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Establishment of a Mass Screening Method of Sand Fly Vectors for *Leishmania* Infection by Molecular Biological Methods

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Abstract. Surveillance of the prevalence of *Leishmania* and its vector, sand fly species, in endemic and surrounding areas is important for prediction of the risk and expansion of leishmaniasis. In this study, a method for the mass screening of sand flies for *Leishmania* infection was established. This method was applied to 319 field-captured specimens, and 5 positive sand flies were detected. Sand fly species were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of the18S rRNA gene, and all the positive flies were *Lu. hartmanni*. Furthermore, cytochrome *b* (*Cyt* b) gene sequence analyses identified all the parasites as *Endotrypanum* species including a probable novel species. Because the method requires minimum effort and can process a large number of samples at once, it will be a powerful tool for studying the epidemiology of leishmaniasis.

INTRODUCTION

Leishmaniasis is a protozoan disease caused by the genus *Leishmania.* It is distributed worldwide, especially in tropical and subtropical areas, and affects at least 12 million people. More than 20 species of *Leishmania* are described as causative of human leishmaniases, and clinical features are largely associated with the species.[1,2] Thus, identification of the parasite species in endemic areas is important for both appropriate treatment and estimation of the prognosis. Female phlebotomine sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World are the vectors of *Leishmania* protozoa.[3] The spread of leishmaniases depends on the distribution of the vectors and reservoir animals. Nearly 1,000 sand fly species has been described, but only a few are medically important.[3] In addition, it has been suggested that only a restricted number of sand fly species can support the development of specific species of *Leishmania* and consequently transmit them. Thus, the detection of *Leishmania* species within sand flies and identification of both *Leishmania* and sand fly species is important for prediction of the risk and expansion of the disease in endemic and surrounding areas.

The infection of sand flies with *Leishmania* promastigotes has usually been examined by dissecting individual sand flies under a microscope. The sand flies should be fresh, and considerable skill and expertise is needed for the study of tiny individuals. Although the procedure takes a relatively long time, a large number of specimens have to be examined to obtain informative data for each area, because the rate of infection of sand flies with *Leishmania* is generally very low (0.01–1%), even in endemic areas.[4,5] Similarly, sand fly species were identified principally based on morphologic characteristics: mainly internal structures, such as the spermatheca, cibarium, and pharynx for females and terminal genitalia for males. This method requires refined storage conditions for samples, a highly skilled technique, and taxonomic expertise. Thus, the development of alternative ways that can process a large number of specimens with limited effort is awaited. Currently, molecular biologic techniques are used for the detection and identification of *Leishmania* species in sand flies and patient specimens.[6–8] In our recent study, a method of detecting *Leishmania* protozoa within naturally infected individual sand flies by polymerase chain reaction (PCR) with minicircle kinetoplast DNA-specific primers was established.[9] The method is easy and sensitive; however, several steps were needed for the preparation of template DNA samples, and thus improvements were essential for practical use in the mass screening of sand fly vectors.

In this study, a method of mass screening sand fly vectors for *Leishmania* infection was established. The method was applied to 319 field-captured specimens and its usability was confirmed. In addition, the sand fly species were identified by PCR- restriction fragment length polymorphism (RFLP) of the 18S rRNA gene using the same specimens. The method requires minimum effort and thus will be a powerful tool for research on prevalent sand fly species and the relationships between *Leishmania* species and the vectors.

MATERIALS AND METHODS

Parasites. A WHO reference strain of *L.* (*Leishmania*) *major* (MHOM/SU/73/5ASKH) was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Cansera International, Ontario, Canada), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 25°C.

Sand fly collection. Sand flies were collected on August 2006 in endemic areas of Ecuador where cutaneous leishmaniasis caused by *L.* (*Viannia*) *panamensis* and *L.* (*V.*) *guyanensis* is prevalent.[4,5,10,11] The sites were as follows:

Portoviejo (Province of Manabi): San Sebastian (Province of Manabi) and surrounding areas (1°20′ S, 80°05′ W), ~80 km from Potoviejo city, at an altitude of 600 m. Collections using CDC light traps were made in a banana and cacao plantation.

