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Genetic diversity of the mitochondrial cytochrome *b* gene in *Lutzomyia* spp., with special reference to *Lutzomyia peruensis*, a main vector of *Leishmania (Viannia) peruviana* in the Peruvian Andes

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Abstract

The genetic divergence caused by genetic drift and/or selection is suggested to affect the vectorial capacity and insecticide susceptibility of sand flies, as well as other arthropods. In the present study, cytochrome *b* (*cyt* b) gene sequences were determined in 13 species circulating in Peru to establish a basis for analysis of the genetic structure, and the intraspecific genetic diversity was assessed in the *Lutzomyia (Lu.) peruensis*, a main vector species of *Leishmania (Viannia) peruviana* in Peruvian Andes. Analysis of intraspecific genetic diversity in the *cyt* b gene sequences from 36 *Lu. peruensis* identified 3 highly polymorphic sites in the middle region of the gene. Haplotype and gene network analyses were performed on the *cyt* b gene sequences of 130 *Lu. peruensis* in 9 Andean areas from 3 Departments (Ancash, Lima and La Libertad). The results showed that the populations of La Libertad were highly polymorphic and that their haplotypes were distinct from those of Ancash and Lima, where dominant haplotypes were observed, suggesting that a population bottleneck may have occurred in Ancash and Lima, but not in La Libertad. The present study indicated that the middle region of the *cyt* b gene is useful for the analysis of genetic structure in sand fly populations.

Keywords: Sand fly; *Lutzomyia*; cytochrome *b*; haplotype

1. Introduction

Phlebotomine sand flies are insects of the family Psychodidae in the order Diptera. Approximately 800 sand fly species have been recorded; however, only some serve as vectors and transmit zoonotic and human diseases such as leishmaniasis (Munstermann, 2004; Kato et al., 2010). Sand flies of the genus *Lutzomyia* are primarily responsible for the transmission of leishmaniasis in the New World (Munstermann, 2004; Kato et al., 2010). It is becoming obvious that a restricted number of species support the development of specific *Leishmania* species and consequently transmit them. Therefore, surveillance of circulating sand flies is important for predicting 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); however, morphological classifications are not always reliable because of closely-related species and intraspecific variation. Consequently, other characteristics like molecular markers have been explored for the development of reliable tools for species identification, as well as an understanding of intraspecific genetic diversity and population structure (Torgerson et al., 2003; Beati et al., 2004; Terayama et al., 2008; Kuwahara et al., 2009; Fujita et al., 2012). Although discrepancies between morphologic groupings and the phylogenetic relationships were occasionally found, ribosomal RNA (rRNA) genes were shown to be useful genetic markers for identifying sand flies at the species level (Beati et al., 2004; Terayama et al., 2008; Fujita et al., 2012). The rRNA internal transcribed spacer (ITS) 1 and ITS2 sequences were also analyzed in sand flies, and their utility for taxonomic purposes, but not for intraspecific analysis, was reported (Kuwahara et al., 2009; Hamarsheh et al., 2009; Belen et al., 2011; Florin et al., 2011).

It has been suggested that genetic divergence caused by genetic drift and/or selection may affect the vectorial capacity and insecticide susceptibility of sand flies, as well as other arthropods (Lanzaro et al., 1993; Hassan et al., 2012). Maternally inherited mitochondrial genes reflect the evolutionary history more accurately because of their clonal inheritance, the lack of recombination and higher mutation rate compared with nuclear DNA (Avise, 1994; Rokas et al., 2003). Thus, mitochondrial genes have been used in population genetics and evolutionary study of vector species including phlebotomine sand flies, and several mitochondrial genes have been applied to estimate the population structure of sand fly species (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). Of these, the 3'-end of the cytochrome *b* (*cyt* b) gene has proven to be a suitable marker to evaluate genetic structure in *Lutzomyia (Lu.) whitmani* (Ishikawa et al., 1999), *Lu. longipalpis* (Hodgkinson et al., 2003; Rocha et al., 2011) and *Lu. verrucarum* (Cohnstaedt et al., 2012). In the present study, *cy t* b gene sequences including the 5' region were determined in 13 sand fly species circulating in Peru to establish a basis for the analysis of population structure, and the intraspecific genetic diversity was assessed for *Lu. peruensis*, a main vector species of *Leishmania (Viannia) peruviana* in the Peruvian Andes (Perez et al., 1991, 2007; Kato et al., 2008, 2011).

2. Materials and methods

2.1.Sand fly collection

Sand flies were collected with CDC light traps and Shannon traps peridomiciliary and/or extradomiciliary in an Amazonian area, Tarapoto, Department of San Martin, and 17 Andean areas of Peru; San Ignacio, La Capilla and Santa Cruz de Toledo, Department of Cajamarca; Chanchamayo, La Cuesta and Pedregal, Department of La Libertad; San Pedro de Chonta, Department of Huanuco; Colcap Bajo, Curcuy, Huanchoc, Atocshay and Pucuhuayllan, Department of Ancash; Ambar and Jalcan, Department of Lima; Pullo, Department of Ayacucho; and Ocobamba and Maranura, Department of Cusco (Fujita et al., 2012). All the flies were captured throughout the night between 18:00 p.m. and 6:00 a.m. with CDC light traps and between 18:00 p.m. and 22:00 p.m. with Shannon traps. The sand flies were morphologically identified based on measurements of wing veins, the ratio of palpus length to length of antenna and the color of the thorax (Young and Duncan, 1994). *Lutzomyia (Lu.) peruensis* captured in 9 Andean areas (La Cuesta and Pedregal, Department of La Libertad; Colcap Bajo, Curcuy, Huanchoc, Atocshay and Pucuhuayllan, Department of Ancash; Ambar and Jalcan, Department of Lima) (Fig. 1) were subjected to haplotype analysis. The sand flies were fixed in 100% ethanol and stored at room temperature.

2.2.DNA extraction

Individual ethanol-fixed sand flies were 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 for 12 h, 25 μl of distilled water was added and 0.5 μl portions were directly used as the

templates for PCR amplification.

2.3.PCR amplification and sequence analysis of Lutzomyia *cytochrome* b *genes*

Since *Lutzomyia cyt* b gene sequences have been determined only in the 3' region, a forward primer cyt-2S (5'-TGATGAAATTTTGGWTCTTTA-3') was designed based on the sequences conserved among *Aedes aegypti, Anopheles gambiae, Culicoides arakawa* and *Drosophila melanogaster*. A reverse primer cyt-R (5'-AGTTTCTATCTAATGTTTTC-3') was prepared on the basis of *cyt* b gene sequences conserved among sand fly species. Another forward primer, cyt-4S (5'-ATAGCMTTTAATAGTGTAAC-3'), was prepared based on the conserved sequences among *Lutzomyia cyt* b genes obtained in this study using cyt-2S and cyt-R primers. PCR amplification using cyt-2S and cyt-R primers or cyt-4S and cyt-R primers 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 (50˚C, 1 min) and polymerization (72˚C, 1 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), and plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN, Hamburg, Germany). 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). For the haplotype analysis, *Lu. peruensis cyt* b genes were amplified with a set of species-specific primers (Lu.peru cyt S: 5'-AGGAACAGCCTTTATAGGAT-3'

and Lu.peru cyt R: 5'-ATTTGACCTGTGAGGATATA-3') with 35 cycles of denaturation (95˚C, 1 min), annealing (55˚C, 1 min) and polymerization (72˚C, 1 min). PCR products were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics) to remove excessive primers, and the sequences were directly determined with a Lu.peru cyt S primer.

