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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)

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ABSTRACT. *Leptomonas podlipaevi* n. sp., a new trypanosomatid species, is described herein based on light microscopic, ultrastructural, and molecular phylogenetic data. The organism is pleomorphic both in host and culture, with two predominant forms—a typical promastigote with a long flagellum and a shorter promastigote with a small or barely extending flagellum. Several spliced leader RNA repeat sequences obtained from the original cultures and the clonal lines representing two types of cells were all nearly identical. These sequences formed a tight cluster in the neighbor-joining tree well separated from other trypanosomatid species. Glyceraldehyde phosphate dehydrogenase gene sequences were determined for *L. podlipaevi* and 10 previously described trypanosomatid species. Molecular phylogenetic analysis has demonstrated that the new species is most closely related to *Leptomonas seymouri* and *Leptomonas pyrrhocoris*. The analysis has also highlighted the polyphyly of the genus *Leptomonas*.

Key Words. GAPDH, glyceraldehyde phosphate dehydrogenase, Kinetoplastida, phylogeny, species description, spliced leader RNA, ultrastructure.

TRYPANOSOMATID protozoa (class Kinetoplastea) include 900 named species and nine genera (Podlipaev 1990; Vickerman 1976). The current taxonomic system of the family was developed in the 1960s (Hoare and Wallace 1966; Wallace 1966). Members of the genus *Trypanosoma* are parasites of vertebrates wherein they are transmitted by blood-sucking arthropods and leeches. The rest of the family includes monoxenous parasites of insects (i.e. *Blastocrithidia*, *Crithidia*, *Leptomonas*, *Herpetomonas*, and *Wallaceina*), and dixenous insect-transmitted parasites of mammals and reptiles (i.e. *Leishmania* and *Endotrypanum*), as well as plants (i.e. *Phytomonas*). In addition, each genus is defined by a set of cell morphotypes, which are distinguished by relative position of the kinetoplast-mitochondrion and the nucleus. Currently, the prevailing view is that this system is not adequate. As all genera are found in insects, in which many of these parasites acquire a promastigote morphology, a correct genus identification is often difficult. Most importantly, the current system does not take into consideration genetic and evolutionary relationships of the organisms and thus is expected to produce artificial assemblages even if followed rigorously.

At the species level, host occurrence and any observable morphological differences were the major taxonomic criteria, although it is clear that their taxonomic significance is uncertain at best. Host specificity is not known in most cases. Morphology can be either misleading due to a considerable degree of pleomorphism or uninformative by not showing sufficient variation. Thus, the validity of most described trypanosomatid species is questionable, with the notable exception of the medically important trypanosomes and leishmanias that have been studied extensively.

In an attempt to overcome these limitations, a group of experts in the field of trypanosomatid taxonomy proposed to use a variety of biochemical, ultrastructural, serological, and nutritional approaches to discriminate taxa (Newton 1976; Wallace et al. 1983). Since then, most new species descriptions and comparative analyses usually involve several methods, such as multilocus enzyme electrophoresis (MLEE), lectin agglutination, analysis of proteolytic profiles, and composition of acidocalcisomes often combined with DNA-based analyses, such as RFLP and RAPD (Bañuls et al. 2000; Barnabé, Brisse, and Tibayrenc 2000; Bulat, Mokrousov, and Podlipaev 1999; Miranda et al. 2004; Müller et al. 1997; Podlipaev 1985; Podlipaev, Frolov, and Kolesnikov 1990; Santos et al. 2005; vila-Levy et al. 2004). However useful in individual cases, these new criteria have not been applied uniformly. Moreover, their taxonomic interpretation can be subjective. Finally, the problem of validating species has not been alleviated by application of these methods, because this analysis would require a direct side-by-side comparison of an organism in question against the reference panel, and that is not always possible. No comprehensive taxonomic system has emerged from these methods in spite of the 20-year history of their application.

A resolution of this problem resides in molecular phylogenetic analysis becoming an indispensable part of a new taxon characterization (Grisard, Sturm, and Campbell 2003; Podlipaev et al. 2004a). By revealing hierarchical relationships of the organisms in question, phylogenies have direct taxonomic implications. In Trypanosomatidae, a large dataset of small subunit (SSU) rRNA genes and smaller datasets for heat shock protein 90 (hsp90) and glycosomal glyceraldehyde phosphate dehydrogenase (GAPDH) have been extensively analyzed for phylogenetic and taxonomic purposes (Fothergill-Gilmore and Michels 1993; Gadelha et al. 2005; Hannaert et al. 2003b; Hamilton et al. 2004; Hamilton et al. 2005; Hollar, Lukeš, and Maslov 1998; Hollar and Maslov 1997; Lukeš et al. 1997; Merzlyak et al. 2001; Simpson, Lukeš, and Roger 2002; Simpson and Roger 2004; Stevens et al. 1999). These analyses have demonstrated that the existing classification at the genus level is artificial. Several well-supported monophyletic clades have been identified that would represent natural genera after a future taxonomic revision. At the species level, however, the SSU rRNA or protein-coding genes do not provide a sufficient resolution for closely related species due to a relatively slow rate of sequence divergence, making them suitable only for studying evolution over large time scales. In their place, spliced leader RNA (SL RNA) gene repeats, which contain a fast-evolving intergenic region, have been used to distinguish closely related species and natural clones (Dollet, Sturm, and Campbell 2001; Podlipaev et al. 2004b; Westenberger et al. 2004). Other potentially useful molecular markers for species- and subspecies-level analyses include rRNA gene spacers (Da Silva et al. 2004; Schönian et al. 2001) and microsatellite loci (Jamjoom et al. 2002). In addition, molecular sequences serve as species

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vouchers, especially when type cultures or specimens are not available. Comparisons of species described by different laboratories or over long time periods thus become possible.

In this work, we have combined the morphological and molecular approaches to a new species description. Our goal is to demonstrate this as a basis for developing a uniform taxonomic procedure. We used two types of molecular markers, a fast evolving SL RNA gene repeat and a slowly evolving GAPDH gene. Towards this end, the GAPDH dataset was significantly expanded to include representatives of the major groups of trypanosomatids. Some of the GAPDH sequences obtained in this work were placed in the public domain earlier and have been employed in several works (Hannaert et al. 2003a; Hamilton et al. 2004, 2005).

