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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).

71 However, this morphological classification is laborious, time-consuming and often complicated
72 by phenotypic plasticity and cryptic species complexes, as well as demanding considerable skill
73 and taxonomic expertise. To overcome these technical limitations, in the last three decades
74 molecular approaches have been increasingly employed to explore the taxonomy, population
75 structure and phylogeny in insect vectors including sand flies (Mukhopadhyay et al., 2000; Uribe
76 Soto et al., 2001; Depaquit et al., 2002; Beati et al., 2004; Khalid et al., 2010; Fujita et al., 2012;
77 Yamamoto et al., 2013; Nzelu et al., 2014; Gomez et al., 2014; Kato et al., 2015).

78 Recently, DNA barcoding has been widely shown to be an effective molecular tool for
79 species identification of many insects (Hebert et al., 2003a, b). Among the mitochondrial genes,
80 cytochromo c oxidase I (COI) is reported to be the most conserved gene and hence has distinct
81 advantages for taxonomic studies (Knowlton and Weigt, 1998). Studies have demonstrated the
82 use of DNA barcoding as a valid molecular tool for identifying dipterans; mosquitoes (Cywinska
83 et al., 2006), black flies (Pramual et al., 2011) and sand flies (Azpurua et al*.,* 2010; Kumar et al.,
84 2012; Contreras Gutiérrez et al., 2014; Nzelu et al., 2014), and revealing sand fly cryptic species
85 (Cohnstaedt et al., 2011; Scarpassa et al., 2012; Kumar et al., 2012). Construction of DNA
86 barcodes for each species of sand fly would provide an efficient tool for their identification. The
87 aim of the present study was to explore the utility of the DNA barcode approach for
88 identification of sand fly species in Peru, where leishmaniasis is endemic, by analyzing
89 morphologically identified adult specimens.

90

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'-GGTCAACAAATCATAAAGATATTGG3') and HCO 2198

113 (5'TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The reaction was carried
114 out in a volume of 15 µl using a pair of primers (0.4 µM each), Ampdirect Plus (Shimadzu
115 Biotech, Tsukuba, Japan), and BIOTaq HS DNA polymerase (Bioline, London, UK). After an
116 initial denaturation at 95 °C for 5 min, amplification was performed with 35 cycles consisting of
117 denaturation at 95 °C for 1 min, annealing at 49 °C for 30 sec, extension at 72 °C for 2 min,
118 followed by a final extension at 72 °C for 10 min. PCR products were purified using a FastGene
119 Gel/PCR Extraction kit (NIPPON Genetics) and sequenced with a forward primer by the dideoxy
120 chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied
121 Biosystems, Foster City, CA). To confirm the authenticity of the COI sequences obtained, some
122 individuals from each sand fly species (in total 40 specimens) were subjected to PCR using a high
123 fidelity DNA polymerase (KOD-Plus-ver.2; TOYOBO, Tokyo, Japan), and the sequences were
124 determined as described above.
125
126 *2.4 Data analysis*
127 The COI sequences were aligned by CLUSTAL W software (Thompson et al., 1997)
128 incorporated into MEGA (Molecular Evolutionary Genetics Analysis) version 5.2 (Tamura et al.,
129 2011). The nucleotide compositions and sequence divergences within and between species were
130 calculated using the distance model Kimura 2-Parameter (K2P) (Kimura, 1980). This model
131 provides the best estimate of divergence when genetic distances are low, as in recently diverged
132 taxa (Nei and Kumar, 2000). The 'best match' method in the SpeciesIdentifier module of
133 TaxonDNA software v1.7.8 (Meier et al., 2006) was used to test the frequency of successful
134 identification. A Neighbor-joining (NJ) tree of K2P distances was created to provide a graphic
135 representation of the clustering pattern among different species (Saitou and Nei, 1987). Branch

136 support for NJ was calculated using the bootstrapping method with 1,000 replicates in MEGA
137 5.2 (Tamura et al., 2011). All sequences and other specimen information are available in the
138 dataset project 'DBSFP' (Process IDs: DBSFP001-15 to DBSFP159-15) on the Barcode of Life
139 Data System (BOLD) at http://www.boldsystems.org. Sequence data are also available in the
140 DDBJ/EMBL/GenBank databases (http://www.ncbi.nlm.nih.gov/genbank/) under the accession
141 numbers AB984357–AB984520 (Table 1).
142

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 straight forward. 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

