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Genetic divergence in populations of *Lutzomyia ayacuchensis*, a vector of Andean-type cutaneous leishmaniasis, in Ecuador and Peru

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ABSTRACT

Haplotype and gene network analyses were performed on mitochondrial cytochrome oxidase I and cytochrome *b* gene sequences of *Lutzomyia* (*Lu.*) *ayacuchensis* populations from Andean areas of Ecuador and southern Peru where the sand fly species transmit *Leishmania* (*Leishmania*) *mexicana* and *Leishmania* (*Viannia*) *peruviana*, respectively, and populations from the northern Peruvian Andes, for which transmission of *Leishmania* by *Lu. ayacuchensis* has not been reported. The haplotype analyses showed higher intrapopulation genetic divergence in northern Peruvian Andes populations and less divergence in the southern Peru and Ecuador populations, suggesting that a population bottleneck occurred in the latter populations, but not in former ones. Importantly, both haplotype and phylogenetic analyses showed that populations from Ecuador consisted of clearly distinct clusters from southern Peru, and the two populations were separated from those of northern Peru.

1. Introduction

Phlebotomine sand flies are insects of the family Psychodidae in the order Diptera. Approximately 800 sand fly species have been recorded; of these, fewer than 10% have been confirmed as vector species of leishmaniasis. These are in two currently defined genera, *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World (Munstermann, 2004; Bates, 2007; Kato et al., 2010; Alvar et al., 2012). Only a restricted number of species support the development of specific *Leishmania* species and consequently transmit the parasites (Kato et al., 2010; Ready, 2013). Therefore, surveillance of circulating sand flies is important for predicting the risk and expansion of the disease in endemic and surrounding areas. Sand flies are generally identified by morphologic characteristics; mainly internal structures such as the spermatheca, cibarium and pharynx in females, and terminal genitalia in males (Young and Duncan, 1994). Genetic information on sand flies is accumulating, and several genetic markers have been used to examine the systematics, relationships and evolution among sand fly species (Kato et al., 2010). The molecular taxonomy of sand flies mostly supports the traditional morphological classification, and can be applied to the surveillance of circulating species as well as identification of the species responsible for the transmission of *Leishmania* parasites in given endemic areas (Aransay et al., 1999; Beati et al., 2004; Kato et al., 2005, 2007, 2008; Terayama et al., 2008; Kuwahara et al., 2009; Fujita et al., 2012).

In addition to the species differences, intraspecific population divergence caused by multiple environmental factors such as climate, distance, altitude, and geographic barriers is suggested to influence vector competence (Lanzaro et al., 1993; Hamarsheh et al., 2009; Ready, 2013). Since the maternally inherited mitochondrial genes reflect the evolutionary history more accurately because of their clonal inheritance, lack of recombination and higher mutation rate compared with nuclear DNA (Avise and Bowen, 1994; Rokas et al., 2003), these genes have been used to estimate the population structure of arthropod vectors, and their

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geographical variation among populations has been reported in sand flies (Esseghir et al., 1997; Ishikawa et al., 1999; Hodgkinson et al., 2003; Hamarsheh et al., 2007; Belen et al., 2011; Florin et al., 2011; Rocha et al., 2011; Cohnstaedt et al., 2012; Yamamoto et al., 2013; Pech-May et al., 2013).

Lutzomyia (*Lu.*) *ayacuchensis* is a unique sand fly species distributing mainly in the Andean highlands of Ecuador and Peru (Takaoka et al., 1990; Caceres et al., 2004; Kato et al., 2005, 2008; Gomez et al., 2014a,b). The species is a proven vector of *Leishmania* (*Leishmania*) *mexicana* in the Ecuadorian Andes (Takaoka et al., 1990; Hashiguchi et al., 1991; Kato et al., 2005, 2008; Gomez et al., 2014a,b), whereas the same species transmits *Leishmania* (*Viannia*) *peruviana* in Andean areas of southern Peru (Caceres et al., 2004). The sand fly populations from Ecuador and Peru were indistinguishable by morphological observation and genomic analysis of the 18S rRNA genes and rRNA internal transcribed spacer (ITS) sequences (Kuwahara et al., 2009). *Lu. ayacuchensis* is also distributed in the northern Peruvian Andes where cutaneous leishmaniasis is endemic; however, no transmission of *Leishmania* parasites by sand fly species has been reported in these areas. In the present study, mitochondrial cytochrome oxidase I (COI) and cytochrome *b* (*cyt* b) genes were compared in *Lu. ayacuchensis* populations from Andean areas of Ecuador and northern and southern Peru to assess genetic divergence among populations with different vector competence.