MATERIALS AND METHODS

Isolation and cultivation of trypanosomatid cells. Western box elder bugs, *Boisea rubrolineata* Barber, were captured on the ground and building walls within the perimeter of the University of California–Riverside campus during the period between September 2002 and January 2003. Out of 11 insects examined (three nymphs, four males, four females), numerous parasites were found in all but one insect from each category (eight infection cases total). Insects were dissected and their gut contents were examined for the presence of flagellates as described by Westenberger et al. (2004). Primary cultures of the parasites were established in the semi-defined medium SDM-79 (Brun and Schonenberger 1979) supplemented with fetal bovine serum (10%), hemin (10 μg/ml), ampicillin (100 μg/ml), and tetracycline (50 μg/ml) in stationary 1.5-ml Eppendorf tubes at 26 °C for two days. Thereafter, cells were transferred into a new tube with the same medium where no bacteria could be detected two days later. Cells were maintained in 5–10 ml of the brain heart infusion (BHI) medium containing hemin and antibiotics in stationary T25 flasks. Antibiotics were omitted from the medium after several passages. Cells were cloned by plating 200 μl of diluted (10^{-5} to 10^{-6}) cultures onto BHI-agar medium supplemented with hemin (10 μg/ml) and folic acid (80 μg/ml) (Cruz, Coburn, and Beverley 1991; Kar 1997).

Light and electron microscopy. Cells were resuspended in phosphate-buffered saline (PBS), and the suspension was spotted onto poly-L-lysine-coated glass microscope slides. After allowing the cells to adhere for 20 min, the slides were fixed for 5 min in methanol and then either stained with Giemsa stain for 5 min or with 0.1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole; Sigma, St. Louis, MO) in PBS for 3 min. The slides were examined with an Olympus IX70 microscope, and the measurements of Giemsa-stained cells were made with an image analyzer Camedia 5060 equipped with Quick PhotoPro 2.0.

For transmission electron microscopy, cells were washed in 0.1 M PBS and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate (SC) buffer, pH 7.4, for 1 h at 4 °C, then washed in 0.1 M PBS and post-fixed with 2% (w/v) osmium tetroxide in the SC buffer for 1 h at room temperature. After dehydration in ethanol, the cells were embedded in Epon-Araldite. Thin sections, stained with lead citrate and uranyl acetate, were examined in a JEOL JEM 1010 microscope.

For scanning electron microscopy, cells fixed in 2.5% (v/v) glutaraldehyde in the SC buffer overnight at 4 °C were spotted onto poly-L-lysine-coated glass cover slips and post-fixed in 2% (w/v) OsO_4 in the SC buffer for 1 h at room temperature, and finally washed 3 × 15 min in the same buffer. After dehydration in a graded series of acetone, cells were critical point-dried using CO_2, coated with gold palladium in a Polaron PS sputter coater, and examined using a JEOL SEM 6300 microscope.

DNA isolation, PCR amplification, and sequencing. Total cell DNA was isolated after SDS-pronase lysis of cells followed by phenol–chloroform extraction (twice) and ethanol precipitation essentially as described by Maslov et al. (1996). PCR amplification of GAPDH genes was done using Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, Indiana) with the conserved sequence primers: GAPDH_dir, 5′-agaggatccATGGCTCCG(A/C)TCAAGGTTGGC-3′, and GAPDH_rev, 5′-agaggatccTTACATCTTCGAGCTCGCG(C/G)(C/G)GTC-3′, wherein the lower case letters indicate non-genomic nucleotides. The thermal cycling profile consisted of the following steps: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min 30 s; final extension at 72 °C for 10 min. PCR amplification of miniexon gene repeats was performed as described by Westenberger et al. (2004). The amplified PCR products were purified using QIAquick gel extraction kit (QIAGEN, Valencia, CA), cloned into pGEM-T Easy (Promega, Madison, Wisconsin) or pT7Blue (EMD Bioscience, San Diego, CA) plasmid vectors and sequenced using an ABI Prism Big Dye Terminator Sequencing kit and a ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The GAPDH sequences determined in this work were deposited in GenBank™ under the following accession numbers: *Blastocrithidia gerricola* (AF322391), *Herpetomonas megaseliae* (DQ092547), *Herpetomonas muscarum* (DQ092548), *Leishmania tarentolae* (DQ092549), *Leptomonas peterhoffi* (AF322390), *Leptomonas podlipaevi* sp. n. (DQ019000, DQ019001), *Leptomonas pyrrhocoris* (AY029072), *Leptomonas* sp. Cfm (AF320820), *Leptomonas* sp. Nfm (AF339451), *Leptomonas* sp. F2 (AF375664), and *Wallaceina brevicula* (AF316620). In addition, the following SL RNA gene repeat sequences have been deposited: *L. podlipaevi* sp. n. (DQ140171–DQ140175); *L. pyrrhocoris* (DQ140169); *Leptomonas* sp. F2 (DQ140170).

Phylogenetic analysis. The following GAPDH sequences were retrieved from GenBank™: *Crithidia fasciculata* (AF047493, AF053739), *Crithidia luciliae* (AF053740), *Euglena gracilis* (L39772), *Herpetomonas pessoai* (AF047494), *Leishmania major* (AF047497), *Leishmania mexicana* (X65226), *Leptomonas lactosovorans* (AF053741), *Leptomonas seymouri* (AF047495), *Phytomonas* sp. (AF047496), *Trypanoplasma borreli* (X74535), *Trypanosoma brucei brucei* (X59955), *Trypanosoma cruzi* (X52898), *Trypanosoma rangeli* (AF053742), and *Trypanosoma vivax* (AF047500). Together with the new sequences, these sequences formed a representative dataset encompassing most of the known natural groups in the family.

