Leptomonas costaricensis sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus *Leishmania*

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SUMMARY
A flagellate isolated from the intestinal tract of a reduviid bug *Ricolla simillima* (Heteroptera) in Costa Rica was found to represent a new trypanosomatid species by the phylogenetic analysis of small subunit ribosomal RNA (SSU rRNA), glyceraldehyde phosphate dehydrogenase (GAPDH) and large subunit of RNA polymerase II (RPOIILS) genes. The phylogenetic position of this trypanosomatid, together with its typical promastigote morphology and the host identity, allowed classifying it as a species that belong to the polyphyletic genus *Leptomonas*. Interestingly, the new species was revealed as a member of the novel phylogenetic clade representing the closest known relative of *Leishmania*. With the new species used as an outgroup to root the *Leishmania* RPOIILS phylogenetic tree, the lineage of the Neotropical species *L. enriettii* was found branching off early during the evolution of this genus. The subsequent branching order was resolved differently by the minimum evolution and maximum likelihood analyses. In both cases, however, the rooted tree topology is consistent with the hypothesis that the initial transition to dixenous parasitism in this group occurred in the Neotropics.

Key words: *Leptomonas costaricensis*, phylogeny, Trypanosomatidae, *Leishmania*, evolution of parasitism, dixenous parasitism.

INTRODUCTION

Origin of a two-host parasitism in the Trypanosomatidae represents one of the most interesting problems of the evolution of these protozoa. Among several groups which constitute this family of the predominantly single-host (usually, an insect) parasites, two genera, *Leishmania* and *Trypanosoma*, stand apart by virtue of their ability to parasitize vertebrates, as well as respective haematophagous insects which serve as transmission vectors (Vickerman 1976; Vickerman 1994). It is thought that dixenous parasites of vertebrates and insects evolved from monoxenous parasites of insects when the latter had developed haematophagy, which resulted in a repeated exposure of the intestinal parasites of insects to the environment of vertebrate blood (reviewed in (Lainson and Shaw 1987; Maslov and Simpson 1995)). Nonetheless, an accidental transmission of monoxenous trypanosomatids into a vertebrate would only extremely rarely leave surviving

descendants (Simpson, Stevens and Lukeš 2006), explaining why there are only two aforementioned groups of dixenous parasites of vertebrates, each appearing to be monophyletic. At least with respect to *Leishmania*, the secondary origin of dixenous parasitism is supported by the molecular phylogenetic evidence which shows that this group emerged from monoxenous parasites relatively late in the evolution of the Trypanosomatidae (Fernandes, Nelson and Beverley 1993; Maslov and Simpson 1995; Maslov *et al.* 1996; Hollar, Lukeš and Maslov 1998; Merzlyak *et al.* 2001). However, additional details of this emergence, including the geographic origin and the nature of the monoxenous ancestors, have not yet been elucidated.

In the phylogenetic trees, the genus *Leishmania* forms a paraphyletic group. In addition to the New World and Old World species of the subgenera *L. (Viannia), L. (Leishmania)* and *L.* (*Sauroleishmania)* and a group of the Neotropical species collectively referred to as the paraleishmania, the *Leishmania* clade also includes several isolates currently assigned to the separate genus *Endotrypanum* (Noyes, Camps and Chance 1996; Noyes *et al.* 1997; Croan, Morrison and Ellis 1997; Noyes *et al.* 2002). It is likely that this assignment is erroneous and the isolates in question actually represent one of the paraleishmania species (Cupolillo *et al.* 2000). The phylogenetic tree of the entire *Leishmania/Endotrypanum* clade was rooted at the paraleishmania branch (Noyes *et al.* 1997; Croan, Morrison and Ellis 1997; Noyes *et al.* 2002). Based on the phylogenetic evidence, a hypothesis was proposed according to which the genus *Leishmania* originated in the Neotropics and subsequently dispersed into the Old World through the Bering Land Bridge (Noyes 1998). An alternative view in favor of the Palaearctic origin was also presented based on biogeographical and paleontological evidence, and it was also suggested that the observed root of the *Leishmania* tree at the long paraleishmania branch could represent an artifact caused by the choice of an inadequate outgroup (Kerr, Merkelz and Mackinnon 2000; Kerr 2000). The Palaearctic hypothesis was contested on several grounds including the absence of an independent phylogenetic support (Noyes *at al.* 2000), although the problem of finding an explicit root of the *Leishmania* tree still remained.

During the ongoing survey of the trypanosomatid biodiversity in Costa Rica (Westenberger *et al.* 2004), we encountered an organism which, according to the genotyping and cluster analysis of the Spliced Leader RNA gene, was clearly different from other trypanosomatid species. In this work we have provided a detailed morphological and molecular phylogenetic characterization of this isolate that we have described herein as a new species of the Trypanosomatidae. Remarkably, we have found that the new species is a member of the novel phylogenetic group with a sister-clade relationship to the genus *Leishmania*, and thus represents a close-enough outgroup for the appropriate rooting of the *Leishmania* phylogenetic tree. We also describe the implications of this analysis for our understanding of the origin and evolution of *Leishmania*.

MATERIALS AND METHODS

Isolation of the parasites

Insect dissection, microscopic examination of intestinal content in the field and establishing and maintaining of parasite cultures were performed as described earlier (Westenberger *et al.* 2004).

Light and electron microscopy

Morphological characterization of cells in culture by light and transmission and scanning electron microscopy followed the protocols described previously (Yurchenko *et al.* 2006), with the exception that for scanning electron microscopy, cells were examined using a JEOL JSM-7401F microscope.

Sources of DNA

Total cell DNA was isolated from axenic cultures of the new isolate (described below as *L. costaricensis* sp. n., strain 15EC) and *Leptomonas podlipaevi* (clonal line 5-10-2) (Yurchenko *et al.* 2006) by a standard phenol-chloroform procedure. The following *Leishmania* DNA samples were kindly provided by H. Noyes via D. A. Campbell: *L. colombiensis* L1245 (IGOM/PA/1985/E582.34), *L. deanei* LV402 (MCOE/BR/XXXX/M808), *L. enriettii* L2434 (MCAV/BR/1945/L88), *L. equatorensis* L888 (MCHO/EC/1982/Lsp1), and *L. herreri* LV341 (ISHA/CR/1974/Sh-1).