2.4.Data analysis

The *cyt* b sequences were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the MEGA program (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). A mathematical measure of entropy was used to evaluate the sequence diversity of the *Lu. peruensis cyt* b gene sequences. Entropy values for each nucleotide position were calculated by using the Shannon Heterogeneity in Alignments Tool (SHiAT) (http://evolve.zoo.ox.ac.uk/evolve/SHiAT.html). The pairwise genetic distances between groups were analyzed by MEGA 4.0 using the Kimura two-parameter (Tamura et al., 2007). Phylogenetic analyses were performed by the neighbor-joining (NJ) method and maximum parsimony (MP) method with the distance algorithms available in the MEGA package (Tamura et al., 2007). The number of haplotypes per population, segregating sites, and number of substitutions between haplotypes were determined using DnaSP 5.0 (Rozas et al., 2003). A haplotype network was constructed based on statistical parsimony using TCS 1.21 (Clement et al., 2000).

3. Results

3.1.Sequence analyses of the sand fly cytochrome b *gene*

To further establish a basis for analysis of intraspecific genetic structure in sand fly populations, *cyt* b gene sequences including the 5' region were determined in 13 species, *Lutzomyia (Lu.) caballeroi, Lu. castanea, Lu. munaypata, Lu. noguchii, Lu. nuneztovari, Lu. ayacuchensis, Lu. peruensis, Lu. nevesi, Lu. robusta, Lu. maranonensis, Lu. verrucarum, Lu. tejadai* and *Warileya (W.) phlebotomanica*, captured in 18 areas of Peru (Fig. 1). PCR using cyt-2S and cyt-R primers successfully amplified *cyt* b genes of 8 sand fly species, *Lu. caballeroi, Lu. castanea, Lu. munaypata, Lu. nuneztovari, Lu. peruensis, Lu. robusta, Lu. verrucarum* and *W. phlebotomanica*. The *cyt* b gene fragments of the other 5 species were amplified using the cyt-R primer and cyt-4S forward primer newly prepared from the sequences of the 8 species obtained in this study. The PCR amplified amplicon by primers cyt-2S and cyt-R was 1,058 bp, and that amplified by primers cyt-4S and cyt-R was 966 bp. Based on the mitochondrion complete genome sequences from *Drosophila melanogaster* (GenBank accession number: NC_001709) and *Aedes aegypti* (GenBank accession number: NC_010241) each containing a 1,137bp-long *cyt* b gene, the 2S-R and 4S-R fragments corresponded to the nucleotide positions (nt) 112-1,137 and nt204-1,137 of *cyt* b gene, respectively, followed by approximately 30bp of the tRNA-Ser gene. The fragments were expected to cover approximately 90% and 82% of the coding region of the *Lutzomyia cyt* b gene, respectively. The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB761134-AB761146.

A phylogenetic analysis was performed on the newly determined 13 *cyt* b gene

sequences to observe the relationships among species. As shown in Fig. 2, most species that belong to the same subgenus were located in the same cluster; that is, *Lu. caballeroi, Lu. castanea, Lu. tejadai, Lu. munaypata, Lu. ayacuchensis, Lu. peruensis* and *Lu. noguchii* composed a clade of the subgenus *Helcocyrtomyia* whereas *Lu. robusta, Lu. maranonensis* and *Lu. nevesi* made up another clade of the subgenus *Pifanomyia* (Fig. 2). On the other hand, two *Verrucarum* group species, *Lu. nuneztovari* and *Lu. verrucarum,* were located in a clade of the subgenus *Helcocyrtomyia* and subgenus *Pifanomyia*, respectively (Fig. 2). *Warileya phlebotomanica* was positioned in a separate branch from *Lutzomyia* species (Fig. 2).

3.2.Haplotype analysis of the Lutzomyia peruensis *cytochrome* b *gene*

Intraspecific genetic diversity was assessed in *Lu. peruensis.* As a first step, *cyt* b gene sequences of 36 *Lu. peruensis* collected from 8 Andean areas (5 flies each from La Cuesta, Pedregal, Curcuy, Huanchoc, Atocshay, Pucuhuayllan and Jalcan, and 1 from Colcap Bajo) were determined by direct sequencing using cyt-2S or cyt-R primer and the 920bp fragments (nt31-950 in 2S-R fragment of *Lu. peruensis cyt* b gene) were aligned. The amount of variability at each position of the alignment was evaluated by an entropy plot using SHiAT, and highly polymorphic sites were observed at nt330-400, nt480-580 and nt620-750 (Fig. 3). Therefore, the *cyt* b gene sequence of the 580bp-fragment (nt 315-894) including 3 polymorphic sites was further analyzed in 130 *Lu. peruensis* collected from 9 Andean areas (Fig. 1). The sequence analysis identified the presence of 62 haplotypes with 52 segregating sites (Fig. 4). No more than two different nucleotides were identified at each segregating site, and neither nonsense mutations nor insertions/deletions were observed (Fig. 4). Among the

haplotypes, transitions outnumbered transversions by 52 to 2 (Fig. 4). The number of segregating sites (S), the number of haplotypes (H), the genetic diversity estimated from segregating sites (θs), and the nucleotide diversity (π) were calculated for each population (Table 1). Haplotype analysis showed that 37 sand flies from Huanchoc, Department of Ancash, belonged to 27 haplotypes, and 31 individuals from Ambar, Department of Lima, consisted of 12 haplotypes (Fig. 4, Table 1). In these areas, dominant haplotypes were noted; haplotype Hap5 in Ancash and haplotype Hap3 in Lima (Fig. 4). Interestingly, populations belonging to Hap5 were also observed in Lima and populations of Hap3 were also distributed in Ancash (Fig. 4). On the other hand, 37 sand flies from La Cuesta, Department of La Libertad, composed 27 haplotypes each including less than 3 individuals in the absence of apparently dominant haplotype, showing distinct haplotype patterns from Ancash and Lima (Fig. 4). A higher genetic diversity of the La Cuesta population (θs=1.4%, π=0.67%) was supported by statistical analyses when compared to other populations (θs=0.25-0.71%, π=0.21-0.52%) (Table 1). Most flies from Pedregal, a neighboring community of La Cuesta in Department of La Libertad, belonged to the haplotypes of the La Cuesta population (Hap13, Hap21, Hap22 and Hap23), indicating distribution of similar populations in these areas (Fig. 4). On the contrary, all populations except for one from Ancash and Lima belonged to different haplotypes from those of La Libertad (Fig. 4). A Tajima's D neutrality test was performed to verify population expansions, and the statistics were zero or negative for all populations with some having significant values, suggesting recent population expansion or genetic bottlenecks (Table 1). The genetic divergence between populations was the lowest between Ambar and Jalcan (0.21%), and the highest between La Cuesta and Colcap Bajo (0.98%) (Table 2). The

divergences among populations within Departments were the highest (0.60%) in La Libertad and the lowest (0.21%) in Lima (Table 2). When the genetic divergence between Departments was analyzed, the populations between Ancash and Lima (0.44%) were closer when compared those between La Libertad and Ancash (0.82%) and between La Libertad and Lima (0.63 %). Haplotype variation visualized as a gene network showed that the populations of Ancash and Lima had closer relationships, whereas La Libertad populations were apart from those of Ancash and Lima and their genetic structure was highly polymorphic (Fig. 5). The haplotype Hap3 seemed to be an ancestor for other haplotypes as it occupied the deep center of the network.

