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Genotyping of sand fly species in Peruvian Andes where leishmaniasis is endemic

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Abstract

Genotyping of sand fly species circulating in Peru was established on the basis of PCR-restriction fragment length polymorphisms (RFLPs) of the 18S ribosomal RNA (rRNA) gene. The sequences of 18S rRNA gene fragments from 12 *Lutzomyia* and 1 *Warileya* species were determined and their RFLP-patterns were analyzed. Consequently, RFLP analysis with the restriction enzyme *Afa*I and then *Hap*II or *Kpn*I, followed by *Xsp*I successfully differentiated them. Intraspecific genetic diversity affecting RFLP-patterns was not detected in the specimens collected from 24 areas of 8 departments. The genotyping was applied to the surveillance of sand flies collected from Andean areas where leishmaniasis is endemic, and its usability was verified. The present method promises to be a powerful tool for the classification and surveillance of sand flies circulating in Peru.

Keywords: genotyping, sand fly, *Lutzomyia*, PCR-RFLP, 18S rRNA gene

1. Introduction

Phlebotomine sand flies are insects of the family Psychodidae in the order Diptera, 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). The majority of the species play no part in the transmission of human pathogens. However, some species belonging to the genera *Phlebotomus* and *Lutzomyia* have been identified as vectors of leishmaniasis, though each vector can transmit only certain species of *Leishmania* (Munstermann, 2004; Bates, 2007; Kato et al., 2010a). Since the spread of leishmaniasis largely depends on the distribution of the vector species, the identification of circulating sand flies in endemic and surrounding areas is important for predictions of the risk and expansion of the disease. Sand flies are typically identified based on morphologic characteristics; mainly internal structures such as the cibarium, pharynx, spermatheca of females and terminal genitalia of males (Young and Duncan, 1994). Morphological classification requires considerable skill and taxonomic expertise, and has the potential risk of misidentification. To overcome these problems, molecular markers have been explored to develop easier and accurate ways of classifying sand flies, and most results of DNA sequence analyses support the morphology-based grouping (Aransay et al., 1999; Torgerson et al., 2003; Beati et al., 2004; Kato et al., 2007, 2008; Terayama et al., 2008; Kuwahara et al., 2009; Cohnstaedt et al., 2011). Since a DNA sequence analysis requires no special storage conditions for samples and less expertise with minimum risk of different interpretations among researchers, the usability of this method for sand fly taxonomy is now widely accepted (Kato et al., 2010a).

Recently, we developed a genotyping method to classify 7 sand fly species

distributed in Ecuador by using a PCR-restriction fragment length polymorphism (RFLP) analysis of 18S rRNA genes (Terayama et al., 2008). The procedure was applied successfully to the differentiation of 3 sand fly species in Peru (Kato et al., 2008). In Peru, approximately 160 species of Phlebotomine sand flies have been reported, most of which were recorded from Eastern Valleys and Amazonian regions (Caceres and Galati, 2001). In the present study, to further advance this technique for countrywide surveillance in Peru, a PCR-RFLP assay of the 18S rRNA gene was optimized for the differentiation of 13 species, mainly distributing in Andean areas, and applied to the surveillance of sand flies in Andean areas of Peru where cutaneous leishmaniasis caused by *Leishmania (Viannia) peruviana* is endemic.

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 23 Andean areas of Peru; San Ignacio, Coro Alto, La Capilla, Chota, San Miguel and Santa Cruz de Toledo, Department of Cajamarca; Chanchamayo, La Cuesta, Nambuque and Padregual, Department of La Libertad; San Pedro de Chonta, Department of Huanuco; Colcap Bajo, Curcuy, Huanchoc, Atocshay and Pucuhuayllan, Department of Ancash; Ambar, Jalcan and Huacan, Department of Lima; Higosniyocc and Pullo, Department of Ayacucho; and Ocobamba and Maranura, Department of Cusco (Fig. 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 based on measurements of wing veins, the ratio of length of palpus to length of antenna and the color of the thorax (Young and Duncan, 1994), and then fixed in 70% ethanol. The species and number of sand flies subjected to the PCR-RFLP analyses for assessing genetic variation at each collection site are shown in Fig. 1. For the large-scale field surveillance of prevalent species, sand flies were captured with CDC light traps and Shannon traps in 11 Andean areas; Loma Grande, La Cuesta, Nambuque and Padregual, Department of La Libertad; Atocshay, Colcap Bajo, Curcuy, Huanchoc and Pucuhuayllan, Department of Ancash; and Ambar and Huarochiri, Department of Lima. The sand flies were fixed in 70% ethanol and stored at room temperature.

