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NOTE *Parasitology*

Identification of *Endotrypanum* Species from a Sloth, a Squirrel and *Lutzomyia* Sandflies in Ecuador by PCR Amplification and Sequencing of the Mini-Exon Gene

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ABSTRACT. PCR amplification and nucleotide sequencing of the mini-exon gene revealed that four strains isolated from a sloth (*Choloepus hoffmanni*), a squirrel (*Sciurus granatensis*) and two sandflies (*Lutzomyia hartmanni*) in Ecuador were indistinguishable from *Endotrypanum monterogeii*. Another strain isolated from *Lu. hartmanni* showed the high sequence similarity to *E. schaudinni*. Since three of these strains have been previously identified as *Leishmania (Viannia) equatorensis*, the results demonstrate that *L. (V.) equatorensis* is genetically closely related to the genus *Endotrypanum*. The present study also indicates that *Endotrypanum* species are distributed in arboreal animals and sandflies in Ecuador, and that mini-exon gene amplification is useful for epidemiological studies of *Leishmania* and *Endotrypanum* in the New World.

KEY WORDS: *Endotrypanum*, *Leishmania*, mini-exon gene.

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The genus *Endotrypanum* is known as a trypanosomatid parasite of the sloths, which is transmitted by sandfly vectors, in South and Central America [14]. The *Endotrypanum* parasites are found within the erythrocytes of two-toed sloths of the genus *Choloepus* and three-toed sloths of the genus *Bradypus*. Currently, only two species of *Endotrypanum schaudinni* and *E. monterogeii* have been described [12, 14], and the intraerythrocytic forms of these species assume trypomastigotes and epimastigotes, respectively. However, erythrocytic parasites are rarely seen in naturally infected sloths. Parasites from *in vitro* culture of the blood of infected sloths and from the alimentary tract of *Lutzomyia* sandflies are promastigotes, which are indistinguishable from the *Leishmania* promastigotes. Sloths also play a role as reservoirs of *Leishmania (Viannia) braziliensis, L. (V.) guyanensis* and *L. (V.) panamensis* [6], which are also transmitted by sandfly vectors. Therefore, the correct classification of *Leishmania* and *Endotrypanum* is particularly important for epidemiological studies on reservoir hosts and sandfly vectors of leishmaniasis in the New World.

We have developed a PCR method based on amplification of the *Leishmania* mini-exon gene for the diagnosis of visceral and cutaneous leishmaniasis [10, 11]. Mini-exon gene or spliced leader RNA gene is present in all trypanosomatid parasites, including vertebrate-infecting genera of *Trypanosoma, Leishmania* and *Endotrypanum*, but is absent from mammalian hosts and sandfly vectors [4, 5]. The mini-exon gene is present as tandem repeated 100–200 copies in the parasite nuclear genome. The gene consists of transcribed and non-transcribed spacer regions. The transcribed region contains exon and intron sequences. In *Leishmania*, the exon sequence consists of 39 nucleotides, which is trans-spliced to nuclear derived RNAs, and is common to all *Leishmania* species. The intron sequence consists of 55 to 57 nucleotides, which is spliced out during the trans-splicing procedure, and is highly conserved [1]. However, the non-transcribed spacer region is distinct in length and in sequence among different *Leishmania* species. The size of the mini-exon gene was approximately 0.25 kb in *L. (V.) braziliensis, L. (V.) guyanensis* and *L. (V.) panamensis*, 0.3 kb in *L. (Leishmania) mexicana* and *L. (L.) amazonensis*, and 0.45 kb in *L. (L.) donovani, L. (L.) infantum, L. (L.) chagasi, L. (L.) tropica, L. (L.) major* and *L. (L.) aetiopica*, respectively [5, 11]. With respect to *E. schaudinni* and *Endotrypanum* isolates, the size of mini-exon gene was about 0.45 kb, and the 39-nucleotide exon sequence was identical to that of *Leishmania* [4].

In our long-term epidemiological study of leishmaniasis in Ecuador, we have characterized a total of 125 *Leishmania* isolates from patients, reservoir hosts and sandfly vectors between 1982 and 1995. By monoclonal antibody reactivity, these stocks were classified into seven species, such as *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis, L. (L.) mexicana, L. (L.) amazonensis, L. (L.) major*-like and *L. (V.) equatorensis* [7]. Among these stocks, two parasite strains isolated from a sloth (*Choloepus hoffmanni*) and a squirrel (*Sciurus granatensis*) in 1983 were distinguishable from other known *Leishmania* species by monoclonal antibody reactivity, isoenzyme electrophoresis profiles and restriction enzyme fragment patterns of kinetoplast DNA (kDNA). Thereby, the new name of *L. (V.) equatorensis* sp.