PCR amplification, cloning and sequencing

PCR amplification of the glyceraldehyde phosphate dehydrogenase (GAPDH) genes, gel-purification, cloning and sequencing were performed as described previously (Yurchenko *et al.* 2006). Small subunit (SSU) rRNA genes were amplified using Expand High Fidelity PCR system (Roche, Indianapolis, IN) and the oligonucleotides SSU1 (5'-GACTTTTGCTTCCTCTA(A/T)TG) and SSU2 (5'-CATATGCTTGTTTCAAGGAC) as follows: initial denaturation at 95 ^{o}C for 3 min followed by 30 amplification cycles (95 ^{o}C for 30 s, 55 ^{o}C for 1 min, 72 ^{o}C for 2 min 30 s), and the final extension at 72 ^{o}C for 10 min. The amplified products were sequenced either directly (for *L. costaricensis*) or following a cloning into a plasmid vector pGEM-T Easy (Promega, Madison, WI) (for *L. podlipaevi*). Internal primers for sequencing were as described previously (Maslov *et al.* 1996).

PCR amplification of the partial RNA polymerase II largest subunit (RPOIILS) gene was done using the oligonucleotides M172 (5'-CGACACAGCCGTCAAGACGTCCGAC) and M173 (5'-GGACGCAGCCGCACAATGCGCTGG). These oligonucleotides were designed based on the alignment of the complete sequences from *Leishmania major*, *Leishmania donovani*, *Leptomonas seymouri* and *Trypanosoma brucei*, available in the databases, to include the central 1.3 Kb region of the gene that corresponds to the amplicon analyzed previously (Croan *et al.* 1997). Amplification was performed with *Taq* polymerase and the following cycling profile: initial denaturation at 95 ^{o}C for 5 min, followed by 35 high-stringency cycles (95 ^{o}C for 30 s, 70 ^{o}C for 1 min, 72 ^{o}C for 2 min 30 s), and the final extension at 72 ^{o}C for 10 min. The products were gel-purified, cloned and sequenced as described earlier.

The following gene sequences of *L. costaricensis* sp. n. determined in this work have been deposited in GenBank^{TM} under the following accession numbers: GAPDH (DQ383650), SSU rRNA (DQ383648), partial RPOIILS (DQ383651). In addition, the following partial RPOIILS sequences have been deposited: *L. colombiensis* (DQ383652), *L. deanei* (DQ383653), *L. enriettii* (DQ383654), *L. equatorensis* (DQ383655), and *L. herreri* (DQ383656). The SSU rRNA sequence from *L. podlipaevi* has been deposited under the accession number DQ383649.

Phylogenetic analysis

In addition to the sequences determined in this work, the RPOIILS dataset used included the sequences determined by Croan et al. (Croan *et al.* 1997). The GAPDH dataset was largely as described previously (Yurchenko *et al.* 2006). After primer removal, the sequences in the RPOIILS and GAPDH datasets were unambiguously aligned over the entire length using CLUSTALX, version 1.81 (Thompson *et al.* 1997). The alignments were 1266 and 1050 nt long, respectively. A general time-reversible model (GTR + Γ) of sequence evolution was selected for the RPOIILS dataset by the hierarchical and AIC tests of MODELTEST, version 3.06 (Posada and Crandall 1998). The proportion of invariable sites was 0, and the gamma-distribution shape parameter for variable sites was 0.3098. The respective parameters of the best-fitting model (GTR + *I* + Γ) selected for the GAPDH dataset were 0.2625 and 0.7971. Maximum likelihood,

distance and parsimony analyses were performed using PAUP* 4.0 beta version (Swofford 1998). Bootstrap analyses were done using 100 replicates (likelihood) or 1000 replicates (distance and parsimony). Statistical evaluation of the unconstrained and constrained likelihood trees was performed using the Kishino-Hasegawa test (Kishino and Hasegawa 1989) with full optimization bootstrap (1000 replicates) implemented by PAUP.

The SSU rRNA dataset was based on the sequences included in the "slowly-evolving" clade described earlier (Merzlyak *et al.* 2001), as well as the trypanosomatid G755 sequence (Noyes *et al.* 1997). After the initial alignment by CLUSTAL, the ambiguously aligned regions were manually selected and removed from the analysis using the interactive alignment editor SEAVIEW (Galtier, Gouy and Gautier 1996). The alignment contained 1954 nt. The MODELTEST analysis yielded the TrNef + I + Γ model with the proportion of invariable sites equal to 0.7406, and the gamma-distribution shape parameter for variable sites equal to 0.5839. Maximum likelihood analysis was performed with PAUP using the MODELTEST-derived model, and also by using the GTR + I + Γ model with the tree parameters estimated by likelihood (proportion of invariable sites equal to 0.7382, and the gamma-distribution shape parameter for variable sites equal to 0.5682).

RESULTS

Isolation of the new trypanosomatid species

A low-level trypanosomatid infection was detected by light microscopy in the gut of one out of twelve investigated specimens of *Ricolla simillima* (Heteroptera, Reduviidae) collected near the western boundary of the Braulio Carrillo National Park in Costa Rica in March 2003. The trypanosomatids appeared as elongated promastigotes some of which were free-moving solitary cells and some were aggregated. The material of the gut smear was used to establish the primary culture of the parasite. The parasites grew readily in the BHI medium supplemented with hemin and an axenic culture was obtained after several passages.