2.2. DNA extraction

Ethanol-fixed specimens were individually lysed in 50 μl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1 % sodium dodecyl sulfate (SDS)] in the presence of proteinase K (100 μg/ml). The samples were incubated overnight at 37˚C, and 0.5-μl portions were directly used as templates for PCR amplification without purification (Kato et al., 2007, 2008).

2.3. Molecular cloning and nucleotide sequencing

The amplification of 18S rRNA gene fragments was performed with sand fly 18S rRNA gene-specific primers (Lu.18S 1S: 5'-TGCCAGTAGTTATATGCTTG-3' and Lu.18S AR: 5'-CACCTACGGAAACCTTGTTAC-3') (Terayama et al., 2008; Kato et al., 2007, 2008). The PCR was carried out in a volume of 15 μl with the primers (0.4 μM each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and *Taq* polymerase (*Ex Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95˚C for 5 min, the amplification was performed with 40 cycles of denaturation (95˚C, 1 min), annealing (50˚C, 1 min) and polymerization (72˚C, 2 min), followed by a final extension at 72˚C for 10 min. The PCR products were directly cloned into the plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI), 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).

2.4. Phylogenetic analysis

The sequences obtained were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). Phylogenetic trees were constructed by the neighbor-joining method with the distance algorithms in the MEGA package. Bootstrap values were determined with 1,000 replicates of the data sets.

2.5. Restriction fragment analysis

Each PCR product was digested with the restriction enzyme, *Afa*I, *Hap*II, *Kpn*I or *Xsp*I (Takara Bio). The digested samples were separated by electrophoresis in a 2 or 3% agarose gel to produce DNA fragments.

3. Results

3.1. Sequence analyses of the sand fly 18S rRNA gene

To establish a PCR-RFLP-based method of genotyping sand fly species circulating in Peru, 18S rRNA gene sequences 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*. The DNA inserts were 1,965-2,048bp long and showed 92% or greater homology with the 18S rRNA sequences recorded for each of the *Lutzomyia* species registered in GenBank. The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB638299-AB638311. A phylogenetic analysis was performed on the newly determined 18S rRNA gene sequences to observe the relationships among species. As shown in Fig. 2, most species that belonged to the same subgenus (Young and Duncan, 1994; Beati et al., 2004) 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. Genotyping of sand fly species