After primer removal, the sequence representing both clones was unambiguously aligned with the remaining GAPDH sequences over the entire length using CLUSTALX, version 1.81 (Thompson et al. 1997). The alignment was 1,050 nt long. A general time-reversible substitution model with a mixed model for among-site rate variation (GTR+I+Γ) was chosen as the best fitting model of sequence evolution based on the hierarchical and AIC analyses of MODELTEST, version 3.06 (Posada and Crandall 1998). The estimated proportion of invariable sites was 0.2810, and the gamma-distribution shape parameter for variable sites was 0.8699. These parameters and the MODELTEST-derived nucleotide frequencies and substitution rate matrix were employed for the maximum likelihood and distance analyses using PAUP* 4.0 beta version (Swofford 1998). Optimality criteria for the distance analyses included minimum evolution and unweighted least squares. Parsimony analyses were performed using PAUP. Likelihood and distance searches involved a heuristic approach, while for parsimony a branch-and-bound search was employed. Bootstrap analyses involved heuristic searches with 100 replicates (maximum likelihood) or 1,000 replicates (other methods). Preliminary minimum evolution analyses were also performed using

MEGA, version 3.0 (Kumar, Tamura, and Nei 2004). The entire analysis was also repeated using an alignment with excluded third codon positions (750 nt long).

For the across-the-family cluster analysis of SL RNA genes, we employed the sequence set described previously by Westenberger et al. (2004). Most of the intergenic region (except for positions − 100 to − 1) was excluded as unalignable. Phenetic clustering of the sequences was routinely performed by neighbor-joining using CLUSTALX. Bootstrap with neighbor-joining was performed using PAUP.

RESULTS

Morphological characterization of the parasites and isolation of axenic cultures. Most parasites represented typical

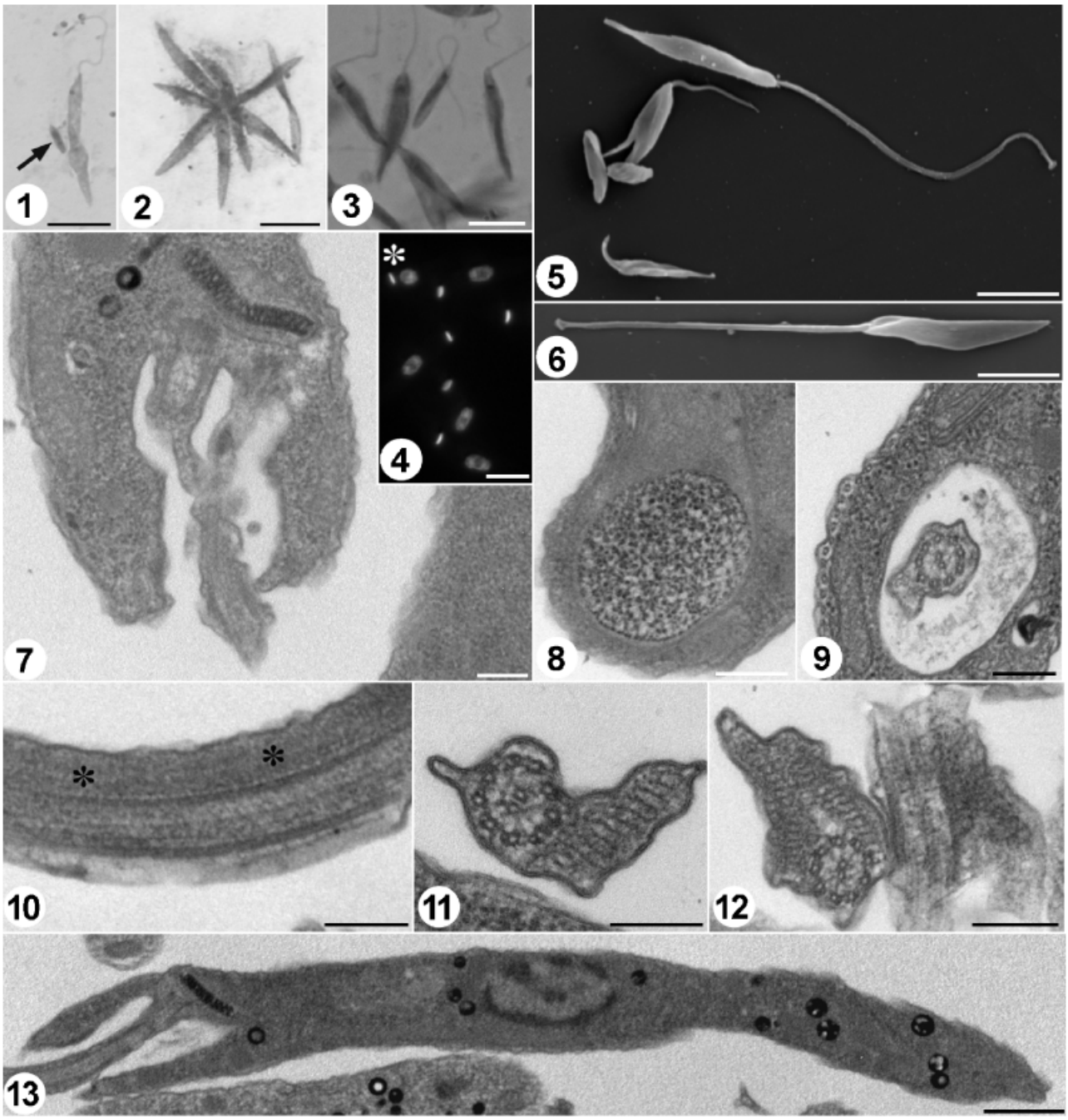

Fig. **1–13.** Light (1–4), scanning (5, 6), and transmission (7–13) electron microscopy of *Leptomonas podlipaevi* n. sp., the long morphotype. **1** and **2.** Free promastigote and a rosette composed of variably sized promastigotes in the smeared gut contents of Host 4. A single short-form cell is indicated by arrow. **3.** Same isolate after multiple rounds of cultivation in liquid media. **4.** DAPI-stained cultured promastigotes, a single short cell is indicated by asterisk. **5, 6.** Highly variable population composed of slender cells with long flagellum and clearly visible flagellar pocket, short promastigotes with inconspicuous flagellum and rarely visible rostrum, and a cell with a rod-shaped extension. **7.** Longitudinal section of the flagellar pocket and the kinetoplast disk undergoing replication characterized by its width and the presence of two flagellar basal bodies. **8.** Transverse section parallel to the face of the kinetoplast disk. **9.** Transverse section of the flagellum inside the flagellar pocket. Note a small paraflagellar rod. Corset of regularly spaced, cross-sectioned, subpellicular microtubules is visible on the periphery of the cell. **10.** Longitudinal view of the paraflagellar rod (marked with asterisks). **11, 12.** Transverse sections of the free flagellum with the adjacent conspicuous flagellar rod with its parallel filaments. **13.** Typical elongated promastigote containing multiple dense acidocalcisomes and relatively deep flagellar pocket. Bar = 5 μm (Fig. 1–6), 250 nm (Fig. 7–12) and 1 μm (Fig. 13).

promastigotes with an elongated and twisted body, a pointed posterior end, and the kinetoplast positioned close to the anterior end (Fig. 1, 14). Several rosette-like aggregates were also seen (Fig. 2). In addition to promastigotes, a few cells with a smaller size and a very short or apparently missing flagellum were observed (Fig. 1, 14, indicated by arrows). In some cases, parasites attached to the gut epithelium were seen (data not shown).