4. Discussion

In the present study, *cy t* b gene sequences were determined from 13 sand fly species circulating in Peru and the genetic diversity among and within species was assessed. Phylogenetic analyses of the *cyt* b gene among species generally supported the morphological classification; however, discrepancies between the morphological classification and phylogenetic relationship of the sequences were noted in the *Verrucarum* groups as reported in the analyses of rRNA genes (Beati et al., 2004; Terayama et al., 2008; Kuwahara et al., 2009; Fujita et al., 2012). This result suggested that the *cyt* b gene analysis will be a useful tool for identification of *Lutzomyia* species, although careful reconsideration is required for the classification of several species, especially in the *Verrucarum* group. The newly designed primers, cyt-4S and cyt-R, successfully amplified *cyt* b gene fragments covering approximately 82% of the coding region from 12 *Lutzomyia* and 1 *Warileya* species. The results suggest that the primer set will work for amplification of *cyt* b genes from most sand fly species.

The genetic background of individual arthropod species and populations within those species are suggested to influence vectorial capacity and insecticide susceptibility (Lanzaro et al., 1993; Hassan et al., 2012). Since the larvae live in soil and the adults fly mostly less than 1 km (Alexander, 1987; Alexander and Young, 1992; Morrison et al., 1993). Therefore, a limited range of activity may result in a site-dependent genetic diversity in sand fly populations. In the present study, the *cyt* b gene was applied for the first time to estimate the population structure of *Lu. peruensis*, a species responsible for transmission of *L. (V.) peruviana* distributed in the Peruvian Andes (Perez et al., 1991, 2007; Kato et al., 2008, 2011). Direct sequence analysis of a 920bp fragment

from 36 *Lu. peruensis* in various geographical locations identified 3 polymorphic sites at nt330-400, nt480-580 and nt620-750 on the *Lu. peruensis cyt* b 2S-R fragment. In previous studies, a 261bp region of the 3'-end of the *cyt* b gene was studied for the population analysis in *Lu. whitmani* and *Lu. longipalpis* (Ishikawa et al., 1999; Hodgkinson et al., 2002, 2003). In addition, a recent study characterized a 653bp fragment encoding parts of *cyt* b, tRNA-Ser and NADH dehydrogenase subunit I genes of *Lu. verrucarum* (Cohnstaedt et al., 2012). These regions corresponded to the nt769-1,029 and nt573-1,029 in the *Lu. peruensis cyt* b gene 2S-R fragment, respectively. Although genetic variation at nt951-1,029 was not analyzed, 3 highly polymorphic sites were identified in the middle region of the *cyt* b gene in *Lu. peruensis*. Therefore, analysis of a longer *cyt* b gene fragment including the middle region is considered to be useful for the population genetics of sand fly species.

The haplotypes of the 580bp *cyt* b gene fragments including 3 polymorphic sites were evaluated in 130 sand flies in 3 Departments and 62 haplotypes were identified. The high number of haplotypes is largely reflected by higher genetic divergence observed in the population from the Department of La Libertad (32 haplotypes from 42 individuals). In general, higher genetic diversity within a geographical location is found in populations that have not undergone drastic size reductions over evolutionary time, while the effect of genetic drift is reduced when the population size is expanding. Therefore, our results suggested that a population bottleneck may have occurred in Ancash and Lima, but not in La Libertad. Further analysis using a larger number of samples from different areas will be required to confirm this point. The genetic divergence between Departments showed that the populations of La Libertad were distant from those of Ancash and Lima. This may simply be attributable to the

isolation distance; the distance of the study areas between Ancash and Lima is approximately 50 km, whereas the areas of La Libertad are approximately 350 km and 400 km apart from those of Ancash and Lima, respectively. Alternatively, some environmental factors may affect the sand fly fauna. Although the environment such as altitude, weather, temperature and degree of dryness do not seem to be very different among the three areas, the distribution of sand fly species is different between La Libertad and the other two: the main species in La Cuesta and Pedregal of La Libertad are *Lu. peruensis* and *Lu. caballeroi* while *Lu. peruensis*, *Lu. verrucarum* and *Lu. noguchii* are the dominant species in the study areas of Ancash and Lima (Kato et al. 2008, 2011; Fujita et al., 2012). Thus, some environmental factors affecting the sand fly fauna possibly influence the population difference and the effect of genetic drift of *Lu. peruensis*.

In the present study, *cyt* b gene sequences were determined on 13 sand fly species in Peru to establish a basis for the analysis of the population structure. In addition, 3 highly polymorphic sites were identified in the *Lu. peruensis cyt* b gene and the utility of *cyt* b gene analysis for population genetics was verified through its application to the haplotype analysis of *Lu. peruensis* from 9 Andean areas. Further study with larger numbers of sand flies from various areas will clarify the involvement of the genotype in the vectorial capacity and insecticide susceptibility of sand flies in each endemic area.

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References

Alexander, J.B. 1987. Dispersal of *phlebotomine* sand flies (Diptera: Psychodidae) in a Colombian coffee plantation. J. Med. Entomol. 24, 552-558.

Alexander, B., Young, D.G. 1992. Dispersal of phlebotomine sand flies (Diptera: Psychodidae) in a Colombian focus of *Leishmania (Viannia) braziliensis*. Mem. Inst. Oswaldo Cruz. 87, 397-403.

Avise, J.C., Bowen, B.W. 1994. Investigating sea turtle migration using DNA markers. Curr. Opin. Genet. Dev. 4, 882-886.

Beati, L., Caceres, A.G., Lee, J.A., Munstermann, L.E. 2004. Systematic relationships among *Lutzomyia* sand flies (Diptera: Psychodidae) of Peru and Colombia based on the analysis of 12S and 28S ribosomal DNA sequences. Int. J. Parasitol. 34, 225-234.

Belen, A., Kucukyildirim, S., Alten, B. 2011. Genetic structures of sand fly (Diptera: Psychodidae) populations in a leishmaniasis endemic region of Turkey. J. Vector Ecol. 1, 32-48.

Clement, M., Posada, D., Crandall, K.A. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657-1659.

Cohnstaedt, L.W., Caceres, A.G., Beati, L., Munstermann, L.E. 2012. The population structure of *Lutzomyia verrucarum* (Diptera: Psycodidae), a *Bartonella bacilliformis* and *Leishmania peruviana* vector in Peru. J. Med. Entomol. 49, 77-84.