The restriction fragment patterns of the 18S rRNA gene fragments from 13 sand fly species were examined by using GENETYX software (GENETYX CORPORATION, Tokyo, Japan), and a RFLP analysis with the restriction enzyme *Afa*I and then *Hap*II or *Kpn*I, followed by *Xsp*I was expected to differentiate them (Fig. 3A). Two closely related species, *Lu. caballeroi* and *Lu. castanea,* were indistinguishable (Fig. 3A). Then, the genotyping of 13 morphologically identified sand fly species was performed by RFLP analysis of the 18S rRNA gene using *Afa*I, *Hap*II, *Kpn*I and *Xsp*I. When digested with *Afa*I, *Lu. maranonensis, Lu. verrucarum, Lu. tejadai* and *W. phlebotomanica* showed unique RFLP-patterns, but *Lu. caballeroi, Lu. castanea, Lu. munaypata, Lu. noguchii* and *Lu. nuneztovari* were not distinguishable from each other on the gel (Fig. 3B). Similarly, *Lu. ayacuchensis* and *Lu. peruensis* as well as *Lu. robusta* and *Lu. nevesi* showed indistinctive RFLP-patterns (Fig. 3B). *Hap*II-digestion divided *Lu. caballeroi, Lu. castanea, Lu. munaypata, Lu. noguchii* and *Lu. nuneztovari* into two groups, one comprising *Lu. caballeroi* and *Lu. castanea,* and the other, *Lu. munaypata, Lu. noguchii* and *Lu. nuneztovari* (Fig. 3C). In addition, *Xsp*I-digestion of the latter three species gave species-specific RFLP-patterns (Fig. 3D). On the other hand, *Lu. ayacuchensis* and *Lu. peruensis* were distinguished by *Kpn*I-digestion (Fig. 3E), and *Lu. robusta* and *Lu. nevesi* by *Hap*II-digestion (Fig. 3C). Two closely related species, *Lu. caballeroi* and *Lu. castanea*, were indistinguishable by the present genotyping targeting the 18S rRNA gene. The genotyping was performed on morphologically-identified sand flies captured at different sites in Peru to examine if genetic diversity affecting RFLP-patterns was evident. To this end, 3 of *Lu. castanea*, 2 of *Lu. munaypata,* 2 of *Lu. nuneztovari,* 2 of *Lu. tejada,* 2 of *Lu. nevesi,* and 3 of *Lu. robusta,* each from one area, 13 of *Lu. caballeroi* and 7 of *Lu. maranonensis*, both

from 2 areas, 7 of *Lu. ayacuchensis* from 3 areas, 9 of *Lu. noguchii* from 4 areas, 28 of *Lu. verrucarum* from 9 areas, 38 of *Lu. peruensis* from 10 areas, and 6 of *W. phlebotomanica* from 2 areas were tested (Fig. 1). No genetic diversity affecting the RFLP-patterns obtained with *Afa*I, *Hap*II, *Kpn*I or *Xsp*I-digestion was observed within each of the 13 species.

The present genotyping was applied to the surveillance of sand flies in 4 northern and 7 central Andean areas where leishmaniasis mainly caused by *L. (V.) peruviana* is endemic. A total of 1,240 sand flies were examined, and dominant species were identified as *Lu. peruensis* and *Lu. caballeroi* in the northern areas, and *Lu. verrucarum* and *Lu. peruensis* in the central Andes (Table 1). Although the genotyping cannot differentiate between *Lu. caballeroi* and *Lu. castanea*, the sand flies captured in Nambuque and Padregual, Department of La Libertad were classified as *Lu. caballeroi* on the basis of their distribution (Kato et al., 2011). The distribution of *Lu. peruensis*, a main vector species of *L. (V.) peruviana* in the Peruvian Andes (Perez et al., 1991, 1994, 2007; Kato et al., 2008, 2011), was verified in 10 of the 11 endemic areas surveyed. Intraspecific genetic diversity affecting the RFLP-patterns was not observed.

4. Discussion

Previously, 3 sand fly species, *Lu. verrucarum*, *Lu. peruensis* and *Lu. noguchii*, from the Peruvian Andes were successfully classified by a simple PCR-RFLP-based genotyping (Kato et al., 2008). In the present study, the technique was further applied to the countrywide surveillance of sand flies circulating in the Peruvian Andes. The 18S rRNA gene sequences of 13 sand fly species were determined, and PCR-RFLP analyses of the gene fragments with *Afa*I, *Hap*II, *Kpn*I and *Xsp*I enzymes successfully differentiated the species from 24 endemic areas in the absence of intraspecific genetic diversity affecting the RFLP-patterns. The usability of the present genotyping was confirmed through its application to large-scale surveillance in Andean areas endemic for cutaneous leishmaniasis caused by *L. (V.) peruviana*.