Detailed measurements were performed for cells observed in Hosts 4 and 5. The body length of cell varied between 7.9 and 23.9 μm (mean ± SD: 15.9 ± 2.6 μm) and between 7.9 and 21.9 μm (16.0 ± 2.7 μm), respectively (n = 50). For both strains the distance between the nucleus and the posterior end ranged from 1.5 to 10.8 μm (5.8 ± 1.4 μm) and from 2.3 to 12.2 μm (6.2 ± 1.7 μm), and the distance between the nucleus and the kinetoplast ranged from 1.0 to 12.0 μm (3.9 ± 1.6 μm) and from 1.1 to 7.7 μm (4.1 ± 1. 4 μm), respectively. Most conspicuous is the variation in the size of the flagellum. In Host 4, it varied between 1.0 and 22.9 μm (9.5 ± 5.3 μm), whereas in Host 5 the variability was even higher (from 0.7 to 28.7 μm; 6.3 ± 4.3 μm).

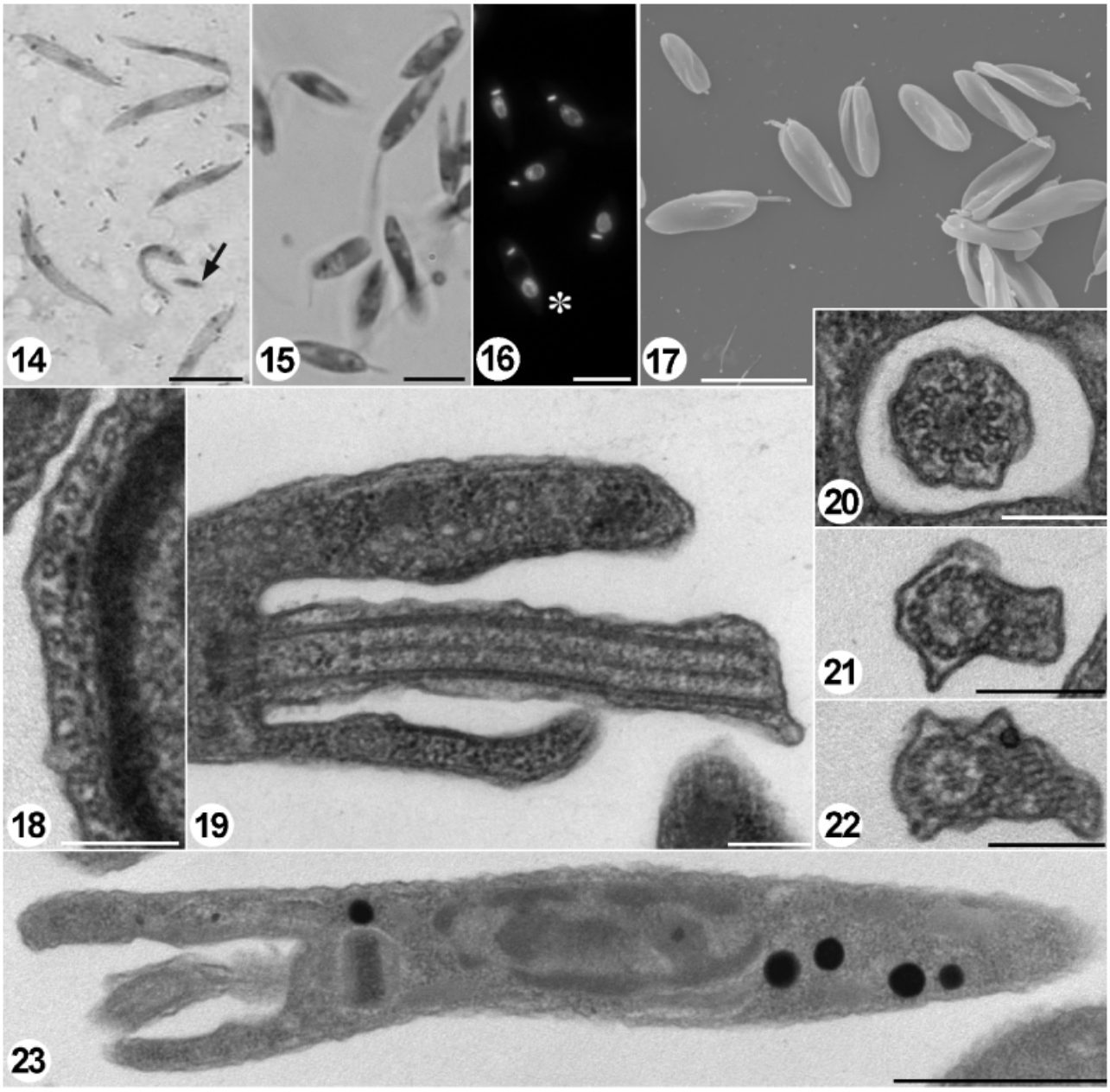

Fig. **14–23.** Light (14–16), scanning (17), and transmission (18–23) electron microscopy of *Leptomonas podlipaevi* n. sp., the short morphotype. **14.** Original cells in Host 5, most cells are long type, a single short-type cell is indicated by arrow. **15.** Shorter cells observed after transfer to liquid medium and multiple rounds of cultivation. **16.** DAPI-stained short-type cells, a single long cell is indicated by asterisk. **17.** Slightly twisted short cells with a very short flagellum. **18.** Cross-sectioned subpellicular microtubules of the corset. **19.** Flagellar pocket with a very short flagellum that abruptly terminates. **20.** Cross-sectioned flagellum lacking any paraflagellar rod inside the pocket. **21, 22.** The paraflagellar rod increases in size towards the end of the short flagellum. **23.** Longitudinally sectioned promastigote with kinetoplast located close to the nucleus. Bar = 5 μm (Fig. 14–17), 250 nm (Fig. 18–22) and 1 μm (Fig. 23).

