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Molecular operational taxonomic units of mosquitoes (Diptera: Culicidae) collected in high Andean mountain ecosystems of Antioquia, Colombia

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Abstract

Accurate taxonomic identification of highland mosquito species may be complicated because of the lack of comprehensive regional morphological keys and taxonomic specialists, particularly for mosquitoes of medical or ecological importance. We applied a multi-locus approach to explore the diversity of genera/species collected, to define the Molecular Operational Taxonomic Units (MOTUs) and to perform phylogenetic clustering. Twenty MOTUs and three single sequences were revealed from 78 concatenated *cox1* + ITS2 sequences, and the species name was allocated for five of these. This study provides molecular taxonomic information of culicid fauna present in high Andean mountain ecosystems in Antioquia, Colombia. However, future morphological and integrative taxonomic studies should be conducted to achieve the specific identity of all detected MOTUs.

Keywords

molecular operational taxonomic units; mosquitoes; *cox1*; highlands; ITS2; Colombia

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Author contributions

DRG, JEC, MMC and SUS participated in study design, data analysis and writing the manuscript. DRG and SB performed laboratory work. DRG, JDS and CP collected mosquitoes. JDS and CP identified specimens based on morphology. All authors critically reviewed the manuscript and approved the final version.

Introduction

There are 3,554 mosquito species recorded worldwide (Harbach, 2017) of which 353 are estimated to be present in Colombia (WRBU, 2017). In this country, mosquito studies have focused mainly on the malaria vector species in lowland areas (Gutiérrez *et al.*, 2009; Naranjo-Díaz *et al.*, 2014; Ahumada *et al.*, 2016), dengue (Santacoloma-Varón *et al.*, 2010; Quintero *et al.*, 2014; Castrillón *et al.*, 2015) and arbovirus vectors (Barajas *et al.*, 2013; Hoyos-López *et al.*, 2015; Suaza-Vasco *et al.*, 2015). Regarding mosquito species in highland ecosystems, there are few records in Colombia and most pertain to the tribe Aedini. Recently, *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) was recorded at an elevation of 2,252 m and was found infected with dengue virus in Bello, Antioquia Department at 1,980 m (Ruiz-López *et al.*, 2016). In addition, *Anopheles* (*Anopheles*) *pseudopunctipennis* (Theobald), a secondary vector of malaria in Colombia, has been recorded at 2,005 m and 2,153 m in the Caldas Department (Galvis, 1943). There are also records of mosquitoes of medical or ecological importance in high Andean mountain ecosystems in other South American countries, e.g. Bolivia (Rutar *et al.*, 2004), Ecuador (Pinault & Hunter, 2011) and Venezuela (Navarro *et al.*, 2010).

Characterizing the mosquito fauna present in ecosystems above 2,000 m is important because it may provide baseline data for future comparisons and contribute to insect inventories (Barratt *et al.*, 2003; Monaghan *et al.*, 2005). In addition, these species may reflect differences in gradient distribution patterns related to environmental changes and/or anthropic activities (Pemola Devi & Jauhari, 2004). For example, global warming may cause the movement of some species to higher elevations where temperatures have or may become suitable for survival, whereas other species may respond differently to these changes by becoming locally extinct because of their inability to adapt to new, perhaps more extreme, conditions (Feo *et al.*, 2009; Githeko *et al.*, 2009). However, evaluating the mosquito diversity above 2,000 m can be challenging, because of the limited knowledge of the species, lack of taxonomic specialists and presence of cryptic or species complexes in which morphological characters alone cannot distinguish species (Hajibabaei *et al.*, 2006; Bickford *et al.*, 2007; Wolff *et al.*, 2016). Finally, taxonomic keys may not be available, making it difficult to determine whether the specimen under study belongs to a previously described species or a new one.