2. Materials and methods

2.1. Sand fly collection

Sand flies were collected in four Andean areas of Ecuador; Huigra (1200 m above sea level), Chanchan (1500 m a.s.l.), and Alausi (2300 m a.s.l.), the Province of Chimborazo; and Paute (2750 m a.s.l.), Province of Azuay (Kato et al., 2008; Kuwahara et al., 2009); and 4 Andean areas of Peru; Higosniyocc, Province of Lucanas, and Saquihuacca (2250 m a.s.l), Province of Parinacochas, Department of Ayacucho; Zapote (340 m a.s.l.), Province of Lambayeque, Department of Lambayeque; Viza (1750 m) and El Paraiso (1400 m a.s.l.), Province of Cutervo, Department of Cajamarca; and La Perla (1930 m a.s.l.), Province of Huancabamba, Department of Piura (Kato et al., 2008; Kuwahara et al., 2009; Fujita et al., 2012) (Fig. 1). All flies were captured between 18:30 and 21:00 by protected human bait, between 18:00 and 22:00 with Shannon traps, and between 19:00 and the next morning at 6:00 by CDC light traps. The sand flies were identified based on the morphology of their spermathecae, measurements of wing veins, the ratio of the palpus length to antenna and the thorax color (Young and Duncan, 1994). These morphologically identified specimens were fixed in absolute ethanol and stored at room temperature for further molecular analysis.

2.2. DNA extraction

Ethanol-fixed sand flies were individually lysed in 50 μl of 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. The samples were incubated at 37 °C overnight, heated at 95 °C for 5 min, and then 0.5 μl portions were directly used as the templates for PCR amplification.

2.2.1. PCR amplification and sequence analysis of *Lu. ayacuchensis* cytochrome oxidase I and cytochrome b genes

The *Lu. ayacuchensis* COI gene fragment was amplified with universal COI primers (LCO1490: GGTCAACAAATCATAAAGATATTGG and HCO2198: TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994), and the *cyt* b gene fragment was amplified with primers prepared based on the *Lu. ayacuchensis cyt* b gene sequences (Lay cyt S: TGTCGAGATGTTAACTATGG and Lay cyt R: TGCTATTTAAGCTTATTAAC) (Yamamoto et al., 2013). PCR amplification was carried out in a volume of 15 μl with the primers (0.4 μM each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and high fidelity DNA polymerase (KOD-Plus-ver.2; TOYOBO, Tokyo, Japan). After an initial denaturation at 95 °C for 5 min, amplification was performed with 35 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and polymerization (72 °C, 1 min), followed by a final extension at 72 °C for 10 min. The PCR products were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics, Tokyo, Japan) to remove excessive primers, and the sequences were directly determined with a forward primer by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

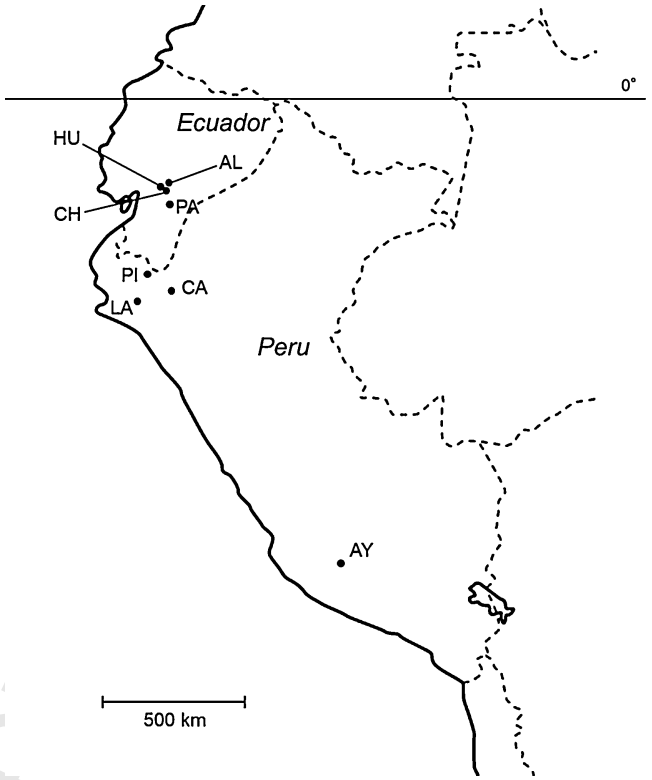

Fig. 1. (A) Map of Ecuador and Peru showing the geographic locations where *Lutzomyia ayacuchensis* were collected. HU, Huigra; CH, Chanchan; AL, Alausi; PA, Paute; PI, Piura; CA, Cajamarca; LA, Lambayeque; AY, Ayacucho.

2.3. Data analysis

The sequences were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the MEGA program (Molecular Evolutionary Genetics Analysis) version 5.2 (Tamura et al., 2011). The pairwise genetic distances between groups were analyzed by MEGA 5.2 using the Kimura two-parameter (Tamura et al., 2011). Phylogenetic analyses were performed by the Maximum Likelihood (ML) method with the distance algorithms available in the MEGA package (Tamura et al., 2011). The number of segregating sites, number of haplotypes, haplotype diversity, average number of differences, and nucleotide diversity for each population were calculated using DnaSP 5.0 (Rozas et al., 2003), and genetic diversity between populations was determined using MEGA 5.2 (Tamura et al., 2011). A haplotype network was constructed using the median-joining methods as implemented in the program

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