Axenic cultures were established from Hosts 4 and 5. At the end of the second passage in the SDM-79 medium, the cultures contained a heterogeneous population of cells: in addition to the two types described above, there were cells of intermediate morphology. During the next few passages in the BHI medium, the cultures almost exclusively contained the small-sized cells, while a variable number of large, fast swimming cells reappeared later. It was possible to enrich for the latter type by growing cultures in vertical 15-ml tubes—most cells in the fluid column and near the surface represented the long type.

Cells derived from Host 4 were cloned by colony formation. Cells in the original culture and on the agar plates were invariably of the short type. However, the morphological variability reappeared in all five clonal lines inspected, including Line 45 selected for subsequent analysis (Fig. 3–6). Most cells in this line represented typical promastigotes with an elongated (10.3–14.4 μm; 12.4 ± 1.1 μm) and flattened body, often twisted up to one full gyre (n = 25). The distance between the nucleus and the posterior end was 4.1–7.8 μm (5.5 ± 0.9 μm), whereas the kinetoplast was 2.2–3.7 μm (2.8 ± 0.4 μm) distant from the nucleus. The flagellum was invariably long in these cells, ranging from 12.9 to 19.9 μm (15.0 ± 2.5 μm).

Cells in 10 clonal lines derived from Host 5 by two rounds of plating were also variable. One clonal line (5102) was selected that showed predominantly the short type with only rare occurrence of the long-type cells (Fig. 17). The short type represented oval promastigotes (body size 6.8–10.2 μm; 8.2 ± 0.9 μm) with a flattened and indented center (n = 25). Their slightly twisted body bore a short (3.2–7.8 μm; 6.0 ± 1.4 μm) flagellum that sometimes barely extended from the flagellar pocket. The pointed posterior end was from 2.3 to 12.2 μm (6.2 ± 0.6 μm) distant from the nucleus, while the distance between the nucleus and the kinetoplast varied in these cells from 1.1 to 2.5 μm (1.8 ± 0.5 μm).

Some cells were intermediate between these two types, and some displayed a rod-shaped extension emerging from a medial part of the body and directed posteriorly (Fig. 5). In DAPI-stained cells of Lines 45 and 5102, the kinetoplast appeared as a prominent disk-shaped structure with a staining intensity higher than in the nucleus (Fig. 4, 16). In the short-type cells (indicated by asterisks in the figures) it was located closer to the nucleus compared to the long-type cells.

Ultrastructure. The differences between the two morphotypes were further investigated by scanning (Fig. 5, 6, 17) and transmission electron microscopy (Fig. 7–13, 18–23). Slender long promastigotes observed in Line 45 have a long straight flagellum (Fig. 5, 6). Most cells also display a small bulb-like expansion at the tip of the flagellum. Analysis of both longitudinally and cross-sectioned cells showed that the flagellum is contained in a rather deep flagellar pocket and its axoneme displays typical 9+2 microtubules. From the point where the flagellum emerges from the flagellar pocket (Fig. 9), a prominent paraflagellar rod appears (Fig. 10–12) with filaments arranged in parallel (Fig. 11, 12).

The short promastigotes of Line 5102 are somewhat twisted and flattened, sometimes exhibiting a hint of a groove (Fig. 17). They bear a very short flagellum that seems to be abruptly terminated shortly after its exit from a deep flagellar pocket (Fig. 19). Although this flagellum also contains a paraflagellar rod with discernible filaments (Fig. 21, 22), it is less conspicuous (Fig. 19, 20) than the same structure in the long morphotype.

Both types of cells contain numerous dense acidocalcisomes (Fig. 13, 23), a corset of evenly spaced subpellicular microtubules (Fig. 9, 18), and a morphologically similar flagellar pocket (Fig. 7, 13, 19, 23). No endosymbionts were observed. The kinetoplast is located in the periflagellar region of the mitochondrion and has identical morphology in both strains, with DNA strands densely packed in parallel to the transverse axis of the disk (Fig. 7, 8, 13, 23). The disk's thickness indicates that both studied strains contain small sized minicircles (Lukeš and Votýpka 2000).

Genotyping of the parasites using SL RNA gene repeats. To determine if genetic heterogeneity of the parasites is associated with morphological variability, the initial uncloned populations of cells derived from Hosts 4 and 5, as well as three clonal lines derived from each culture were genotyped using sequences of the SL RNA gene repeats. The same band of ~0.5 Kb was observed in all cases (data not shown), suggesting that all samples represent the same organism. Three individual SL RNA repeats from the clonal Line 45, and two repeats from the Line 5102 were cloned and sequenced. The sequences, each 455–457 nt long, were nearly identical (98.9–99.6% identity levels in pairwise comparisons), with a few nucleotide differences localized in the intergenic region. The SL repeat sequences from *Leptomonas pyrrhocoris* (1 kb), *Leptomonas* sp. F2 (0.9 kb) or any other known SL sequences were all clearly different, especially within the highly divergent intergenic region.

Phenetic clustering analysis of SL sequences was performed for the most conserved stretch thereof encompassing positions from − 100 upstream of the first nucleotide of the SL exon to the T-tract downstream of the intron. The analysis showed that the new isolates form a distinct well-separated cluster (Fig. 24), most closely related with *L. seymouri* and *L. pyrrhocoris*, although the bootstrap support for this affiliation is moderate. The analysis recovered the *Leishmania-Endotrypanum* (LE) and *Phytomonas* (P) clades, and the large clade of monoxenous parasites (SE) observed previously (Merzlyak et al. 2001).