In the latter context, multiple approaches to provide taxonomic information are desirable (Rubinoff, 2006; Krishna-Krishnamurthy & Francis, 2012). The 5′ end of the cytochrome c oxidase subunit 1 gene (*cox1*) is frequently sequenced to confirm or to complement the identity of mosquito species from South American countries (González *et al.*, 2010; Arregui *et al.*, 2015; López-Rubio *et al.*, 2016). One interesting use of *cox1* barcode sequences is the definition of Molecular Operational Taxonomic Units (MOTUs) in which a cluster of sequences that differ from each other are proposed as taxonomic units of differentiation (Floyd *et al.*, 2002; Blaxter, 2004). In Colombia, for the Albitarsis Group of *Anopheles* (*Nyssorhynchus*), two differentially distributed MOTUs were detected, *An. albitarsis* I in the northwest and northeast and *An. albitarsis* E and F in the northeast (Gómez *et al.*, 2013). In addition, seven MOTUs were identified using *cox1* + ITS2 markers in *Anopheles* species of the Neotropical Arribalzagia Series (subgenus *Anopheles*) (Gómez *et al.*, 2015). It is well

known that the internal transcribed spacer 2 (ITS2) of ribosomal DNA is informative for molecular differentiation of species belonging the genera *Anopheles*, *Culex*, *Culiseta* and *Ochlerotatus*, among others (Wesson *et al.*, 1992; Bargues *et al.*, 2006; Cienfuegos *et al.*, 2011; Khoshdel-Nezamiha *et al.*, 2016).

Considering that the reliance on a single genetic marker may give misleading results (Dupuis *et al.*, 2012), in the present study we evaluated a multi-locus (*cox1* + ITS2) approach for its effectiveness in the identification of MOTUs in mosquitoes collected in high Andean mountain ecosystems. We hypothesized that the combined sequences would identify Molecular Operational Taxonomic Units (MOTUs) more accurately than a single locus analysis within each genus of mosquitoes present above 2,000 m in Antioquia, Colombia.

Material and methods

Mosquito sampling and morphological identification of genera and sub genera

Mosquitoes were collected from six sites within three localities in Antioquia Department. The municipality of Belmira (BEL) (06° 36′ 18″ N, 75° 39′ 57″ W), Jardín (JAR) (05° 35′ 03″ N, 75° 46′ 02″ W) and Jericó (JER) (05° 47′ 18″ N, 75° 47′ 26″ W). The altitudinal range was between 2,009−3,098 m and a minimum of two field collections were performed each year in each locality (Fig. 1). Adult mosquitoes were collected using entomological nets, mouth aspirators and Shannon traps, and larvae and pupae were sampled with pipettes and transported to the laboratory and reared to obtain adults (Belkin *et al.*, 1969; Louton *et al.*, 1996). Representative larval exuviae and male genitalia were mounted on microscope slides in Euparal (Pecor & Gaffigan, 1997), and at least five voucher specimens selected based on apparent morphospecies were deposited in the collection of the Entomological Museum Francisco Luis Gallego, Universidad Nacional de Colombia, sede Medellín.

Mosquitoes were identified using morphological keys and reviews available for Neotropical Culicidae (Lane, 1953; Mattingly, 1971; Forattini, 2002) and for the genera and subgenera of *Anopheles* (González & Carrejo, 2009; Harrison *et al.*, 2012), *Culex* (Bram, 1967), *Haemagogus* (Levi-Castillo, 1954; Arnell, 1973), *Aedes* (*Howardina*) (Berlin, 1969), *Aedes* (*Ochlerotatus*) (Arnell, 1976), *Trichoprosopon* (Stone, 1944; Zavortink, 1979) and *Wyeomyia* (Judd, 1998; Motta & Lourenço-de-Oliveira, 2000). The generic and subgeneric names of aedine mosquitoes follow the more recent classification proposed by Wilkerson *et al.* (2015), available at Systematic Catalog of Culicidae, Walter Reed Biosystematics Unit (WRBU, 2017).

DNA isolation, PCR amplification and sequencing

Genomic DNA was extracted from one or two legs of each adult mosquito and from the abdominal segments III–V of each larva. The extraction was carried using a QIAcube instrument and the DNeasy Blood & Tissue Handbook protocol (Qiagen, CA, USA) following the manufacturers' instructions. An approximate 700 bp fragment of the *cox1* gene was amplified using universal primers LCO 1490F and HCO 2198R (Folmer *et al.*, 1994) and modified PCR conditions (Batovska *et al.*, 2016). A rDNA ITS2 region of

