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

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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 rangeliana, 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 (Feliciangeli 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; Feliciangeli et al. 1993; Pérez & Ongusuku 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 Acandi (Department of Chocó), “Primates” (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 (Floh & Abonnenc) (0.006) (Table 2). The maximal intra-specific K2P distance was 0.031, detected in specimens of L. dubitans and L. rangelianna (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 (%) |
|--------------------|-------------------------|------------------------------------|--------------------------|-----------------------------------|---------------------------------------------|-----------|
| L. atroclavata     | Tb, Tt, Tc              | Sincelejo                          | 9                        | 5                                 | ---                                         | 14 (9.86) |
| L. migonei         | Tb, Tt, Tr              | Primates                           | 13                       | ---                               | ---                                         | 13 (9.15) |
| L. micropyga       | Tb, Ll, Tc, Tr          | Sincelejo, Primates, El Aguacate, Rio Claro | 6                        | 2                                 | ---                                         | 8 (5.63)  |
| L. serrana         | Tb, Th, Tt              | Primates                           | 3                        | 2                                 | ---                                         | 5 (3.52)  |
| L. dubitans        | TM, Th, Cv              | Sincelejo, Primates                | 4                        | 2                                 | ---                                         | 6 (4.23)  |
| L. evansi          | Tb, Ll                  | Sincelejo, Primates                | 5                        | ---                               | ---                                         | 5 (3.52)  |
| L. c. cayennensis  | Tb, Ll, Tc              | Sincelejo, Primates                | 6                        | 1                                 | ---                                         | 7 (4.93)  |
| L. rangeliana      | Tb, Tc, Tn              | Sincelejo                          | 2                        | 2                                 | ---                                         | 4 (2.82)  |
| L. ovallesi        | Ll                      | Primates                           | 2                        | ---                               | ---                                         | 2 (1.41)  |
| L. shannoni        | Th                      | Primates                           | 1                        | 1                                 | ---                                         | 2 (1.41)  |
| L. trinidadensis   | Tr                      | Rio Claro                          | 2                        | ---                               | ---                                         | 2 (1.41)  |
| L. pilosa          | Tr                      | Rio Claro                          | 2                        | ---                               | ---                                         | 2 (1.41)  |
| L. gorbitzi        | Th                      | Primates                           | 1                        | ---                               | ---                                         | 1 (0.70)  |
| B. hamata          | Th, Tr                  | El Aguacate, Rio Claro             | 4                        | 2                                 | ---                                         | 6 (4.23)  |
| B. mesai           | Tr                      | El Aguacate                        | ---                      | 1                                 | ---                                         | 1 (0.70)  |
| **Subtotal (%)**   | **---**                 | **---**                            | **60 (42.25)**           | **18 (12.67)**                    | **---**                                      | **78 (54.92)** |
| Lutzomyia spp.     | Tb, Th, Tt, Ll, Tc, Tr  | Sincelejo, Primates, Rio Claro     | ---                      | ---                               | 38                                          | 38 (26.76) |
| Brumptomyia 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.

| Species            | n  | 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 |
|--------------------|----|--------|--------------------------------------|----------------------------------------------------------|------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------|
| L. atroclavata     | 5  | Larva  | 4 (4–5nt)                            | KR907851, KR907852, KR907853, KR907854, KR907855          | GU909477–82                                                 | 8 (C*T) 3                                                    | 4 (C*T) 3                                                    | 0.002–0.011         |
| L. rangeliana      | 2  | Pupa-Larva | 2 (2nt)                              | KR907849, KR907850                                       | GU909493–97                                                 | 17 (C*T/A*G) 3                                                | 13 (A*G) 3                                                  | 0.002–0.031         |
| L. dubitans        | 2  | Larva  | 1                                    | KR907860, KR907861                                       | GU909446–50                                                 | 15 (C*T/A*G) 3                                               | 0                                                             | 0.002–0.031         |
| L. micropyga       | 2  | Larva  | 2 (2nt)                              | KR907856, KR907857                                       | GU909464–66                                                 | 3 (C*T) 3/2                                                  | 0                                                             | 0.002–0.006         |
| L. serrana         | 2  | Larva  | 1                                    | KR907858, KR907859                                       | GU909501–FJ437274                                           | 1 (G*A) 3                                                   | 0                                                             | 0.002               |
| L. shannoni        | 1  | Larva  | 1                                    | KR907862                                                 | GU909467–71                                                 | 9 (C*T/A*G) 2                                                | 2 (A*G) 2                                                   | 0.006–0.013         |
| L. c. cayennensis  | 1  | Larva  | 1                                    | KR907863                                                 | GU909472–76                                                 | 13 (C*T/A*G) 1/2/3                                           | 5 (C*T) 3                                                   | 0.006–0.021         |
| B. hamata          | 2  | Larva  | 1                                    | KR907865, KR907866                                       | Azpurua et al., 2010                                         | 10 (C*T) 3                                                  | 0                                                             | 0.019               |
| B. mesai           | 1  | Larva  | 1                                    | KR907867                                                 | GU909506–Bmesace01                                           | 17 (G*A/C*T/T*A) 3                                            | 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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