Esseghir, S., Ready, P.D., Killick-Kendrick, R., Ben-Ismail, R. 1997. Mitochondrial haplotypes and phylogeography of *Phlebotomus* vectors of *Leishmania major*. Insect Mol. Biol. 6, 211-225.

Florin, D.A., Davies, S.J., Olsen, C., Lawyer, P., Lipnick, R., Schultz, G., Rowton, E., Wilkerson, R., Keep, L. 2011. Morphometric and molecular analyses of the sand fly species *Lutzomyia shannoni* (Diptera: Psychodidae: Phlebotominae) collected from seven different geographical areas in the southeastern United States. J. Med. Entomol. 46, 154-166.

Fujita, M., Kato, H., Caceres, A.G., Gomez, E.A., Velez, L., Mimori, T., Zhang, F., Iwata, H., Korenaga, M., Sakurai, T., Katakura, K., Hashiguchi, Y. 2012. Genotyping of sand fly species in Peruvian Andes where leishmaniasis is endemic. Acta Trop. 121, 93-98.

Hamarsheh, O., Presber, W., Abdeen, Z., Sawalha, S., Al-Lahem, A., Schonian, G. 2007. Genetic structure of Mediterranean populations of the sandfly *Phlebotomus papatasi* by mitochondrial cytochrome *b* haplotype analysis. Med. Vet. Entomol. 21, 270-277.

Hamarsheh, O., Presber, W., Al-Jawabreh, A., Abdeen, Z., Amro, A., Schonian, G. 2009. Molecular markers for *Phlebotomus papatasi* (Diptera: Psychodidae) and their usefulness for population genetic analysis. Trans. R. Soc. Trop. Med. Hyg. 103, 1085-1086.

Hassan, M.M., Widaa, S.O., Osman, O.M., Numiary, M.S., Ibrahim, M.A., Abshama, H.M. 2012. Insecticide resistance in the sand fly, *Phlebotomus papatasi* from Khartoum State, Sudan. Parasit. Vectors. 5, 46.

Hodgkinson, V.H., Birungi, J., Haghpanah, M., Joshi, S., Munstermann, L.E. 2002. Rapid identification of mitochondrial cytochrome *b* haplotypes by single strand conformation polymorphism in *Lutzomyia longipalpis* (Diptera: Psychodidae) populations. J. Med. Entomol. 39, 689-694.

Hodgkinson, V.H., Birungi, J., Quintana, M., Dietze, R., Munstermann, L.E. 2003. Mitochondrial cytochrome *b* variation in populations of the visceral leishmaniasis vector *Lutzomyia longipalpis* across eastern Brazil. Am. J. Trop. Med. Hyg. 69, 386-392.

Ishikawa, E.A., Ready, P.D., de Souza, A.A., Day, J.C., Rangel, E.F., Davies, C.R., Shaw, J.J. 1999. A mitochondrial DNA phylogeny indicates close relationships between populations of *Lutzomyia whitmani* (Diptera: Psychodidae, Phlebotominae) from the rain-forest regions of Amazonia and northeast Brazil. Mem. Inst. Oswaldo Cruz. 94, 339-345

Kato, H., Caceres, A.G., Gomez, E.A., Mimori, T., Uezato, H., Marco, J.D., Barroso, P.A., Iwata, H., Hashiguchi, Y. 2008. Molecular mass screening to incriminate sand fly vectors of Andean-type cutaneous leishmaniasis in Ecuador and Peru. Am. J. Trop. Med. Hyg. 79, 719-721.

Kato, H. , Gomez, E.A., Caceres, A.G., Uezato, H., Mimori, T., Hashiguchi, Y. 2010. Molecular epidemiology for vector research on leishmaniasis. Int. J. Env. Res. Pub. Heal. 7, 814-826.

Kato, H., Gomez, E.A., Caceres, A.G., Vargas, F., Mimori, T., Yamamoto, K., Iwata, H., Korenaga, M., Velez, L., Hashiguchi, Y. 2011. Natural infections of man-biting sand flies by *Leishmania* and *Trypanosoma* species in the northern Peruvian Andes. Vector Borne Zoonotic Dis. 11, 515-521.

Kuwahara, K., Kato, H., Gomez, E.A., Uezato, H., Mimori, T., Yamamoto, Y., Calvopina, M., Caceres, A.G., Iwata, H., Hashiguchi, Y. 2009. Genetic diversity of ribosomal RNA internal transcribed spacer sequences in *Lutzomyia* species from areas endemic for New World cutaneous leishmaniasis. Acta Trop. 112, 131-136.

Lanzaro, G.C., Ostrovska, K., Herrero, M.V., Lawyer, P.G., Warburg, A. 1993. *Lutzomyia longipalpis* is a species complex: genetic divergence and interspecific hybrid sterility among three populations. Am. J. Trop. Med. Hyg. 48, 839-847.

Morrison, A.C., Ferro, C., Morales, A., Tesh, R.B., Wilson, M.L. 1993. Dispersal of the sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. J. Med. Entomol. 30, 427-435.

Munstermann, L.E. 2004. Phlebotomine sand flies, the Psychodidae. In: WC. Marquardt, WC. Black, JE. Freier, HH. Hagedorn, J. Hemingway, S. Higgs, AA. James, B. Kondratieff, CG. Moore, (Ed.), Biology of Disease Vectors. Second edition. Elsevier, San Diego CA, 141-151.

Perez, J.E., Villaseca, P., Caceres, A., Lopez, M., Zolessi, A., Campos, M., Guerra, H., Llanos-Cuentas, A. 1991. *Leishmania (Viannia) peruviana* isolated from the sandfly *Lutzomyia peruensis* (Diptera: Psychodidae) and a sentinel hamster in the Huayllacallán Valley, Ancash, Peru. Trans. R. Soc. Trop. Med. Hyg. 85, 60.

Perez, J.E., Veland, N., Espinosa, D., Torres, K., Ogusuku, E., Llanos-Cuentas, A., Gamboa, D., Arevalo, J. 2007. Isolation and molecular identification of *Leishmania (Viannia) peruviana* from naturally infected *Lutzomyia peruensis* (Diptera: Psychodidae) in the Peruvian Andes. Mem. Inst. Oswaldo Cruz. 102, 655-658.

Rocha, L.de S., Falqueto, A., Dos Santos, C.B., Grimaldi, G.J., Cupolillo, E. 2011. Possible implication of the genetic composition of the *Lutzomyia longipalpis* (Diptera: Psychodidae) populations in the epidemiology of the visceral leishmaniasis. J. Med. Entomol. 48, 1016-1022.