Cell morphology and ultrastructure

All cells in the culture are promastigotes, most of which are characterized by a prolonged shape (Figs. 1, 3, 5, 11). The only noticeable heterogeneity is referred to the body length which varied between 7.3 and 15.1 μm (mean ± SD: 11.6 ± 1.2 μm; n = 55). Cells representing the length extremes are shown in Fig. 5 (a more typical elongated promastigote) and Fig. 3 (a shorter form). Cells are somewhat flattened along the entire body length to form a slightly twisted shape with the breadth not exceeding 1.5 μm. The distance between the nucleus and the posterior end ranged from 2.5 to 7.2 μm (4.9 ± 0.8 μm), and that between the nucleus and the kinetoplast ranged from 0.9 to 3.1 μm (2.1 ± 0.5 μm). A relatively short distance between the kinetoplast and the nucleus is apparent in the DAPI-stained cells (Fig. 2). The promastigotes were equipped with an extended flagellum, the size of which varied between 7.4 and 12.3 μm (9.9 ± 1.2 μm).

Examination of the cells by high resolution scanning electron microscopy revealed that the flagellum is relatively massive compared to the cell body. At its exit from the flagellar pocket the flagellum is almost as thick as the anterior end of the cell itself (Fig. 6). The paraflagellar rod is very prominent with an elongated protrusion forming a groove visible almost along the entire length of the flagellum (Figs. 4 and 9).

Transmission electron microscopy revealed that the flagellum has typical 9+2 microtubules (Fig. 9), that it exits from a deep flagellar pocket (Fig. 11) and is furnished with a thick paraflagellar rod composed of parallel filaments (Fig. 7). The kinetoplast is typically disk-shaped with DNA strands arranged in parallel to the transverse axis of the disk (Fig. 11). The

diameter of the kinetoplast disk is 0.59 ± 0.10 μm and its thickness is 0.15 ± 0.03 μm (n = 33). The extremely thin morphology of the cells is reflected in the highly elongated shape of their nuclei (Fig. 10). The promastigotes contain numerous usually electron-dense acidocalcisomes (Fig. 8) and evenly spaced subpellicular microtubules (data not shown). Endosymbionts were absent.

Phylogenetic position of the new isolate

Relationships of the new isolate with the rest of the Trypanosomatidae were investigated by the phylogenetic analysis of the GAPDH and SSU rRNA gene sequences amplified from the axenically grown cells. Fig. 12 shows a GAPDH maximum likelihood tree inferred from the sequences representing most of the family. The new species is found in a close association with the group of *Leishmania* sequences, and bootstrap support for this association was very high. The same result was obtained using parsimony and minimum evolution (data not shown). The topologies of the respective GAPDH trees were similar to the trees described and discussed by us previously (Yurchenko *et al.* 2006).

The analysis of the SSU rRNA sequences allowed us to compare the new isolate to a larger number of *Leishmania* species than the analysis of the GAPDH dataset. We initially performed the analysis using the dataset that included all major phylogenetic clades identified in the Trypanosomatidae (Hollar, Lukeš and Maslov 1998; Merzlyak *et al.* 2001). This analysis (data not shown) confirmed that the new isolate is closely related to the *Leishmania* clade. At the next step we analyzed the subset of sequences representing the so-called "slowly evolving" clade (Merzlyak *et al.* 2001) of which the organisms in question are found to be the members. The SSU rRNA tree (Fig. 13) confirmed that the new isolate represents a sister group to the *Leishmania-Endotrypanum* clade. Interestingly, this group includes the undescribed trypanosomatid species G755 which was also found to be closely related to *Leishmania* (Noyes *et al.* 1997).

Leptomonas costaricensis sp. n.

Higher order taxonomic summary. Phylum Euglenozoa Cavalier-Smith, 1981; class Kinetoplastea Honigberg, 1963; order Trypanosomatida (Kent, 1880) Hollande 1952; family Trypanosomatidae Doflein, 1951.

Generic assignment. This was based on the existing taxonomic system of the Trypanosomatidae (Wallace 1966; Hoare and Wallace 1966), although a future revision of this status is warranted along with the revision of the genus *Leptomonas* itself.

Specific diagnosis. Cells are elongated promastigotes, slightly flattened and twisted. The flagellum is relatively thick with a long prominent paraflagellar rod separated from the flagellar proper by a distinct groove. The cell's anterior end is tapered forming a smooth transition between the cell body and the flagellum.

Differential diagnosis. At present there is not enough information to decide whether the morphology alone can distinguish this species from other leptomonads, including the closely related trypanosomatid G755. However, the organism is clearly distinguishable from other known trypanosomatids by the sequences of SSU rRNA, GAPDH and RPOIILS genes and the inferred phylogenetic position.

Type host. Intestine of *Ricolla simillima* Stål (Heteroptera, Reduviidae)

Type locality. A vicinity of El Ceibo (10^{o} 20' N, 84^{o} 05' W), 10 km South-East of the community La Virgen, Province Heredia, Costa Rica.

Etymology. The new species is named after the country of origin.

Type material. The xenotype (post-dissection remains of the host) is deposited in the UCR Entomology Research Museum (UCRC ENT 130967). The type culture is deposited in the American Type Collection (ATCC PRA-186).

A rooted tree of the Leishmania *clade*

Previously, partial sequences of the RPOIILS genes were found to be informative phylogenetic markers for resolving relationships among the *Leishmania* lineages (Croan and Ellis 1996; Croan, Morrison and Ellis 1997; Noyes *et al.* 2002). We have determined the respective RPOIILS sequences from the new isolate, as well as from several *Leishmania* species with the previously unknown (*L. colombiensis*, *L. equatorensis*) or potentially problematic phylogenetic status (*L. enriettii*, *L. deanei*, *L. herreri*). Initially, the phylogenetic analyses were performed using *T. brucei* and *T. cruzi* as outgroups (data not shown). The maximum likelihood tree had the topology: (Outgroup taxa, (*L. seymouri*, (*L. costaricensis*, (*Leishmania* spp.)))), although bootstrap support for this topology was low due to the large distance between the outgroup and the ingroup taxa. The support was higher when the trypanosome sequences were omitted and the *L. seymouri* sequence was used as an outgroup (data not shown). The analysis confirmed that *L. costaricensis* n. sp. had diverged prior to the radiation within the *Leishmania* clade, and thus can be used as the closest outgroup for *Leishmania*.