approximately 500 bp was amplified for selected specimens of each *cox1* haplotype using 5.8S F and 28S primers (Collins & Paskewitz, 1996) following the protocol below. The total PCR volume was 25 μL and consisted of 1X buffer, 1.5 mM $MgCl_2$, 1.25 mM dNTPs, 0.4 mM of each 10 μM primer, 0.08 U Taq DNA Polymerase and 4 μL template DNA. The final volume was adjusted with 12.6 μL of ddH_2O. Cycling conditions were 94°C for 3 min, 36 cycles of 94°C for 1 min, 46°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 15 min. Each PCR reaction was carried out using the Qiagen *Taq* DNA polymerase kit (Qiagen, CA, USA) and performed on a Bio-RAD C1000 touch™ thermal cycler (Bio-Rad laboratories, Inc., Hercules, CA, USA). PCR products were visualized in 1.0% agarose gels stained with GelRed™ nucleic acid gel stain (Phenix Research Products, NC, USA). PCR products were purified with ExoSAP-IT (USB Corporation, Ohio, USA) and Sanger sequencing was performed at the Applied Genomic Technologies Core (Wadsworth Center, New York State Department of Health, NY, USA) on an ABI PRISM 3700 genetic analyzer (Life Technologies, Thermo Fisher Scientific, MA, USA).

Sequence editing and analysis

All sequences were edited using Geneious Pro 9.1.4 (Kearse *et al.*, 2012). For *cox1* sequences, a scheme to rule out co-amplifications of nuclear mitochondrial DNA (NUMTs) was implemented (Song *et al.*, 2008; Buhay, 2009; Hlaing *et al.*, 2009). ITS2 sequences were checked for insertions and deletions. Each individual sequence of ITS2 was annotated following parameters available in the ITS2 database (Keller *et al.*, 2009). All consensus sequences were aligned with the Muscle algorithm (Edgar, 2004) plugin in Geneious (Biomatters). The identity of each individual *cox1* or ITS2 sequence was estimated using MegaBLAST (Ashfaq *et al.*, 2014). In cases where the match was 98% sequence similarity, the species name for the individual sequence was allocated (Ratnasingham & Hebert, 2007). The *cox1* and ITS2 sequences obtained were submitted to GenBank (KY117238–KY117452).

The number of haplotypes was estimated for each *cox1* and ITS2 dataset separately using DnaSP v. 5.0 (Rozas *et al.*, 2003). Neighbour Joining (NJ) and Bayesian Inference (BI) analyses were performed for the *cox1*, ITS2 and *cox1* + ITS2 datasets. The best-fit model of DNA substitution was determined by jModeltest 2.1.10 (Darriba *et al.*, 2012). The NJ analyses were performed using the Kimura-2-parameter (K2P) recommended for species-level in barcoding analysis (Hebert *et al.*, 2003) with 10,000 bootstrap replicates. The BI analysis was implemented in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001), and the Markov Chain Monte Carlo algorithm was allowed to run four chains for at least 5,000,000 generations, sampling every 1,000 generations, with a burn-in of 25%. *Chironomus kiiensis* (Diptera: Chironomidae) and *Nephrotoma altissima* (Diptera: Tipulidae) were used as “nearest neighbour” taxa and “outgroup”, respectively, to root the trees (GenBank accessions KJ424336 and KR439445), which were selected according to molecular phylogenetic analysis (Friedrich & Tautz, 1997). Genetic distances were calculated using the K2P model in MEGA v. 6 (Tamura *et al.*, 2011).

MOTU determination

Three similarity-based analyses for generating molecular clusters (MOTUs) were compared: Barcode Index Number (BIN) (Ratnasingham & Hebert, 2013), Automated Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012) and the Java program jMOTU (Jones *et al.*, 2011). The Refined Single Linkage (RESL) algorithm adopted in the Barcode of Life Database (BOLD) provided Barcode Index Number (BIN) assignments for each *cox1* sequence (Ratnasingham & Hebert, 2013). This analysis using standard BIN assignments is available at BOLD v3.6 (http://www.boldsystems.org). The ABGD method was implemented through the ABGD source available on the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/) to obtain the partitioning of the *cox1* sequence datasets into clusters (Puillandre *et al.*, 2012). In the jMOTU program a 2.5% cutoff was used and a Basic Local Alignment Search Tool (BLAST) identity filter of 95 was employed. Clusters were arranged using the number of variable nucleotides, equivalent to *p*-distance (Jones *et al.*, 2011).

