

Molecular Typing of Sand Fly Species (Diptera, Psychodidae, Phlebotominae) from Areas Endemic for Leishmaniasis in Ecuador by PCR-RFLP of 18S Ribosomal RNA Gene

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ABSTRACT. Surveillance of the distribution of sand fly species is important for prediction of the risk and expansion of *Leishmania* infection in endemic and surrounding areas. In the present study, a simple and reliable method of typing New World *Lutzomyia* species circulating in endemic areas in Ecuador was established by using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique. PCR-RFLP of 18S ribosomal RNA (rRNA) genes with the restriction enzyme *AfaI* and subsequently *HinfI* successfully identified seven sand fly species in nine endemic areas in Ecuador. Although intraspecific genetic-diversity affecting the RFLP-patterns was detected in a species, the patterns were species specific. The method promises to be a powerful tool for the classification of New World *Lutzomyia* species.

KEY WORDS: *Lutzomyia*, PCR-RFLP, Sand fly, typing, 18S ribosomal RNA gene.

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Phlebotomine sand flies are insects of the family Psychodidae in the order Diptera. More than 800 sand fly species have been described, however, only some serve as vectors and transmit zoonotic and human diseases such as leishmaniasis [13, 17]. The medically important species belong to the genus *Phlebotomus* in the Old World and to *Lutzomyia* in the New World [13, 17]. The surveillance of sand flies is performed through epidemiological research on leishmaniasis, and interestingly, it is becoming obvious that a restricted number of species support the development of specific *Leishmania* species and consequently transmit them. Thus, surveillance of the distribution of sand fly species is important for predictions of the risk and expansion of the diseases in endemic and surrounding areas. Currently, sand flies are identified principally based on morphological characteristics [23]: mainly internal structures such as spermatheca, cibarium, and pharynx in females and terminal genitalia in males. However, the morphological classification requires considerable skill as well as taxonomic expertise and is difficult in many cases. In addition, the presence of intraspecific variation and cryptic species frequently complicates classifications based on morphological features. Further, damage can be caused by improper storage conditions and the mounting process. Therefore, other characteristics like molecular markers have been explored for the development of more simple and accurate ways to identify sand flies [1–3, 12, 20, 22].

Since 1982, we have been conducting epidemiological

research on leishmaniasis in the New World, especially in Ecuador [4, 8, 9]. To date, pathogens have been isolated from hundreds of patients, and causative *Leishmania* species have been identified by multilocus enzyme electrophoresis and recently, molecular biological methods [4, 15, 16]. A molecular biological technique was also used for vector epizootiology, and a method for the detection and identification of *Leishmania* species within naturally infected sand flies was successfully established [12]. Thus, it would be very useful if vector species can be identified simultaneously from the DNA specimens used for the detection and identification of pathogens. Molecular markers are being extensively explored in vector insects as well as pathogens as tools for molecular phylogenetic analyses and population genetics, and rRNA genes and mitochondrial DNA are commonly used for such purposes [1–3, 18–20, 22], indicating these genes to be prospective targets for molecular taxonomy. In the present study, we attempted to establish a method for the molecular typing of *Lutzomyia* species by DNA polymorphisms of the 18S rRNA gene and successfully classified 7 species captured in different areas where leishmaniasis is endemic in Ecuador by PCR-RFLP.

MATERIALS AND METHODS

Sand fly collection: Sand flies were caught with CDC light traps and/or protected human bait in lowland subtropical areas; Portoviejo (Province of Manabi), Puerto Quito (Province of Pichincha), Manta Real, Piedrero, Ocaña (Province of Cañar), and Cumanda (Province of Chimborazo), and in Andean areas; Chanchan, Alausi (Province of Chimborazo), and Paute (Province of Azuay) in Ecuador