A phylogenetic analysis of 12 *Lutzomyia* and 1 *Warileya* species isolated in this study was performed to observe the relationships among species. Most of the results supported the generally accepted classification based on morphological characteristics (Young and Duncan, 1994); however, *Lu. nuneztovari* and *Lu. verrucarum*, both of which belong to the *Verrucarum* group, were closely associated with subgenus *Helcocyrtomyia* and *Pifanomyia* species, respectively, in the analysis (Fig. 2). A similar observation indicating that *Lu. nuneztovari* from Bolivia was located in the *Helcocyrtomyia* clade was made on the basis of the 18S rRNA gene sequence (Terayama et al., 2008). In addition, a discrepancy in the classification of *Lutzomyia* species including the *Verrucarum* group between the morphological examination and phylogenetic analyses of 12S and 28S rRNA gene sequences was reported (Beati et al., 2004), suggesting the necessity for careful reconsideration of the classification of some *Lutzomyia* species. Further DNA sequence analyses targeting rRNA internal

transcribed spacer (ITS) regions and the mitochondrial cytochrome *b* gene may help to clarify the issue.

In the present study, a RFLP analysis using restriction enzymes, *Afa*I, *Hap*II, *Kpn*I and *Xsp*I, successfully differentiated the species although two closely-related species, *Lu. caballeroi* and *Lu. castanea*, were indistinguishable. The present method may seem not to be simple; however, information on the distributions of sand fly species in each area is accumulating. Besides, not many species usually coexist in an area. Therefore, only a few steps will be required for actual field surveillance. In addition, modification of the sampling method can restrict the species captured since the use of light traps usually results in the collection of more species including non-human biting species and cold-blood feeders when compared to the use of protected human bait.

On assessment of the genetic diversity affecting RFLP-patterns in sand flies collected from 24 endemic areas of 8 departments in the Peruvian Andes, no intraspecific variation was observed in the present 13 species although it was reported in several other species (Aransay et al., 1999; Terayama et al., 2008). Further study will be required to clarify the genetic diversity within each sand fly species; however, the 18S rRNA gene of Peruvian species seems to have less DNA polymorphism and therefore would be a suitable target for genotyping.

The present method was applied to the surveillance of sand flies in 4 northern and 7 central Andean areas where cutaneous leishmaniasis caused by *L. (V.) peruviana* is endemic. Consistent with previous reports and the present morphological examination, *Lu. peruensis* and *Lu. caballeroi* were identified as two dominant species in northern Andean areas, especially in Nambuque and Padregual, whereas *Lu. verrucarum* and *Lu. peruensis* were identified as major species in central Andes (Kato et

al., 2008, 2011). No intraspecific genetic diversity affecting RFLP-pattern was detected in the present 1,240 specimens. *Lutzomyia peruensis* known as a main vector species of *L. (V.) peruviana* (Perez et al., 1991, 1994, 2007; Kato et al., 2008, 2011) was identified in most areas, suggesting that this species is associated with the disease transmission in these areas. *Lutzomyia verrucarum*, which was identified as a dominant species in central Andes, was reported to exhibit a capacity for transmission of *L. (V.) peruviana* under experimental conditions (Davies et al., 1993). Therefore, possible transmission of the disease by this species may be considered in the areas where *Lu. verrucarum* is prevalent. *Lutzomyia ayacuchensis*, another proven vector of *L. (V.) peruviana* in the western Andean valleys of the Department of Ayacucho (Caceres et al., 2004), was not identified in the present study.

In conclusion, a PCR-RFLP-based method for genotyping sand fly species was established, and its usability was verified through its application to field surveillance in 11 Andean areas where cutaneous leishmaniasis is endemic. Since the incidence and expansion of leishmaniasis is associated with the distribution of vectors, periodical large-scale surveillance of prevalent sand flies in various endemic areas in different seasons using the present simple genotyping will provide information helpful for the control of vectors and disease expansion. In addition, combined application of the present genotyping with a recently established molecular mass screening for *Leishmania*-infected sand flies (Kato et al., 2007, 2008) will be a powerful tool for the investigation of individual sand flies in each endemic area. Finally, further DNA sequence analyses of sand flies will advance a definitive PCR-RFLP-based molecular taxonomy as well as the molecular evolutionary analysis of sand fly species.