To test whether the MOTUs derived from all analyses represent putative species, the species delimitation plugin for Geneious (Masters *et al.*, 2011) was used to calculate *Rosenberg's* P_{AB} value, which tests for taxonomic distinctiveness based on the null hypothesis that the observed monophyly was found by chance alone. *Rosenberg's* P_{AB} calculates the probability that a MOTU with "A" haplotype is monophyletic to its closest relative with "B" haplotype (Rosenberg, 2007). In addition, sequences from GenBank corresponding to species of a genus previously reported at high altitude in Latin American countries, or in this study based on the morphological assignment, were used to establish the hypothetical position of each MOTU (Ng'endo *et al.*, 2013). Finally, a MOTU was considered valid within a genus if the following requirements were met: (i) concordance among monophyletic groups found in ABGD, jMOTU, NJ and BI; (ii) >2% K2P distance among MOTUs; and (iii) significant ($p<0.05$) *Rosenberg's* P_{AB} values. Concordance of the results of the molecular analysis was evaluated in a matrix constructed in Microsoft® Excel 2013 version. For *cox1* sequences with a match success in BOLD or GenBank above 98%, the species name was allocated.

Results

Morphological identification of genera and subgenera

Four genera and two subgenera were identified in the altitudinal range of 2,009–3,098 m using morphology-based keys (Table 1): *Anopheles* ($n = 10$), *Culex* ($n = 25$), *Aedes* (*Howardina*) ($n = 62$), *Aedes* (*Ochlerotatus*) ($n = 19$), *Trichoprosopon* ($n = 6$) and *Wyeomyia* ($n = 8$). Seven specimens damaged during field sampling were morphologically assigned as *Howardina/Ochlerotatus* ($n = 6$) and *Trichoprosopon*? ($n = 1$). These specimens were also processed to evaluate the correlation between morphological identification and their identity based on their MOTU grouping or by comparison with available sequences.

Sequence analyses

After sequence editing, a 640 bp 5′ *cox1* barcode fragment was obtained for 137 specimens and 117 haplotypes were generated. No indels or stop codons were observed in the barcode sequences, ensuring that they did not constitute NUMTs. ITS2 sequences were obtained for

78 representative specimens from each group defined by the *cox1* NJ dendrogram (Table 2). The ITS2 analysis revealed substantial length variation in ITS2 among representatives of the six groups: *Anopheles* 527 bp, *Culex* 523 bp, *Ae*. (*Howardina*) 379 bp, *Ae*. (*Ochlerotatus*) 422 bp, *Trichoprosopon* 359 bp and *Wyeomyia* 409 bp. Comparison of the sequences with those in GenBank revealed close matches (>98% nucleotide identity) for only three of the 137 *cox1* sequences: two *cox1* sequences of larvae collected from the leaf based of a species of *Xanthosoma* at 2,009 m in Jericó were 98% similar to *Trichoprosopon evansae* (Antunes) (GenBank accession KM593039) collected in Rio Sucio, Caldas, Colombia at 1,960 m (Rozo-Lopez & Mengual, 2015). The other *cox1* sequence corresponding to an adult female reared in the laboratory from a larva collected from a bromeliad at 2,862 m in Belmira was 99% similar to *Culex* (*Microculex*) *imitator* (Theobald) (GenBank accession GU291979) from Espirito Santo, Santa Teresa, Brazil (Demari-Silva *et al*., 2011). The ITS2 results did not reveal any close GenBank sequence matches.

MOTU determination based on DNA barcodes. Barcode Index Number (BIN)

The online classification of each sequence individually was treated as “unknown specimen” in the BOLD database (Fig. 2). Remarkably, 132 *cox1* sequences did not match any records in BOLD, and BINs were assigned only for five *cox1* sequences (Table 2). One *cox1* sequence of an adult female from Jardín collected at 2,403 m (Shannon trap) and one larvae collected from a bromeliad at 3,000 m in Belmira were 97.3% similar to *Ae*. (*Ochlerotatus*) *euiris* (Dyar), previously recorded in Colombia by Rozo-Lopez & Mengual (2015). However, the *Ae. euiris* sequences grouped with *Aedes* (*Howardina*) *quadrivittatus* (Coquillett) from Hidalgo, Mexico and *Aede*s (*Howardina*) *sexlineatus* (Theobald) from Magdalena, Colombia (Fig. 2A). A detailed morphological review of our specimens resulted in verification of members of the subgenus *Howardina*. Three *cox1* sequences of *Anopheles* females collected at 2,403 m (Shannon trap) in Jardín municipality were 98.1% similar to *Anopheles* MBI-06 (Fig. 2B) collected in Merida, Venezuela, which were in turn part of a group called ″Boliviensis″: *Anopheles boliviensis* (Theobald) and *Anopheles rollai* (Cova García, Pulido F. & Escalante de Ugueto).