Phylogenetic position of the new species. The determined GAPDH sequence was identical in the two lines except for a single mismatch corresponding to a degenerate primer position. Thus, in the phylogenetic analysis both lines were represented by the same sequence. The best least-squares distance tree (Fig. 25A) and the parsimony trees (two trees, 1,696 steps, differed from each other by the topology of the NR clade, not shown) were congruent with each other and showed the split between trypanosomes (T) and non-trypanosome lineages as the earliest divergence within the Trypanosomatidae. However, the best minimum-evolution trees (two trees, minimum evolution score 3.08038, differed by position of the L1 clade with respect of the C and NR clades, not shown) showed the root attached at the *H. pessoai* branch. This topology was not supported by the bootstrap analysis, and all three majority consensus trees had the root attached as shown in Fig. 25A and also demonstrated a high support for most clades. The maximum-likelihood consensus tree (Fig. 25B) had the root attached to the lineage of *H. muscarum* and *H. megaseliae*, although the bootstrap support for this topology was low.

All analyses performed showed that the new trypanosomatid forms a distinct lineage within a well-supported late-diverging monophyletic clade that also contains two *Leptomonas* species described earlier: *L. seymouri* Wallace 1977 and *L. pyrrhocoris* Zotta 1912. The sequences of GAPDH genes clearly distinguish members of this clade from each other. The trees also highlights the polyphyly of the current genus *Leptomonas*, members of which are found in several positions in the tree.

For each method tested, exclusion of codon's third nucleotide position did not change the respective general tree topology (not shown). Variations were restricted to reduction in bootstrap support values and to relationships within some clades (NR and C) composed of nearly identical sequences.

DISCUSSION

New species description. We have isolated a new trypanosomatid species and provided its morphological and molecular phylogenetic characterization. The organism is dimorphic,

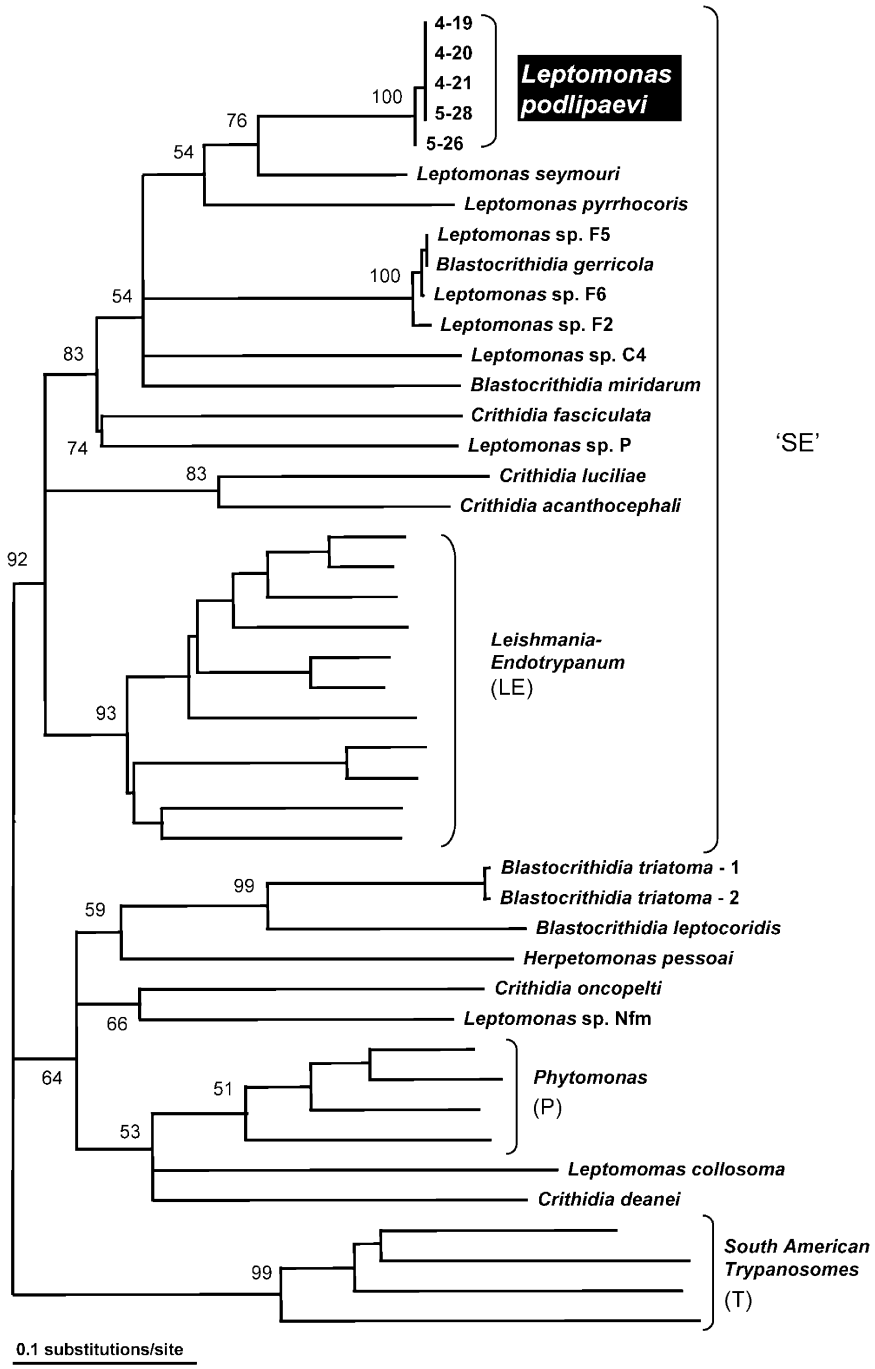

Fig. **24.** Neighbor-joining clustering of the spliced leader (SL) RNA repeats from *Leptomonas podlipaevi* n. sp. and a set of previously described trypanosomatid species. The aligned SL repeat region encompassed positions from − 100 to the T-tract at the 3′-end of the intron. Alignment was performed using CLUSTALX with gap opening weight = 12 and gap extension weight = 5. Kimura 2-parameter distance analysis with bootstrapping (250 replicates, the values are shown at the selected nodes preserved in the 50% majority-rule consensus tree) was implemented with PAUP. ''SE'' denotes the ''slowly evolving'' clade according to the SSU rRNA analysis (Merzlyak et al. 2001), LE, *Leishmania-Endotrypanum*; P, *Phytomonas*; T, *Trypanosoma*.