166 the subgenus *Lutzomyia* (*Lu. bicornuta* and *Lu. battistinii*), and the *Verrucarum* group (*Lu.*
167 *verrucarum* and *Lu. nuneztovari*) were 8.88 and 12.27%, respectively, whereas the distances
168 between species belonging to the subgenus *Pifanomyia* (*Lu. maranonensis*, *Lu. robusta* and *Lu.*
169 *nevesi*) ranged from 11.46 to 15.43%. On the other hand, the diversity within the subgenus
170 *Helcocyrtomyia* (*Lu. munaypata*, *Lu. quillabamba*, *Lu. pescei*, *Lu. noguchii*, *Lu. castanea*, *Lu.*
171 *caballeroi, Lu. tejadai*, *Lu. ayacuchensis* and *Lu. peruensis*) ranged from 8.39 to 16.5%. Using
172 SpeciesIdentifier, the overall percentage of correct species identification was 100% as
173 recognized by traditional taxonomy based on morphological features. Generally, the COI
174 divergence within species did not exceed the genetic distance between species. As a result, there
175 was a distinction between intraspecfic and interspecfic genetic divergences, which is critical for
176 DNA barcoding success in taxa discrimination (Fig. 2).

177

178 *3.2 Neighbor-joining tree*

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

188 monophyletic, but the individuals were divided into two clades: Cajamarca I (northwest) clade,
189 and Cajamarca II (northwest) clade (Fig. 3).
190

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 intraspecifc divergence), *Lu*. (*Verrucarum*) *verrucarum* (3.48%), and *Lu*. (*Helcocyrtomyia*) *ayacuchensis* (2.76%) (Table 2). Overall, there were no overlaps between the intra- and inter-specific 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;

214 Pramual et al., 2011; Kumar et al., 2012). The Neighbor-joining (NJ) tree analysis distinctively
215 clustered the COI sequences obtained from individual *Lutzomyia* sand flies as previously
216 determined by morphological features; specimens of single species formed barcode clusters with
217 tight cohesion that were clearly distinct from those of allied species. However, in this study four
218 taxa, *Lu. peruensis*, *Lu. verrucarum*, *Lu. maranonensis* and *Lu. robusta* were found to be
219 complexes of at least 2 genetically distinct groups. The NJ tree revealed two deep-divergence
220 reciprocally monophyletic clades in *Lu. peruensis*, the main vector of *L.* (*V.*) *peruviana* (Perez et
221 al., 1991, 2007; Kato et al., 2008, 2011) that clustered by geographic collection area (Fig. 3), and
222 indicated that the *Lu. peruensis* group from Cusco (southeast) was genetically distinct from those
223 from Lima (central) and La Libertad (northwest). A similar scenario was previously observed in
224 *Lu. peruensis* based on the analysis of *cyt* b gene, among specimens from two regions, Ancash
225 and Lima (central), and La Libertad (northwest) (Yamamoto et al., 2013). Additionally, we also
226 observed a high genetic distance between the Lima (central) and Piura (northwest) groups of *Lu.*
227 *verrucarum*. Therefore, these observed genetic distances among *Lu. peruensis* and *Lu.*
228 *verrucarum* groups indicated that the groups in each taxa were highly isolated, with little or no
229 genetic exchange between the groups.

230 Phlebotomine sand flies are known to have limited sustained flight and tend to remain
231 associated with their emergence sites, typically not dispersing more than a half kilometer
232 (Alexander, 1987; Killick-Kendrick, 1990; Morrison et al., 1993). Such putatively low flight
233 capacity and dispersal range of sand flies coupled with the heterogeneous terrain of the Andes
234 may cause the isolation of these populations throughout their distributions, thereby producing
235 genetically distinct but morphologically similar individuals. Consequently, genetic
236 differentiation observed within some species, may be ascribable to local adaptation or genetic

237 drift. Moreover, the observation of genetic differentiation among the vector species (*Lu*.
238 *peruensis*, *Lu*. *verrucarum*, and *Lu*. *ayacuchensis*) may have serious implications for
239 epidemiological studies and vector control strategies. Indeed it has been shown that insecticide
240 susceptibility and vector capacity can vary within evolutionary lineages, even within the same
241 species (Lanzaro et al., 1993; O' Loughlin et al., 2008; Hassan et al., 2012). Collectively, the fact
242 that some species showed higher conspecific divergence does not compromise the use of COI
243 sequences for their identification; rather, it allows delineation of the regional lineages that
244 constitute them (Hebert et al., 2003b). Nonetheless, further investigations with more specimens
245 from various localities will be required to provide additional information on the levels of
246 divergence within these vector species and other prevalent sand fly species in Peru. Such studies
247 may also contribute to more effective leishmaniasis control in endemic areas.