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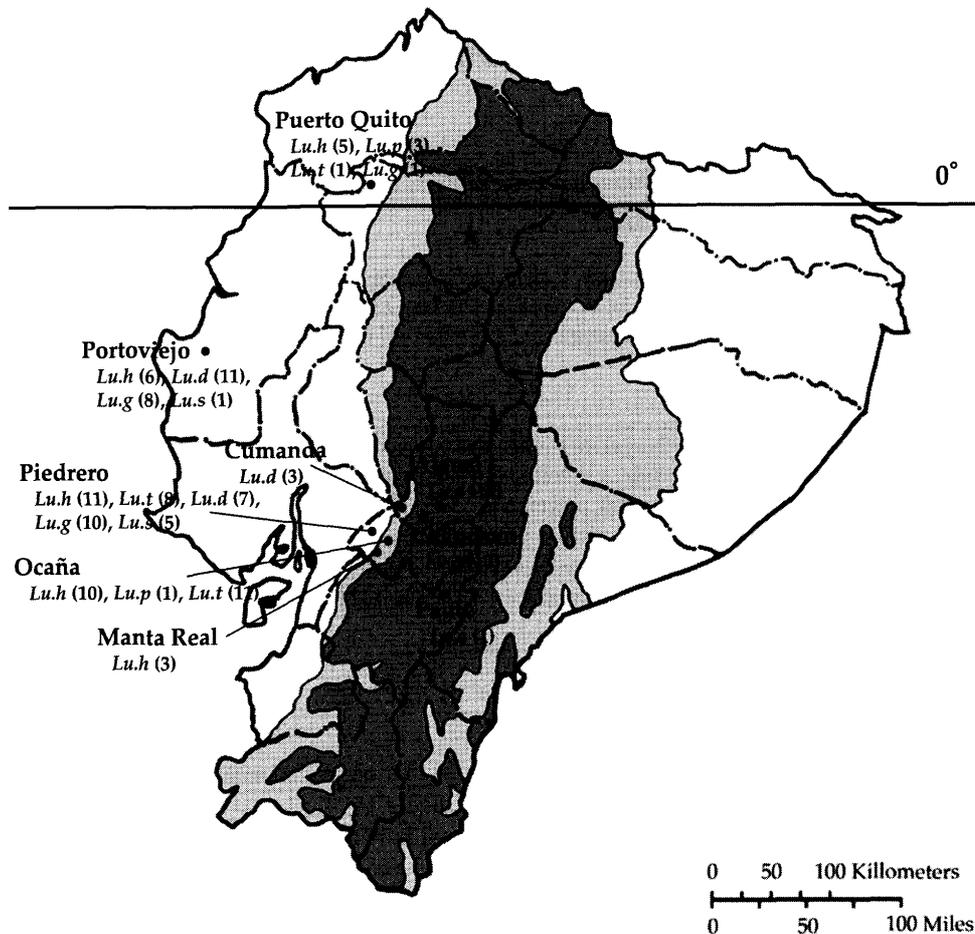


Fig. 1. Map of Ecuador showing the geographic locations where sand flies were collected along with the respective species and numbers (in parentheses) examined for the genetic diversity in this study. The dark gray areas show the Andean plateau (>1,000 m altitude), and the light gray areas show highland jungle or Andean slopes (400–1,000 m elevation). The tropical rainforests of Pacific coastal and Amazon regions are shown in white. The asterisk denotes the capital city, Quito. *Lu.a*, *Lu. ayacuchensis*; *Lu.h*, *Lu. hartmanni*; *Lu.p*, *Lu. panamensis*; *Lu.t*, *Lu. trapidoi*; *Lu.g*, *Lu. gomezi*; *Lu.d*, *Lu. dysponeta*; *Lu.s*, *Lu. serrana*.

(Fig. 1). Cutaneous leishmaniasis caused by *Leishmania (Viannia) panamensis* and *L. (V.) guyanensis* is dominant in the subtropical areas, and Andean-type cutaneous leishmaniasis caused by *L. (L.) mexicana* and *L. (L.) major*-like is prevalent in the highland areas [4, 5]. Soon after their collection, the sand flies were dissected, and then the species was identified based mainly on the morphology of spermathecae [23]. Once classified, the samples were fixed individually in 70% ethanol for PCR-RFLP analysis. The species and number of sand flies subjected to the PCR-RFLP analyses in each collection site are shown in Fig. 1.

DNA extraction: Ethanol-fixed individual sand flies were homogenized and lysed in DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] with 100 µg/ml of proteinase K at 37°C for 12 hr. These samples were then extracted with phenol and chloroform followed by ethanol precipitation. DNA pellets were resuspended in 10 µl of distilled water, and 1-µl portions of the DNA extracts were subjected to

PCR amplification.