both in host and culture, with typical promastigotes observed along with short cells with a short flagellum. Morphological heterogeneity is frequently reported for insect trypanosomatids and is usually assumed to be due to intrinsic pleomorphy of the organisms, although it can also be attributed to mixed infections (Fiorini et al. 2001; Podlipaev 1985, 1999; Podlipaev, Malysheva, and Kolesnikov 1991; Romeiro et al. 2000). We have found that double-cloned culture cells still maintained the dimorphism. No evidence for a substantial genetic heterogeneity was obtained by SL RNA-based genotyping of two clonal lines. A small level of the heterogeneity observed among the five analyzed repeats was of the same order of magnitude or even less than the differences between individual SL repeats within each of the several investigated trypanosomatid species (D.A.M. and Westenberger, S.,

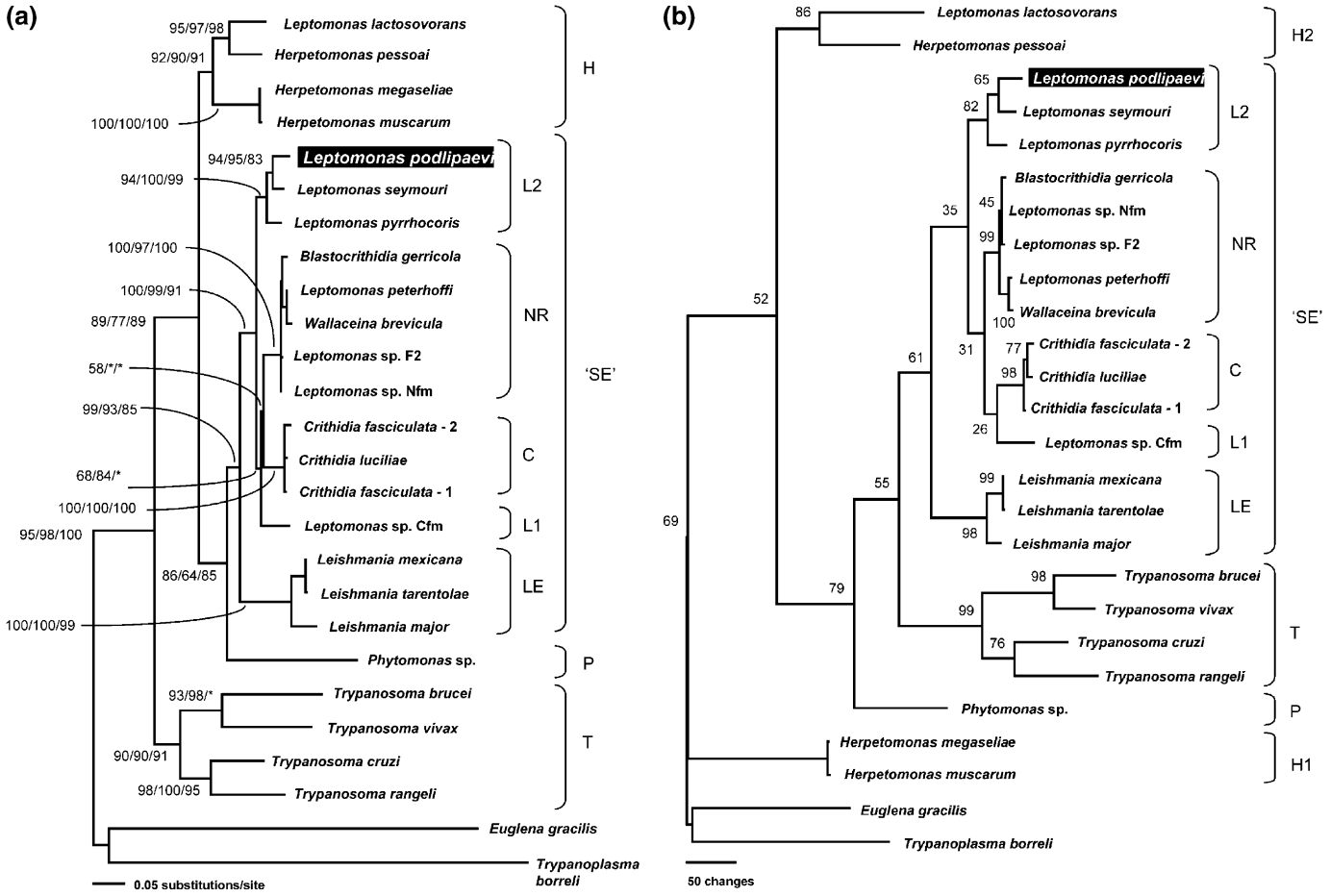

Fig. **25.** Glyceraldehyde phosphate dehydrogenase (GAPDH) phylogenetic trees of the Trypanosomatidae showing the position of *Leptomonas podlipaevi* n. sp. Major clade designations were as established earlier by the SSU rRNA analysis (Merzlyak et al. 2001): H, endosymbiont-free *Herpetomonas*; P, *Phytomonas*; ''SE,'' ''slowly-evolving''; T, trypanosomes. Additional clades are: C, endosymbiont-free *Crithidia*; H1 and H2, subgroups of clade H; L1, *Leptomonas* 1, L2, *Leptomonas* 2; LE, *Leishmania-Endotrypanum*; NR, Northern Russia. The GAPDH sequences were aligned over the entire length using CLUSTALX with gap opening weight = 12 and gap extension weight = 5. After exclusion of the primers the alignment was 1,050 nt long. (A) Unweighted least-squares distance tree inferred by a heuristic search under the GTR+I+Γ model (proportion of invariable sites was 0.2810, γ-distribution shape parameter was 0.8699). The tree's sum-of-squares was 0.68756. Bootstrap values (1,000 replicates) shown at most nodes represent analyses performed using unweighted least squares (the first value) or minimal evolution (the second value) as optimality criteria. The third bootstrap value shown represents results of the bootstrap analysis (1,000 replicates) performed with parsimony. Asterisks indicate that a particular clade was recovered less than in 50% of cases by the respective analysis. (B) Maximum likelihood tree inferred by a heuristic under the GTR+I+Γ model with the same parameters as in the distance analysis. *Ln*-likelihood of the tree was − 8590.93147. Bootstrap analysis involved 100 replicates.

unpubl. data). Thus, we have concluded that both types of cells must represent the same organism capable of a morphological differentiation. Factors triggering this differentiation remain unknown, except that cells found in confined growth conditions, such as on agar plates, are invariably of the short type, whereas cells with a ready access to nutrients and oxygen are often of the long type (data not shown).