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

254

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261

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409

410 **Figure Legends**

411 **Fig. 1.** Map of Peru showing the geographic locations where sand flies were collected along with
412 the respective species analyzed in this study. Bat: *Lu. battistinii*; Bic: *Lu. bicornuta*; Ver: *Lu.*
413 *verrucarum*; Nun: *Lu. nuneztovari*; Mar: *Lu. maranonensis*; Nev: *Lu. nevesi*; Rob: *Lu. robusta*;
414 Tej: *Lu. tejadai*; Qui: *Lu. quillabamba*; Cab: *Lu. caballeroi*; Cas: *Lu. castanea*; Pes: *Lu. pescei*;
415 Aya: *Lu. ayacuchensis*; Per: *Lu. peruensis*; Mun: *Lu. munaypata*; Nog: *Lu. noguchii*; Fis: *Lu.*
416 *fischeri*; Gor: *Lu. gorbitzi*; Eun: *Warileya euniceae*.

417

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

420

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

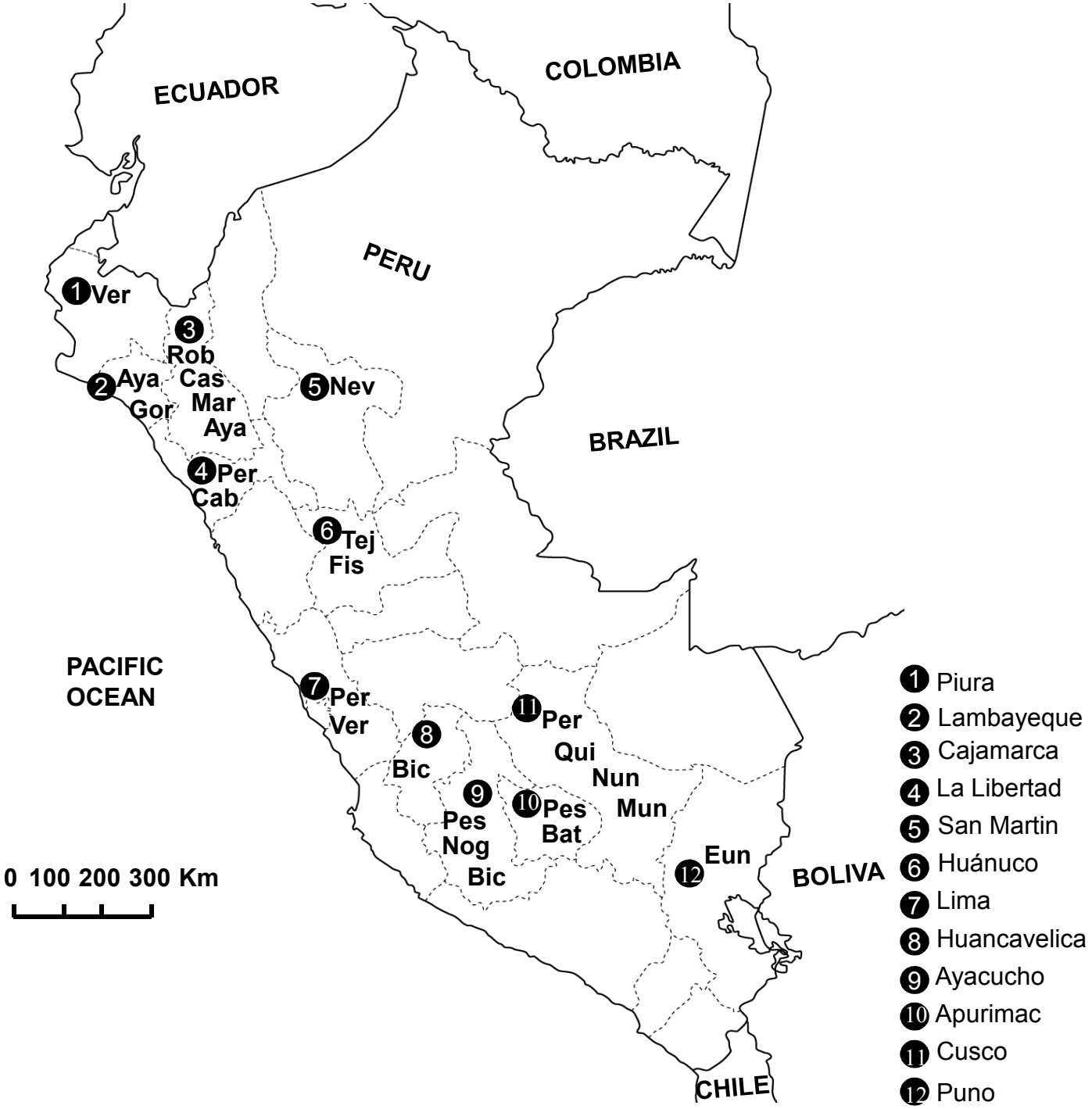

ECUADOR
COLOMBIA
PERU
BRAZIL
BOLIVA
CHILE
PACIFIC OCEAN
1 Ver
2 Aya Gor
3 Rob Cas Mar Aya
4 Per Cab
5 Nev
6 Tej Fis
7 Per Ver
8 Bic
9 Pes Nog Bic
10 Pes Bat
11 Per Qui Nun Mun
12 Eun
0 100 200 300 Km
1 Piura
2 Lambayeque
3 Cajamarca
4 La Libertad
5 San Martin
6 Huánuco
7 Lima
8 Huancavelica
9 Ayacucho
10 Apurimac
11 Cusco
12 Puno