Automated Barcode Gap Discovery (ABGD) and jMOTU

The species delimitation analysis based on 117 *cox1* haplotypes using ABGD produced 23 groups with 21 single sequences, and with jMOTU resulted in 11 MOTUs and 102 single sequences (Table 2). The single sequences were not considered to be MOTUs and additional sampling is desirable at the locations in Jardín and Belmira, along with complementary analyses for MOTU detection. Both ABGD and jMOTU methods were congruent for two MOTUs within each of the genera *Anopheles* and *Culex* and *Ae*. (*Howardina*), and for one MOTU within the genus *Trichoprosopon*. For *Ae*. (*Ochlerotatus*) and the genus *Wyeomyia*, no MOTUs were congruent between ABGD and jMOTU analyses. The results revealed a striking difference in performance between methods for MOTU determination. The difference between groups was only evident for members of the genus *Anopheles* where the ABGD and jMOTU results were congruent (Table 2).

Neighbour Joining (NJ) and Bayesian Inference (BI)

NJ analyses detected 117 *cox1* haplotypes, resulting in 25 groups that were supported by bootstrap values ranging from 56–100% (Table 2; Fig. 3). Interestingly, the four genera and two subgenera based on morphological identification were also represented in the molecular analysis as six different groups with moderate to high bootstrap values: *Ae*. (*Howardina*) (74%), *Ae*. (*Ochlerotatus*) (98%), *Culex* (83%), *Wyeomyia* (65%), *Trichoprosopon* (99%) and *Anopheles* (100%) (Fig. 3). In the NJ analysis of ITS2 sequences, the specimens formed 18 groups with bootstrap values between 51–100% (data not shown). The groupings with ITS2 sequences confirmed the four genera and two subgenera previously detected by morphology and *cox1* data. In addition, results of the NJ dendrogram that included 78 concatenated sequences (*cox1* + ITS2), depicted 22 groups (Table 2) supported by bootstrap values between 56–100% (data not shown).

In the BI analysis for each of the *cox1* and ITS2 datasets (data not shown), the sequences of *Ae*. (*Ochlerotatus*) formed a clearly separate group; however, in the BI analysis of the *cox1* + ITS2 dataset only one group was detected that included both *Ae*. (*Howardina*) and *Ae*. (*Ochlerotatus*) (Fig. 4). In the multi-locus analysis (*cox1* + ITS2), 20 monophyletic groups were detected and supported by 0.71–1.00 posterior probability plus five single sequences (Fig. 4). BI analysis confirmed the groups supported by the NJ analysis within *Anopheles*, *Culex*, *Trichoprosopon* and *Wyeomyia* (Table 2).

Four specimens of *Howardina*/*Ochlerotatus* were assigned to *Ae*. (*Ochlerotatus*), and one specimen was grouped within *Ae*. (*Howardina*). The specimen morphologically identified as *Trichoprosopon*? was molecularly assigned to the genus *Trichoprosopon*, but the closest match to any species in GenBank was 87% or lower.

MOTUs within each genus

Rosenberg's P_{AB} values were significant for all 20 groups ($p<0.05$). Each of the 20 MOTUs detected was named according to its associated genus and the number of MOTUs within each, as follows: *Anopheles* (A1 and A2), *Culex* (C1), *Ae*. (*Howardina*) (H1–H9), *Ae*. (*Ochlerotatus*) (O1–O5), *Trichoprosopon* (T1) and *Wyeomyia* (W1 and W2). Localities, geographic coordinates, altitude, collecting method and collection year for each MOTU are detailed in Table 3. The overall mean nucleotide diversity for the barcode sequences was 0.117. The mean uncorrected *p*-distances between MOTUs were generally higher than the divergence within the MOTUs, except for subgenus *Howardina*. Within each MOTU, sequence divergences varied from 0.5 (H9) to 9.7% (C1), whereas differences between MOTUs ranged from 3.8 to 26.9% (Table 2).