Piedrero (Province of Cañar): Piedrero (2°50′ S, 79°20′ W), ~20 km from La Troncal city (Province of Cañar), at an

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altitude of 500 m. Collections using CDC light traps were made in a banana and cacao plantation.

Ocaña (Province of Cañar): Ocaña (2°50′ S, 79°10′ W), ~5 km from La Troncal city, at an altitude of 400 m. Collections were made using protected human bait in a subtropical forest.

The sand flies were fixed in 70% ethanol and stored at room temperature. Ethanol-fixed sand flies experimentally infected with *L.* (*L.*) *major* were kindly provided by Dr. Jesus G. Valenzuela (NIH, Rockville, MD).

DNA extraction. For the preparation of parasite DNA, 10,000 parasites were suspended in 50 μL of DNA extraction buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 8.0], 10 mmol/L EDTA, and 0.1% sodium dodecyl sulfate [SDS]) in the presence of proteinase K (200 μg/mL) and serially diluted 10-fold in the same buffer. For the extraction of DNA from sand flies, ethanol-fixed specimens were placed individually in each well of 96-well plates and lysed in 50 μL DNA extraction buffer without homogenization. The samples were incubated at 37°C for 12 hours, 25 μL distilled water was added, and 0.5-μL portions were directly used as the templates for PCR amplification. The DNA samples were stored at –20°C for further use.

Identification of sand fly species. For the identification of sand fly species by PCR-RFLP, PCR amplification was performed with *Lutzomyia* 18S rRNA gene-specific primers.[12,13] The primer sequences were 5′-TGCCAGTAGTTATATGCTTG-3′ (Lu.18S 1S) and 5′-CACCTACGGAAACCTTGTTAC-3′ (Lu.18S AR). PCR was carried out in a volume of 20 μL using the primers (0.4 μmol/L each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and *Taq* polymerase (*Ex Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95°C for 5 minutes, PCR amplification was performed with 40 cycles of denaturation (95°C, 1 minute), annealing (50°C, 1 minute), and polymerization (72°C, 2 minutes), followed by a final extension at 72°C for 10 minutes.

Each PCR product (5 μL) was digested with the restriction enzyme, *Afa*I (Takara Bio) or *Hin*fI (Takara Bio) in 96-well V-bottom plates. The digested samples were separated by electrophoresis in a 3% agarose gel to produce DNA fragments.

Detection and identification of *Leishmania* species. For detection of *Leishmania* parasites within sand flies, PCR was performed with primers specific for *Leishmania* minicircle kinetoplast DNA.[9] The primer sequences were 5′-CTRGGGGTTGGTGTAAAATAG-3′ (L.MC-1S) and 5′-TWTGAACGGGRTTTCTG-3′ (L.MC-1R). PCR was carried out in a volume of 20 μL using the primers (0.4 μmol/L each), Ampdirect Plus (Shimadzu Biotech), and *Taq* polymerase (Nova*Taq* Hot Start DNA Polymerase; Novagen, Darmstadt, Germany). After an initial denaturation at 95°C for 10 minutes, PCR amplification was performed with 35 cycles of denaturation (95°C, 1 minute), annealing (55°C, 1 minute), and polymerization (72°C, 1 minute), followed by a final extension at 72°C for 10 minutes. The PCR products were analyzed on a 2% agarose gel.