Fig. 1

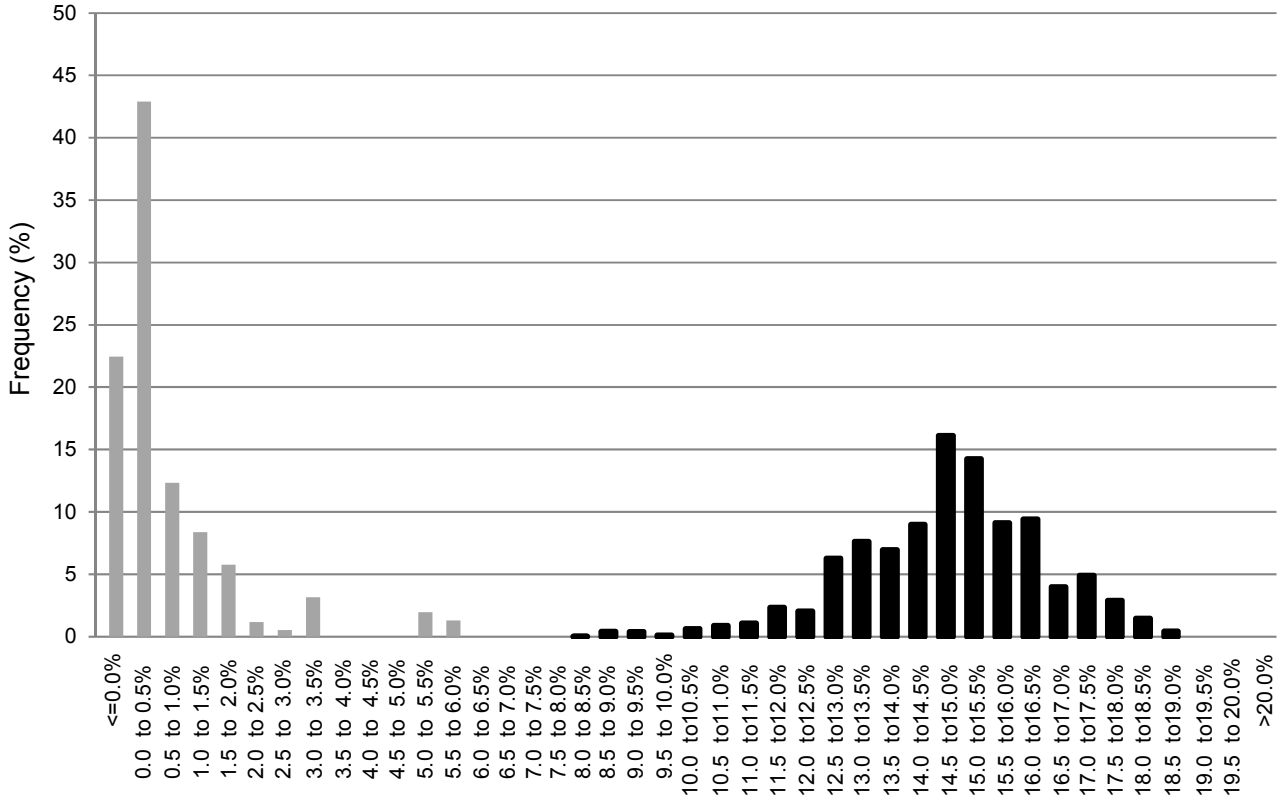

Frequency (%)
50
45
40
35
30
25
20
15
10
5
0
<=0.0%
0.0 to 0.5%
0.5 to 1.0%
1.0 to 1.5%
1.5 to 2.0%
2.0 to 2.5%
2.5 to 3.0%
3.0 to 3.5%
3.5 to 4.0%
4.0 to 4.5%
4.5 to 5.0%
5.0 to 5.5%
5.5 to 6.0%
6.0 to 6.5%
6.5 to 7.0%
7.0 to 7.5%
7.5 to 8.0%
8.0 to 8.5%
8.5 to 9.0%
9.0 to 9.5%
9.5 to 10.0%
10.0 to10.5%
10.5 to11.0%
11.0 to11.5%
11.5 to12.0%
12.0 to12.5%
12.5 to13.0%
13.0 to13.5%
13.5 to14.0%
14.0 to14.5%
14.5 to15.0%
15.0 to15.5%
15.5 to16.0%
16.0 to16.5%
16.5 to17.0%
17.0 to17.5%
17.5 to18.0%
18.0 to18.5%
18.5 to19.0%
19.0 to19.5%
19.5 to 20.0%
>20.0%