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Table 1. *Leishmania* and *Endotrypanum* strains used in the present study

International Code[a)]	Stain abbreviation	Species	Source[b)]
MHOM/BR75/M4147	M4147	*L. (V.) guyanensis*	1
MHOM/EC/87/G-05	G-05	*L. (V.) panamensis*	2
MCHO/EC/82/Lsp-1	Lsp-1	*L. (V.) equatorensis*	2
MSCI/EC/82/Lsp-2	Lsp-2	*L. (V.) equatorensis*	2
IHAR/EC/93/OC-2	OC-2	*L.* sp	2
IHAR/EC/93/OC-3	OC-3	*L.* sp	2
IHAR/EC/93/OC-4	OC-4	*L. (V.) equatorensis*	2
MCHO/CR/62/A9	A9	*E. monterogeii*	3
MCHO/BR/80/M6159	E	*E. schaudinni*	3
MBRA/PA/00/415P0	LV-58	*E.* sp	3
MBRA/PA/81/1222P82	LV-59	*E.* sp	3

a) M, Mammalia; I, Insecta; HOM, *Homo*; CHO, *Choloepus*; SCI, *Sciurus*; HAR, *Lutzomyia hartmanni*; BRA, *Bradypus*; BR, Brazil; EC, Ecuador; CR, Costa Rica; PA, Panama. b) 1, WHO reference strain; 2, the present study [7, 8]; 3, J. J. Shaw, The Wellcome Parasitology Unit, Institute Evandro Chagas, Belem, Para, Brazil.

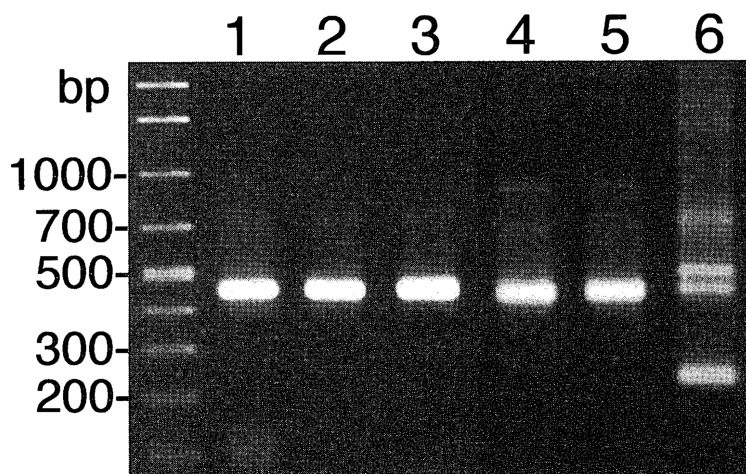

Fig. 1. PCR amplification of the mini-exon genes for *Leishmania* and *Endotrypanum* species. 1, *L. (V.) equatorensis* (Lsp-1); 2, *L. (V.) equatorensis* (Lsp-2); 3, *L.* sp (OC-2); 4, *L.* sp (OC-3); 5, *L. (V.) equatorensis* (OC-4); 6, *L. (V.) panamensis* (G-05).

n. was proposed for these parasites [9]. In addition, three isolates isolated from *Lu. hartmanni* in 1993 were also markedly different from *Leishmania* species. Although one of them was identified as *L. (V.) equatorensis*, the other two isolates are still unidentified [8].