For the identification of *Leishmania* species using a molecular biologic method, PCR amplification was performed with primers specific for *Leishmania Cyt* b.[9,14] The primer sequences were 5′-GGTGTAGGTTTTAGTYTAGG-3′ (L.cyt-S) and 5′-CTACAATAAACAAATCATAATATRCAATT-3′ (L.cyt-R). The conditions for PCR amplification were the same as for the *Leishmania* minicircle kinetoplast DNA. The products were electrophoresed on a 2% agarose gel and directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). *Escherichia coli*, JM109 cells, were transformed with the ligation mixture and plated onto LB agar containing ampicillin (50 μg/mL), 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal; 36 μg/mL), and isopropyl β-D-thiogalactoside (IPTG; 40 μg/mL). Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN, Tokyo, Japan). The inserts of the plasmids were sequenced by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Phylogenetic analysis. The *Leishmania* and *Endotrypanum Cyt* b gene sequences were aligned with CLUSTAL W software[15] and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 3.1.[16] Neighbor-joining (NJ) trees were constructed with the distance algorisms available in the MEGA package. The database for phylogenetic analyses consisted of *Cyt* b gene sequences from six prevalent *Leishmania* spp., *L.* (*L.*) *amazonesis*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *major-like*, *L.* (*V.*) *panamensis*, *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis,* in Ecuador and two *Endotrypanum* spp., *E. schaudinni* and *E. monterogeii*.

RESULTS

Sensitivity and specificity of the PCR. For the detection of *Leishmania* within sand flies, a pair of primers specific for *Leishmania* minicircle kinetoplast DNA, which were designed in our recent study,[9] were applied in this study. However, there are many differences between the previous and present assay: 1) with or without homogenization of individual specimens during the extraction of the DNA, 2) the absence or presence of biologic substances and reagent for DNA extraction in each DNA solution, 3) the final volume of each DNA sample (10 versus 75 μL), and 4) the reagent for PCR amplification. Therefore, the sensitivity and specificity of the primers for the detection of *Leishmania* were assessed in this assay. As shown in Figure 1, we could amplify minicircle kinetoplast DNA if only one parasite existed in a sample. In the next step, PCR was performed using sand fly samples infected experimentally with *Leishmania* to test the sensitivity and specificity of the present assay. When amplified with *Leishmania* minicircle kinetoplast DNA-specific primers, a distinct DNA band of ~700 bp corresponding to minicircle DNA was detected in a *Leishmania*-positive sand fly sample (Figure 2, lane 3) but not in a negative one (Figure 2, lane 1). On the other hand, a ~2,000-bp fragment corresponding to the sand fly 18S rRNA genes was amplified in both samples (Figure 2, lanes 2 and 4). Thus, target genes were successfully amplified with good specificity and sensitivity, and minimum effort.

Mass screening of sand flies from areas where leishmaniasis is endemic. The newly established method was applied to the mass screening of sand flies from areas where leishmaniasis is endemic. Ethanol-fixed sand fly samples were lysed in DNA extraction buffer without homogenization for 12 hours in 96-well plates and directly used as templates for PCR. For the detection of *Leishmania*, amplification was performed with minicircle DNA-specific primers in 96-well PCR plates, and