The majority consensus minimum evolution RPOIILS tree rooted with the sequence of *L. costaricensis* n. sp. is shown in Fig. 14. The best distance and parsimony trees (not shown) had the same relative branching order as the presented tree. This analysis revealed a deep split of the genus on two major clades corresponding to the euleishmania and the paraleishmania sections. The tree also showed the Old World species as emerging relatively late compared to most Neotropical groups. The notable exception from a clear separation of the New World and Old World species from each other is a close association of *L. (L.) mexicana* and *L. (L.) amazonensis* with the Old World *L. (Leishmania)*.

The best maximum likelihood tree (not shown) also had two major clades. However, because *L. (Viannia)* was a part of the paraleishmania clade instead of the euleishmania clade, these two clades roughly represented the Old World and the New World species with exception of the *mexicana* group as described above. Bootstrap support for the association of *L. (Viannia)* with the paraleishmania was low and the majority-rule consensus tree (not shown) showed a trichotomy for the clades of *L. (Viannia),* paraleishmania and the remaining euleishmania.

In all trees, the Neotropical species *L. enriettii* was found to form the earliest separating branch. When the analysis was done under a topological constraint enforcing the monophyly of *L. enriettii* with the other euleishmania, the inferred tree (*Ln*-likelihood = -5820.96082) was significantly inferior ($P = 0.000$) compared to the best unconstrained tree (*Ln*-likelihood = -5692.86354).

DISCUSSION

The new species reported herein was found in only one out of twelve specimens of the reduviid host *R. simillima*. With the total number of the Heteroptera specimens from Costa Rica analyzed so far being close to 400, this remains the only encounter of this species, albeit other trypanosomatids have been observed in *R. simillima* (D. A. M., unpublished observations). Phylogenetically, the new species is most closely related to the trypanosomatid isolate G755 found in a sandfly in Guatemala (Noyes *et al.* 1997). Both organisms form a sister clade to *Leishmania,* a group of dixenous parasites most of which are known (or thought) to be transmitted by sandflies. Thus, it is possible that the infection of *R. simillima* with *L.*

costaricensis sp. n. might have been a result of the predation of an infected sandfly by the bug. Additional situations when an insectivorous host might have acquired the parasites of its prey were recognized and discussed previously (Carvalho and Deane 1974; Podlipaev 2003).

Due to the aforementioned phylogenetic affinity to *Leishmania*, there is an intriguing possibility that the newly discovered organism along with the isolate G755 may represent a heretofore unknown group of dixenous parasites. However, because dixenous organisms have been extensively studied, it is more likely that the novel clade represents monoxenous parasites. As *L. costaricensis* sp. n. descended from the recent common ancestor with *Leishmania*, comparative studies of these two organisms may shed light on the evolutionary transition to dixenous parasitism.

The Neotropics have been proposed as a place of the origin of the genus *Leishmania* based on the tree topology which shows the Neotropical species branching off close to the base of the tree (Noyes *et al.* 1997; Croan, Morrison and Ellis 1997; Stevens *et al.* 2001). An additional support for this view may be found in a higher diversity of the Neotropical *Leishmania* species indicating that this region may represent the center of origin of this group. According to the alternative view, the genus emerged in the Old World with the subgenus *L. (Sauroleishmania)* placed at the basal node of the tree (Kerr 2000; Kerr 2006; Kerr, Merkelz and Mackinnon 2000). The discovery of the Central American clade of monoxenous parasites closely related to *Leishmania* is consistent with the idea that the initial transition to dixenous parasitism occurred in Neotropics. This notion is also supported by the phylogenetic tree which is rooted at the branch of *L. enriettii*, an "enigmatic" Neotropical species (Lainson 1997). The subsequent evolutionary scenario would involve the diversification of the ancestral dixenous organisms into the lineages of the New World and Old World parasites at some point. The species of the *mexicana* group would be reintroduced in the New World more recently. The new rooted tree topology does not lend any support for the Palaearctic hypothesis.

An additional hypothesis was proposed which postulates the Neotropical origin for *L. (Viannia)* and the paraleishmania on one hand and the African origin of *L. (Leishmania)* and *L. (Sauroleishmania)* on the other (Momen and Cupolillo 2000; Pratlong *et al.* 2001). This scenario implies an ancient separation of the respective lineages, possibly triggered by the continental split, as was proposed for the separation of the South American and African trypanosomes (Stevens *et al.* 1999; Stevens *et al*. 2001). This also implies that the initial transition to dixenous parasitism predated the separation of South America and Africa. By showing a deep split between the Old World and the New World groups, the topology of the maximum likelihood RPOIILS tree in particular is consistent with this scenario.

Finding an ROPIILS tree rooted at *L. enriettii* was unexpected. According to the SSU rRNA analysis, the root of the *Leishmania* tree is placed between the paraleishmania and euleishmania clades (Noyes *et al.* 2002; D. A. M., unpublished observations), while *L. enriettii* could be expected to branch off within the euleishmania together with *Leishmania* sp. MAR1. A resolution of the incongruence between the RPOIILS and SSU analyses awaits for the availability of full length SSU rRNA sequences of *L. enriettii* and additional species, especially paraleishmania, to make the two datasets directly comparable.

Although *L. enriettii* is formally placed in the subgenus *L. (Leishmania)* as a member of the *mexicana* complex, this species is atypical in many aspects (Lainson 1997; Lainson and Shaw 1987). The wild host and the vector of this species remain unknown (Thomaz-Soccol *et al.* 1996; Lainson 1997). The same applies to *Leishmania* sp. MAR1, initially presumed to be a monoxenic trypanosomatid (Boisseau-Garsaud *et al.* 2000; Garin *et al.* 2001) and subsequently

found to branch off together with *L. enriettii* (Noyes *et al.* 2002) (D.A.M., unpublished observations). Given the phylogenetic position of these species at the base of the tree, an identification of their natural hosts becomes very important for reconstruction of the initial host of the group.

