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DNA barcode for identification of immature stages of sand flies (Diptera: Psychodidae) collected from natural breeding sites

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Abstract

Although phlebotomine sand flies breeding sites have been identified and recorded by several studies, the microhabitats exploited by these insects remain little-known and hard to find. In this context, the difficulty of finding immature stages, and the limited number of taxonomic studies to identify immature stages of phlebotomine sand flies, are considered the major obstacles when attempting a complete inventory of *Lutzomyia* species. The objective of this study is to validate *Cytochrome Oxidase I* (Barcode region) as a marker for the identification of immature stages of *Lutzomyia* species recovered from natural breeding sites in Colombia. Among 142 collected sand flies, 18 immature individuals that did not complete their life cycle were identified to species level through sequencing of the *COI* gene. Values of K2P genetic distance between 0.002–0.031 allowed the identification of larvae at species level. The bootstrap support values (96%) in the Neighbor-Joining dendrogram were consistent for the majority of the established MOTUS of *Lutzomyia atroclavata*, *Lutzomyia micropyga*, *Lutzomyia serrana*, *Lutzomyia cayennensis*, *Lutzomyia rangelifiana*, *Lutzomyia shannoni* and some species of the genus *Brumptomyia*. The *COI* gene is validated as a marker for the identification of immature stages of the genus *Lutzomyia*.

Key words: *Brumptomyia*, *Lutzomyia*, Colombia, *Cytochrome Oxidase I*, mitochondrial DNA

Introduction

Females of *Lutzomyia* França are hematophagous insects and some species are capable of transmitting parasites of *Leishmania* Ross (Kinetoplastida: Trypanosomatidae) (Bates 2015). This fact raises an epidemiological issue for human communities dwelling in tropical regions, where such insects occur in great diversity. In most cases, the available entomological records for *Lutzomyia* species are based only on adult sampling surveys. Such insect sampling would be improved if the immature specimens are studied as well (Vivero *et al.* 2015).

The lack of taxonomic information that limits the production of taxonomic keys for the immature stages of *Lutzomyia* species, as well as the difficulty to find the natural breeding sites (Felicangeli 2002), are the main barriers for the complete and representative inventories of sand flies in a determined area. In the scientific literature, scanning electron microscopy appears to be the most appropriate technique for studying the chorionic structure of eggs of sand flies. With this technique, some authors have contributed with the description of morphological characteristics of eggs and the chaetotaxy of larvae associated to species of medical importance and description of the mouthparts to differentiate some *Lutzomyia* species (Hanson 1968; Endris *et al.* 1987; Ward 1976; Felicangeli *et al.* 1993; Pérez & Ogusuku 1997; Fausto *et al.* 1998; Arrivillaga *et al.* 1999; Sierra *et al.* 2000). However, this methodology is considered complex and expensive. Though there are a few contributions on morphological traits of immatures in *Lutzomyia* (Ward 1976; Arrivillaga *et al.* 1999), they are considered isolated and exploratory studies (Sierra *et al.* 2000).

In an epidemiological context, when an inventory of species is required, a survey should include not only information about the adults but also about immature forms. For example, the systematic control of other insect vectors such as mosquitoes (vectors of malaria and dengue), is often aimed at the larval stages, in aquatic breeding sites, and adult stage (Feliciangeli 2004). However, this strategy has not been possible in the control of sand flies. This premise and the problems previously discussed have motivated new initiatives and methodologies directed toward the study of immature stages of *Lutzomyia*. In recent years, genes with different substitution rates and types of inheritance, such as mitochondrial (*Cytochrome Oxidase I*, *Cytochrome B*, *NADH 1* and *NADH 4*, *Serine tRNA*) and nuclear (*Period*, *Cacophony*, *Paralytic*), have been used for the differentiation of *Lutzomyia* species, mainly in adult stage (Vivero *et al.* 2007; Depaquit 2014; Dvorak *et al.* 2014; Pinto *et al.* 2015).