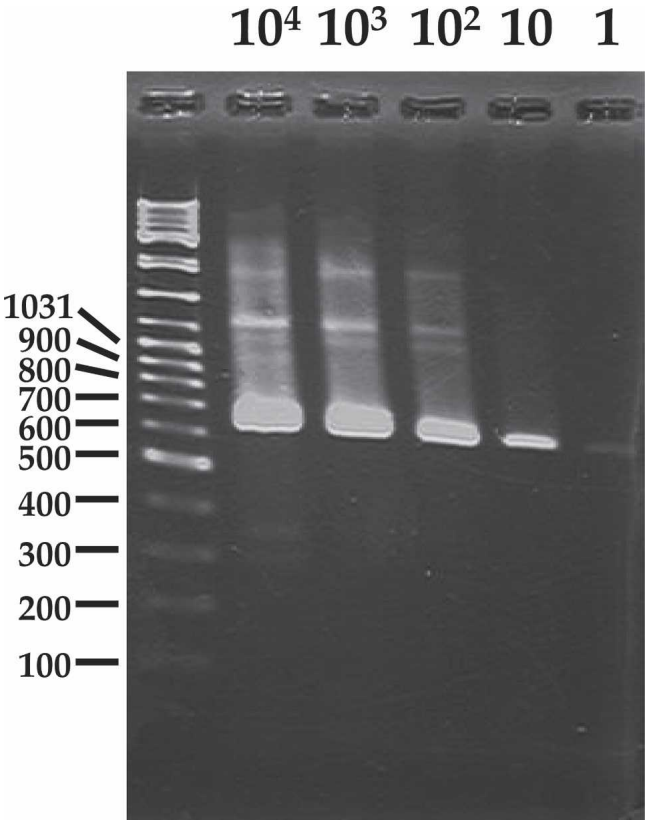

FIGURE 1. Specificity and sensitivity of PCR with primers specific for *Leishmania* minicircle kinetoplast DNA. Ten thousand parasites were suspended in 50 μL DNA extraction buffer and serially diluted 10-fold in the same buffer. The samples (10^4, 10^3, 10^2, 10, or 1 parasites) were incubated at 37°C for 12 hours, 25 μL of distilled water was added, and 0.5-μL portions were directly used as the templates for PCR amplification.

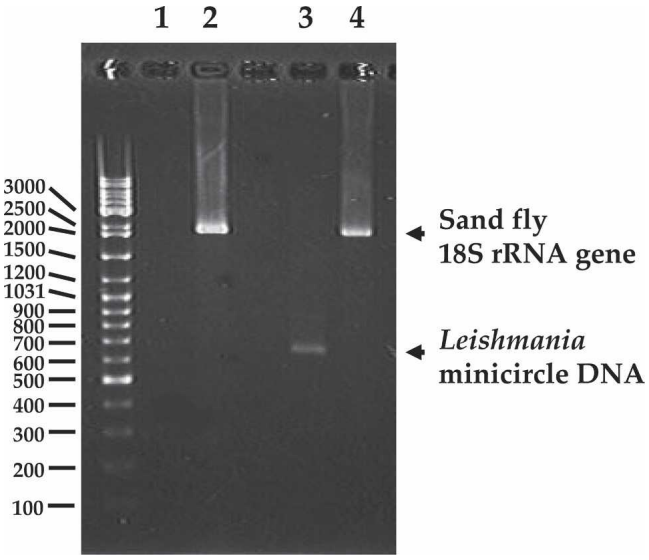

FIGURE 2. Detection of *Leishmania* minicircle kinetoplast DNA within *Leishmania*-negative (lane 1) or -positive (lane 3) sand flies by PCR. The *Lutzomyia* 18S rRNA gene was also amplified in these samples (lanes 2 and 4, respectively).

the PCR products were analyzed on 2% agarose gels. Figure 3 shows the results for 96 samples from Piedrero, 2 of which were positive. In this way, 113, 76, and 130 samples from Piedrero, Portoviejo, and Ocaña, respectively, were tested and two, zero, and three positive sand flies, respectively, were detected (Table 1). The sand fly species were also examined by a recently established method using the PCR-RFLP of 18S rRNA genes.[13] PCR amplification was performed with sand fly 18S rRNA gene-specific primers using the same samples in 96-well PCR plates and the products were digested with *Afa*I or *Hin*fI in 96-well plates for typing of the species. Figure 4 shows the results for 16 samples from Piedrero. *Lu. hartmanni* was identified by digestion with *Afa*I. Subsequent treatment with *Hin*fI revealed *Lu. trapidoi*, *Lu. gomezi,* and *Lu. dysponeta* in these samples (Figure 4). In this way, all 113, 76, and 130 sand fly species from Piedrero, Portoviejo, and Ocaña, respectively, were successfully classified (Table 1). Thus, the distribution of *Lu. hartmanni, Lu. trapidoi*, *Lu. gomezi, Lu. dysponeta,* and *Lu. serrana* from Piedrero and Portoviejo and *Lu. hartmanni* and *Lu. trapidoi* from Ocaña was confirmed. The results corresponded to those obtained by the morphologic identification of species in the same sand fly pool conducted during the field research activities. All the minicircle DNA-positive sand flies were identified as *Lu. hartmanni*.