Fig. 2

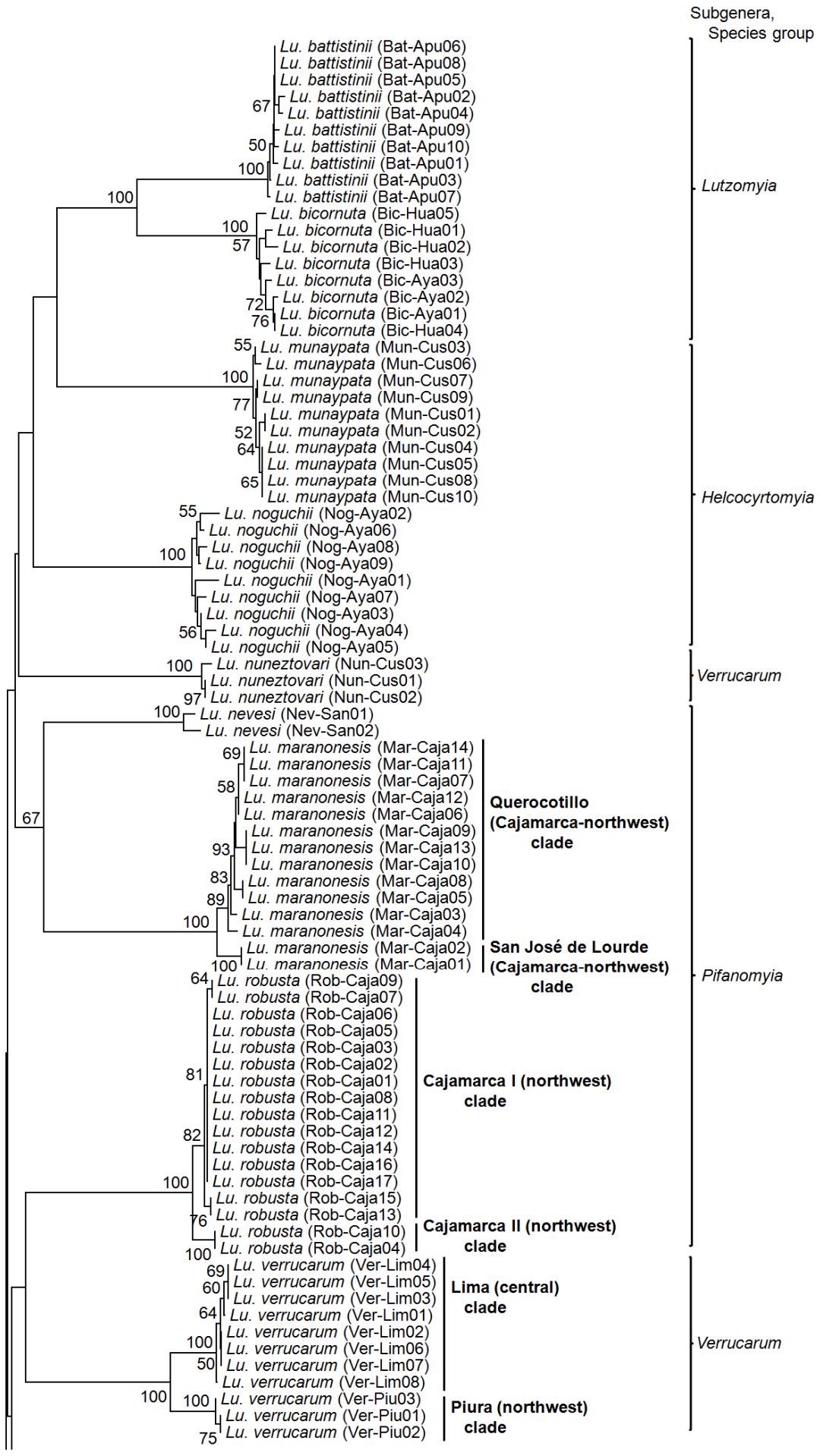

Subgenera,
Species group
Lu. battistinii (Bat-Apu06)
Lu. battistinii (Bat-Apu08)
Lu. battistinii (Bat-Apu05)
Lu. battistinii (Bat-Apu02)
Lu. battistinii (Bat-Apu04)
Lu. battistinii (Bat-Apu09)
Lu. battistinii (Bat-Apu10)
Lu. battistinii (Bat-Apu01)
Lu. battistinii (Bat-Apu03)
Lu. battistinii (Bat-Apu07)
Lu. bicornuta (Bic-Hua05)
Lu. bicornuta (Bic-Hua01)
Lu. bicornuta (Bic-Hua02)
Lu. bicornuta (Bic-Hua03)
Lu. bicornuta (Bic-Aya03)
Lu. bicornuta (Bic-Aya02)
Lu. bicornuta (Bic-Aya01)
Lu. bicornuta (Bic-Hua04)
Lutzomyia
Lu. munaypata (Mun-Cus03)
Lu. munaypata (Mun-Cus06)
Lu. munaypata (Mun-Cus07)
Lu. munaypata (Mun-Cus09)
Lu. munaypata (Mun-Cus01)
Lu. munaypata (Mun-Cus02)
Lu. munaypata (Mun-Cus04)
Lu. munaypata (Mun-Cus05)
Lu. munaypata (Mun-Cus08)
Lu. munaypata (Mun-Cus10)
Helcocyrtomyia
Lu. noguchii (Nog-Aya02)
Lu. noguchii (Nog-Aya06)
Lu. noguchii (Nog-Aya08)
Lu. noguchii (Nog-Aya09)
Lu. noguchii (Nog-Aya01)
Lu. noguchii (Nog-Aya07)
Lu. noguchii (Nog-Aya03)
Lu. noguchii (Nog-Aya04)
Lu. noguchii (Nog-Aya05)
Lu. nuneztovari (Nun-Cus03)
Lu. nuneztovari (Nun-Cus01)
Lu. nuneztovari (Nun-Cus02)
Verrucarum
Lu. nevesi (Nev-San01)
Lu. nevesi (Nev-San02)
Lu. maranonesis (Mar-Caja14)
Lu. maranonesis (Mar-Caja11)