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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 and numbers (in parentheses) examined in this study. The numbers represent the following locations: 1. San Ignacio, 2. Coro Alto, 3. La Capilla, 4. Chota, 5. San Miguel, and 6. Santa Cruz de Toledo, Department of Cajamarca; 7. Chanchamayo, 8. La Cuesta, Nambuque and Padregual, Department of La Libertad; 9. Tarapoto, Department of San Martin; 10. San Pedro de Chonta, Department of Huanuco; 11. Colcap Bajo, Curcuy, Huanchoc, Atocshay and Pucuhuayllan, Department of Ancash; 12. Ambar, Jalcan and Huacan, Department of Lima; 13. Higosniyocc and 14. Pullo, Department of Ayacucho; 15. Ocobamba and 16. Maranura, Department of Cusco. Cab, *Lutzomyia (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 18S rRNA gene sequences among sand fly species. The 18S rRNA genes of 13 species were amplified from morphologically identified sand fly samples, and sequenced. The scale bar represents 0.005% divergence. Bootstrap values are shown above branches.

Fig. 3. PCR-RFLP analyses of 18S rRNA genes from 13 sand fly species in Peru. (A) Schematic representation for genotyping of 13 sand fly species by PCR-RFLP of

18S rRNA genes. The restriction enzymes used for classification are shown above branches. (B)-(E) PCR amplification was performed with sand fly 18S rRNA gene-specific primers, and the PCR products were digested with (B) *Afa*I, (C) *Hap*II, (D) *Kpn*I and (E) *Xsp*I. Cab, *Lutzomyia (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*.

ECUADOR
COLOMBIA
PERU
BRAZIL
BOLIVIA
PACIFIC OCEAN
1 Rob(3)
2 Aya(5)
Cas(3), Mar(4)
3 4 Mar(3)
9 Nev(2)
Per(1), Ver(2)
5
6 Wph(3)
7 Aya(1)
8 Nog(3), Per(2); Cab(13), Per(5)
10 Tej(2)
Per(3), Ver(5); Per(5), Ver(3); Per(4), Ver(3);
11 Per(4), Ver(1); Per(5), Ver(4)
12 Nog(2), Per(4), Ver(4);
Nog(1), Per(5), Ver(1); Ver(5)
15 Mun(2)
16 Nun(2)
13 Aya(1)
14 Nog(3), Wph(3)
0 100 200 300 km