It becomes increasingly clear that no single gene phylogeny is fully adequate for resolving phylogeny of the Trypanosomatidae or its subgroups (Philippe 1998; Simpson, Stevens and Lukeš 2006). The resolution of this problem can be achieved with the aid of a detailed and reliable phylogenetic tree based on a large amount of molecular data, a comprehensive set of taxa and rooted with the closest outgroup possible. The new species described herein is particularly useful for the last purpose (with respect to phylogeny of *Leishmania*), as illustrated by the analyses presented in this work.

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CAPTIONS TO FIGURES

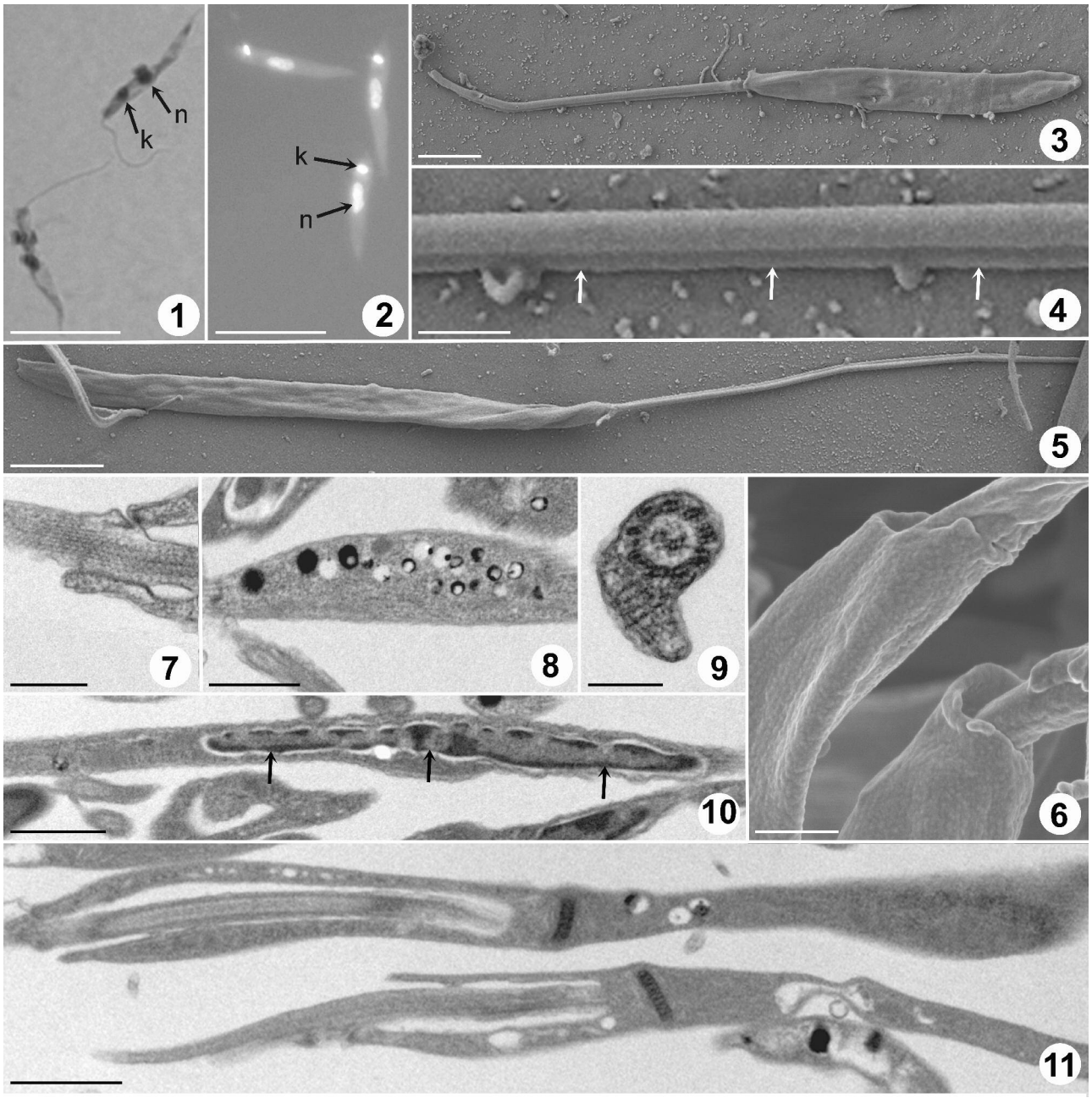
Figs. 1-11. Light (Figs. 1, 2), scanning (Figs. 3-6) and transmission (Figs. 7-11) electron microscopy of *Leptomonas costaricensis* sp. n. in culture. Fig. 1. Giemsa-stained promastigotes with well-visible kinetoplast, nucleus and flagellum. The positions of kinetoplast (k) and nucleus (n) are indicated. Fig. 2. DAPI-stained promastigotes with a prominent kinetoplast located close to the nucleus. The positions of kinetoplast (k) and nucleus (n) are indicated. Fig. 3. A shorter promastigote cell with a relatively short flagellum. Fig. 4. The paraflagellar rod visible along the flagellum as a thin elongated protrusion (arrows). Fig. 5. Typical slender promastigote with a long flagellum. Fig. 6. Anterior parts of a cell with a prominent flagellum at its exit from the flagellar pocket. Fig. 7. Longitudinal section of the flagellum with well-visible parallel filaments of the paraflagellar rod. Fig. 8. Part of the cell with acidocalcisomes. Fig. 9. Transverse section of a flagellum with a prominent paraflagellar rod. Fig. 10. Extremely elongated nucleus (arrows). Fig. 11. Longitudinal section of "squid-like" promastigotes revealing a very deep flagellar pocket. Bar = 10 μm (Figs. 1 and 2), 1 μm (Figs. 8, 10 and 11), 2 μm (Figs. 3 and 5), 500 nm (Figs. 4, 6 and 7) and 200 nm (Fig. 9).