In the present study, we amplified and determined nucleotide sequences of the mini-exon genes for three strains of *L. (V.) equatorensis* and unidentified two strains from sandflies, in comparison with those of *Endotrypanum* species. Parasites used in this study are listed in Table 1. DNA isolation and PCR assay was performed as described previously [10, 11]. In short, promastigotes of the parasites were grown in Schneider's *Drosophila* medium at 26°C. Parasite DNA was extracted using a DNA extraction kit (Blood & Cell culture DNA Kit, QIAGEN Inc., Chatsworth, CA). PCR amplification was carried out in a reaction mixture of 20 μl, containing 10 mM Tris-HCl (pH 9.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of four kinds of deoxynucleoside triphosphates, 10% dimethylsulfoxide, 0.5 μM primers, 20 *ng* parasite DNA and 0.05 units/μl *Taq* DNA polymerase (Amersham Pharmacia Biotech Ltd., Uppsala, Sweden). Primers used were S-1629 (5'-gggaattCAATAT/AAGTACAGAAACTG) and S-1630 (5'-gggaagcTTCTGTACTT/ATATTGGTA) [4, 10, 11], in which lowercase letters indicate non-complementary bases. Using a thermocycler (Perkin-Elmer Corp., Norwalk, CT), the samples were denatured at 95°C for 5 min and were then subjected to 35 cycles of 95°C for 1 min, 50°C for 30 s and 72°C for 1 min. After the final extension at 72°C for 10 min, PCR products in 10-μl aliquots were electrophoresed in 2% agarose in 1x TAE (40 mM Tris-acetate and 1 mM EDTA) at 100 V. The gels were stained with 0.5 μg/m*l* of ethidium bromide and photographed.

As shown in Fig. 1, amplified PCR products from three strains of *L. (V.) equatorensis* (Lsp1, Lsp2 and OC-4) and two unidentified strains (OC-2 and OC-3) (Fig. 1, lanes 1–5)

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A9     GTATGCGAAA CTTCCGGAAA CCCTATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTACA TTTTTTTTTT
LV-58  GTATGCGAAA CTTCCGGAAA CCCTATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTACA TTTTTTTTTT
Lsp-1  GTATGCGAAA CTTCCGGAAA CC-TATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTACA TTTTTTTTTT
Lsp-2  GTATGCGAAA CTTCCGGAAA CC-TATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTACA TTTTTTTTTT
OC-3   GTATGCGAAA CTTCCGGAAA CC-TATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTATA TTTTTTTTTT
OC-4   GTATGCGAAA CTTCCGGAAA CC-TATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTACA TTTTTTTTTT
E      GTATGCGAAA CTTCCGGAAT CCGTATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTACA TTTTTTTTTT
LV-59  GTATGCGAAA CTTCCGGAAT CCGTATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCT--A TTTTTTTTTT
OC-2   GTATGCGAAA CTTCCGGAAT CCGTATTCCG GCAAGATTTT GGAAGCGCGC AAGCGCTAT- TTTTTTTTGT  70

A9     T--GTCATGT GCGGGTGCGC GCCGCCCCTC CTCCACTGGG GGGGG----C TTCCCACACG CGCTGTGGTC
LV-58  TTTGTCATGT GCGGGTGCGC GCCGCCCCTC CTCCACTGGG GGGGG----C TTCCCACACG CGCTGTGGTC
Lsp-1  --TGTCATGT GCGGGTGCGC GCCGCCCCTC CTCCACTGGG GGGGGGG-CC TTCCCACACG CGCTGTGGTC
Lsp-2  ---GTCATGT GCGGGTGCGC GCCGCCCCTC CTCCACTGGG GGGGGGG-CC TTCCCACACG CGCTGTGGTC
OC-3   --TGTCATGT GCGGGTGCGC GCCGCCCCTC CTCCACTGGG GGGGGGG-CC TTCCCACACG CGCTGTGGTC
OC-4   ---GTCATGT GCGGGTGCGC GCCGCCCCTC CTCCACTGGG GGGGGGGGCC TTCCCACACG CGCTGTGGTC
E      ---GTCATGT GTGAGTGTGC GCCCCCCCTC CTCCACCGGG GGG---GGTC TTCCCACGCG CGCGGTGGTC
LV-59  ---GTCATGT GTGAGTGTGC GCCCCCCCTC CTCCACCGGG GGG---GGTC TTCCCACGCG CGCGGTGGTC
OC-2   ---GTCATGT GTGAGTGTGC GCCCCCCCTC CTCCACCGGG GGG---GGTC TTCCCACGCG CGCTGTGGTC 140