Rokas, A., Melika, G., Abe, Y., Nieves-Aldrey, J.L., Cook, J.M., Stone, G.N. 2003. Lifecycle closure, lineage sorting, and hybridization revealed in a phylogenetic

analysis of European oak gallwasps (Hymenoptera: Cynipidae: Cynipini) using mitochondrial sequence data. Mol. Phylogenet. Evol. 26, 36-45.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinfrmatics. 19, 2496-2497.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 123, 585-595.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

Terayama, Y., Kato, H., Gomez, E.A.L, Uezato, H., Calvopina, M., Iwata, H., Hashiguchi, Y. 2008. Molecular typing of sand fly species from areas endemic for leishmaniasis in Ecuador by PCR-RFLP of 18S ribosomal RNA gene. J. Vet. Med. Sci. 70, 907-913.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Torgerson, D.G., Lampo, M., Velazquez, Y., Woo, P.T. 2003. Genetic relationships among some species groups within the genus *Lutzomyia* (Diptera: Psychodidae). Am. J. Trop. Med. Hyg. 69, 484-493.

Young, D.G., Duncan, M.A. 1994. Guide to the Identification and Geographic Distribution of *Lutzomyia* Sand Flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae), Memoirs of the American Entomological Institute, 54, Associated Publishers—American Entomological Institute, Gainsville, FL.

Figure Legends

Fig. 1. Map of Peru showing the geographic locations where sand flies were collected along with the respective species analyzed in this study. The numbers represent the following locations: 1. San Ignacio, 2. La Capilla, 3. Santa Cruz de Toledo, Department of Cajamarca; 4. Tarapoto, Department of San Martin; 5. Chanchamayo, 6. La Cuesta and Pedregal, Department of La Libertad; 7. San Pedro de Chonta, Department of Huanuco; 8. Colcap Bajo, Curcuy, Huanchoc, Atocshay and Pucuhuayllan, Department of Ancash; 9. Ambar and Jalcan, Department of Lima; 10. Ocobamba and 11. Maranura, Department of Cusco; 12. Pullo, Department of Ayacucho. Unshaded circles represent the areas where *Lutzomyia (Lu.) peruensis* subjected to the haplotype analysis were captured. Cab, *Lu. caballeroi*; Cas, *Lu. castanea*; Mun, *Lu. munaypata*; Nog, *Lu. noguchii*; Nun, *Lu. nuneztovari*; Aya, *Lu. ayacuchensis*; Per, *Lu. peruensis*; Nev, *Lu. nevesi*; Rob, *Lu. robusta*; Mar, *Lu. maranonensis*; Ver, *Lu. verrucarum*; Tej, *Lu. tejadai*; Wph, *Warileya phlebotomanica*. Adapted from a map available at http://english.freemap.jp/.

Fig. 2. Phylogenetic tree of cytochrome *b* gene sequences among sand fly species. The cytochrome *b* genes of 13 species were amplified from morphologically identified sand fly samples and sequenced. The scale bar represents 0.02% divergence. Bootstrap values are shown above or below branches.

Fig. 3. The entropy score (a mathematical measure of variability) at each position of the alignment of 920bp cytochrome *b* gene sequences of 36 *Lutzomyia peruensis* collected from 8 Andean areas. The entropy score was calculated by using the Shannon

Heterogeneity in Alignments Tool (SHiAT) and each bar represents variability at a single nucleotide position. The nucleotide position is numbered based on the *Lutzomyia peruensis* cytochrome *b* gene 2S-R fragment obtained in this study. The 580bp fragment sequences including 3 polymorphic sites (dotted frame) was used for the haplotype analysis.

Fig. 4. Variable nucleotides found in the alignment of the *Lutzomyia peruensis* cytochrome *b* gene. The cytochrome *b* gene sequence of a 580bp-fragment including 3 polymorphic sites was analyzed in 130 *Lu. peruensis* collected from 9 Andean areas (LCU, La Cuesta; PDL, Pedregal; CLB, Colcap Bajo; CRC, Curcuy; HUA, Huanchoc; ATC, Atocshay; PCH, Pucuhuayllan; AMB, Ambar; JLC, Jalcan). Dots denote identical sequences and numbers show their corresponding positions from the *Lutzomyia peruensis* cytochrome *b* gene 2S-R fragment obtained in this study.

Fig. 5. Haplotype network of the cytochrome *b* sequences of *Lutzomyia peruensis* collected from 9 Andean areas of 3 Departments (Ancash, Lima and La Libertad). The number of specimens in each haplotype is shown in parentheses. Each haplotype is represented by a circle sized in proportion to the frequency of the haplotypes, and populations are differentiated by color. Each crossbar represents one nucleotide substitution. Small black circles indicate one nucleotide substitution between haplotypes.

Table 1. Summary statistics of the *Lutzomyia peruensis* population group

Department	Locality	N	S	H	θs	π	Tajima's D
La Libertad	La Cuesta	37	34	27	0.01404	0.00668	-1.84528*
La Libertad	Pedregal	5	7	5	0.00579	0.00517	-0.74682
Ancash	Colcap Bajo	1	-	-	-	-	-
Ancash	Curcuy	5	5	3	0.00414	0.00414	0.00000
Ancash	Huanchoc	36	17	17	0.00707	0.00385	-1.50549
Ancash	Atocshay	5	7	5	0.00579	0.00517	-0.74682
Ancash	Pucuhuayllan	5	6	4	0.00497	0.00448	-0.66823
Lima	Ambar	31	13	12	0.00561	0.00230	-2.05155*
Lima	Jalcan	5	3	3	0.00248	0.00207	-1.04849
Total		130	52	62	0.01648	0.00569	-2.07537*

N, number of individuals sequences; S, number of segregating sites; H, number of haplotypes; θs, genetic diversity estimated from segregating sites; π, nucleotide diversity; *$p < 0.05$.

Table 2. Genetic divergence between populations of *Lutzomyia peruensis* using a cytochrome *b* gene fragment

Locality*	LCU	PDL	CLB	CRC	HUA	ATC	PCH	AMB	JLC
La Cuesta									
Pedregal	0.0060								
Colcap Bajo	0.0098	0.0091							
Curcuy	0.0086	0.0079	0.0024						
Huanchoc	0.0082	0.0076	0.0039	0.0042					
Atocshay	0.0088	0.0084	0.0063	0.0058	0.0047				
Pucuhuayllan	0.0077	0.0069	0.0045	0.0047	0.0042	0.0051			
Ambar	0.0064	0.0056	0.0058	0.0054	0.0042	0.0050	0.0036		
Jalcan	0.0061	0.0054	0.0056	0.0052	0.0041	0.0049	0.0034	0.0021	
*Lu. ayacuchensis***	0.1505	0.1528	0.1496	0.1485	0.1502	0.1520	0.1504	0.1517	0.1504

*LCU, La Cuesta; PDL, Pedregal; CLB, Colcap Bajo; CRC, Curcuy; HUA, Huanchoc; ATC, Atocshay; PCH, Pucuhuayllan; AMB, Ambar; JCL, Jalcan.

