Title
DNA barcoding for identification of sand fly species (Diptera: Psychodidae) from leishmaniasis-endemic areas of Peru

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Abstract

Phlebotomine sand flies are the only proven vectors of leishmaniases, a group of human and animal diseases. Accurate knowledge of sand fly species identification is essential in understanding the epidemiology of leishmaniasis and vector control in endemic areas. Classical identification of sand fly species based on morphological characteristics often remains difficult and requires taxonomic expertise. Here, we generated DNA barcodes of the cytochrome c oxidase subunit 1 (COI) gene using 159 adult specimens morphologically identified to be 19 species of sand flies, belonging to 6 subgenera/species groups circulating in Peru, including the vector species. Neighbor-joining (NJ) analysis based on Kimura 2-Parameter genetic distances formed non-overlapping clusters for all species. The levels of intraspecific genetic divergence ranged from 0 to 5.96%, whereas interspecific genetic divergence among different species ranged from 8.39 to 19.08%. The generated COI barcodes could discriminate between all the sand fly taxa. Besides its success in separating known species, we found that DNA barcoding is useful in revealing population differentiation and cryptic diversity, and thus promises to be a valuable tool for epidemiological studies of leishmaniasis.

Keywords: DNA barcode, sand flies, leishmaniasis, vector, Peru
1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) are of significant public health importance in many parts of the world as vectors of the causative agents of leishmaniasis, bartonellosis and sand fly fever (Maroli et al., 2013). Leishmaniasis remains one of the world’s most neglected diseases; endemic in 98 countries, putting 350 million people at risk, with some two million new cases every year (WHO, 2010; Alvar et al., 2012). The disease is caused by several species of cell-infecting parasites of the genus *Leishmania*, and presents three major distinct clinical manifestations: cutaneous, mucocutaneous and visceral forms (Bates, 2007).

Sand flies are the only proven vectors of leishmaniases and approximately 800 species have been recorded in five major genera; *Phlebotomus* and *Sergentomyia* in the Old World, and *Lutzomyia*, *Brumptomyia* and *Warileya* in the New World (Munstermann, 2004). However, only species belonging to the genera *Phlebotomus* and *Lutzomyia* are the putative vectors of *Leishmania* (Munstermann, 2004; Bates, 2007; Kato et al., 2010). Therefore, correct sand fly species identification is very important to design strategies for surveillance and control of leishmaniasis in given endemic areas.

In Peru, three sand fly species, *Lutzomyia* (*Lu.*) *peruensis*, *Lu.* *ayacuchensis* and *Lu.* *verrucarum* have been incriminated as the vectors of *Leishmania* (*Viannia*) *peruviana*, the aetiological agent of Andean-type cutaneous leishmaniasis in the endemic areas (Davies et al., 1993; Perez et al., 1991, 1994, 2007; Caceres et al., 2004; Kato et al., 2008, 2011). Although these sand fly species role in disease transmission has been well studied, detailed molecular taxonomic knowledge of each species is far from complete. Presently, sand flies are typically identified based on morphological features; mainly by internal structures such as the cibarium, pharynx, spermatheca of females and terminal genitalia of males (Young and Duncan, 1994).
However, this morphological classification is laborious, time-consuming and often complicated by phenotypic plasticity and cryptic species complexes, as well as demanding considerable skill and taxonomic expertise. To overcome these technical limitations, in the last three decades molecular approaches have been increasingly employed to explore the taxonomy, population structure and phylogeny in insect vectors including sand flies (Mukhopadhyay et al., 2000; Uribe Soto et al., 2001; Depaquit et al., 2002; Beati et al., 2004; Khalid et al., 2010; Fujita et al., 2012; Yamamoto et al., 2013; Nzelu et al., 2014; Gomez et al., 2014; Kato et al., 2015).