Nevertheless, the usefulness of these genetic markers to identify immature stages of sandflies collected on natural breeding sites has not been evaluated. Currently, there are online platforms such as DDBJ, EMBL-EBI and NCBI, bio-informatics tools such as BLAST, Clustal Omega and several methods, that could be used to perform alignments, and assigning the taxonomic identity, in a rapid manner and with good support and precision at species level (Hebert *et al.* 2003). The *Cytochrome Oxidase I* (COI) mitochondrial gene is used as a genetic barcode to assign taxonomic identity to many insects, among them, the medically important insect groups (Contreras *et al.* 2014; Romero *et al.* 2016), thus appearing as a potential gene for the identification of immature phlebotomine sand fly stages collected from breeding sites (Romero *et al.* 2016). The COI gene has also been used for the delimitation of *Lutzomyia* species in leishmaniasis transmission areas in the Andean and Caribbean regions of Colombia (Contreras *et al.* 2013; Romero *et al.* 2016). Other studies have shown the usefulness of COI gene to explore the diversity of sand flies species (Azpurua *et al.* 2010), and to reconstruct the phylogeny of sandflies in Peru, Colombia and Ecuador (Cohnstaedt *et al.* 2011; Kato *et al.* 2015; Nzelu *et al.* 2015). All these sequences from these studies can be used to compare with sequences of COI gene of immature stages.

Considering the importance of studying the immature stages of phlebotomine sandflies in the context of epidemiology and control of leishmaniasis, the objective of this study is to assign the taxonomical identity to immature stages of sandflies, by using the sequences of the mitochondrial gene COI.

Material and methods

Study area. We conducted entomological surveys in three Natural Reserves in Colombia: “Río Claro” (5°49’59.37”N, 74°52’00.62”W, 418 m a.s.l.) located in the municipality of San Francisco (Department of Antioquia), “El Aguacate” (8°36’53.85”N, 77°19’39.15”W, 13 m a.s.l.) in the municipality of Acandí (Department of Chocó), “Primates” (09°31’48.0”N, 75°21’4.3”W, 220 m a.s.l.) in the municipality of Colosó, and in the urban area of the City of Sincelejo (9°18’09.75”N, 75°23’42.22”W, 209 m a.s.l.), located in the Department of Sucre.

Isolation and processing of immature sandflies. A total of 160 possible breeding sites were explored. The isolation of larvae, pupae and exuviae was performed by direct examination. Adults were also collected, with emergence traps and incubation of soil samples of breeding sites in the laboratory (Vivero *et al.* 2015). The immature stages recovered dead during isolation and laboratory breeding were kept dried in 1.5 mL vials at -20°C for molecular identification using the COI genetic barcode sequence.

DNA extraction and partial amplification of the *Cytochrome Oxidase I* gene. The DNA extraction of immature stages of sandflies was performed according to the protocol of high salt concentration (Vivero *et al.* 2007). In the case of the larvae, the head was separated as a taxonomic support, while the pupae were processed completely. The 5’ fragment of the mitochondrial gene COI (680 pb) was amplified using the oligonucleotides LCO1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) and HCO2198 (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’) (Folmer *et al.* 1994). The amplified products were purified using Wizard PCR Preps (Promega) and sequenced in both senses, in an automatic sequencer ABI 3730XL of capillary electrophoresis.

Molecular analysis of the *Cytochrome Oxidase I* gene. The obtained chromatograms were edited with BioEdit (Hall 1999) software to generate a partial consensus sequence of the COI gene for each one of the immature specimens. The multiple alignment was performed with Clustal W (Higgins *et al.* 1992) algorithm incorporated in MEGA 5.05 (Tamura *et al.* 2011), with a posterior manual edition for the delimitation of the codifying fragment size to be analyzed. The mitochondrial genetic code of invertebrates was used to guarantee the

correct reading frame of the codons and the identification of variable sites corresponding to nucleotides and aminoacids using the MEGA 5.05 software. To verify the identity of the partial sequences of the *COI* gene obtained of immature stages, a previous analysis was performed in BLAST (Altschul *et al.* 1997) with sequences of adults. At the same time, a sequence matrix for the *COI* gene corresponding to adults of *Lutzomyia*, *Brumptomyia* and *Bruchomyiinae* was built with the sequences of adults from different regions of Colombia, retrieved from Genbank. The analyzed fragment of the *COI* gene corresponded to the positions 1567 to 2144 on the mitochondrial genome used as reference (*L. umbratilis* Ward & Fraiha; Genbank accession no. KP702938).