***Lutzomyia ayacuchensis*, a member of the subgenus *Helcocyrtomyia*, was used as an outgroup.

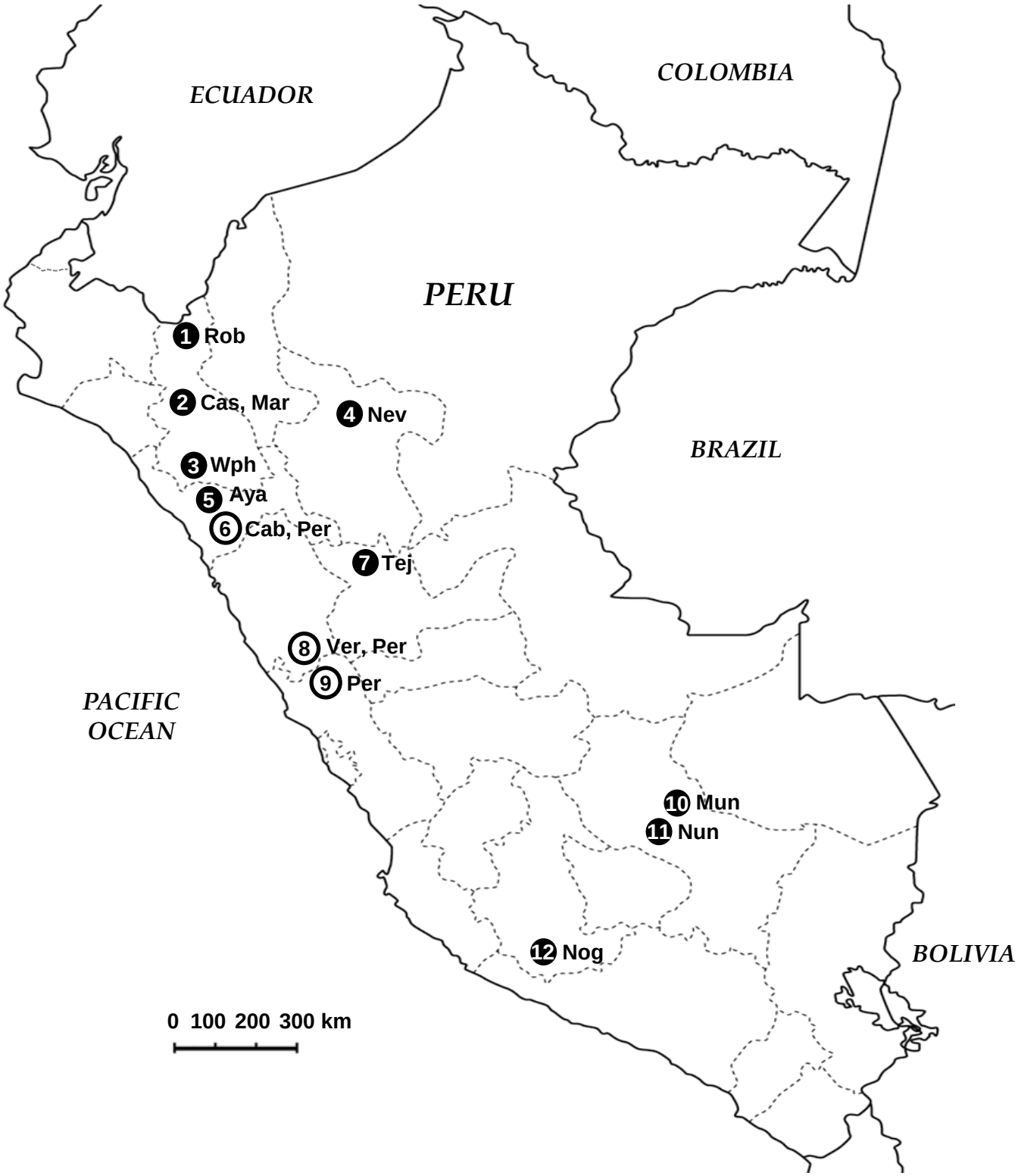

ECUADOR
COLOMBIA
PERU
BRAZIL
BOLIVIA
PACIFIC OCEAN
1 Rob
2 Cas, Mar
3 Wph
4 Nev
5 Aya
6 Cab, Per
7 Tej
8 Ver, Per
9 Per
10 Mun
11 Nun
12 Nog
0 100 200 300 km