Fig. 1

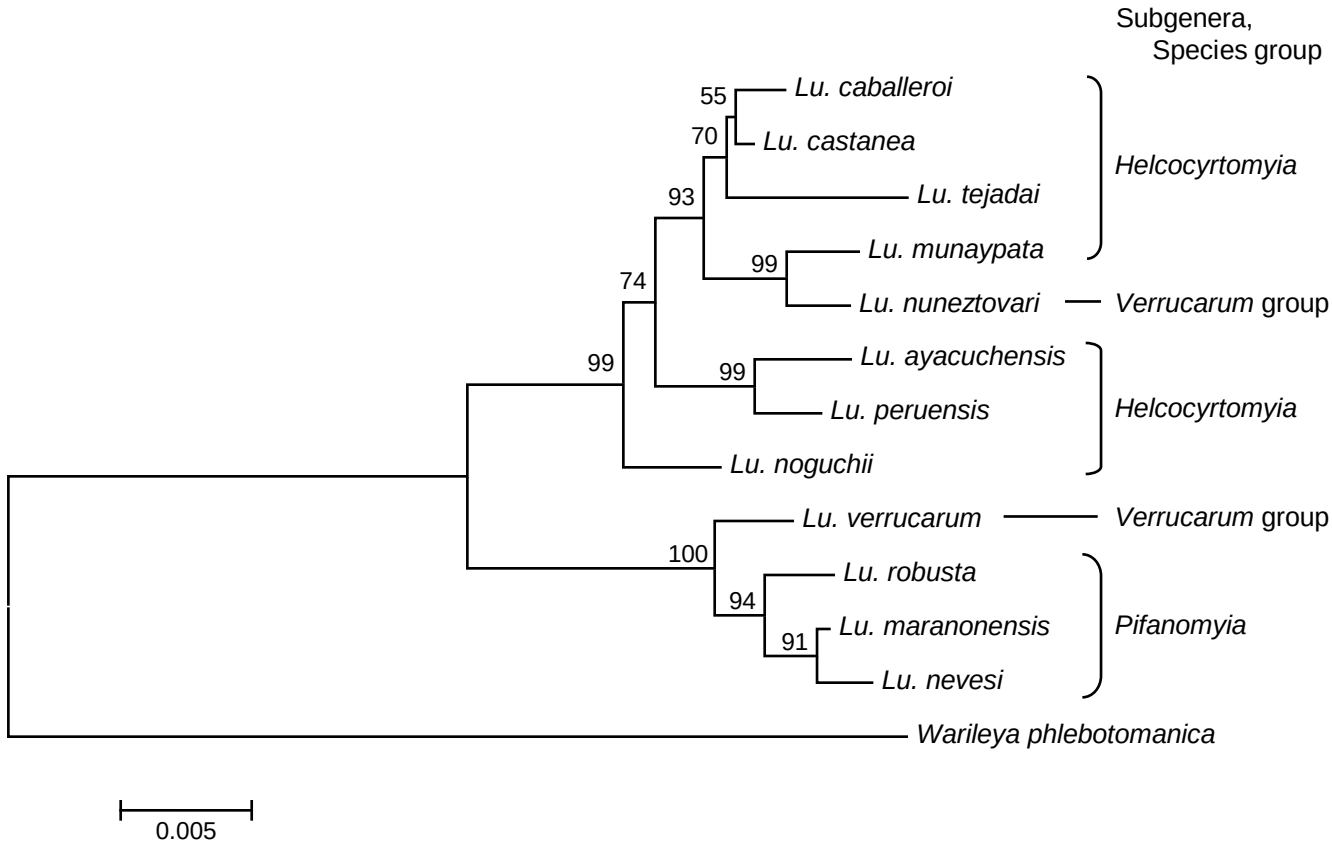
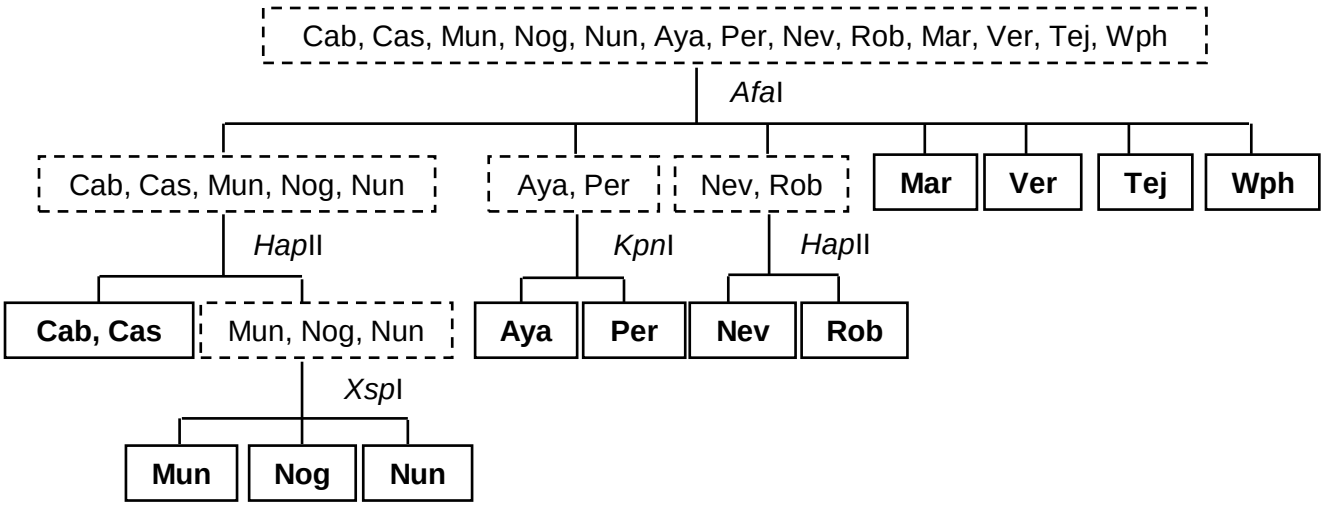