The genetic divergences among sequences from immature specimens of sandflies were evaluated with MEGA 5.05, as well as the nucleotide composition, haplotype variability, variable sites and parsimonious sites. The absence of NUMTs was also verified. The NUMT in these sequences of immature stages, was examined with BLAST to search the regions of similarity with our mitochondrial sequences. Strategies to help identify these alien sequences include search for ambiguity among sequences, noise, or double peaks in the electropherogram; sequence translation in search for additional termination codons and the comparison of the amplified sequences with other published sequences from closely related species. Verification of recombination events and the presence of chimeras was performed with RDP4 (Recombination Detection Program version 4) software, using all sequences of *COI* obtained in our study in order to ensure the accuracy of the nucleotide variability with respect to previously reported sequences in GenBank.

With the final alignment of *COI* from immature and adult stages with taxonomic identity known, the paired genetic intra/interspecific distances were calculated under the bi-parametrical model of Kimura (K2P) (Kimura 1980), which gives different mutation rates to transitions and transversions. The sequence matrix of *COI* gene fragments was exported to MEGA 5.05, to generate a Neighbor-Joining dendrogram (Saitou & Nei 1987), using the K2P genetic distances, and following the standard recommendations of the Barcode of Life (Hebert *et al.* 2003; Lanteri 2007; <http://www.boldsystems.org/index.php/Resources>). The sequences from the partial segment of the *COI* gene reported for immature stages were submitted to GenBank.

Results

Genetic analysis of sequences for identification of immature stages

13 species of *Lutzomyia* and two of *Brumptomyia* França & Parrot genera were identified (Table 1). A total of 18 immature specimens (12.67%) were identified through analysis of the 680bp fragment of the *COI* gene (Table 1). These immature specimens, did not complete their development to adult stage.

The average nucleotide composition of the *COI* gene of the immature specimens corresponded to a 67.8% of Adenine-Thymine relation and a 32.2% Guanine-Cytosine relation (Table 2), denoting differences in genetic composition with respect to the species. In the 18 sequences of immature sand flies from this study, a total of 14 haplotypes, and a low number of variable sites, were obtained (Table 2). The majority of variable changes were Cytosine by Thymine in the third codon position.

The minimal intra-specific K2P distance was of 0.002 in the *Lutzomyia* species, except for *L. shannoni* (Dyar) and *L. cayennensis cayennensis* (Floch & Abonnenc) (0.006) (Table 2). The maximal intra-specific K2P distance was 0.031, detected in specimens of *L. dubitans* and *L. rangelifana* (Table 2). In *Brumptomyia*, intra-specific K2P values varied from 0.002 to 0.033. This showed consistency for this marker at the taxonomical level, as well as for the identification of the species *B. hamata* (Fairchild & Hertig) and *B. mesai* Sherlock (Table 2). The inter-specific values of K2P distances for the different *Lutzomyia* species (>0.1), between *Brumptomyia* and *Lutzomyia* (0.130–0.194) and upon comparison of *Lutzomyia* species and specimens from *Bruchomyiinae* (0.142–0.235), showed higher values which is in agreement with the percentages previously established in the Barcode initiative for inter-specific values.

The Neighbor-Joining dendrogram obtained under the K2P model, corroborated the identification of immature stages of *Lutzomyia* and *Brumptomyia* in our study (Figure 1). The groups defined as molecular operational taxonomic units (MOTUs) clarify the differentiation between species according to high bootstrap values detected (95–99%) (Figure 1).

FIGURE 1. Neighbor-joining analysis of mitochondrial COI sequences of immature stages of sand flies isolated from natural breeding sites, contrasted with COI gene sequences from adults from GenBank. The black triangles represent the larvae and pupae identified.

TABLE 1. Sandfly species isolated from natural breeding sites, identified with the analysis of mitochondrial COI sequences and reared under laboratory conditions.