A9     CCTTTTCCCC TTGCTGCTGG GAGGGGGGG- -CGCGCCGCT GCGGGTGGTT TCCTCCTGTC GGCGTCCCCT
LV-58  CCTTTTCCCC TTGCTGCTGG GAGGGGGGGG GCGCGCCGCT GCGGGTGGTT TCCTCCTGTC GGCGTCCCCT
Lsp-1  CCTTTTCCCC TTGCTGCTGG GAGGGGGGGG GCGCGCCGCT GCGGGTGGTT TCCTCCTGTC GGCGTCCCCT
Lsp-2  CCTTTTCCCC TTGCTGCTGG GAGGGGGGGG GCGCGCCGCT GCGGGTGGTT TCCTCCTGTC GGCGTCCCCT
OC-3   CCTTTTCCCC TTGCTGCTGG GAGGGGGGGG GCGCGCCGCT GCGGGTGGTT TCCTCCTGTC GGCGTCCCCT
OC-4   CCTTTTCCCC TTGCTGCTGG GAGGGGGGGG GCGCGCCGCT GCGGGTGGTT TCCTCCTGTC GGCGTCCCCT
E      TCTTTTTCCC TTGCTGCTGG GCCGGGGGGG -CGCGCCGCT GCGGGTGTTC TCCTCTTGTC GGCGCCCCCT
LV-59  TCTTTTTCCC TTGCTGCTGG GCCGGGGGGG -CGCGCCGCT GCGGGTGTTC TCCTCTTGTC GGCGCCCCCT
OC-2   TCTTTTTCCC TTGCTGCTGG GCCGGGGGGG GCGCGCCGGT GCGGGTGTTT TCCTCTTGTC GGCGCCCCCT 210

A9     CGGGCCTGCC CGGCATCCTT GCTGGGGGCT CTGCCGGCGC GATTCCGGCA CCCCATGCCC AGTGGCCGGG
LV-58  CGGGCCTGCC CGGCATCCTT GCTGGGGGCT CTGCCGGCGC GATTCCGGCA CCCCATGCCC AGTGGCCGGG
Lsp-1  CGGGCCTGCC CGGCATCCTT GCTGGGGGCT CTGACGGCGC GATTCCGGCA CCCCATGCCC AGTGGCCGGT
Lsp-2  CGGGCCTGCC CGGCATCCTT GCTGGGGGCT CTGACGGCGC GATTCCGGCA CCCCATGCCC AGTGGCCGGT
OC-3   CGGGCCTGCC CGGCATCCTT GCTGGGGGCT CTGACGGCGC GATTCCGGCA CCCCATGCCC AGTGGCCGGT
OC-4   CGGGCCTGCC CGGCATCCTT GCTGGGGGCT CTGACGGCGC GATTCCGGCA CCCCATGCCC AGTGGCCGGT
E      CGGGCCTGCC TGGCCTCCTT GCAAGGGGCC CTGC--ATGC GCACCCGGCA CCCCGCGCAC GGTGGCCGGG
LV-59  CGGGCCTGCC TGGCCTCCTT GCAAGGGGCC CTGC--ATGC GCACCCGGCA CCCCGCGCAC GGTGGCCGGG
OC-2   CGCGCCCGCC CCGCCTCCTT GCAAGGGGCC CTAC--GTGC GCTCCCGGCA CCCCGCGCAC AGTGGCCGGG 280

A9     ACCCCCCC-C TCTGGTCCCC CCCCC---AT GATCCGGCGC ACGCCTGACA CGCGGTTAGT GCATGCCAAA
LV-58  ACCCCCCC-C TCTGGTCCCC CCCCCCC-AT GATCCGGCGC ACGCCTGACA CGCGGTTAGT GCATGCCAAA
Lsp-1  CCCCCCCCTC TCTGGTCCCC CCCCCCCCAT GATCCGGCGC ACGCCTGACA CGCGGTTAGT GCATGCCAAA
Lsp-2  CCCCCCCCCC TCTGGTCCCC CCCCCCC-AT GATCCGGCGC ACGCCTGACA CGCGGTTAGT GCATGCCAAA
OC-3   CCCCCCCC-C TCTGGTCCCC CCCCC--CAT GATCCGGCGC ACGCCTGACA CGCGGTTAGT GCATGCCAAA
OC-4   CCCCCCCCCC TCTGGTCCCC CCCCC--CAT GATCCGGCGC ACGCCTGACA CGCGGTTAGT GCATGCCAAA
E      ACCCCCC-TA TCCCCCCCCC CCCCCTCCTT GGTCCGGCGC ACGCCTGACA CGCGGTTAAA GCATGCCAAA
LV-59  ACCCCCC-TA TCCCCCCCCC CCC-----TT GGTCCGGCGC ACGCCTGACA CGCGGTTAAA GCATGCCAAA
OC-2   GACCCCC-CC TATCCCCCCC CTCCC-CCGT GGTCCGGCGC ACGCCTGACA CGCGGATAAA GCATGCCAAA 350