Fig. 12. Glyceraldehyde phosphate dehydrogenase (GAPDH) phylogenetic tree of the Trypanosomatidae. Clade designations are given in accordance with the previous works (Merzlyak *et al.* 2001; Yurchenko *et al.* 2006): L – *Leptomonas*, LE – *Leishmania-Endotrypanum*, NR – Northern Russia, 'SE' – 'slowly-evolving', T – trypanosomes. 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 1050 nt long. Maximum likelihood analysis was peformed by a heuristic search under the GTR + I + Γ model (proportion of invariable sites was 0.2625, gamma-distribution shape parameter was 0.7971). *Ln*-likelihood of the best tree (not shown) was -8738.23926, the value for the presented bootstrap 50% majority-rule consensus tree was -8766.10637. Bootstrap values shown at most nodes and the first value shown at the *L. costaricensis* node represent analyses performed using maximum likelihood. The remaining values at the *L. costaricensis* node were derived by unweighted least squares (the second value), minimal evolution (the third value) and parsimony (the first value) analyses. GenBank accession numbers of the sequences used are: *Blastocrithidia gerricola* (AF322391), *Crithidia fasciculata* (AF047493), *Crithidia luciliae* (AF053740), *Euglena gracilis* (L39772), *Herpetomonas megaseliae* (DQ092547), *Herpetomonas muscarum* (DQ092548), *Herpetomonas pessoai* (AF047494), *Leishmania major* (AF047497), *Leishmania mexicana* (X65226), *Leishmania tarentolae* (DQ092549), *Leptomonas lactosovorans* (AF053741), *Leptomonas peterhoffi* (AF322390), *Leptomonas podlipaevi* (DQ019000), *Leptomonas pyrrhocoris* (AY029072), *Leptomonas seymouri* (AF047495), *Leptomonas* sp. Cfm (AF320820), *Leptomonas* sp. Nfm (AF339451), *Leptomonas* sp. F2 (AF375664), *Phytomonas* sp. (AF047496), *Trypanoplasma borreli* (X74535), *Trypanosoma brucei brucei* (X59955), *Trypanosoma cruzi* (X52898), *Trypanosoma rangeli* (AF053742), *Trypanosoma vivax* (AF047500), and *Wallaceina brevicula* (AF316620).

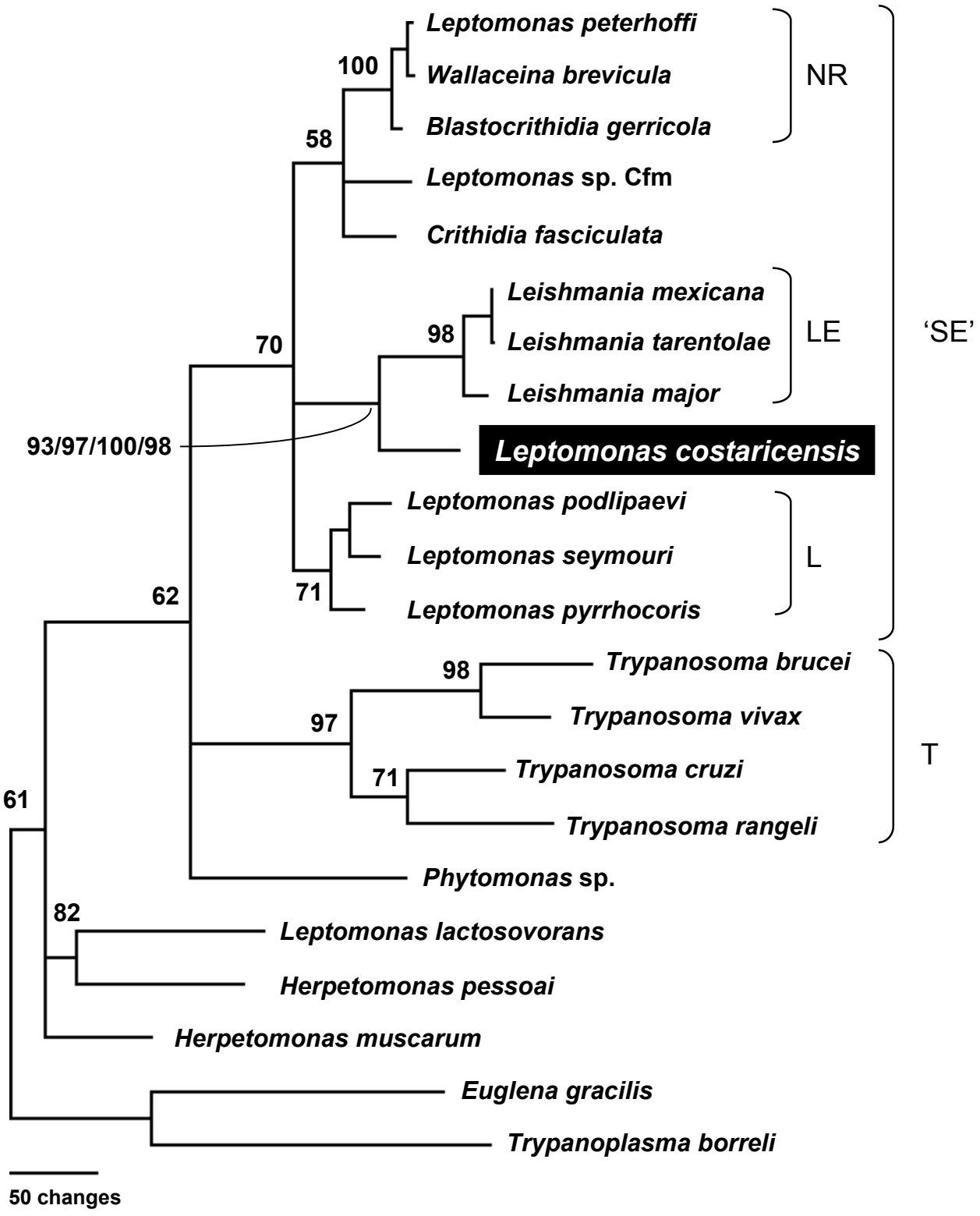
Fig. 13. Small subunit ribosomal RNA (SSU rRNA) phylogenetic tree of the 'slowly-evolving' clade (Merzlyak *et al.* 2001) of the Trypanosomatidae. After the initial alignment by CLUSTAL, the ambiguously aligned regions were manually selected and removed from the analysis using the interactive alignment editor SEAVIEW. The alignment, containing 1954 nucleotides, lacks