Species	Breeding sites	Study areas	Number of emerged adults	Number of immatures (DNA barcode)	Number of immatures identified only to genus	Total (%)
<i>L. atroclavata</i>	Tb, Tt, Tc	Sincelejo	9	5	---	14 (9.86)
<i>L. migonei</i>	Tb, Tt, Tr	Primates	13	---	---	13 (9.15)
<i>L. micropyga</i>	Tb, Ll, Tc, Tr	Sincelejo, Primates, El Aguacate, Rio Claro	6	2	---	8 (5.63)
<i>L. serrana</i>	Tb, Th, Tt	Primates	3	2	---	5 (3.52)
<i>L. dubitans</i>	TM, Th, Cv	Sincelejo, Primates	4	2	---	6 (4.23)
<i>L. evansi</i>	Tb, Ll	Sincelejo, Primates	5	---	---	5 (3.52)
<i>L. c. cayennensis</i>	Tb, Ll, Tc	Sincelejo, Primates	6	1	---	7 (4.93)
<i>L. rangelliana</i>	Tb, Tc, Tn	Sincelejo	2	2	---	4 (2.82)
<i>L. ovallesi</i>	Ll	Primates	2	---	---	2 (1.41)
<i>L. shannoni</i>	Th	Primates	1	1	---	2 (1.41)
<i>L. trinidadensis</i>	Tr	Rio Claro	2	---	---	2 (1.41)
<i>L. pilosa</i>	Tr	Rio Claro	2	---	---	2 (1.41)
<i>L. gorbitzi</i>	Th	Primates	1	---	---	1 (0.70)
<i>B. hamata</i>	Th, Tr	El Aguacate, Rio Claro	4	2	---	6 (4.23)
<i>B. mesai</i>	Tr	El Aguacate	---	1	---	1 (0.70)
Subtotal (%)	---	---	60 (42.25)	18 (12.67)	---	78 (54.92)
<i>Lutzomyia</i> spp.	Tb, Th, Tt, Ll, Tc, Tm, Tr	Sincelejo, Primates, Rio Claro	---	---	38	38 (26.76)
<i>Brumptomyia</i> spp.	Tr	El Aguacate	---	---	26	26 (18.30)
Total (%)			60 (42.25)	18 (12.67)	64 (45.07)	142 (100)

Abbreviations for natural breeding sites Tb. Tree-bases; Tt. Trunk of tree; Bk. Tree-cortex; Ll. Leaf litter; Tr. Tabular roots; Th. tree-holes; CV. Cave; TM. Termite-nests.

Discussion

Taxonomic studies of immature insects are scarce and complex because knowledge of morphological characters is limited. A particular case is presented in Phlebotominae Rondani, where the absence of taxonomical keys for immature stages symbolizes how partial the available inventories are, including only information of adult stages (Vivero *et al.* 2013). This study validated in a consistent way, the use of the mitochondrial gene *COI* as a molecular marker for the identification of immature stages collected from natural breeding sites.

In this sense, the comparative analysis of partial sequences of the *COI* gene between immature and adult stages of sand flies, and the use of morphology, suggests a high congruency in the taxonomical assignation of *Lutzomyia* and *Brumptomyia* species. The use of *COI* gene for the identification of immature stages of *Lutzomyia* and *Brumptomyia* species are supported by the well-differentiated values of intra-specific genetic distance values (0.2–3.1%) compared to the inter-specific (12.4–20.1%) divergence values. The “Barcode Gap” effect is evidenced as an adequate criteria for the genetic variation between species (Hebert *et al.* 2003), assuming that the lines diversify faster between species than inside of them. This affirmation is coherent with the estimation of a bigger number of synonym substitution (transition) in the *COI* mitochondrial gene in the third position, upon comparison on taxonomical level (Blouin *et al.* 1998).

Other argument supporting the use of *COI* gene is the absence of stop codons or NUMTs sequences inside the analyzed fragment. Also, the bootstrap support (99%) in the Neighbor-Joining dendrogram demonstrated the

TABLE 2. Immature sand flies identified by the mitochondrial gene cytochrome oxidase I (COI) and comparison with sequences of adult reported in GenBank.
Abbreviations: A. Adenine, T. Thymine, G. Guanine, C. Cytosine.