Lu. maranonesis (Mar-Caja07)
Lu. maranonesis (Mar-Caja12)
Lu. maranonesis (Mar-Caja06)
Lu. maranonesis (Mar-Caja09)
Lu. maranonesis (Mar-Caja13)
Lu. maranonesis (Mar-Caja10)
Lu. maranonesis (Mar-Caja08)
Lu. maranonesis (Mar-Caja05)
Lu. maranonesis (Mar-Caja03)
Lu. maranonesis (Mar-Caja04)
Querocotillo (Cajamarca-northwest) clade
Lu. maranonesis (Mar-Caja02)
Lu. maranonesis (Mar-Caja01)
San José de Lourde (Cajamarca-northwest) clade
Pifanomyia
Lu. robusta (Rob-Caja09)
Lu. robusta (Rob-Caja07)
Lu. robusta (Rob-Caja06)
Lu. robusta (Rob-Caja05)
Lu. robusta (Rob-Caja03)
Lu. robusta (Rob-Caja02)
Lu. robusta (Rob-Caja01)
Lu. robusta (Rob-Caja08)
Lu. robusta (Rob-Caja11)
Lu. robusta (Rob-Caja12)
Lu. robusta (Rob-Caja14)
Lu. robusta (Rob-Caja16)
Lu. robusta (Rob-Caja17)
Lu. robusta (Rob-Caja15)
Lu. robusta (Rob-Caja13)
Cajamarca I (northwest) clade
Lu. robusta (Rob-Caja10)
Lu. robusta (Rob-Caja04)
Cajamarca II (northwest) clade
Lu. verrucarum (Ver-Lim04)
Lu. verrucarum (Ver-Lim05)
Lu. verrucarum (Ver-Lim03)
Lu. verrucarum (Ver-Lim01)
Lu. verrucarum (Ver-Lim02)
Lu. verrucarum (Ver-Lim06)
Lu. verrucarum (Ver-Lim07)
Lu. verrucarum (Ver-Lim08)
Lima (central) clade
Lu. verrucarum (Ver-Piu03)
Lu. verrucarum (Ver-Piu01)
Lu. verrucarum (Ver-Piu02)
Piura (northwest) clade
Verrucarum