A9     TGAGCCACCC CCCTCAGACC CACTTGGCAT GTGTTAACTT TT........
LV-58  TGAGCCACCC CCCTCAGACC CACTTGGCAT GTGTTAACTT TT........
Lsp-1  TGAGCCACCC CACTCAGACC CACTTGGCAT GTGTTAACTT TT........
Lsp-2  TGAGCCACCC CCCTCAGACC CACTTGGCAT GTGTTAACTT TT........
OC-3   TGAGCCACCC CCCTCAGACC CACTTGGCAT GTGTTAACTT TT........
OC-4   TGAGCCACCC CCCTCAGACC CACTTGGCAT GTGTTAACTT CT........
E      AAAGCCACCC CCCTCAGACC CACTTGGCAT GTGTAAACTT TT........
LV-59  AAAGCCACCC CCCTCAGACC CACTTGGCAT GTGTAAACTT TT........
OC-2   AAAGCCACCC CCCTCAGACC CACTTGGCAT GTGTAAACTT TC........
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Fig. 2. Alignment of intron and non-transcribed spacer sequences of the mini-exon genes for *Leishmania* and *Endotrypanum* species. The sequences are derived from PCR products of *E. monterogeii* (A9, DDBJ accession number AB092602), *E.* sp. (LV-58, AB092600), *L. (V.) equatorensis* (Lsp-1, AB092595), *L. (V.) equatorensis* (Lsp-2, AB092596), *L.* sp (OC-3, AB092598), *L. (V.) equatorensis* (OC-4, AB092599), *E. schaudinni* (E, AB092603), *E.* sp (LV-59, AB092601) and *L.* sp (OC-2, AB092597). The alignment was made with DNA-SIS-Mac v.3.4 and identical nucleotides at the same position are highlighted.

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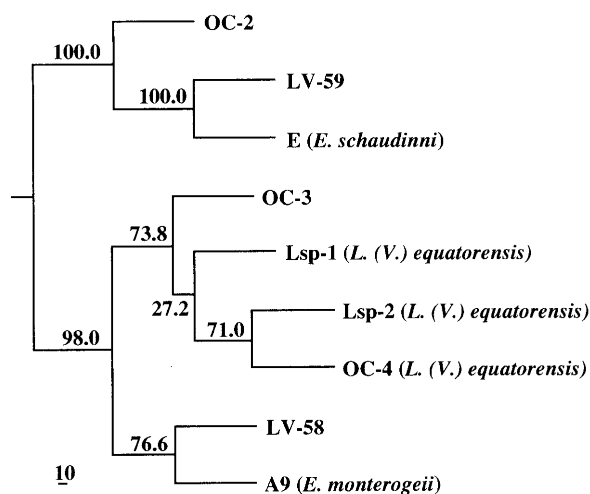

Fig. 3. The parsimonious tree of *Endotrypanum* species inferred from mini-exon gene. The number at each branching point indicates the bootstrap percentage based on 100 replicates.

were almost identical in size. The size was approximately 450 bp, which was in agreement with that reported with *Endotrypanum* species [4]. PCR products of a similar size were also detected with DNA samples from strains A9, E, LV-58 and LV-59 (data not shown). Whereas, approximately 250-bp amplification products were generated from DNA samples of *L. (V.) panamensis* (G-05) (Fig. 1, lane 6) and *L. (V.) guyanensis* (M4147) (data not shown), as reported previously [11]. Since the mini-exon gene is tandem repeated copies, one unit or multimeric forms of the mini-exon gene can be amplified [4, 5, 10]. Approximately 900-bp PCR products corresponding to dimeric forms were clearly observed in strains OC-3 and OC-4 (Fig. 1, lanes 4 and 5). PCR products corresponding to dimeric forms of about 500 bp and trimeric forms of 750 bp were observed in strain G-05 (Fig. 1, lane 6), although some additional DNA bands were also generated.

