism represents a well-separated phylogenetic lineage. Sequence divergence between this species and its closest known relatives (*L. costaricensis* and the genus of *Leishmania*) is greater than divergence that is usually observed between closely related species of trypanosomatids.

Type host. Intestine of *C. oleosa* Distant (Hemiptera, Miridae). The xenotype (post-dissection remains of the original host of isolate 21 EC) is deposited in the UCR Entomology Museum (UCRC ENT 113060).

Type locality. In the vicinity of El Ceibo on the slope of volcano Barva at the altitude 500 m (10°20′N, 84°05′W) near the western boundary of the Braulio Carrillo National Park, province Heredia, Costa Rica.

Type material. A culture of isolate 21 EC deposited in the American Type Culture Collection (ATCC PRA-303) is the hapantotype. The fungal component of the culture is excluded from the type.

Etymology. The chosen species name reflects the collection area, so far the only place from which this organism is known to exist.

Gene sequence. The new species is established on the basis of the sequences of SL RNA gene repeats (GenBank™ accession numbers DQ864310, DQ864311, DQ864312, DQ864313), SSU rRNA gene (FJ968532), and gGAPDH gene (FJ968529).

***Blastocrithidia largi* n. sp. Maslov and Lukeš**

Generic assignment. The morphotypes of the organism in culture are predominantly epimastigotes with pointed anterior and posterior ends, lateral opening of the flagellar pocket, and flagellum closely paralleling the cell body and extending beyond the anterior end. However, an undulating membrane is either missing or inconspicuous. Oval amastigotes (cysts) are also observed. By the current nomenclature (Hoare et al. 1966; Wallace 1966) the presence of epimastigotes and amastigote cysts corresponds to the genus *Blastocrithidia* Laird, 1959. However, because the

Fig. **20.** A maximum likelihood tree ($-Ln$-likelihood = $-8{,}254.31979$) derived from small subunit ribosomal RNA (SSU rRNA) gene sequences of the Trypanosomatidae and showing the position of the two new species, *Leptomonas barvae* n. sp. and *Blastocrithidia largi* n. sp. Most of the bootstrap values shown are derived by maximum likelihood (100 replicates). When three values are shown, the first value corresponds to maximum likelihood, the second value to minimal evolution (GTR distances, 1,000 replicates), and the third value to parsimony (1,000 replicates). Asterisks denote the clades with bootstrap support below 50%. Bootstrap values within SE clade are not shown. The tree is rooted using bodonids *Bodo saltans* and *Parabodo caudatus*. Scale bar represents 0.01 substitutions per site. GenBank™ accession numbers of the retrieved SSU rRNA sequences are: *Blastocrithidia culicis* (U05679), *Blastocrithidia triatomae* (AF153037), *Bodo caudatus* (X53910), *Crithidia abscondita* (EU079126), *Crithidia fasciculata* (Y00055), *Crithidia insperata* (EU079125), *Crithidia oncopelti* (AF038025), *Crithidia permixta* (EU079127), *Herpetomonas muscarum* (L18872), *Herpetomonas roitmani* (AF038023), *Herpetomonas ztiplika* (AF416560), *Leishmania guyanensis* (X53913), *Leishmania m. amazonensis* (X53912), *Leptomonas acus* (DQ910923), *Leptomonas bifurcata* (DQ910925), *Leptomonas collosoma* (AF153038), *Leptomonas costaricensis* (DQ383648), *Leptomonas jaculum* (EF184218), *Leptomonas neopamerae* (DQ910924), *Leptomonas podlipaevi* (DQ383649), *Leptomonas seymouri* (AF153040), *Leptomonas tarcoles* (EF546786), *Phytomonas serpens* (AF016323), *Phytomonas* sp. EM1 (AF016322), *Phytomonas* sp. HART1 (L35076), *Sergeia podlipaevi* (DQ394362), *Trypanosoma avium* (U39578), T*rypanosoma scelopori* (U67182).