Subgenera,
Species group
Lu. caballeroi
Lu. castanea
Lu. tejadai
Lu. munaypata
Lu. nuneztovari
Lu. ayacuchensis
Lu. peruensis
Lu. noguchii
Lu. verrucarum
Lu. robusta
Lu. maranonensis
Lu. nevesi
Warileya phlebotomanica
55
70
93
99
74
99
99
100
94
91
Helcocyrtomyia
Verrucarum group
Helcocyrtomyia
Verrucarum group
Pifanomyia
0.005

Fig. 2

A


Cab, Cas, Mun, Nog, Nun, Aya, Per, Nev, Rob, Mar, Ver, Tej, Wph
AfaI
Cab, Cas, Mun, Nog, Nun
Aya, Per
Nev, Rob
Mar
Ver
Tej
Wph
HapII
KpnI
HapII
Cab, Cas
Mun, Nog, Nun
Aya
Per
Nev
Rob
XspI
Mun
Nog
Nun

B

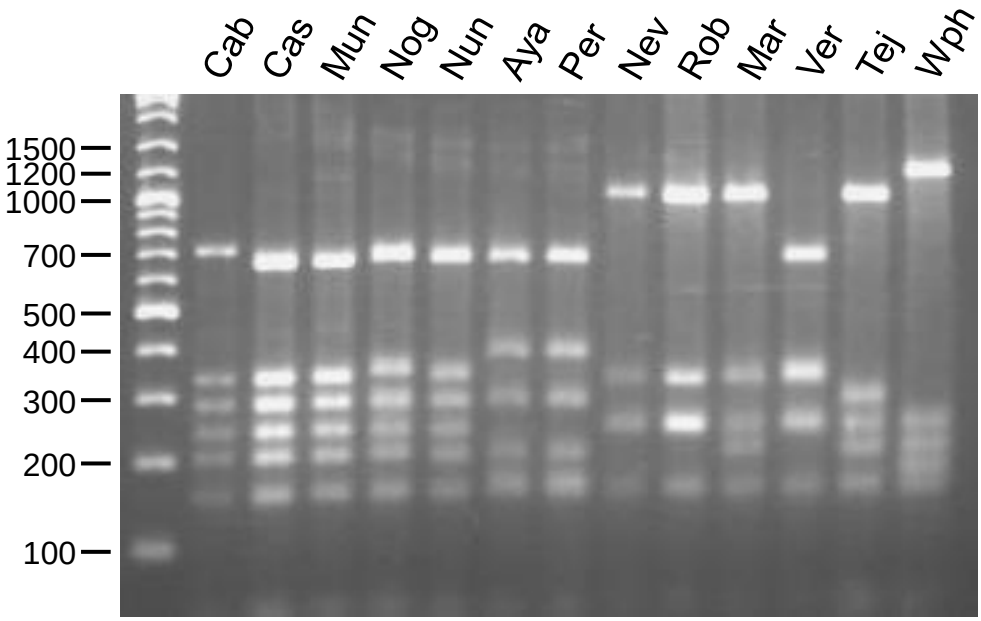
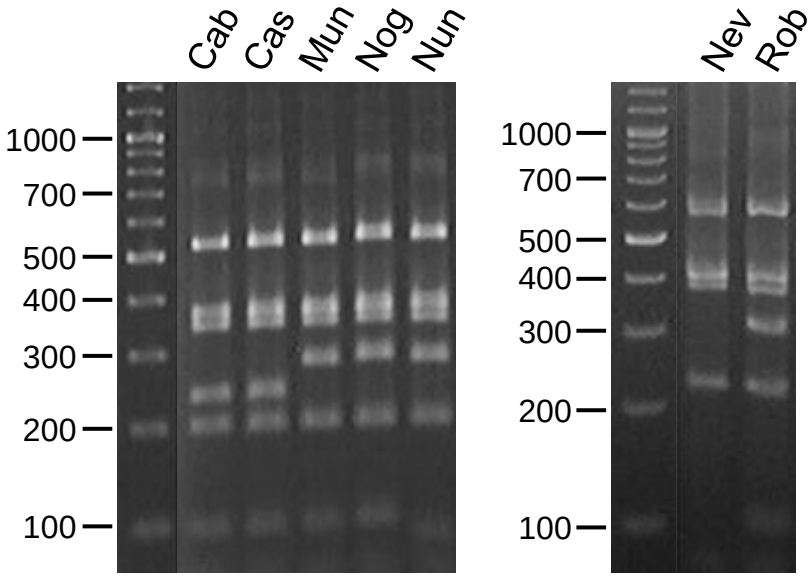
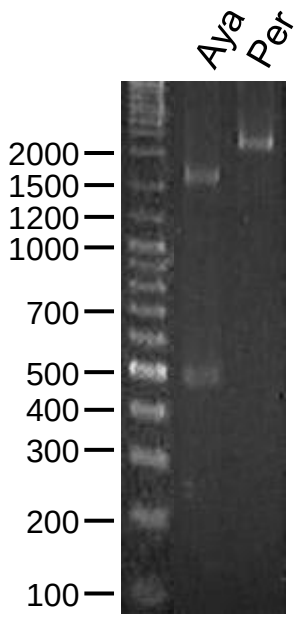