The PCR products were size-excised from agarose gels and cloned into the pCR2.1 vector (TA-cloning kit, Invitrogen Corp., Carlsbad, CA). Several clones were isolated for each parasite strain, and one of the clones was performed for nucleotide sequencing for both strands by the dideoxy termination method (PE Applied Biosystems, Warrington, UK). Alignment of intron and spacer sequences (375–388 nucleotides) of the mini-exon genes of nine strains is shown in Fig. 2. Phylogenetic analysis was performed using the Dnapars in the PHYLIP phylogeny package, which implements the parsimony method for DNA sequences. The parsimony phylogram of *Endotrypanum* species is shown in Fig. 3, which is the majority-rule bootstrap consensus tree. The bootstrap percentage was obtained from 100 replicates using the Seqboot and Consense program in the PHYLIP package. The mini-exon gene sequence of the *L. (L.) chagasi* (GenBank accession number X69446) were taken as an out-group for this analysis. DNA sequence data revealed that these strains were divided into two groups. The first group includes strains A9, LV-58, Lsp-1, Lsp-2, OC-3 and OC-4, and the DNA sequences were 96–99% identical to each other. Since strain A9 is a representative strain of *E. monterogeii*, strains Lsp-1, Lsp-2 and OC-4, which were previously identified as *L. (V.) equatorensis*, and unidentified OC-3 are probably variants of *E. monterogeii.* The second group contains strains E, LV-59 and OC-2, and these strains showed 93–98% nucleotide sequence identity. Strains LV-59 and OC-2 are most likely variants of *E. schaudinni*, since strain E is a representative strain of this species. The overall mini-exon gene sequences between the first group of *E. monterogeii* and the second group of *E. schaudinni* showed 84–86% identity. We also determined the nucleotide sequence (223 nucleotides) of the PCR-amplified mini-exon gene of *L. (V.) panamensis* G-05 (DDBJ accession no. AB093592), showing only 46–53% identity to those of *Endotrypanum* strains described above.

The findings of the present study clearly suggest that PCR amplification of the mini-exon gene provides a useful method to distinguish *Leishmania* from *Endotrypanum* in samples from sloths and sandflies. Sloths are known as important reservoirs of human-pathogenic parasites of *Leishmania (Viannia)* species and *Trypanosoma cruzi* [6]. This study also demonstrated the geographical distribution of *Endotrypanum* species in the sloth (*C. hoffmanni*) and squirrel (*S. granatensis*) as well as the sandfly (*Lu. hartmanni*) in Ecuador. As far as we know, *Endotrypanum* infection in animals other than sloths has not previously been reported. In Brazil, *Endotrypanum* parasites have been detected in *Lu. shannoni, Lu. umbratilis* and *Lu. anduzei* by microscopic examination or by kDNA probes [2, 13]. Based on this study *Lu. hartmanni* in Ecuador should be included in a list of natural sandfly vectors of *Endotrypanum.*

Trypanosomatid parasites are very complicated organisms in their classification. Representative strains and species of the parasites were recently re-examined by sequencing of the DNA polymerase, RNA polymerase and small subunit rRNA genes, and by isoenzyme electrophoresis analyses [3]. The results strongly suggested that several *Leishmania* species were phylogenetically more closely related to *Endotrypanum* than to the other species of *Leishmania* [3]. These species include *L. (V.) equatorensis, L. (L.) hertigi* isolated form sloths, *L. (L.) deanei* and *L. (L.) hertigi* from porcupines (*Coendu* spp.), and *L. (V.) colombiensis*, which has been isolated from various hosts, including humans. Furthermore, considerable genetical diversity among *Endotrypanum* isolates has been demonstrated using various molecular techniques [6]. Since only two species of *E. schaudinni* and *E. monterogeii* have been described at present, the genus *Endotrypanum* may be subdivided into several species or re-classified in the future. The present sequence data of the mini-exon gene showed that *L. (V.) equatorensis* is most likely a parasite within the genus *Endotrypanum*. We also observed extreme similarity between *L. (V.) equatorensis* (Lsp-1 and Lsp-2) and *E.*

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monterogeii (A9) using different PCR analyses (unpublished data). However, demonstration of intraerythrocytic trypomastigotes or epimastigotes from sloths experimentally infected with *L. (V.) equatorensis*, might be required to establish taxonomical status within the genus *Endotrypanum*.

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