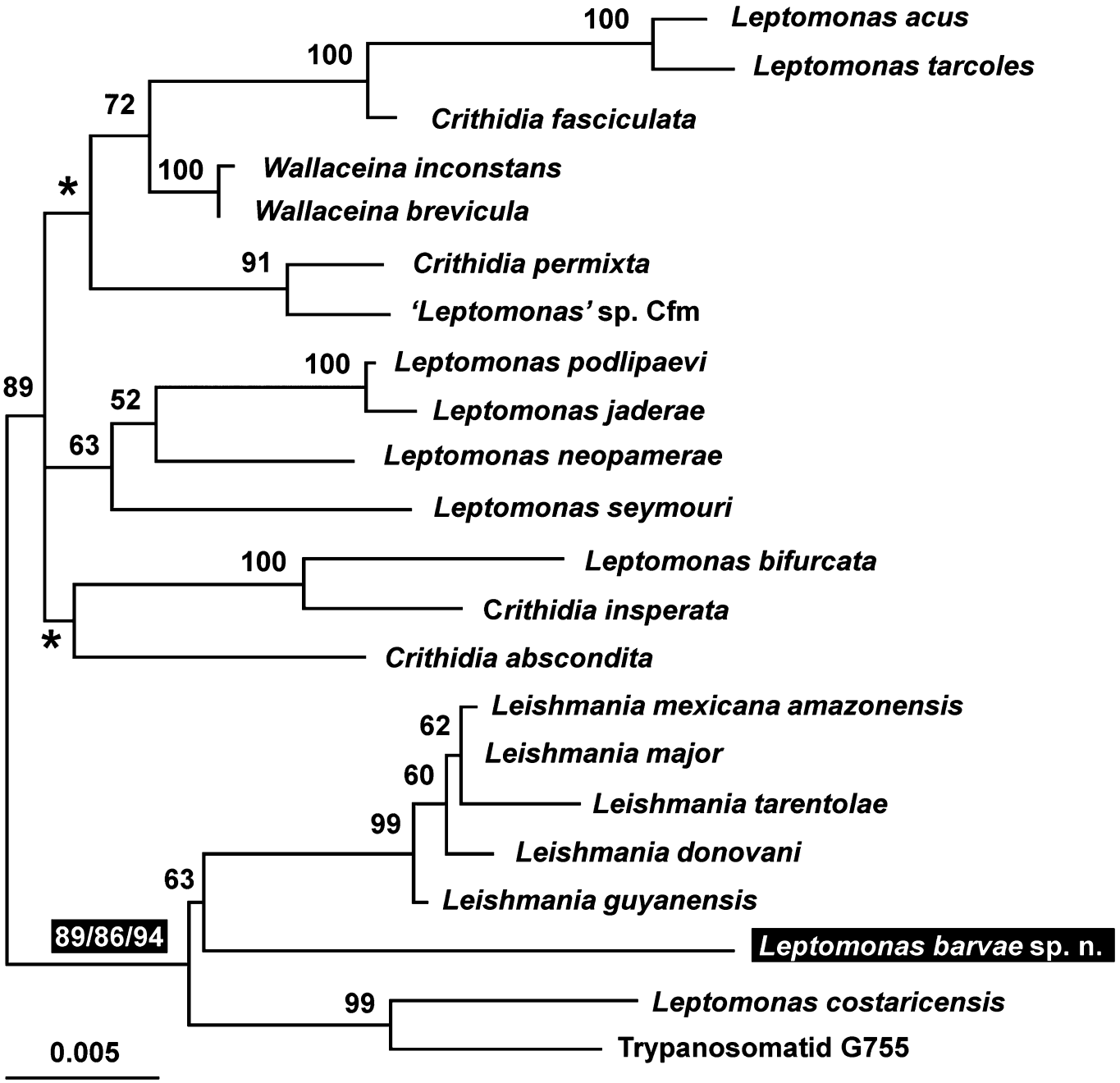


Fig. **21.** An unrooted maximum likelihood tree ($-$ *Ln*-likelihood = $-$4,811.65099.) derived from small subunit ribosomal RNA (SSU rRNA) gene sequences of the SE clade of trypanosomatids and showing the position of the two new species, *Leptomonas barvae* n. sp. and *Blastocrithidia largi* n. sp. Most of the bootstrap values shown are derived by maximum likelihood (100 replicates). When three values are shown, the first value corresponds to maximum likelihood, the second value to minimal evolution (GTR distances, 1,000 replicates), and the third value to parsimony (1,000 replicates). Scale bar represents 0.005 substitutions per site. GenBank^{TM} accession numbers of the retrieved SSU rRNA sequences are listed in the legend to Fig. 20. Additional sequences are: *Leishmania donovani* (X07773), *Leishmania major* (X53915), *Leishmania tarentolae* (M84225), *Leptomonas jaderae* (EU079123), ''*Leptomonas*'' sp. Cfm (AF153041), Trypanosomatid G755 (U59491), *Wallaceina brevicula* (AF153045), and *Wallaceina inconstans* (AF153044).

phylogenetic position of the type species for this genus, *Blastocrithidia gerridis*, is unknown, and the genus has proven to be polyphyletic, this generic assignment should be regarded as provisional.

Species diagnosis. Cells are epimastigotes, often twisted with a relatively long flagellum. The elongated and flexible anterior end closely adheres to the flagellum but no undulating membrane is noticeable. About 1% of epimastigotes carry oval straphangers attached to the basal portion of their flagellum. The thick (312.9 $\pm$ 77.7 nm) and relatively narrow (744.0 $\pm$ 155.9 nm) kDNA disk is unusually dense at its upper and lower face. The species is mainly defined phylogenetically. As follows from the SSU rRNA gene analysis, the most closely related species is *B. triatomae*. However, *B. largi* n. sp. differs from *B. triatomae* by

the sequences of SL RNA gene repeats (69.6% similarity). This level is below the usual $\sim 90\%$ level of intraspecies variability, and that, along with the host and geographic origin, separates the new species from *B. triatoma*.

Type host. Intestine of *L. cinctus* Herrich-Schaeffer (Hemiptera, Largidae). The xenotype (post-dissection remains of the original host) is deposited in the UCR Entomology Museum (UCRC ENT 138276).

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

Type material. A culture of the isolate 67 VL deposited in the American Type Culture Collection (ATCC PRA-304) is the hapantotype.

Etymology. The species name is given after the genus name (*Largus*) of the host species.

Gene sequence. The sequences used to define the species were: SL RNA gene repeat (GenBank™ accession number FJ968530), SSU rRNA gene (FJ968531), and gGAPDH gene (FJ968528).

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