Fig. 1

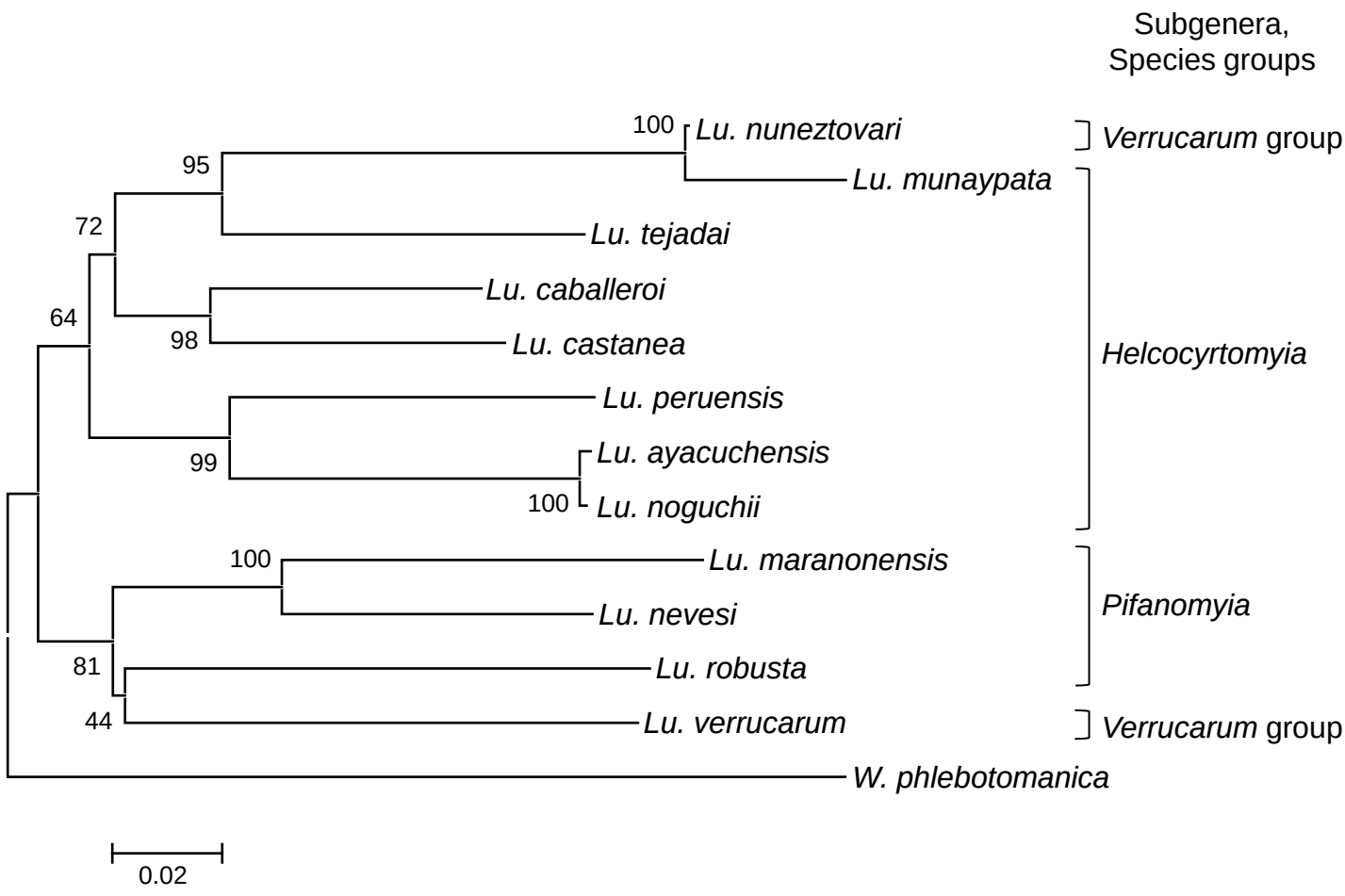

Subgenera,
Species groups
100
Lu. nuneztovari
Verrucarum group
95
Lu. munaypata
72
Lu. tejadai
Lu. caballeroi
64
98
Lu. castanea
Helcocyrtomyia
Lu. peruensis
99
Lu. ayacuchensis
100
Lu. noguchii
100
Lu. maranonensis
Lu. nevesi
Pifanomyia
81
Lu. robusta
44
Lu. verrucarum
Verrucarum group
W. phlebotomanica
0.02

Fig. 2

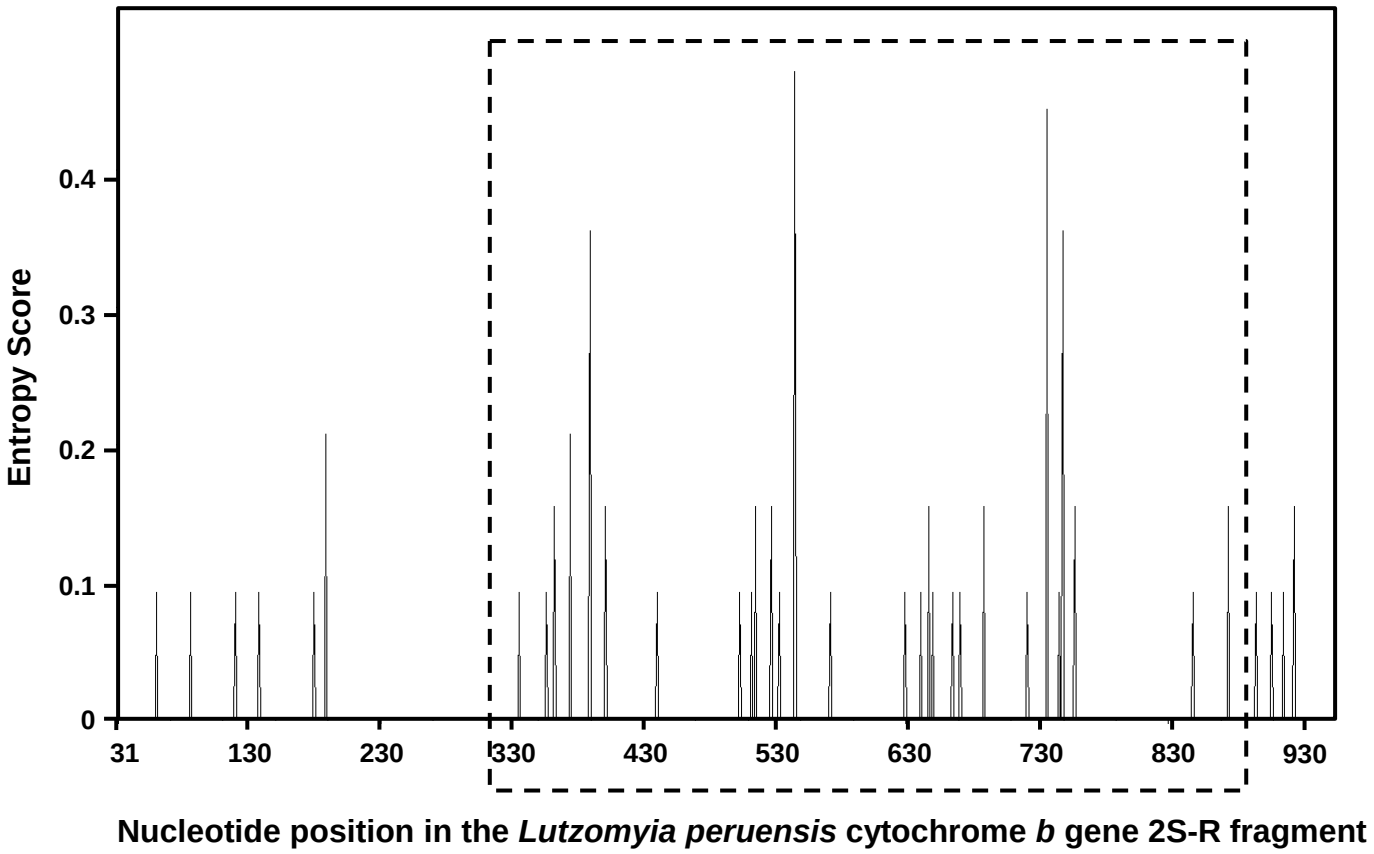

Entropy Score
0
0.1
0.2
0.3
0.4
31
130
230
330
430
530
630
730
830
930
Nucleotide position in the *Lutzomyia peruensis* cytochrome *b* gene 2S-R fragment