Molecular analysis of the GAPDH and SL RNA genes has allowed us to unambiguously identify this organism as a distinct new species most closely related to two species of the genus *Leptomonas* (Wallace 1977; Zotta 1912). The overall GAPDH tree topology produced by the distance and parsimony analyses is consistent with the SSU rRNA-based tree in which mutational saturation and long branch attraction artifacts were minimized (Hollar et al. 1998; Lukeš et al. 1997; Merzlyak et al. 2001; Stevens et al. 1999), and with the published protein-based reconstructions (Hannaert et al. 2003b; Simpson et al. 2002). It needs to be mentioned, however, that the maximum likelihood and minimum evolution distance analyses produced a tree with the root attached within the H-clade, as in the previous work (Hamilton et al. 2004), although the bootstrap support for these alternative topologies was low. The reason for the discrepancy in the root position is unclear but the problem deserves further investigation including incorporation of additional ingroup and closer outgroup taxa.

Based on the phylogenetic affinity, and also on the fact that the cell morphology is consistent with the diagnosis for this genus (Vickerman 1976; Wallace 1966), the new species is assigned to the genus *Leptomonas* in its current sense. It must be noted, however, that validity of this genus is questionable. The type species, *Leptomonas bütschlii*, described in 1880 from a nematode, has never been reinvestigated and its relationship with promastigote parasites of insects or other trypanosomatids is uncertain (Wallace 1966, 1979). Even if the genus name is valid, the group itself is polyphyletic (Merzlyak et al. 2001) and its revision is warranted in the future.

Another trypanosomatid species, *Blastocrithidia leptocoridis*, was isolated previously from a related host species, *Boisea trivittata* (formerly *Leptocoris trivittatus*), in Minnesota (Todd

and Wallace 1962). The SL gene sequence from *B. leptocoridis* (Podlipaev et al. 2004b), as well as occurrence of epimastigotes and flagellar cysts clearly distinguish this species from *L. podlipaevi* n. sp.

Molecular approach to kinetoplastid taxonomy. The main advantage of the molecular sequence analysis over other types of molecular and biochemical characterization, such as RFLP, RAPD or MLEE, is that one does not need to assemble in the same laboratory a reference panel of cultures or DNA samples for a side-to-side comparison. Moreover, since it is possible to amplify trypanosomatid DNA directly from environmental samples, such as insect gut material, plant sap or animal tissues, the integrated molecular-morphological approach can be used to include species that are currently refractory to cultivation. Molecular sequences serve as permanent vouchers or ''barcodes'' (Stoekle 2003) to validate the taxonomic status of the organisms in question and allow for a future comparison with other species or isolates. Thus, an adequate description of trypanosomatid biodiversity becomes feasible.

Results of the molecular phylogenetic approach have obvious taxonomic implications. More slowly evolving sequences, such as GAPDH or SSU rRNA genes, can serve to assign species to species groups or genera, whereas faster evolving sequences, such as SL RNA gene repeats, are useful as a convenient ''first-pass'' marker, as well as for discrimination of closely related species. Multilocus microsatellite typing is appropriate for further distinctions at the subspecies (natural clone) level (Jamjoom et al. 2002; Oliveira et al. 1998).

It must be stressed that for a biologically meaningful species description, the molecular genetic data must be accompanied by investigations of morphology, hosts and other properties as prescribed by the traditional taxonomy. This integrated approach should allow for development of a natural and comprehensive taxonomic system of Trypanosomatidae.

Taxonomic Summary
Phylum Euglenozoa Cavalier-Smith, 1981
Class Kinetoplastea Honigberg, 1963
Order Trypanosomatida (Kent, 1880) Hollande 1952
Family Trypanosomatidae Doflein, 1901
Leptomonas podlipaevi n. sp.

Generic assignment. According to the existing taxonomic system of the Trypanosomatidae (Hoare and Wallace 1966; Wallace 1966), the flagellate described herein is assigned to the genus *Leptomonas*.

Specific diagnosis. Cells in the hosts and culture are pleomorphic, with two predominant forms—typical promastigotes (10–16 μm) with a long (up to 23 μm) flagellum and short promastigotes (up to 8 μm) with a very small or almost inconspicuous flagellum. The body of long-type cells was slightly flattened and twisted, in some cells up one full gyre. In short cells the body was usually flat and either indented longitudinally as a groove or also twisted, either slightly or up to half a gyre. Intermediate size-and-shape cells were often present, including cells with a lateral rod-like extension. Both types of cells appear to be dividing. The flagellum of long-type cells has a prominent paraflagellar rod, while in short cells this structure is much less pronounced. The flagellum terminates abruptly, with a small bulb-like extension at the tip in long cells. Endosymbionts were not observed. Cysts were not found.

Differential diagnosis. The organism is clearly distinguished by many nucleotide positions of sequences of GAPDH genes and SL RNA gene repeats and by the inferred phylogenetic tree position from all other known species or isolates of Trypanosomatidae including the two most closely related species, *L. seymouri* and *L. pyrrhocoris*. No cells similar to the short form with short flagellum were reported for the related species.

Type host. Intestine of *Boisea rubrolineata* Barber (Hemiptera: Rhopalidae).

Type locality. Riverside, California, USA.

Etymology. The species is named in honor of Dr. Sergei A. Podlipaev, a prominent Russian protozoologist who had devoted his life-time efforts to investigations of insect trypanosomatids.

Type material. The hapantotype and the paratype (Giemsa-stained microscope slides of the gut smear representing the parasites in Hosts 4 and 5, respectively) are deposited in the U.S. National Parasite Collection (USNPC 97118, USNPC 97119). The xenotypes (post-dissection remains of the hosts) are stored in the UCR Entomology Research Museum (Host 4—UCRC ENT 117850, Host 5—UCRC ENT 117851). Axenic cultures representing Line 45 and Line 5102 are deposited in the American Type Culture Collection (ATCC PRA-171 and ATCC PRA-172, respectively).

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