Recently, DNA barcoding has been widely shown to be an effective molecular tool for species identification of many insects (Hebert et al., 2003a, b). Among the mitochondrial genes, cytochrome c oxidase I (COI) is reported to be the most conserved gene and hence has distinct advantages for taxonomic studies (Knowlton and Weigt, 1998). Studies have demonstrated the use of DNA barcoding as a valid molecular tool for identifying dipterans; mosquitoes (Cywinska et al., 2006), black flies (Pramual et al., 2011) and sand flies (Azpurua et al., 2010; Kumar et al., 2012; Contreras Gutiérrez et al., 2014; Nzelu et al., 2014), and revealing sand fly cryptic species (Cohnstaedt et al., 2011; Scarpassa et al., 2012; Kumar et al., 2012). Construction of DNA barcodes for each species of sand fly would provide an efficient tool for their identification. The aim of the present study was to explore the utility of the DNA barcode approach for identification of sand fly species in Peru, where leishmaniasis is endemic, by analyzing morphologically identified adult specimens.
2. Materials and methods

2.1 Sand fly collection

Sand flies were caught using CDC light traps and Shannon traps peridomiciliary, intradomiciliary and extradomiciliary at 12 Departments; Piura, Lambayeque, Cajamarca, La Libertad, San Martin, Huánuco, Lima, Huancavelica, Ayacucho, Apurimac, Cusco and Puno in Peru between 2001 and 2013 (Fig. 1, Table 1). 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 mainly 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), and then fixed in absolute 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 (pH8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] with 100 µg/ml proteinase K. The samples were incubated at 37 °C overnight. Afterwards, 25 µl of distilled water was added, heated at 95 °C for 5 min, and then, 0.5 µl portions were directly used as templates for PCR amplification.

2.3 Polymerase chain reaction and sequence analysis

The mitochondrial COI was amplified using the primers LCO 1490 (5'-GGTCAACAATCATAAGATATTGG3') and HCO 2198
(5'TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The reaction was carried out in a volume of 15 µl using a pair of primers (0.4 µM each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and BIOTaq HS DNA polymerase (Bioline, London, UK). After an initial denaturation at 95 °C for 5 min, amplification was performed with 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 49 °C for 30 sec, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. PCR products were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics) and sequenced with a forward primer by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). To confirm the authenticity of the COI sequences obtained, some individuals from each sand fly species (in total 40 specimens) were subjected to PCR using a high fidelity DNA polymerase (KOD-Plus-ver.2; TOYOBO, Tokyo, Japan), and the sequences were determined as described above.

2.4 Data analysis

The COI sequences were aligned by CLUSTAL W software (Thompson et al., 1997) incorporated into MEGA (Molecular Evolutionary Genetics Analysis) version 5.2 (Tamura et al., 2011). The nucleotide compositions and sequence divergences within and between species were calculated using the distance model Kimura 2-Parameter (K2P) (Kimura, 1980). This model provides the best estimate of divergence when genetic distances are low, as in recently diverged taxa (Nei and Kumar, 2000). The ‘best match’ method in the SpeciesIdentifier module of TaxonDNA software v1.7.8 (Meier et al., 2006) was used to test the frequency of successful identification. A Neighbor-joining (NJ) tree of K2P distances was created to provide a graphic representation of the clustering pattern among different species (Saitou and Nei, 1987). Branch
support for NJ was calculated using the bootstrapping method with 1,000 replicates in MEGA 5.2 (Tamura et al., 2011). All sequences and other specimen information are available in the dataset project ‘DBSFP’ (Process IDs: DBSFP001-15 to DBSFP159-15) on the Barcode of Life Data System (BOLD) at http://www.boldsystems.org. Sequence data are also available in the DDBJ/EMBL/GenBank databases (http://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers AB984357–AB984520 (Table 1).
3. Results

3.1 Sequence analysis

A total of 159 COI sequences from 19 morphologically identified species of phlebotomine sand flies belonging to 6 subgenera/species groups were obtained. Each species was analyzed using 1 to 17 specimens (Table 1, 2). The COI sequences obtained varied from 650 to 672 bp by direct sequencing. No deletions, insertions or stop codons were found indicating that all sequences constituted functional mitochondrial products (Funk and Omland, 2003). As these 159 COI sequences contained no indels, alignments were straightforward. The COI sequences from 19 species of sand flies had high adenosine and thymine (AT)-rich content bias (65.9%), similar to that observed for other insects (Simon et al., 1994), with an average nucleotide composition of A=28.2%, T=37.7%, C=18.0%, and G=16.2%.