the fastest evolving sites which resulted in a reduced support and polytomies observed in the consensus tree. Maximum likelihood analysis was performed by a heuristic search under the GTR + *I* + Γ model (proportion of invariable sites was 0.738193, gamma-distribution shape parameter was 0.568177, estimated via likelihood). *Ln*-likelihood of the best tree (not shown) was -4153.39053, the value for the presented bootstrap 50% majority-rule consensus tree was -4174.52421. Bootstrap values show results of the maximum likelihood (the first value), minimum evolution (the second value) and parsimony (the third value) analyses. Asterisks indicate that a particular clade was recovered in less than 50% of cases by the respective analysis. GenBank accession numbers of the sequences used are: *Blastocrithidia gerricola* (AF153036), *Crithidia fasciculata* (Y00055), *Endotrypanum monterogeii* (X53911), *Leishmania amazonensis* (X53912), *Leishmania donovani* (X07773), *Leishmania guyanensis* (X53913), *Leishmania major* (X53915), *Leishmania tarentolae* (M84225), *Leptomonas seymouri* (AF153040), *Leptomonas* sp. (X53914), *Leptomonas* sp. Cfm (AF153041), *Leptomonas* sp. F6 (AF153042), *Leptomonas* sp. Nfm (AF153043), trypanosomatid G755 (U59491), *Wallaceina inconstans* (AF153044).

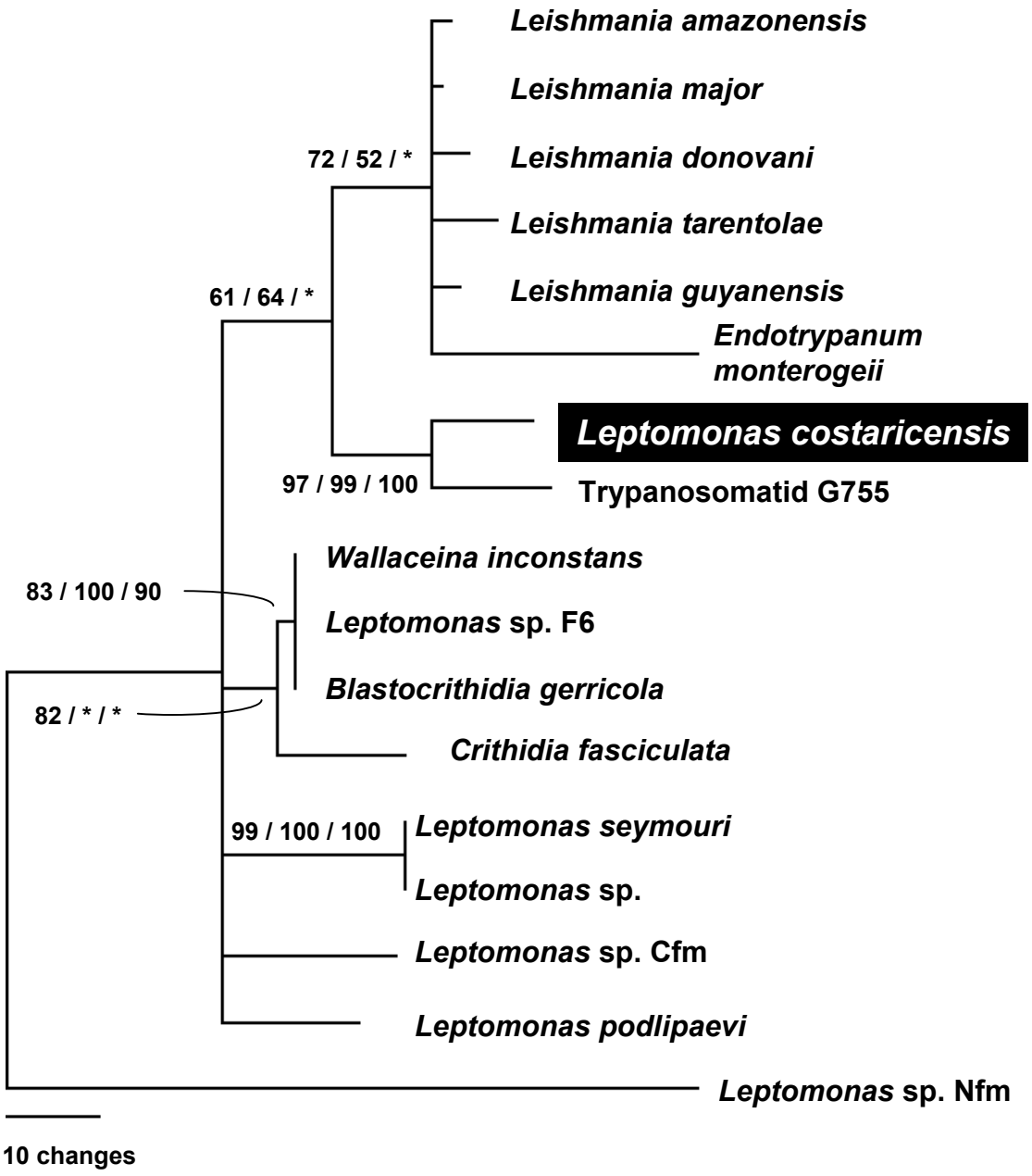
Fig. 14. A rooted large subunit of RNA polymerase II (RPOIILS) phylogenetic trees of the *Leishmania/Endotrypanum* clade. The partial RPOIILS sequences were unambiguously aligned over the entire length. The alignments were 1266 nt long. The new sequences of *L. deanei*-2, *L. enrietti*-2 and *L. herreri*-2 are labeled as such to distinguish them from the previously determined sequences of the same species. GenBank accession numbers of the remaining sequences are: *Endotrypanum monterogeii* (AF009158), *Leishmania adleri* (AF009153), *Leishmania amazonensis* (AF009154), *Leishmania braziliensis* (AF009155), *Leishmania deanei* (AF009156), *Leishmania donovani* (AF009157), *Leishmania enriettii* (AF151727), *Leishmania gymnodactyli* (AF009159), *Leishmania herreri* (AF009160), *Leishmania hertigi* (AF009161), *Leishmania hoogstraali* (AF009162), *Leishmania major* (AF009163), *Leishmania mexicana* (AF009164), *Leishmania panamensis* (AF009165), *Leishmania tarentolae* (AF009166), *Leishmania tropica* (AF009167). The terms ‘Euleishmania’ and ‘Paraleishmania’ refer to the major groups (‘Sections’) previously identified within the genus (Cupolillo *et al*. 2002). Minimum evolution distance and maximum likelihood analyses were performed by a heuristic search under the GTR + Γ model (proportion of invariable sites was 0, gamma-distribution shape parameter was 0.3098). The score of the best distance tree (not shown) was 0.86383 and the *Ln*-likelihood value of the best maximum likelihood (not shown) tree was -5692.86354. The figure shows a 50% majority-rule consensus distance tree. Bootstrap values represent results of the maximum likelihood (the first value), minimum evolution (the second value) and parsimony (the third value) analyses. An asterisk indicates that a clade was recovered in less than 50% of cases by the respective analysis.