Analysis of *Cyt* b genes in the positive samples. To identify the parasite species within the minicircle DNA-positive sand flies, *Cyt* b was analyzed because the gene has been shown to be good indicator for the classification of *Leishmania* species.[9,14,17,18] *Cyt* b genes from four of five positive samples were successfully amplified, and the sequences were determined. The sequences were compared with those from six prevalent *Leishmania*, *L.* (*L.*) *amazonesis*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *major-like*, *L.* (*V.*) *panamensis*, *L.* (*V.*) *braziliensis,* and *L.* (*V.*) *guyanensis,* in Ecuador and two *Endotrypanum* spp., *E. schaudinni* and *E. monterogeii,* both of which had identical *Cyt* b sequences, and all the samples tested had the

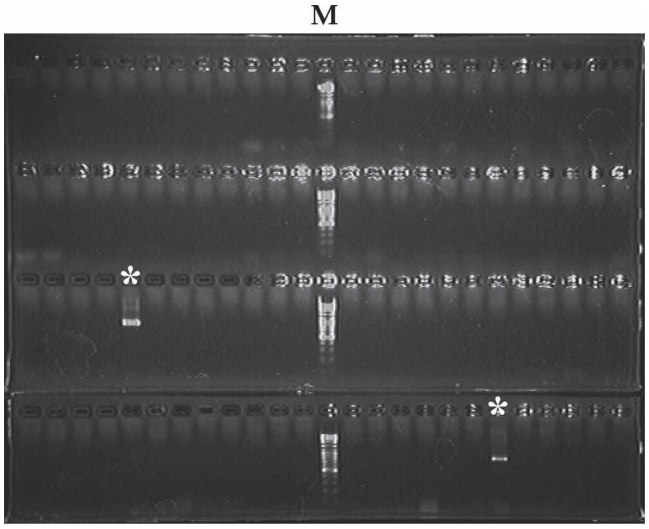

FIGURE 3. Mass screening of sand fly vectors for *Leishmania* infection. Ethanol-fixed sand fly samples were lysed in DNA extraction buffer without homogenization for 12 hours in 96-well plates and directly used as the templates for PCR with *Leishmania* minicircle DNA-specific primers. The PCR products were analyzed by electrophoresis on a 2% agarose gel. The figure shows the results for 96 samples from Piedrero. Asterisks denote minicircle DNA-positive specimens. Lane M, DNA molecular weight marker.

TABLE 1
Mass screening of sand flies from endemic areas of leishmaniasis in Ecudor

Locality	Captured by	*Lu. h*	*Lu. t*	*Lu. g*	*Lu. d*	*Lu. s*	Total	Positive
Piedrero	CDC trap	15	10	78	9	1	113	2
Portoviejo	CDC trap	6	2	29	36	4	76	0
Ocaña	Human bait	113	17	0	0	0	130	3

Lu. h, Lu. hartmanni; Lu. t, Lu. trapidoi; Lu. g, Lu. gomezi; Lu. d, Lu. dysponeta; Lu. s, Lu. serrana.

highest level of homology with *Endotrypanum* species (Table 2). A phylogenetic tree was also constructed based on those sequences to see the relationships among species. As shown in Figure 5, all four positive samples divided into the same clade as *E. schaudinni* and *E. monterogeii* but not *Leishmania* species. These results indicated that all the positive sand flies were infected with *Endotrypanum* species. In the *Cyt* b gene analysis, one sample (Ocaña 1-12G) had a relatively low level of homology (93.3%), whereas the other three (Ocaña 1-11B, Piedrero 7E, and Piedrero 12C) were almost identical