Intraspecific genetic distances based on Kimura 2-Parameter values for the sand fly species ranged from 0 to 5.96% (Table 2). Maximum intraspecific genetic diversity was observed in 7 of the 19 species, especially within the three vector species of *Leishmania (Viannia) peruviana*; *Lutzomyia (Lu.) peruensis* (5.96%), *Lu. verrucarum* (3.48%) and *Lu. ayacuchensis* (2.76%) (Table 2). The high level of intraspecific divergence was related to geographical distribution in some species. The mean intraspecific K2P distance ranged from 0.05% (*Lu. caballeroi*) to 3.36% (*Lu. peruensis*) (Table 2). Interspecific K2P genetic distances for the sand fly species included in the study ranged from 8.39 to 19.08%. The overall nucleotide diversity recorded among the 19 species under the Subfamily Phlebotominae included in the study was found to be 13.9%. Low levels of minimum interspecific divergence typically occurred in closely related species, such as *Lu. tejadai* and *Lu. quillabamba* (8.39%), *Lu. bicornuta* and *Lu. battistinii* (8.88%), and *Lu. castanea* and *Lu. caballeroi* (10.15%). Collectively, the distances between species belonging to
the subgenus Lutzomyia (Lu. bicornuta and Lu. battistinii), and the Verrucarum group (Lu. verrucarum and Lu. nuneztovarii) were 8.88 and 12.27%, respectively, whereas the distances between species belonging to the subgenus Pifanomyia (Lu. maranonensis, Lu. robusta and Lu. nevesi) ranged from 11.46 to 15.43%. On the other hand, the diversity within the subgenus Helcocyrtomyia (Lu. munaypata, Lu. quillabamba, Lu. pescei, Lu. noguchii, Lu. castanea, Lu. caballeroi, Lu. tejadai, Lu. ayacuchensis and Lu. peruensis) ranged from 8.39 to 16.5%. Using SpeciesIdentifier, the overall percentage of correct species identification was 100% as recognized by traditional taxonomy based on morphological features. Generally, the COI divergence within species did not exceed the genetic distance between species. As a result, there was a distinction between intraspecific and interspecific genetic divergences, which is critical for DNA barcoding success in taxa discrimination (Fig. 2).

3.2 Neighbor-joining tree

The Neighbor-joining tree showed shallow intraspecific and deep interspecific divergences (Fig. 3). Specimens of the same species were grouped closely together, regardless of the collection site. However, in exceptional cases, obvious geographic differences in sequences within the same species were found in three species, namely, Lu. peruensis, Lu. verrucarum and Lu. maranonensis. Each of these species was monophyletic, but the individuals were divided into two clades corresponding to geographic origin (Fig. 3), namely, Lu. peruensis: Cusco (southeast) clade, and Lima (central) and La Libertad (northwest) clade; Lu. verrucarum: Lima (central) clade, and Piura (northwest) clade; Lu. maranonensis: Querocotillo (Cajamarca-northwest) clade, and San José de Lourdes (Cajamarca-northwest) clade. Additionally, the Lu. robusta cluster was
monophyletic, but the individuals were divided into two clades: Cajamarca I (northwest) clade, and Cajamarca II (northwest) clade (Fig. 3).
4. Discussion

Adequate knowledge of the ecological and medical importance of sand flies relies on correct identification of these minute and fragile insects. The gold standard for any robust taxonomic system is its ability to deliver reliable and accurate species identifications (Hebert et al., 2003a). In the present study, we were able to recover and align the targeted COI fragment from all sand fly species examined. The strongly biased AT-richness noted within the DNA barcode sequences was consistent with results from other insects including different sand fly genera (Cywinska et al., 2006; Pramual et al., 2011; Kumar et al., 2012; Contreras Gutiérrez et al., 2014). The range of intraspecific variation was between 0 and 5.96%, while the interspecific variability for species differentiation ranged between 8.39 and 19.08%. The range of intraspecific genetic divergence in this study, 0 to 5.96%, was very similar to values reported for New World sand flies in Colombia (0 to 6%; Contreras Gutiérrez et al., 2014), whereas the 5.96% maximum intraspecific divergence was higher than that earlier reported for Old World sand flies in India (2%; Kumar et al., 2012). High intraspecific sequence divergence was found among the three vector species of *Leishmania* (*Viannia*) *peruviana* in Peru (Davies et al., 1993; Perez et al., 1991, 1994, 2007; Caceres et al., 2004; Kato et al., 2008, 2011); *Lu.* (*Helcocyrtomyia*) *peruensis* (5.96% maximum intraspecific divergence), *Lu.* (*Verrucarum*) *verrucarum* (3.48%), and *Lu.* (*Helcocyrtomyia*) *ayacuchensis* (2.76%) (Table 2). Overall, there were no overlaps between the intra- and interspecific divergence levels, despite the high values of intraspecific divergences observed in some species, supporting the utility of mtDNA COI in discriminating species (Hebert et al., 2003b).