Polymerase chain reaction (PCR): For amplification of the 18S rRNA gene fragments from various *Lutzomyia* species, PCR was performed with primers designed based on the sand fly 18S rRNA gene sequence conserved among *Lutzomyia* species. The primer sequences were 5'-TGC-CAGTAGTTATATGCTTG-3' (*Lu.18S 1S*) and 5'-CAC-CTACGGAAACCTTGTTAC-3' (*Lu.18S AR*). PCR was carried out in a volume of 20 µl of PCR solution (Premix *Taq*; Takara Bio Inc., Shiga, Japan). After an initial denaturation at 95°C for 5 min, amplification was performed with 40 cycles of denaturation (95°C, 1 min), annealing (50°C, 1 min) and polymerization (72°C, 2 min), followed by a final extension at 72°C for 10 min.

Molecular cloning and nucleotide sequencing: The PCR products were analyzed by electrophoresis on a 1% agarose gel and then directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI, U.S.A.). *Escherichia coli* (*E. coli*) JM109 cells were transformed

with the ligation mixture and plated onto LB agar plates 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 K.K., 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, U.S.A.).

Restriction fragment analysis: Each PCR product was digested with the restriction enzymes *AfaI* (Takara Bio Inc.), *HapII* (Takara Bio Inc.), *HinfI* (Takara Bio Inc.) or combination of *AfaI* and *HapII*. The digested samples were separated by electrophoresis in a 2 or 3% agarose gel to produce DNA fragments.

Phylogenetic analysis: The sand fly 18S rRNA gene sequences were aligned with CLUSTAL W software [21] and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 [14]. Neighbor-joining (NJ) trees were constructed with the distance algorithms available in the MEGA package. The database for phylogenetic analyses consisted of 7 *Lutzomyia* species isolated in this study and 8 *Lutzomyia* species, *Lu. neivai*, *Lu. shannoni*, *Lu. geniculata*, *Lu. vattieri*, *Lu. toroensis*, *Lu. verrucarum*, *Lu. nuneztovari* and *Lu. longipalpis*, that have been registered in DDBJ/EMBL/GenBank under the accession numbers AB214970, U48382, AJ391734, AJ391733, AJ391735, AJ391732, AJ391731 and AJ244428.

RESULTS

Sequence analyses of sand fly 18S rRNA gene: To establish a method for the molecular typing of New World sand flies, we tried to build on a technique used to classify the Old World species from Greece and Cyprus that is based on DNA polymorphisms of the 18S rRNA gene [1]. However, the primers did not work on some of the New World specimens. Hence, *Lu.18S 1S* and *Lu.18S AR* were designed based on sequences conserved among *Lutzomyia* species, and 18S rRNA genes were amplified from seven morphologically identified species, *Lu. ayacuchensis*, *Lu. hartmanni*, *Lu. panamensis*, *Lu. trapidoi*, *Lu. dysponeta*, *Lu. gomezi* and *Lu. serrana*, captured in areas (Chanchan, Ocaña, Puerto Quito, Ocaña, Cumanda, Piedrero and Puerto Quito, respectively) where leishmaniasis is endemic in Ecuador (Fig. 1). The fragments were cloned into pGEM-T Easy Vector, and their nucleotide sequences were determined. The DNA inserts were 1964–1980 bp long and showed 93.0–99.3% homologies with 18S rRNA genes from *Lutzomyia* species, *Lu. neivai*, *Lu. shannoni*, *Lu. geniculata*, *Lu. vattieri*, *Lu. toroensis*, *Lu. verrucarum*, *Lu. nuneztovari* and *Lu. longipalpis*. These results indicated that the DNA fragments were 18S rRNA genes of *Lu. ayacuchensis*, *Lu. hartmanni*, *Lu. panamensis*, *Lu. trapidoi*, *Lu. dysponeta*, *Lu. gomezi* and *Lu. serrana*. The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB288336,

AB288337, AB288338, AB288339, AB288340, AB288341 and AB288342, respectively.

A phylogenetic tree based on the newly determined 18S rRNA gene sequences together with sequences from the New World sand fly species registered in DDBJ/EMBL/GenBank was used to observe the phylogenetic relationships among species. As shown in Fig. 2, most of the sand flies that belong to the same subgenus [23] were divided into the same cluster. For example, *Lu. trapidoi* showed a closer relationship with the same subgenus *Nyssomyia*, *Lu. neivai* and *Lu. intermedia*. Similarly, *Lu. ayacuchensis*, subgenus *Helcocyrtomyia*, was closer to the same subgenus species *Lu. hartmanni*, and *Lu. panamensis* divided into the same clade as the subgenus *Psychodopygus* species, *Lu. geniculata* (Fig. 2). In contrast, although *Lu. serrana* and *Lu. verrucarum*, which belongs to the *Verrucarum* group, were classified into the same clade, a same group species, *Lu. nuneztovari* was classified into a separate branch from the two species. In addition, a subgenus *Lutzomyia* species, *Lu. gomezi* was distant from a same subgenus species, *Lu. longipalpis* in this analysis.

