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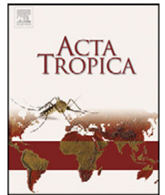

Genetic diversity of ribosomal RNA internal transcribed spacer sequences in *Lutzomyia* species from areas endemic for New World cutaneous leishmaniasis

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ABSTRACT

In this study, each of 60 rRNA internal transcribed spacer (ITS) 1 and ITS2 sequences was determined from 44 individuals of 14 morphologically identified New World sand fly *Lutzomyia* species in Ecuador, and their interspecies and intraspecies genetic diversity was compared. Distinguishing between related species based on the ITS1 sequence was difficult because of variability, while the genetic diversity of ITS2 was distinct even among closely related species. Further, an assessment of intraspecies ITS sequence diversity in the subgenus *Helcocyrtomyia* revealed no correlation between sequence variation and geographic distribution. The results strongly suggested ITS2 to be a more suitable marker than ITS1 for the taxonomic analysis of *Lutzomyia* species including closely related species. Moreover, neither ITS sequence may be useful for the analysis of population structures in *Lutzomyia* species.

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1. Introduction

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 (Munstermann, 2004). The genus *Lutzomyia* sand flies are primarily responsible for the transmission of leishmaniasis in the New World (Munstermann, 2004). It is becoming obvious that a restricted number of species support the development of specific *Leishmania* species and consequently transmit them. Therefore, 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. Sand flies are identified principally based on morphological characteristics (Young and Duncan, 1994; Munstermann, 2004); however, morphological classifications are not always reliable because of closely related species and intraspecies variation. Consequently, other characteristics like genetic markers have been explored for the development of reliable tools for species identification as well as an understanding of intraspecies genetic diversity and population structure (Aransay et al., 1999; Di Muccio et al., 2000; Torgerson et al., 2003; Beati et al., 2004; Barroso et al., 2007; Depaquit et al., 2002, 2008; Terayama et al., 2008; Barón et al., 2008). Recently, we analyzed 18S ribosomal RNA (rRNA) gene sequences of *Lutzomyia* species circulating in Ecuador, and found discrepancies in the classification of some *Lutzomyia* species between generally accepted morphologic groupings and the phylogenetic relationships of the sequences (Terayama et al., 2008). Similar observations were made in analyses of *Lutzomyia* 12S and 28S rRNA gene sequences (Beati et al., 2004). These findings indicate the necessity for further genetic analyses of sand fly taxonomy, especially in the closely related species, as well as analyses of intraspecies variation.

The rRNA internal transcribed spacer (ITS) 1 and ITS2, which are separated by the 5.8S rRNA gene and flanked by the 18S and 28S

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rRNA genes in most eukaryotes, are not subject to the same functional constraints as rRNA genes. As a result, the ITS regions are subject to higher evolutionary rates leading to greater variability in both nucleotide sequence and length, and therefore represent useful tools for the analysis of closely related species, the recognition of new species, and discrimination within a species (Hillis and Dixon, 1991). The spacer genes have been used to analyze the taxonomy and phylogenetic relationship of insect vectors such as mosquito, *Culicoides* and triatomine bugs (Marrelli et al., 2006; Martínez et al., 2006; Gomulski et al., 2006; Perrin et al., 2006; Walton et al., 2007a,b; Zapata et al., 2007; Matson et al., 2008) and shown to be effective markers for analyses of population structure as well as for classification at the species level. In *Anopheles*, variation in ITS1 is readily detectable among individuals of a single species (Beebe et al., 2000; Fairley et al., 2005), and has been used to identify populations within a species, whereas variation in ITS2 is low within a species but greater among species. On the other hand, the utility of both ITS1 and ITS2 was described in *Culicoides* (Gomulski et al., 2006; Perrin et al., 2006). In Old World sand flies, the heterogeneity of ITS2 sequences among subgenus *Larroussius* species was shown (Di Muccio et al., 2000). In addition, the ITS2 was analyzed in *Phlebotomus (P.) sergenti*, and intraspecies variation among populations was reported (Depaquit et al., 2002; Barón et al., 2008). Thus, intensive molecular studies among and within species will lead to a better understanding of differences in vectorial capacity as well as vector taxonomy.

Genetic data on *Lutzomyia* are scarce despite that the genus includes all species involved in the transmission of leishmaniasis in the New World. In addition, the genetic variation among and within species is not well documented. The present study was conducted to evaluate the utility of ITS1 and ITS2 as molecular tools for interspecies and intraspecies analyses in *Lutzomyia* species.