Immature sand flies				Comparison with sequences reported in GenBank				
Species	<i>n</i>	Stages	Number of haplotypes (variable sites)	GenBank accession nos. of sequences from immature stages	GenBank accession nos. of sequences from adult individuals	Nucleotide variable sites (Type of substitution) – Codon position	Informative positions (Type of substitutions) – Codon position	K2P genetic distance
<i>L. atroclavata</i>	5	Larva	4 (4–5nt)	KR907851, KR907852, KR907853, KR907854, KR907855	GU909477–82 Romero <i>et al.</i> , 2011	8 (C*T) <u>3</u>	4 (C*T) <u>3</u>	0.002–0.011
<i>L. rangelifana</i>	2	Pupa–Larva	2 (2nt)	KR907849, KR907850	GU909493–97 Romero <i>et al.</i> , 2011	17 (C*T/A*G) <u>3</u>	13 (A*G) <u>3</u>	0.002–0.031
<i>L. dubitans</i>	2	Larva	1	KR907860, KR907861	GU909446–50 Romero <i>et al.</i> , 2011	15 (C*T/A*G) <u>3</u>	0	0.002–0.031
<i>L. micropyga</i>	2	Larva	2 (2nt)	KR907856, KR907857	GU909464–66 Romero <i>et al.</i> , 2011	3 (C*T) <u>3/2</u>	0	0.002–0.006
<i>L. serrana</i>	2	Larva	1	KR907858, KR907859	GU909501–FJ437274 Romero <i>et al.</i> , 2011 Cohnstaedt <i>et al.</i> , 2011	1 (G*A) <u>3</u>	0	0.002
<i>L. shannoni</i>	1	Larva	1	KR907862	GU909467–71	9 (C*T/A*G) <u>3</u>	2(A*G) <u>3</u>	0.006–0.013
<i>L. c. cayennensis</i>	1	Larva	1	KR907863	GU909472–76 Romero <i>et al.</i> , 2011	13 (C*T/A*G) <u>1/2/3</u>	5 (C*T) <u>3</u>	0.006–0.021
<i>B. hamata</i>	2	Larva	1	KR907865, KR907866	GU001740 Azpurua <i>et al.</i> , 2010	10 (C*T) <u>3</u>	0	0.019
<i>B. mesai</i>	1	Larva	1	KR907867	GU909506–Bmesacc01	17 (G*A/C*T/T*A) <u>3</u>	0	0.002–0.033
Total	18		14		----	---	---	----

cohesiveness of the groupings (MOTUs) that included *COI* sequences from adults and immatures obtained from a wide geographic range including different ecosystems, habitats and micro-habitats.

Getting readable sequences from larvae and pupae collected from different organic substrates with decomposing materials and high concentration of humic acids (Vivero *et al.* 2013) is complex and need DNA extraction methods that have good performance at low pH and that may block chelating substances from the organic substrates. These humic acids may interfere with the DNA amplification (Boom *et al.* 1990). Also, the contents of the gastrointestinal tract of the larvae can include the presence of nematodes, fungi, vegetable material and arthropod rests under decomposing processes that could eventually affect the specificity of the DNA amplification in immature stages of sandflies.

Currently, very little information is available regarding the identification of immature stages of *Lutzomyia*, using the DNA barcoding in Colombia (Contreras *et al.* 2013). In the present study, the utility of the *COI* marker is demonstrated given that a total of 18 specimens were identified, represented by seven species of genus *Lutzomyia* (*L. atroclavata*, *L. c. cayennensis*, *L. dubitans*, *L. micropyga*, *L. rangeliana*, *L. serrana*, *L. shannoni*), and two species from genus *Brumptomyia* (*B. hamata*, *B. mesai*).

Despite the scarcity of molecular taxonomical studies for immature stages of sandflies, it is worth noticing that such contributions exist for other arthropods and insect groups, between these: *COI* sequences comparison to distinguish non identified immature stages of species of *Cicuria* Menge (Araneae: Dictynidae) (Paquin & Hedin 2004); identification of morphologically undistinguishable larvae of species of canegrubs (Coleoptera: Scarabaeidae), using traditional techniques as well as DNA sequences (Miller *et al.* 1999); and the identification of non-described, undistinguishable larvae of *Philodytes umbrinus* (Motschulsky) (Coleoptera: Dytiscidae), through the comparison of *COI* sequences obtained from adults of the same species with known identity (Miller *et al.* 2005).

The analysis of nucleotide sequences complemented with the morphological patterns (e.g. terminal setae) facilitates the identification of immature stages *Lutzomyia* and *Brumptomyia* recovered in natural breeding sites at leishmaniasis transmission areas. The methodological scheme and obtained results set a platform for future identifications of non-identified larvae and the opportunity to apply these procedures for future studies involving morphological and molecular topics. Studies such as the present, are relevant given the lack of knowledge about immature forms of most species of sandflies and the increasing number of studies focused on finding these immatures, some of which remain unidentified because they do not reach adulthood. DNA barcoding use as proposed by us here, appears as an alternative to species determination.

Conclusions

This study was planned as a complementary identification tool in entomological surveys in order to accelerate the finding of vector species in transmission areas where leishmaniasis is prevalent.. In this sense, the *COI* gene was validated as a marker for the identification of immature stages of the *Lutzomyia* recovered from natural breeding sites.

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