Restriction fragment analysis of sand fly 18S rRNA genes: It was reported that analyzing DNA polymorphisms of 18S rRNA genes double-digested with *RsaI* (which cleaves the same sites as *AfaI*) and *HpaII* (which cleaves the same sites as *HapII*) was useful for classifying the Old World sand fly species from Greece and Cyprus [1]. In the present study, 8, 6, 7, 7, 4, 4 and 5 *AfaI* restriction enzyme digestion sites and 7, 8, 7, 7, 8, 7 and 7 *HapII* sites in the 18S rRNA gene fragments of *Lu. ayacuchensis*, *Lu. hartmanni*, *Lu. panamensis*, *Lu. trapidoi*, *Lu. dysponeta*, *Lu. gomezi* and *Lu. serrana*, respectively, were observed (data not shown). Then, typing of sand fly species was performed by restriction fragment analysis of the 18S rRNA gene using *AfaI* and *HapII*. When digested with *AfaI*, *Lu. ayacuchensis*, *Lu. hartmanni* and *Lu. serrana* showed distinct RFLP-patterns, but *Lu. panamensis* and *Lu. trapidoi* as well as *Lu. dysponeta* and *Lu. gomezi* were indistinguishable on the gel (Fig. 3A). On the other hand, *HapII*-digestion gave less variation among the RFLP-patterns, that is, only *Lu. hartmanni* and *Lu. dysponeta* showed unique patterns, whereas the other 5 species were almost identical (data not shown). RFLPs of 18S rRNA genes digested with *AfaI* and *HapII* were assessed. *Lu. ayacuchensis*, *Lu. hartmanni*, and *Lu. gomezi* had species-specific patterns, while the other 4 species were divided into 2 groups; *Lu. panamensis* was almost identical with *Lu. trapidoi*, and *Lu. dysponeta* was indistinguishable from *Lu. serrana* (data not shown). Since species-specific patterns were not obtained with *AfaI* and/or *HapII*, further analysis with another restriction enzyme, *HinfI* was done. As shown in Fig. 3B, less variation was seen; however, patterns were distinguishable between *Lu. trapidoi* and *Lu. panamensis* and between *Lu. dysponeta* and *Lu. gomezi*, which was not the case with *AfaI*. Thus, a single cut with *AfaI* of 18S rRNA gene fragments identified 3 of 7 species, and subsequent digestion with *HinfI* classified the rest distributing in areas where leishmaniasis is endemic in Ecu-