Fig. 3

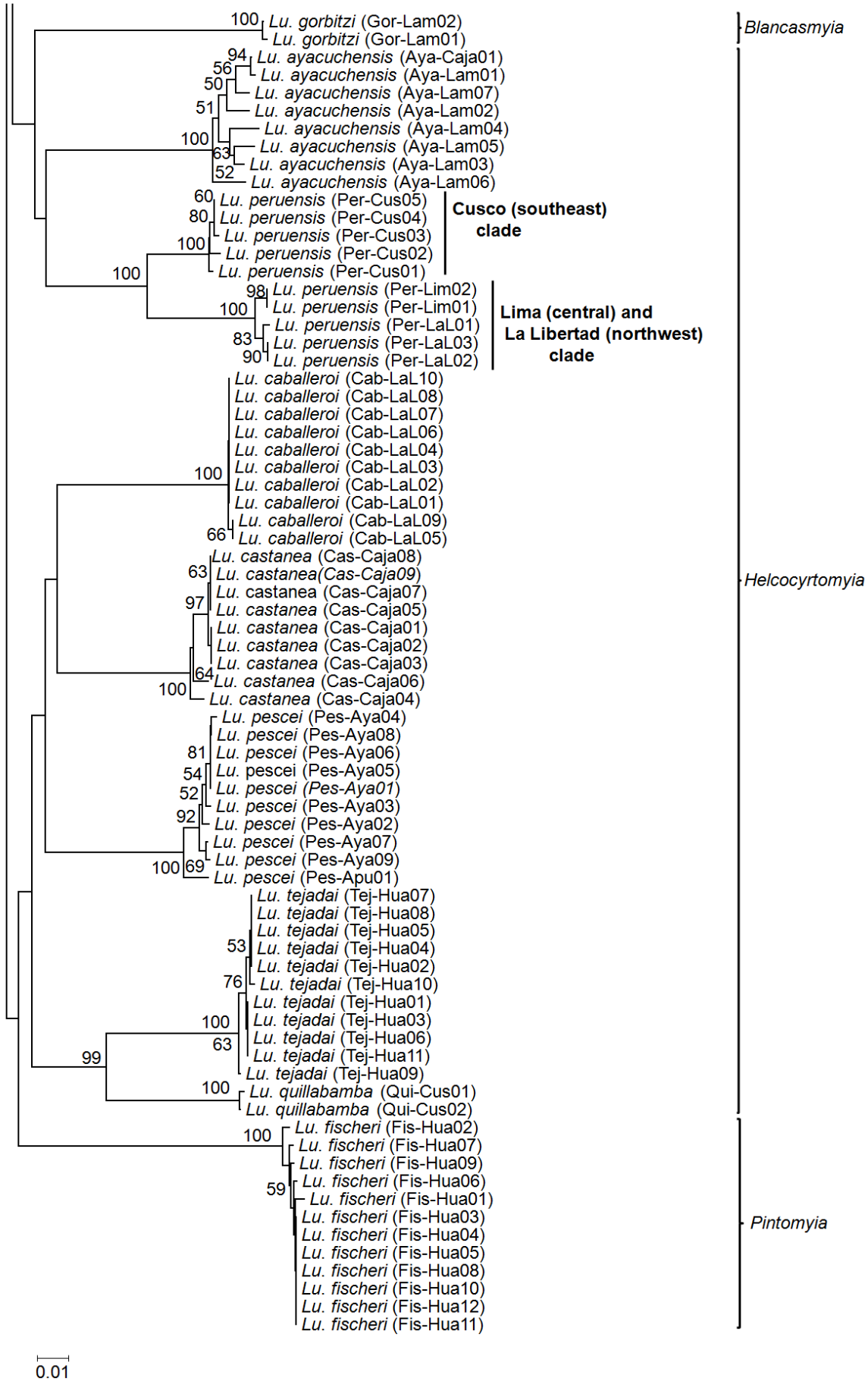


Fig. 3 (Continued)

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.