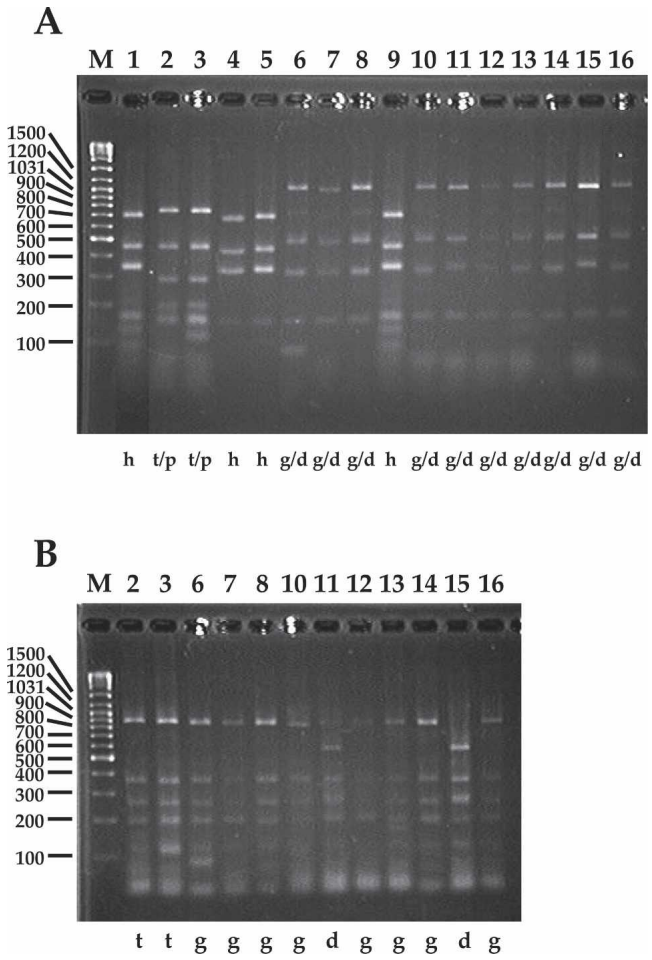

FIGURE 4. Mass screening of sand fly vectors. PCR amplification was performed with sand fly 18S rRNA gene-specific primers in 96-well PCR plates and the products were digested with *Afa*I or *Hin*fI in 96-well plates for typing of the species. The figure shows the results for 16 samples from Piedrero digested with *Afa*I (A) or *Hin*fI (B). Lane M, DNA molecular weight marker; lanes 1–16, sample numbers. The results of the species identification are shown at the bottom. h, *Lu. hartmanni*; t, *Lu. trapidoi*; g, *Lu. gomezi*; d, *Lu. dysponeta*; t/p, *Lu. trapidoi* or *Lu. panamensis*; g/d, *Lu. gomezi* or *Lu. dysponeta*.

(99.6%) with *Endotrypanum* species (*E. schaudinni* and *E. monterogeii*; Table 2). The phylogenetic analysis also showed that Ocaña 1-12G classified into a separate branch from the other three samples and *Endotrypanum* species (Figure 5), strongly suggesting that Ocaña 1-12G belongs to a novel *Endotrypanum* species.

DISCUSSION

In this study, a method of mass screening sand flies for *Leishmania* infection was established. The method was applied to 319 field-captured specimens, and five positive sand flies were detected. In addition, all the species were successfully identified by PCR-RFLP of the 18S rRNA gene, and the positive flies were all *Lu. hartmanni*. Furthermore, *Cyt* b gene sequence analyses identified the parasites as *Endotrypanum* species.

Molecular biologic techniques have been applied to the detection and identification of *Leishmania* species.[6–8] In our recent study, a method of detecting *Leishmania* protozoa within naturally infected individual sand flies by PCR with minicircle kinetoplast DNA-specific primers was established.[9] The method is easy and sensitive; however, some improvements were needed for practical use in the mass screening of sand fly vectors. Therefore, we decided to apply Ampdirect (Shimadzu), a reagent recently produced for genotyping and other purposes, which allows PCR in the presence of inhibitory substances in biologic samples, as well as reagents in DNA extraction buffer. The experimental conditions were successfully optimized and the method was applied to the mass screening of sand fly vectors. The merit of this procedure is that one is able to acquire data on individuals. In some endemic areas, one sand fly species is dominant,[9,19] and information on circulating *Leishmania* species and infection rates in the sand fly population can be obtained from pooled samples.[19] However, several sand fly species co-exist in most areas where leishmaniasis is endemic, and the use of pooled samples is apt to lose some important information on the vector epidemiology such as the prevalent sand fly species as a risk factor and the relationships between *Leishmania* spe-

