Short communication

*Leishmania herreri* (Kinetoplastida; Trypanosomatidae) is more closely related to *Endotrypanum* (Kinetoplastida; Trypanosomatidae) than to *Leishmania*¹

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Since *L. herreri* was first described in 1979 [1], no further isolates have been identified and some doubts have been expressed as to the classification of this parasite. The 16 original strains of *L. herreri* were isolated from sloths (*Bradypus griseus* and *Choloepus hoffmanni*) and sandflies (*Lutzomyia trupidoi*, *Lu. ylephiletor* and *Lu. Shannoni*) in Costa Rica. These parasites were classified with the *Leishmania* principally because they develop as intracellular amastigotes in vitro in primary hamster embryo tissue. They also produce transitory amastigotes (<48 h) at the site of inoculation into hamsters. *L. herreri* were allocated to incertae sedis in a later revision of the *Leishmania* due to the very low nuclear DNA buoyant density of these parasites and the development of sphaeromastigotes in hamster lesions [2].

We have examined the three survivors of the original *L. herreri* isolates, which were deposited in the cryobank at the Liverpool School of Tropical Medicine. A number of methods were used to determine their relationship both to *Leishmania* and to *Endotrypanum* reference strains.

Strains LV341 and LV344 were inoculated into CD1 mouse peritoneal exudate macrophage cultures at a ratio of 10:1 and incubated at 37°C for 24, 48 and 72 h. Both LV341 and LV344 were capable of infecting some macrophages but the infection rate was less than 0.1%, and the only evidence of intracellular multiplication was a single macrophage infected with eight LV341 amastigotes. DNA prepared by standard methods

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from these strains was subjected to PCR with L. braziliensis complex specific primers [4], to determine if the low rate of infection was caused by contamination with L. braziliensis or L. panamensis parasites, since these are also found in Costa Rica. Neither of these species were detected.

The 16S SSU rRNA gene has been used for the identification or classification of all genera of Trypanosomatidae except Rhynchoidomonas [5,6]. In order to develop a method for identifying trypanosomatid genera, phylogenies were compiled from different regions of the SSU rRNA gene until a 561-bp variable region (corresponding to bases 901–1461 of the L. donovani GB:X07773 sequence) was found. When this 561-bp variable region was analyzed using the DNAPARS program in PHYLIP version 3.5c (J. Felsenstein, Dept. of Genetics SK-50, University of Washington, Seattle, Washington 98195, USA) it generated a dendrogram of trypanosomatid genera with a topology in general agreement with published phylogenies of the whole gene [6,7].

The sequence of this variable region of the SSU rRNA gene of LV344 was obtained and a dendrogram was prepared to show the relationship of this sequence with the homologous region of other trypanosomatids (Fig. 1). A matrix of Kimura distances was also prepared from the aligned sequences using the DNADIST programme in PHYLIP. LV344 was found to be most closely related to E. monterogei, an intraerythrocytic parasite of sloths and sandflies (Kimura distance 0.0082), whilst the distance between LV344 and the Leishmania strains was between 0.0440 and 0.0482. LV344 was less closely related to C. fasciculata (0.3488 and 0.3395). This confirmed a recent study of the polymerase II gene of Leishmania which indicated that LV344 was more closely related to E. monterogei (LV88) than to Leishmania [8].

RFLPs of the complete PCR amplified SSU rRNA gene with HaeIII, HpaII, Rsal, Sau961, and of the partial SSU rRNA gene with HhaI and AluI showed that LV344, LV341 and LV342 all had the same restriction pattern with these enzymes. The restriction pattern of the putative L. herreri strains was identical to that produced by E. schaudinni and E. monterogei, the only two named species of Endotrypanum, but different from the pattern produced by Leishmania with all these enzymes (not shown). HaeIII and HpaII digests of the 561-bp region of the SSU rRNA gene provided the clearest markers for the Leishmania and Endotrypanum genera (Fig. 2a). In addition to the species shown in Fig. 2a the following species also showed the same Leishmania specific fingerprint L. infantum, L. donovani, L. major, L. tarentolae, L. hoogstraali, L. gymnodactyli and L. adleri when the 561 bp fragment amplified by primers SSU561F and SSU561R was digested with HaeIII. The only Leishmania species that differed from other members of the genus were L. hertigi and L. deanei (LV42 and LV402) (Fig. 2a).