Inclusion of DNA sequence data in phylogenetic analysis of individuals allows for quick determination of monophyletic groups and recognition of hidden or potential species. In addition to delineation of species, DNA barcoding can reveal cryptic diversity (Hebert et al., 2004;
Pramual et al., 2011; Kumar et al., 2012). The Neighbor-joining (NJ) tree analysis distinctively clustered the COI sequences obtained from individual Lutzomyia sand flies as previously determined by morphological features; specimens of single species formed barcode clusters with tight cohesion that were clearly distinct from those of allied species. However, in this study four taxa, Lu. peruensis, Lu. verrucarum, Lu. maranonensis and Lu. robusta were found to be complexes of at least 2 genetically distinct groups. The NJ tree revealed two deep-divergence reciprocally monophyletic clades in Lu. peruensis, the main vector of L. (V.) peruviana (Perez et al., 1991, 2007; Kato et al., 2008, 2011) that clustered by geographic collection area (Fig. 3), and indicated that the Lu. peruensis group from Cusco (southeast) was genetically distinct from those from Lima (central) and La Libertad (northwest). A similar scenario was previously observed in Lu. peruensis based on the analysis of cyt b gene, among specimens from two regions, Ancash and Lima (central), and La Libertad (northwest) (Yamamoto et al., 2013). Additionally, we also observed a high genetic distance between the Lima (central) and Piura (northwest) groups of Lu. verrucarum. Therefore, these observed genetic distances among Lu. peruensis and Lu. verrucarum groups indicated that the groups in each taxa were highly isolated, with little or no genetic exchange between the groups.

Phlebotomine sand flies are known to have limited sustained flight and tend to remain associated with their emergence sites, typically not dispersing more than a half kilometer (Alexander, 1987; Killick-Kendrick, 1990; Morrison et al., 1993). Such putatively low flight capacity and dispersal range of sand flies coupled with the heterogeneous terrain of the Andes may cause the isolation of these populations throughout their distributions, thereby producing genetically distinct but morphologically similar individuals. Consequently, genetic differentiation observed within some species, may be ascribable to local adaptation or genetic
drift. Moreover, the observation of genetic differentiation among the vector species (*Lu. peruensis, Lu. verrucarum*, and *Lu. ayacuchensis*) may have serious implications for epidemiological studies and vector control strategies. Indeed it has been shown that insecticide susceptibility and vector capacity can vary within evolutionary lineages, even within the same species (Lanzaro et al., 1993; O’Loughlin et al., 2008; Hassan et al., 2012). Collectively, the fact that some species showed higher conspecific divergence does not compromise the use of COI sequences for their identification; rather, it allows delineation of the regional lineages that constitute them (Hebert et al., 2003b). Nonetheless, further investigations with more specimens from various localities will be required to provide additional information on the levels of divergence within these vector species and other prevalent sand fly species in Peru. Such studies may also contribute to more effective leishmaniasis control in endemic areas.

In conclusion, this study provided the COI barcodes for several Peruvian sand flies and showed their effectiveness in discriminating species recognized through prior conventional taxonomic work. Aside from its success in discriminating known species, DNA barcoding was found to be useful in revealing population differentiation. A better understanding of the implication of the genetic differentiation among the vector species will require further molecular (genetic information from multiple loci), ecological and morphological studies.
Acknowledgements

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**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. Bat: *Lu. battistini*; Bic: *Lu. bicornuta*; Ver: *Lu. verrucarum*; Nun: *Lu. nuneztovari*; Mar: *Lu. maranonensis*; Nev: *Lu. nevesi*; Rob: *Lu. robusta*; Tej: *Lu. tejadai*; Qui: *Lu. quillabamba*; Cab: *Lu. caballeroi*; Cas: *Lu. castanea*; Pes: *Lu. pescei*; Aya: *Lu. ayacuchensis*; Per: *Lu. peruensis*; Mun: *Lu. munaypata*; Nog: *Lu. noguchii*; Fis: *Lu. fischeri*; Gor: *Lu. gorbitzi*; Eun: *Warileya euniceae*.