TABLE 2
Homologies (%) of *Cyt* b sequences from minicircle DNA-positive samples with those from reference strains

Locality	Number	*L. a*	*L. me*	*L. p*	*L. g*	*L. m-l*	*L. b*	*E. spp*
Piedrero	7E	88.5	88.6	87.4	87.0	87.3	87.5	99.6
	12C	88.5	88.6	87.4	87.0	87.3	87.5	99.6
Ocaña	1-11B	88.5	88.6	87.4	87.0	87.3	87.5	99.6
	1-12G	88.9	88.4	88.2	88.0	86.5	88.6	93.3

L. a, L. (L.) amazonesis; L. me, L. (L.) mexicana; L. p, L. (V.) panamensis; L. g, L. (V.) guyanensis; L. m-l, L. (L.) major-like; L. b, L. (V.) braziliensis; E. spp, Endotrypanum spp.

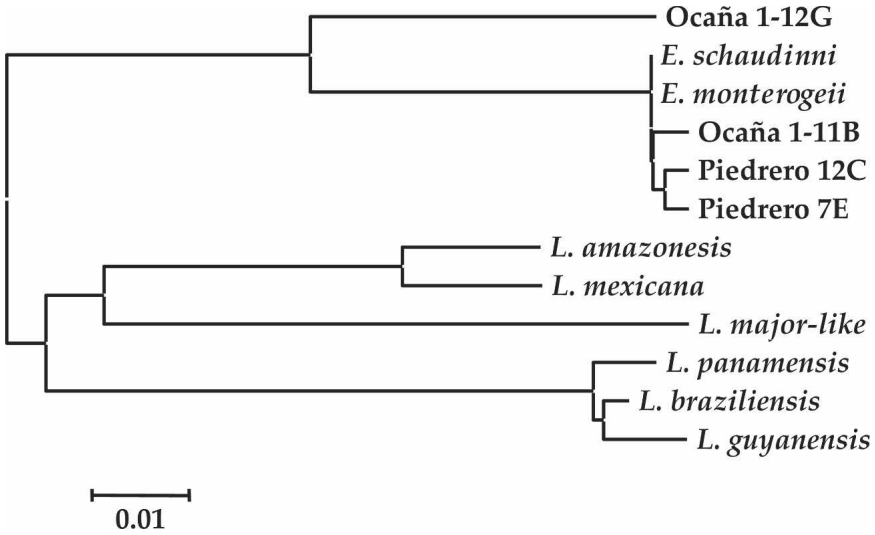

FIGURE 5. Phylogenetic tree of *Cyt* b gene sequences among species. The *Cyt* b genes of the parasites were amplified from the minicircle DNA-positive sand fly samples (Ocaña 1-12G, Ocaña 1-11B, Piedrero 7E, and Piedrero 12C), and the sequences were determined. Analyses were performed based on the sequences together with those from six prevalent *Leishmania* species in Ecuador (*L. amazonesis*, *L. mexicana*, *L. major-like*, *L. panamensis*, *L. braziliensis,* and *L. guyanensis*) and two *Endotrypanum* species (*E. schaudinni* and *E. monterogeii*). The scale bar represents 0.01% divergence.

cies and the vectors. Other advantages of this method are that it minimizes the risk of contamination among samples and loss of DNA during the procedure because of the limited processes for DNA extraction.