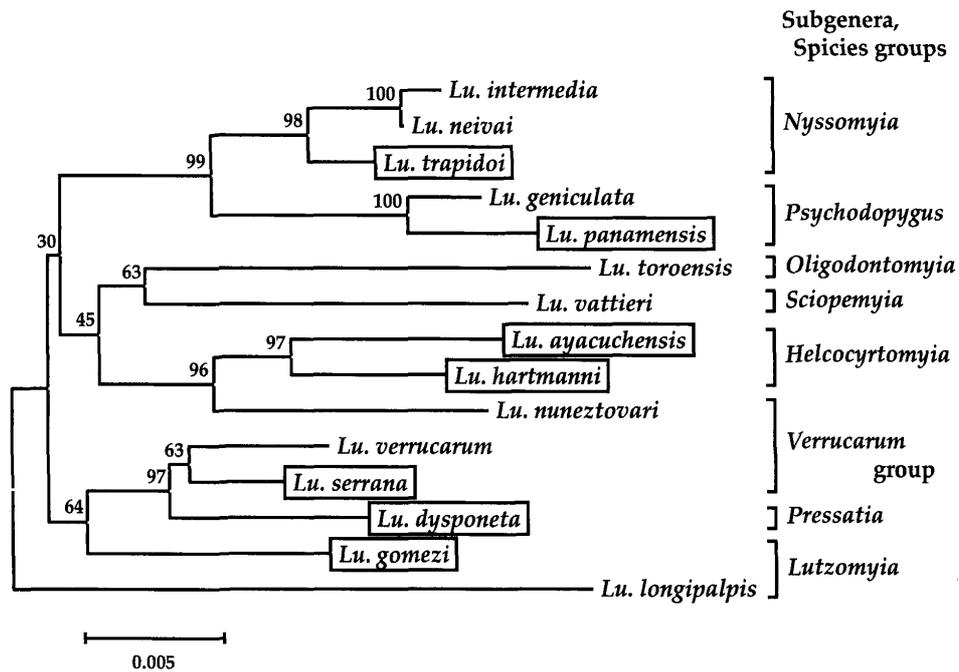


Fig. 2. Phylogenetic tree of 18S rRNA gene sequences among sand fly species. The 18S rRNA genes of 7 species were amplified from species-identified sand fly samples, and the sequences were determined. Analyses of the sequences together with those from 8 *Lutzomyia* species previously registered in DDBJ/EMBL/GenBank were performed. The species whose 18S rRNA gene sequences were determined in this study are boxed. The scale bar represents 0.005% divergence. Bootstrap values are shown above branches.

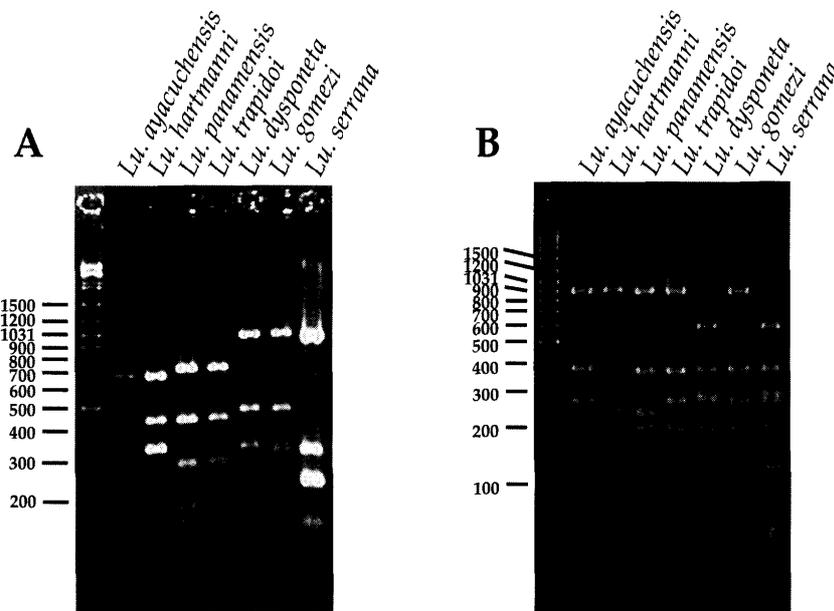


Fig. 3. PCR-RFLP analyses of 18S rRNA genes from 7 *Lutzomyia* species in Ecuador. PCR amplification was performed with sand fly 18S rRNA gene-specific primers, and the PCR products were digested with *Afa*I (A) or *Hinf*I (B).

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Further, a PCR-RFLP analysis with microscopically identified sand flies captured at different sites in Ecuador (Fig. 1) was performed to see if genetic diversity affects the

RFLP-patterns. For this purpose, 25 *Lu. ayacuchensis*, 35 *Lu. hartmanni*, 4 *Lu. panamensis*, 20 *Lu. trapidoi*, 21 *Lu. dysponeta*, 19 *Lu. gomezi* and 6 *Lu. serrana* specimens were tested (Fig. 1). As shown in Fig. 4A, all seven species had