Since the Kimura distance between E. monterogei (LV88) and LV344 (0.0082) was greater than that between any of the Leishmania species
Fig. 2. (A) HaeIII digests of the 561-bp variable region of the SSU rRNA gene of *Endotrypanum* and *Leishmania* species. The 561-bp fragment was amplified using primers SSU561F and SSU561R and 10 μl of crude PCR product was digested with HaeIII. Five microliters of each digest was loaded onto 6% 29:1 polyacrylamide:bisacrylamide gel. The gel was silver stained [13]. The *L. herreri* strains LV341, LV342 and LV344 were compared with *E. monterogeii* (LV88); *E. schaudinni* (LV58 and LV59); *L. hertigi* (LV42); *L. deanei* (LV402); *L. braziliensis* (LbV); *L. panamensis* (004); *L. mexicana* (M379); *L. amazonensis* (LV78). Products at 186 and 260 bp were in all *L. herreri* and *Endotrypanum* strains tested. Products at 171 bp and 212 bp were found in all *Leishmania* species tested except *L. hertigi* and *L. deanei* which were characterised by products at 171 and 286. (B) Agarose gel (1.5%) of RAPD products amplified with primer M13 [14]. Test strains LV344, LV342 and LV341 were compared with *E. monterogeii* (LV88) and *E. schaudinni* (LV58 and LV59) strains.

used (0.0000–0.0040), LV344 may represent a new species of *Endotrypanum*. However, the Kimura distance between the two *T. cruzi* strains used was 0.0712, which was greater than the distance between *E. monterogeii* and the *Leishmania* species (0.0412–0.0482), consequently the Kimura distance between species may not provide a suitable threshold for the establishment of new species.

Previous studies using DNA buoyant densities and RFLPs of whole genomic DNA have identified three groups (A, B and C) within the genus *Endotrypanum* [9,10]. The buoyant density of the nuclear and kinetoplast DNA of LV341 was recalculated from traces prepared when the parasites were first received using standard methods [11] and found to be 1.712 g ml⁻¹ (nDNA) and 1.698 g ml⁻¹ (kDNA), these values are very similar to those reported for *E. monterogeii* (LV88) and *E. schaudinni* (LV58) — 1.712 g ml⁻¹ (nDNA) and 1.697 g ml⁻¹ kDNA [9]. LV58 and LV88 are both group B strains and consequently LV341 may also be a group B strain. LV342 and LV341 were very similar each other by RAPD and more similar to *E. schaudinni* (LV58) and *E. monterogeii* (LV88) than to LV344 or *E. schaudinni* (LV59) (Fig. 2b). The RAPD data was consistent with the pattern of cross hybridisation found using kDNA variable region probes prepared by the method of Bozza et al. [15]. A LV341 kDNA variable region probe hybridised with almost equal intensity to 1 ng of LV342 and LV341 kDNA but not to 1 ng of LV344 or to any *Endotrypanum* strain, and a LV344 kDNA variable region probe hybridised strongly to LV344 kDNA and more weakly but equally to all other *Endotrypanum* and *L. herreri* strains used (not shown). The group affiliation of LV344 could not be determined. However, LV344 may not belong to group B given the relatively large Kimura distance between this strain and LV88 (0.0082) compared with the distance between *Leishmania* sub-genera (< 0.0041). As LV341 and LV342 appear to be more closely related to *E. monterogeii*
(LV88) and E. schaudinni (LV58) than they are to LV344, these three L. herreri strains do not form a monophyletic group and cannot all be included in a new Endotrypanum species should it be found appropriate to create one.

The nDNA and kDNA buoyant densities of LV341 have not been published previously but in the original description of L. herreri it was reported that we had described the buoyant densities as being unlike any other Leishmania or Endotrypanum. It is not clear how this mistake arose.

E. schaudinni develops as an epimastigote in the sloth erythrocyte and is found in tropical forests in South and Central America whilst E. monterogeii develops as a trypomastigote and is found in temperate forests in Costa Rica [12]. It is, therefore, surprising that E. monterogeii and E. schaudinni strains are both in the same group. LV88 is the only E. monterogeii strain that has been characterised by any method except morphology. Consequently it is possible that the apparent contradiction between the morphological and DNA based classifications is due to cross contamination or mislabelling of strains. Since L. herreri has been isolated in both temperate and tropical regions in Costa Rica it would be unwise to re-classify this species until it can be compared with fresh isolates of E. monterogeii and E. schaudinni that have been identified by any method except morphology.

The development of amastigotes in vitro by Endotrypanum spp. does not appear to have been reported and attempts to infect macrophages in vitro with confirmed Endotrypanum strains have failed [1]. The ability to infect macrophages in vitro is not unique to Leishmania since the distantly related trypansomatid T. cruzi also forms amastigotes in vitro and consequently this ability may have evolved independently on various occasions in the Trypanosomatidae. Other trypanosomatids may also be capable of transforming into intracellular amastigotes, a capacity which would have been central to the transition from a monogenetic to digenetic lifecycle, a transition that may have occurred on as many as four different occasions [5]. The strains studied are listed in Table 1.

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References