2. Materials and methods

2.1. Sand fly specimens

Sand flies were caught with CDC light traps and protected human bait in lowland subtropical areas of Ecuador, i.e., Portoviejo (Province of Manabi), Puerto Quito (Province of Pichincha), Manta Real, Piedrero, Ocaña (Province of Cañar), Cumanda (Province of Chimborazo) and Quininde (Province of Esmeraldas); in a tropical area Arajuno (Province of Pastaza); and in Andean areas, i.e., Chanchan, Alausi (Province of Chimborazo), and Paute (Province of Azuay) (Fig. 1). Cutaneous leishmaniasis caused by *Leishmania (Viannia) panamensis* and *L. (V.) guyanensis* is dominant in the subtropical areas, *L. (V.) guyanensis* and *L. (V.) braziliensis* are endemic in the tropical area, and Andean-type cutaneous leishmaniasis caused by *L. (Leishmania) mexicana* and *L. (L.) major*-like is prevalent in the highland areas (Calvopiña et al., 2004, 2006; Kato et al., 2005, 2008a). Soon after their collection, the sand flies were dissected, and identified to the species level based mainly on the morphology of spermathecae (Young and Duncan, 1994). Once classified, the samples were fixed individually in 100% ethanol for molecular biological analysis. *Lu. ayacuchensis* captured in the South of the Province of Parinacochas, Department of Ayacucho, Peru was also used for the comparative study. The collection sites of *Lu. ayacuchensis* in Peru were more than 1200 km from those in Ecuador.

2.2. DNA extraction

Individual ethanol-fixed 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 h. After extraction with phenol and chloroform followed by precipitation with ethanol. DNA pellets were resuspended in 20 μl of distilled water, and 1-μl portions of the DNA extracts were subjected to PCR amplification.

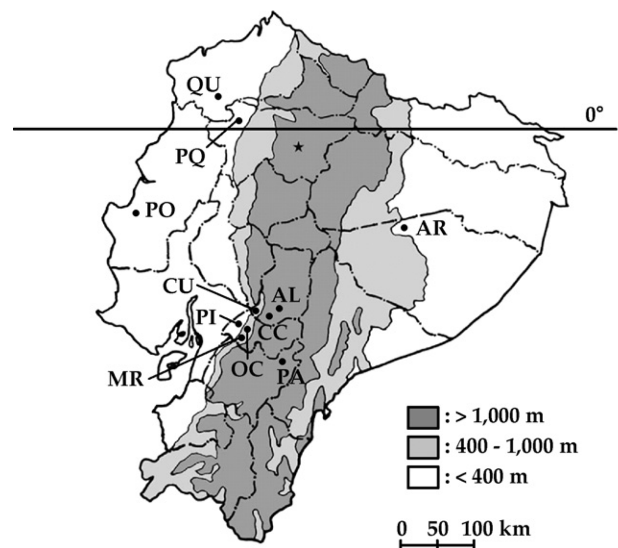

Fig. 1. A map of Ecuador showing the geographic locations where sand flies were collected in this study. Dark gray areas show the Andean plateau (altitude of >1000 m), and light gray areas show highland jungle or Andean slopes (400–1000 m). The tropical rainforests of Pacific coastal and Amazon regions are shown in white (<400 m). The asterisk denotes the capital city, Quito. Sand flies were collected in lowland subtropical areas, Portoviejo (PO), Puerto Quito (PQ), Manta Real (MR), Piedrero (PI), Ocaña (OC), Cumanda (CU) and Quininde (QU); in a tropical area, Arajuno (AR); in Andean areas, Chanchan (CC), Alausi (AL) and Paute (PA), where leishmaniasis is endemic.

2.3. Molecular cloning and sequence analysis of Lutzomyia ITS1 and ITS2 regions

For amplification of the ITS1 and ITS2 sequences from various *Lutzomyia* species, PCR was performed with primers designed based on sequences in the 18S and 28S rRNA genes highly conserved among *Lutzomyia* species. The primer sequences were 5′-CGTAACAAGGTTTCCGTAGGTG-3′ (ITS-1S) and 5′-GTTRGTTTCTTTCCTCCSCT-3′ (ITS-1R). PCR was carried out in 15 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 (55 °C, 1 min) and polymerization (72 °C, 2 min), followed by a final extension at 72 °C for 10 min. The PCR products were directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). The sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed by Genetyx ver.8 software (Genetyx, Tokyo, Japan).

2.4. Phylogenetic analyses

The *Lutzomyia* ITS sequences were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). The pairwise genetic distances between species were analyzed by MEGA 4.0 using the Kimura two-parameter (Tamura et al., 2007). Phylogenetic analyses were performed by the Neighbor-joining (NJ) and maximum parsimony (MP) methods with the distance algorisms available in the MEGA package (Tamura et al., 2007) and by the maximum likelihood (ML) method using the PHYML program (Guindon and Gascuel, 2003).

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