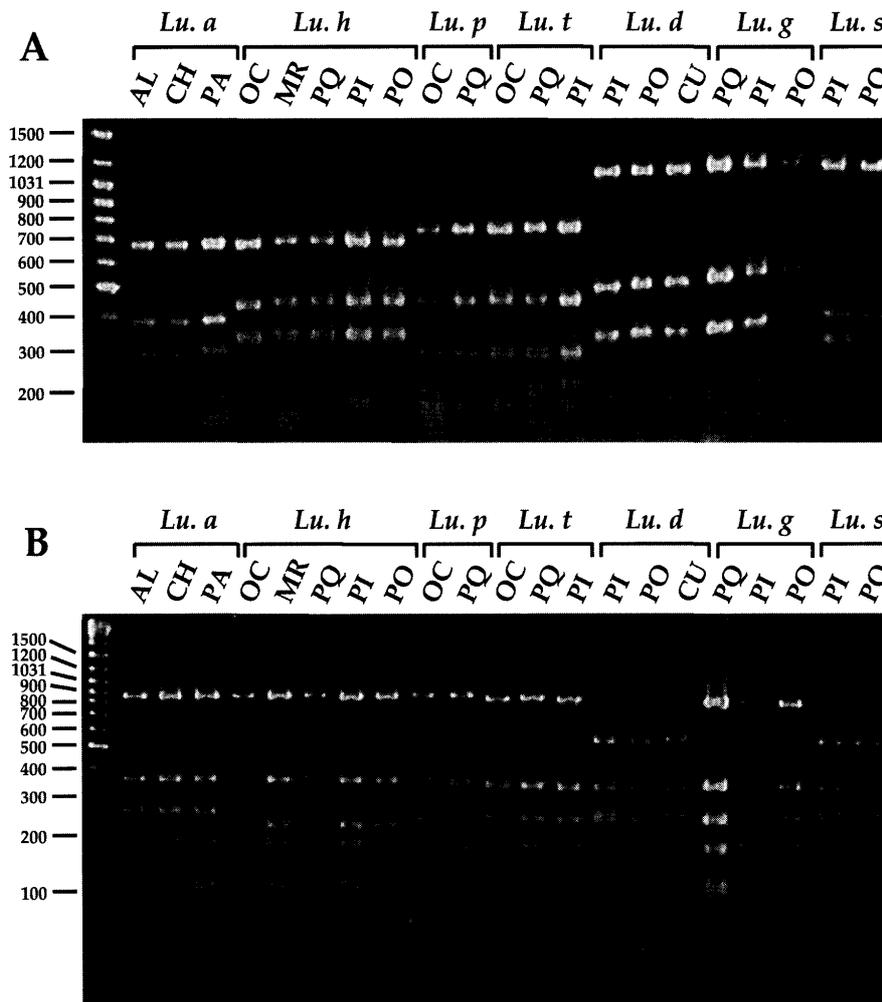


Fig. 4. PCR-RFLP analyses of 18S rRNA genes from species-identified sand fly samples from 9 different endemic areas in Ecuador (AL, Alausi; CH, Chanchan; PA, Paute; CU, Cumanda; OC, Ocaña; MR, Manta Real; PQ, Puerto Quito; PI, Piedrero; PO, Portoviejo). PCR amplified 18S rRNA genes were digested with *AfaI* (A) or *HinfI* (B) to see the genetic diversity affecting RFLP-patterns. *Lu.a*, *Lu. ayacuchensis*; *Lu.h*, *Lu. hartmanni*; *Lu.p*, *Lu. panamensis*; *Lu.t*, *Lu. trapidoi*; *Lu.g*, *Lu. gomezi*; *Lu.d*, *Lu. dysponeta*; *Lu.s*, *Lu. serrana*.

the same RFLP-patterns within species when digested with *AfaI*. On the other hand, 2 RFLP-patterns, which affect *HinfI*-digestion profiles, were observed in *Lu. panamensis* (Fig. 4B).

DISCUSSION

In the present study, we attempted to establish a simple and reliable method for the identification of sand fly species captured in different areas where leishmaniasis is endemic in Ecuador. For this purpose, the 18S rRNA gene was targeted, and DNA sequences were determined for seven species in the areas. Based on these sequences, RFLP-patterns were determined and PCR-RFLP of 18S rRNA genes with the restriction enzymes *AfaI* and *HinfI* identified 7 species, although genetic diversity was observed in a species.

Initially, a method used previously to identify the Old

World sand fly species [1] was applied, but the primers did not work well for some New World specimens. However, with the newly designed primers Lu.18S 1S and Lu.18S AR, specific fragments were amplified, and the sequences of 7 *Lutzomyia* species, *Lu. ayacuchensis*, *Lu. hartmanni*, *Lu. panamensis*, *Lu. trapidoi*, *Lu. dysponeta*, *Lu. gomezi* and *Lu. serrana*, were determined. The sequences of the new set of primers were also conserved in 12 *Phlebotomus* and 9 *Sergentomyia* species, strongly suggesting that these primers will work for amplification of 18S rRNA genes from most sand fly species.