In this study, minicircle kinetoplast DNA could be amplified if only one parasite exists in a sample by using *L.* (*L.*) *major* as a template. The primers were designed based on sequences conserved among species and confirmed to work on all seven species examined in our recent study.[9] Thus, the primers were considered to work for all the *Leishmania* species. In addition, the specificity of the primers was tested using ethanol-fixed *L. major*–infected sand fly samples and no non-specific band was detected, as observed in a recent study using the same primers. In this study, the PCR-RFLP method[13] was applied for mass screening using 96-well plates in each process, and 319 field-captured sand flies were analyzed. As a result, *Lu. hartmanni, Lu. trapidoi, Lu. gomezi*, *Lu. dysponeta,* and *Lu. serrana* were identified in the three subtropical areas where leishmaniasis is endemic. The results were consistent with our previous data obtained by morphologic identification through long-term epidemiologic research.[10] Many *Lu. dysponeta* were identified from Piedrero and Portoviejo, where sand flies were captured with CDC light traps, but not from Ocaña, where sand flies were collected using protected human bait. *Lu. dysponeta* usually do not bite humans for feeding, and the lack of species in specimens from Ocaña probably resulted from the method of collection used. Thus, sampling methods have to be taken into consideration when studying the epidemiology of sand flies.

The mass screening of vectors from the present three endemic areas resulted in the detection of five minicircle DNA-positive sand flies. The five were identified as *Endotrypanum* species based on *Cyt* b gene sequencing. *Endotrypanum* and *Leishmania* are parasites belonging to the family Trypanosomatidae and are the two most closely related genera.[20] *Endotrypanum* parasites infect the erythrocytes of mammalian hosts, and infections have been reported in sloths and squirrels but not humans.[20,21] Both *Endotrypanum* and *Leishmania* are transmitted by sand fly vectors, and therefore, the discrimination of these parasites is important for epidemiologic surveillance of reservoir host and sand fly vectors.[20] In this study, *Leishmania* parasites were not detected in the mass screening because of the very low infection rate (0.01–1%) among sand fly populations, even in the endemic areas. Testing sand fly samples from highly infected populations (1–8%) in Andean areas in Ecuador using this method should give positive results in a certain number of samples.[9] Further surveillance of larger populations using the present mass screening will provide more information on each endemic area. Currently, only two named species, *E. schaudinni* and *E. monterogeii*, have been described in the genus *Endotrypanum,*[20] and both species have identical *Cyt* b sequences (Uezato H and others, unpublished data). Four of five positive samples were successfully sequenced, and three samples were considered to be *E. schaudinni* or *E. monterogeii* on the basis of the *Cyt* b sequencing analysis. On the other hand, the sequence from the rest, Ocaña 1-12G, had relatively low level of homology with sequences from the above-mentioned three samples, *E. schaudinni* and *E. monterogeii*. The phylogenetic analysis classified the Ocaña 1-12G into a separate branch from the others, strongly suggesting that the sample belongs to a novel *Endotrypanum* species. At present, definitive evidence was not given; however, further molecular analyses of other genes may clarify the issue. An attempt to isolate the parasites from sand flies in each PCR-positive area will be necessary. According to a recent study, PCR-RFLP analysis of genes including the small subunit and internal transcribed spacer 1 of rRNA gene can be an effective tool for classification of the main New World *Leishmania* species.[22] The method was applied with a slight modification, and *Endotrypanum* species seemed to have a unique RFLP pattern after *Afa*I or *Hap*II digestion compared with *Leishmania* species distributing in Ecuador (Kato H and others, unpublished data). Thus, *Endotrypanum* species are probably distinguishable in materials

from *Leishmania* species by PCR-RFLP without sequencing of the *Cyt* b gene.

In conclusion, a method of mass screening sand fly vectors was established for the detection of *Leishmania* and identification of sand fly species from individual samples. The method requires minimum effort, and therefore will be a powerful tool for studying the epidemiology of leishmaniasis. Use of the method will disclose the prevalent sand fly species as a risk factor and the relationships between *Leishmania* species and the responsible vectors in a given endemic area.

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