**Fig. 2.** Distribution of intraspecific (grey) and interspecific (black) genetic distances for the cytochrome c oxidase subunit I (COI) sequences of sand fly species from Peru.

**Fig. 3.** Neighbor-joining tree based on Kimura 2-parameter genetic distances of mitochondrial COI sequences of *Lutzomyia* sand fly species from Peru. The bar scale represents 0.01% divergences. Bootstrap values are shown above or below branches. Specimen IDs (in parentheses).
Fig. 1
Fig. 3
Fig. 3 (Continued)
Table 1

Details of sand fly specimens used for DNA barcoding and analysis in the study along with the BOLD Process IDs and GenBank accession numbers.

<table>
<thead>
<tr>
<th>Sand fly species</th>
<th>No. of samples</th>
<th>District</th>
<th>Department</th>
<th>Trapping method</th>
<th>Year of capture</th>
<th>BOLD Process IDs</th>
<th>GenBank accession nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu. battistinii</td>
<td>10</td>
<td>Abancay</td>
<td>Apurimac</td>
<td>CDC</td>
<td>2012</td>
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<td>AB984357-AB984366</td>
</tr>
<tr>
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<td>5</td>
<td>San Marcos de Recheac</td>
<td>Huancavelica</td>
<td>CDC</td>
<td>2011</td>
<td>DBSFP014-15-DBSFP018-15</td>
<td>AB984370-AB984374</td>
</tr>
<tr>
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<td>1</td>
<td>Chacoche</td>
<td>Apurimac</td>
<td>CDC</td>
<td>2012</td>
<td>DBSFP051-15</td>
<td>AB984407</td>
</tr>
<tr>
<td>Lu. caballeroi</td>
<td>10</td>
<td>La Cuesta</td>
<td>La Libertad</td>
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<td>DBSFP052-15-DBSFP061-15</td>
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</tr>
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<td>San José de Lourdes</td>
<td>Cajamarca</td>
<td>ShT</td>
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<td>DBSFP062-15-DBSFP066-15</td>
<td>AB984418-AB984422</td>
</tr>
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<td>Cajamarca</td>
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<td>Huánuco</td>
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<td>Lambayeque</td>
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<td>Cajamarca</td>
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<td>Cajamarca</td>
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<td>2006</td>
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<td>DBSFP142-15-DBSFP143-15</td>
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<td>Alto Inambari</td>
<td>Puno</td>
<td>ShT</td>
<td>2013</td>
<td>DBSFP159-15</td>
<td>AB984520</td>
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Lu: Lutzomyia; Wa: Warileya; CDC: CDC light trap; ShT: Shannon trap; BOLD: Barcode of Life Data System.
### Table 2

Intraspecific Kimura 2-Parameter (K2P) pairwise distances of sand fly species from Peru.

<table>
<thead>
<tr>
<th>Subgenera/Species group</th>
<th>Sand fly species</th>
<th>No. of Specimens</th>
<th>Minimum (%)</th>
<th>Mean (%)</th>
<th>Maximum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lutzomyia</em></td>
<td><em>Lu. battistinii</em></td>
<td>10</td>
<td>0</td>
<td>0.27</td>
<td>0.46</td>
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<tr>
<td></td>
<td><em>Lu. bicornuta</em></td>
<td>8</td>
<td>0</td>
<td>0.76</td>
<td>1.25</td>
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<tr>
<td><em>Verrucarum</em></td>
<td><em>Lu. verrucarum</em></td>
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<td><em>Lu. nuneztovari</em></td>
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<td>0</td>
<td>0.3</td>
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<td>1.85</td>
<td>2.76</td>
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<td>3.36</td>
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</tr>
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</table>

N/A: not applicable.