A phylogenetic analysis consisted of 7 *Lutzomyia* species isolated in this study and 8 species of the subgenus *Lutzomyia* that have been registered in DDBJ/EMBL/GenBank was performed to observe the phylogenetic relationships among species. Most of the results supported the generally accepted classification based on the morphological charac-

teristics [23]; however, subgenus *Lutzomyia* and *Verrucarum* group species positioned separately in the analysis. Recently, *Lutzomyia* 12S rDNA and 28S rDNA were extensively analyzed, and interspecific relationships were assessed [3]. They also reported some discordance on the classification including subgenus *Lutzomyia* and *Verrucarum* group species between morphological and phylogenetic analyses, and suggested the necessity of careful reconsideration on the classification of *Lutzomyia* species. Thus, further genetic analyses will help to clarify the issues.

In a previous study on the classification of the Old World species, PCR-RFLP of 18S rRNA gene fragments double-digested with *RsaI* (which cleaves the same sites as *AfaI*) and *HpaII* (which cleaves the same sites as *HapII*), and subsequent digestion with *AccI* and *BanI* identified 7 *Phlebotomus* and 3 *Sergentomyia* species distributed in Greece and Cyprus [1]. In our study, double-digestion with *AfaI* and *HapII* identified 3 of 7 species with unique RFLP-patterns. The other 4 were divided into 2 groups that can be classified by additional procedures. Although this procedure would be effective, most double-digested fragments were smaller than 400 bp in size and hard to analyze through agarose gel electrophoresis. On the other hand, although digestion with *AfaI* identified 3 species as well, subsequent *HinfI* digestion successfully classified the rest. Further, the single-digested fragments were easier to analyze on the gel than the double-digested fragments, indicating that *AfaI*-digestion and subsequent *HinfI*-digestion of 18S rRNA fragments are appropriate for typing the 7 species identified in Ecuador. In addition, we performed PCR-RFLP analysis on the registered *Lutzomyia* 18S rRNA gene sequences and found that 15 species divided into 11 RFLP-patterns, of which 9 species had species-specific patterns, when digested with *AfaI*. Subsequent *HinfI*-cut identified 4 species, and then further *HapII*-digestion classified the rest. Taken together, our results strongly suggested that the present method would be a powerful tool for the classification of the New World sand fly species, *Lutzomyia* spp.

Assessing the genetic diversity affecting RFLP-patterns in sand fly populations captured in 9 different endemic areas in Ecuador, 2 different patterns were detected in *Lu. panamensis* when digested with *HinfI*, although the variation was species specific. Similar genetic diversity affecting RFLP-patterns was reported in the Old World *Phlebotomus* species [1]. Further study will be necessary to obtain more detailed information on intraspecific DNA polymorphisms. New World *Lutzomyia* species are more numerous than the Old World *Phlebotomus* species. At the species level, 408 *Lutzomyia* species have been morphologically described, whereas 94 *Phlebotomus* species have been classified [17]. It seems to be very difficult to classify all the species only by PCR-RFLP analyses; however, information on the distribution of sand fly species in the endemic areas is accumulating gradually. Therefore, the method established in this study would be useful for epidemiological surveillance of leishmaniasis and its vector, *Lutzomyia* species. Alternatively, additional analyses targeting other genes, such as 12S

and 28S rRNA genes, the mitochondrial cytochrome *b* gene, and internal transcribed spacer 2 (ITS2) regions, promise reliable information [3, 6, 7, 10, 18–20, 22].

In conclusion, PCR-RFLP of 18S rRNA genes with the restriction enzyme *AfaI* and subsequently *HinfI* identified 7 sand fly species in areas where *Leishmania* is endemic in Ecuador based on the presence of genetic polymorphisms. Further study with specimens from more diverse geographical areas will allow us to confirm the species-specific RFLP-patterns. Sustained efforts at genetic analyses of sand flies would be helpful not only for a definitive PCR-RFLP-based molecular taxonomy but also for analyses of the evolutionary relationships among species. Finally, a method for the detection and identification of *Leishmania* species within naturally infected sand fly vectors using molecular biological techniques was developed in our recent study [11, 12]. Thus, a combination of these methods using the same DNA samples of vector sand flies will be a powerful tool for investigating the molecular epidemiology of leishmaniasis.

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