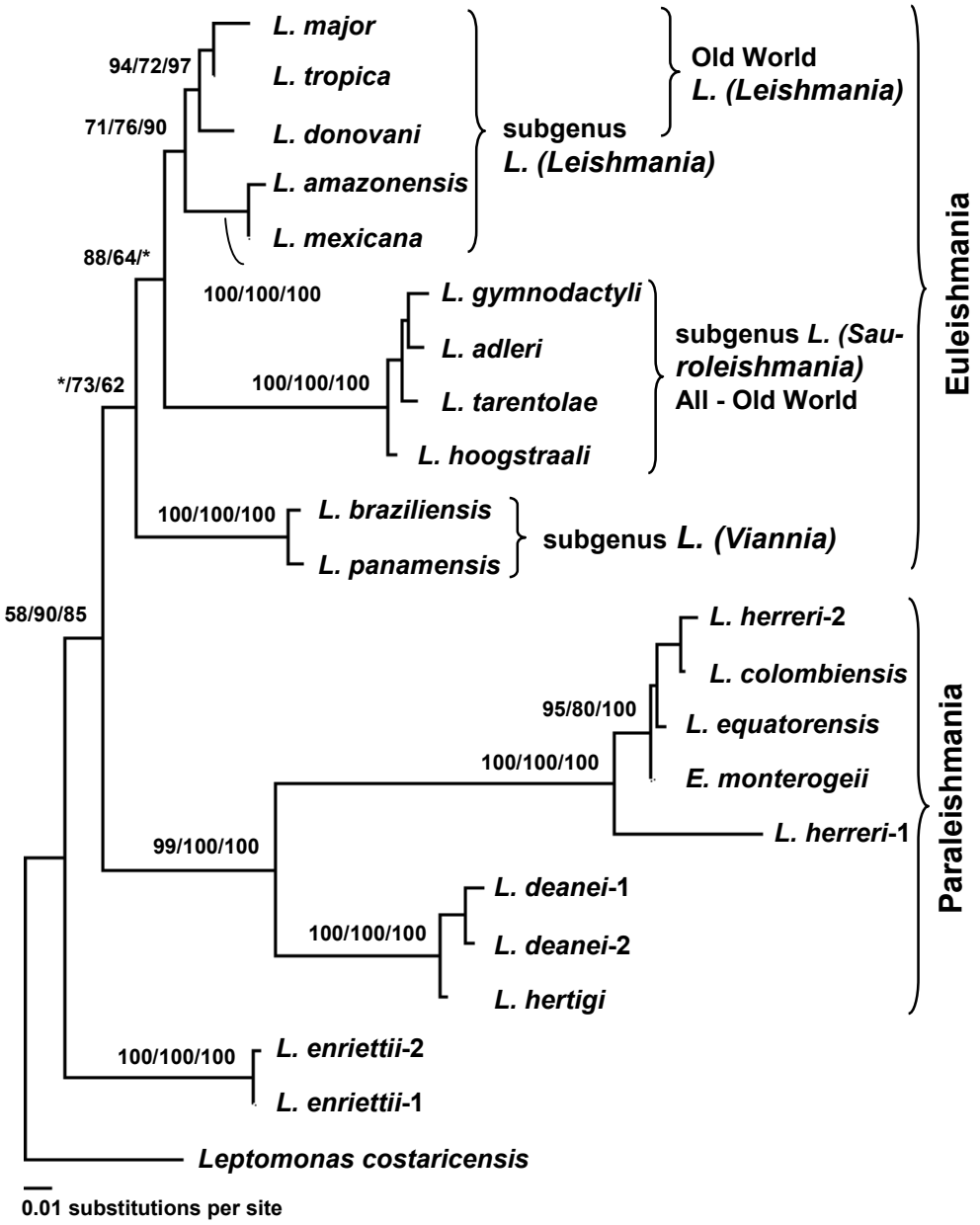
Yurchenko et al. Figs. 1 – 11



Yurchenko et al. Fig. 12



Yurchenko et al. Fig. 13



Yurchenko et al. Fig. 14