Cab
Cas
Mun
Nog
Nun
Aya
Per
Nev
Rob
Mar
Ver
Tej
Wph
1500
1200
1000
700
500
400
300
200
100

Fig. 3

C


Cab
Cas
Mun
Nog
Nun
Nev
Rob
1000
700
500
400
300
200
100

D


Aya
Per
2000
1500
1200
1000
700
500
400
300
200
100

E

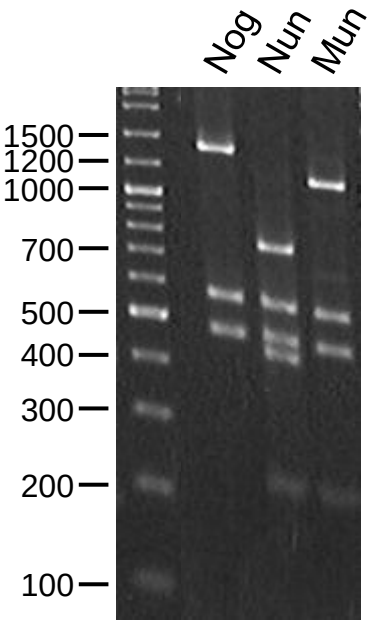

Nog
Nun
Mun
1500
1200
1000
700
500
400
300
200
100

Fig. 3

Table 1. Genotyping of sand flies in Andean areas of Peru

Locality	Ver	Per	Nog	Cab	Total
Department of La Libertad					
Loma Grande	8	12	0	0	20
La Cuesta	0	40	2	0	42
Nambuque	0	9	0	64	73
Padregual	0	74	0	40	114
Department of Ancash					
Atocshay	31	11	0	0	42
Colcap Bajo	124	2	0	0	126
Curcuy	116	38	0	0	154
Huanchoc	98	165	0	0	263
Pucuhuayllan	13	8	0	0	21
Department of Lima					
Ambar	214	142	4	0	360
Huarochiri	20	0	5	0	25

Ver, *Lu. verrucarum*; Per, *Lu. peruensis*; Nog, *Lu. noguchii*; Cab, *Lu. caballeroi*