Fig. 3

Haplotype	Segregating sites	Numbers in population								
		La Libertad		Ancash					Lima	
	222222333333333344444445555555556666666666666777788	L	P	C	C	H	A	P	A	J
	578899002234578934566680135667880011223567889035780 1	C	D	L	R	U	T	C	M	L
	800517392465454987024807915246250658479702143930437 0	U	L	B	C	A	C	H	B	C
Hap1	TTGCTAATGAATCCCTACCTGTCGAAAGTTCATCGTCTACCTTCAGGCATTA									1
Hap2	.....T.....T.C.....								1	1
Hap3	.....T.....					2		1	14	3
Hap4	.....T.....T.....C..					2				
Hap5	.....T.....T.....					10		2	4	
Hap6	.....G.....T.....T.....T.....			1	3	7				
Hap7	.....G.....C..T.....T.....						1			
Hap8	.....T...T.....T.....					1	1			
Hap9	....C.....T.....T.....G.....						1			
Hap10	.....G.....T.....T.....G						1			
Hap11	.....T.....T.....G.....					1	1			
Hap12	.....T...T.....T.....G.....				1					
Hap13	.....T.....T.....C.....	2	1		1					
Hap14	.....G.....T.....T.....CT.....G							1		
Hap15	.....CT.....							1		
Hap16	.....G.....T.....C.....	3								
Hap17	.....C..G.....C.T.....C.....	2								
Hap18	.....T.....C.....T.....C.....	1								
Hap19	.....CT.....G.T..T....C.....	1								
Hap20	.....C..G.....T.....C.....		1							
Hap21	.C.....T.....T.....C.....	1	1							
Hap22	.....C.....A.....T....T...C.....	1	1							
Hap23	.....T.....C.....	3	1							
Hap24	.....G.....T.....C.....	1								
Hap25	.....T.....T.C.....	2								
Hap26	.....G.....CT.....C.....	1								
Hap27	.....T.C.A.....C...A.....	1								
Hap28	.....T.....T.....C.....	1								
Hap29	.....CT.....C.....	1								
Hap30	.....T.....TC.....	2								
Hap31	.....TC.....CT.....C..G.....	1								
Hap32	.....T.....C..G.....	1								
Hap33	.....T....T....C.....	1								
Hap34	.....CT...G.....C.....	1								
Hap35	.....G.....T....C.C...C.....	1								
Hap36	.....T.....C.....C.	3								
Hap37	.....G.....A.T.....T....T....C.....	1								
Hap38	.....CT.G.....C.....	1								
Hap39	.....G.....T.C.....C.....T....	1								
Hap40	...T.....T.....T.C.....	1								
Hap41	.....G.....T...A.....C.....	1								
Hap42	.....T...CT.....C.....	1								
Hap43	.....G.....C..T....A..T.....					2				
Hap44	.....C.....T.....T.....					1				
Hap45	.....T..G....T.....T.....					1				
Hap46	.....G.A.....T.....T.....					1				
Hap47	.....G.....T.....T.....T.....					1				
Hap48	A.....T.....T.....					2				
Hap49	.....T.....T.....T....G...					1				
Hap50	....C.....T.....					1				
Hap51	.....G...G.....T.....T.....T.....					1				
Hap52	....C.....T.....T.....					1				
Hap53	.....G.....T.....T.....G....G.....					1				
Hap54	.....C.....T.....								2	
Hap55	.....T.....T.....G.....								1	
Hap56	..A.....T.....T.....								1	
Hap57	.....C.T.....								3	
Hap58	.....T.....C.....								1	
Hap59	.....G.....T.....T.....								1	
Hap60	.....G.....T.....A.....								1	
Hap61	.....G.....T.....								1	
Hap62	.....T.....T.....T.....								1	
Total		37	5	1	5	36	5	5	31	5

Fig. 4

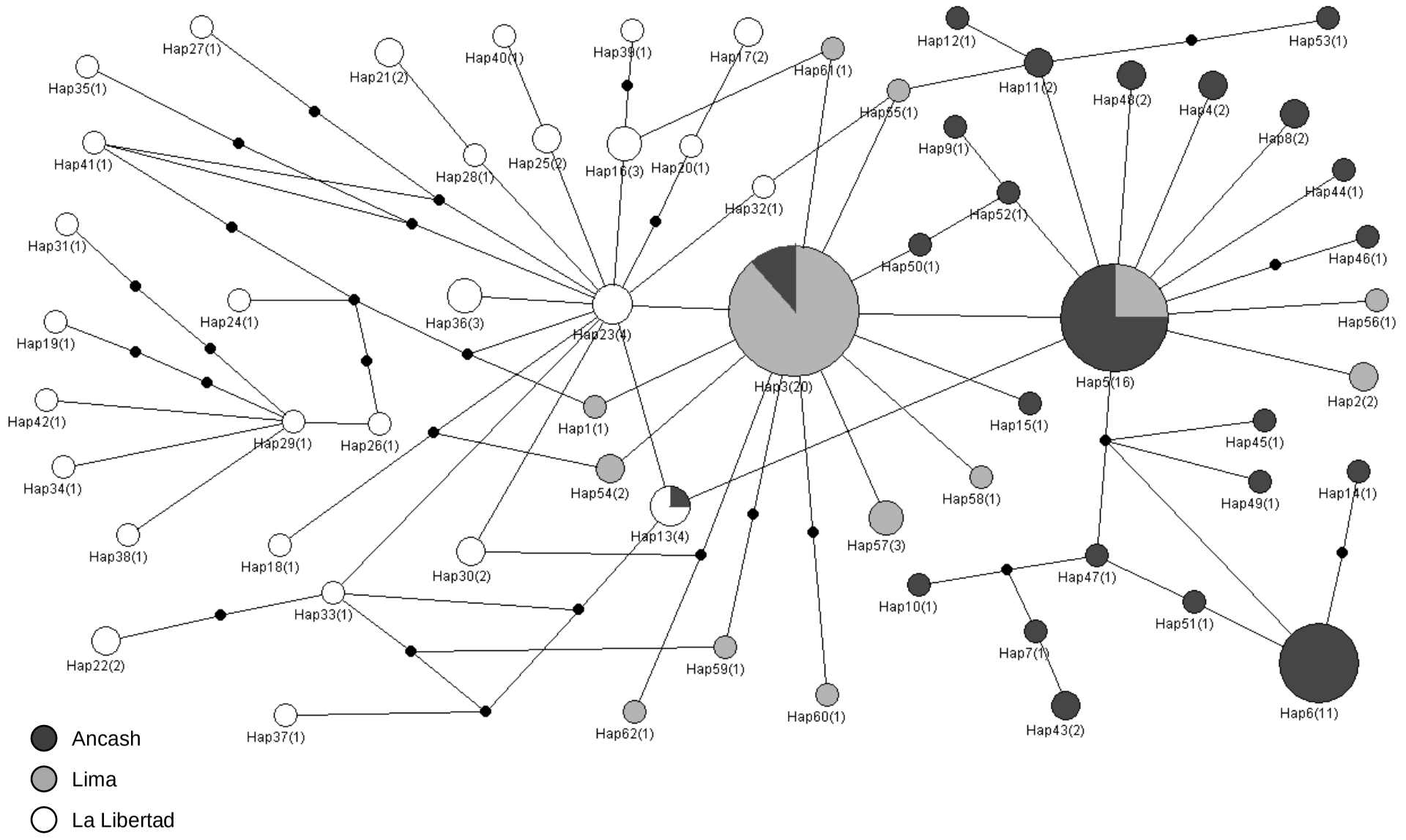

Hap27(1)
Hap35(1)
Hap21(2)
Hap40(1)
Hap39(1)
Hap17(2)
Hap61(1)
Hap12(1)
Hap53(1)
Hap11(2)
Hap48(2)
Hap4(2)
Hap8(2)
Hap55(1)
Hap41(1)
Hap28(1)
Hap25(2)
Hap16(3)
Hap20(1)
Hap9(1)
Hap32(1)
Hap52(1)
Hap44(1)
Hap50(1)
Hap46(1)
Hap31(1)
Hap24(1)
Hap36(3)
Hap23(4)
Hap3(20)
Hap5(16)
Hap56(1)
Hap19(1)
Hap2(2)
Hap42(1)
Hap29(1)
Hap26(1)
Hap1(1)
Hap15(1)
Hap45(1)
Hap34(1)
Hap54(2)
Hap58(1)
Hap49(1)
Hap14(1)
Hap13(4)
Hap38(1)
Hap18(1)
Hap30(2)
Hap57(3)
Hap47(1)
Hap10(1)
Hap33(1)
Hap51(1)
Hap22(2)
Hap59(1)
Hap7(1)
Hap6(11)
Hap60(1)
Hap43(2)
Hap37(1)
Hap62(1)
Ancash
Lima
La Libertad

Fig. 5