Sand fly species	No. of samples	Location		Trapping method	Year of capture	BOLD Process IDs	GenBank accession nos.
		District	Department				
Lu. battistinii	10	Abancay	Apurimac	CDC	2012	DBSFP001-15- DBSFP010-15	AB984357-AB984366
Lu. bicornuta	3	Luricocha	Ayacucho	CDC	2009	DBSFP011-15- DBSFP013-15	AB984367-AB984369
Lu. bicornuta	5	San Marcos de Rocchac	Huancavelica	CDC	2011	DBSFP014-15- DBSFP018-15	AB984370-AB984374
Lu. munaypata	10	Maranura	Cusco	CDC	2007	DBSFP019-15- DBSFP028-15	AB984375-AB984384
Lu. quillabamba	2	Calca	Cusco	CDC	2011	DBSFP029-15- DBSFP030-15	AB984385-AB984386
Lu. tejadai	11	Huánuco	Huánuco	CDC	2007, 2012	DBSFP031-15- DBSFP041-15	AB984387-AB984397
Lu. pescei	9	Luricocha	Ayacucho	CDC	2009	DBSFP042-15- DBSFP050-15	AB984398-AB984406
Lu. pescei	1	Chacoche	Apurimac	CDC	2012	DBSFP051-15	AB984407
Lu. caballeroi	10	La Cuesta	La Libertad	CDC	2007	DBSFP052-15- DBSFP061-15	AB984408-AB984417
Lu. castanea	5	San José de Lourdes	Cajamarca	ShT	2001	DBSFP062-15- DBSFP066-15	AB984418-AB984422
Lu. castanea	4	San Ignacio	Cajamarca	CDC	2007	DBSFP067-15- DBSFP070-15	AB984423-AB984426
Lu. fischeri	12	Ambo	Huánuco	CDC	2013	DBSFP071-15- DBSFP082-15	AB984428-AB984439
Lu. gorbitzi	2	Motupe	Lambayeque	CDC	2013	DBSFP083-15- DBSFP084-15	AB984440-AB984441
Lu. ayacuchensis	7	Motupe	Lambayeque	CDC	2013	DBSFP085-15- DBSFP091-15	AB984442-AB984448
Lu. ayacuchensis	1	San José de Lourdes	Cajamarca	CDC	2013	DBSFP092-15	AB984449
Lu. peruensis	2	Ambar	Lima	CDC	2007	DBSFP093-15- DBSFP094-15	AB984450-AB984451
Lu. peruensis	3	La Cuesta	La Libertad	CDC	2007	DBSFP095-15- DBSFP097-15	AB984452-AB984454
Lu. peruensis	1	Cusipata	Cusco	CDC	2011	DBSFP098-15	AB984455
Lu. peruensis	1	Urubamba	Cusco	CDC	2012	DBSFP099-15	AB984456
Lu. peruensis	3	Ollantaytambo	Cusco	CDC	2012	DBSFP100-15- DBSFP102-15	AB984457-AB984459
Lu. verrucarum	3	Huancabamba	Piura	CDC	2004	DBSFP103-15- DBSFP105-15	AB984460-AB984462
Lu. verrucarum	2	San Antonio de Chaclla	Lima	CDC	2013	DBSFP106-15- DBSFP107-15	AB984463-AB984464
Lu. verrucarum	6	Santa Eulalia	Lima	CDC	2013	DBSFP108-15- DBSFP113-15	AB984465-AB984470
Lu. nuneztovari	3	Maranura	Cusco	CDC	2007	DBSFP114-15- DBSFP116-15	AB984471-AB984473
Lu. noguchii	9	Pullo	Ayacucho	CDC	2007	DBSFP117-15- DBSFP125-15	AB984474-AB984482
Lu. nevesi	2	Tarapoto	San Martin	CDC	2007	DBSFP126-15- DBSFP127-15	AB984483-AB984484
Lu. maranonesis	2	San José de Lourdes	Cajamarca	CDC	2001	DBSFP128-15- DBSFP129-15	AB984485-AB984486
Lu. maranonesis	12	Querocotillo	Cajamarca	CDC	2006	DBSFP130-15- DBSFP141-15	AB984487-AB984498
Lu. robusta	2	San José de Lourdes	Cajamarca	ShT	2001	DBSFP142-15- DBSFP143-15	AB984501-AB984502
Lu. robusta	10	San José de Lourdes	Cajamarca	CDC	2001	DBSFP144-15- DBSFP153-15	AB984503-AB984512
Lu. robusta	5	San Juan de Cutervo	Cajamarca	CDC	2007	DBSFP154-15- DBSFP158-15	AB984513-AB984517
Wa. euniceae	1	Alto Inambari	Puno	ShT	2013	DBSFP159-15	AB984520

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.

Subgenera/Species group	Sand fly species	No. of Specimens	Minimum (%)	Mean (%)	Maximum (%)
Lutzomyia	*Lu. battistinii*	10	0	0.27	0.46
	Lu. bicornuta	8	0	0.76	1.25
Verrucarum	*Lu. verrucarum*	11	0	1.59	3.48
	Lu. nuneztovari	3	0	0.3	0.46
Pifanomyia	*Lu. maranonesis*	14	0	0.81	1.87
	Lu. nevesi	2		0.93	0.93
	Lu. robusta	17	0	0.37	1.55
Helcocyrtomyia	*Lu. tejadai*	11	0	0.16	0.46
	Lu. quillabamba	2		0.15	0.15
	Lu. caballeroi	10	0	0.05	0.15
	Lu. castanea	9	0	0.5	1.24
	Lu. pescei	10	0	0.58	1.69
	Lu. ayacuchensis	8	0.2	1.85	2.76
	Lu. peruensis	10	0	3.36	5.96
	Lu. munaypata	10	0	0.28	0.46
	Lu. noguchii	9	0.2	0.92	1.72
Pintomyia	*Lu. fischeri*	12	0	0.2	0.75
Blancasmyia	*Lu. gorbitzi*	2		0.15	0.15
–	*Wa. euniceae*	1		N/A	

N/A: not applicable.