Taxonomic assignment was performed for four MOTUs (A2, C1, H4 and T1). MOTU A2 was assigned to the "Boliviensis" group based on the BIN results. The only MOTU within *Culex* (C1) consisted of 19 *cox1* sequences from larvae collected in Jardín and one *cox1* sequence of an adult collected in Belmira; all shared the same haplotype and had a 99% nucleotide identity to *Cx. imitator*. For *Ae*. (*Howardina*), nine MOTUs were detected among the specimens collected in Jardín and Belmira between 2,100–3,098 m. However, only sequences of MOTU H4 ($n = 4$) were grouped with *Ae. quadrivittatus* according to BIN

results. Surprisingly, five MOTUs fell within *Ae*. (*Ochlerotatus*) (O1–O5), and two of these, O4 and O5, were from the same collection site in Belmira at 2,928 m. It is noteworthy that all haplotypes of the *Trichoprosopon* specimens were grouped together (T1) and were nearest to *Tr. evansae* based on nucleotide identity in MegaBLAST and by NJ grouping. Finally, two MOTUs were detected within the genus *Wyeomyia* from the three municipalities at different altitudes: 2,044 m in Jericó (W2), 2,253–2,403 m in Jardín (W1 and W2) and 2,927 m in Belmira (W1).

Discussion

The present study used a multi-locus approach (*cox1* + ITS2) and morphology to identify mosquitoes collected above 2,000 m in three municipalities of Antioquia Department, Colombia. Briefly, the MOTU approach for identification indicates that two or more sequences that are similar within a defined cut-off threshold are assigned to the same MOTU (Blaxter, 2004). In the present study, the *cox1* and ITS2 analyses were individually unsuccessful at resolving and identifying the 20 MOTUs detected in the concatenated analysis (*cox1* + ITS2) using BI. In agreement with other studies where this analysis provided a more robust estimate of monophyletic groups (Bourke *et al*., 2013; Conn *et al*., 2013), the multi-locus (*cox1* + ITS2) approach used here was also a useful tool for MOTU clustering of specimens that were previously identified to genus and subgenus based on morphology.

At least one MOTU within *Anopheles* (A2) is a member of the “Boliviensis” group, which consists of three species with high morphological similarity: *An. boliviensis*, *An. gonzalezrinconesi* (Cova García, Pulido F. & Escalante de Ugueto) and *An. rollai*. These species have been reported in sympatry in high Andean ecosystems associated with arboreal bromeliads at 2,232 m in the Parque Nacional Dinira in Venezuela (Prado, 2003; Navarro *et al*., 2007, 2010). In Colombia, third- and fourth-instar larvae of the “Boliviensis” group were previously found in arboreal bromeliads at 2,600 m in the Reserva forestal El Romeral (Suaza-Vasco *et al*., 2013a), and adult females were also collected by Shannon trap in Jardín at 2,400 m (Suaza-Vasco *et al*., 2013b), both localities in the Antioquia Department. The present study supports previous findings by investigators of the Grupo de Investigación en Sistemática Molecular-GSM in Jardín, Antioquia; complementary morphological and molecular observations are currently being performed (Suaza-Vasco *et al*., 2013a, 2013b).

Only one MOTU was detected among specimens of each of the genera *Culex* and *Trichoprosopon* (C1 and T1 respectively); therefore, it can be inferred that the collections included one species per genus. One *cox1* sequence belonging MOTU C1 was 99% similar to *Cx. imitator* of the subgenus *Microculex*, the species of which have a strong preference for choosing natural containers for oviposition, especially bromeliads (Müller & Marcondes, 2006; Ceretti-Junior *et al*., 2016). Therefore, we propose that *Culex* MOTU C1 may to correspond to *Cx. imitator*, a species whose females apparently do not feed on humans (WRBU, 2017). Immature stages of this species were also collected from axils of bromeliads in association with *Ae. quadrivittatus* in El Cielo Biosphere Reserve above 2,500 m in Tamaulipas State, Mexico (Ortega-Morales *et al*., 2015). One larva of *Ae*. (*Howardina*) MOTU H8 and one larva of *Culex* MOTU C1 shared the same bromeliad at 2,862 m in

Belmira; consequently, it is suggested that *Ae. quadrivittatus* is present in this municipality in association with *Cx. imitator*.

In the present study, and according to sequence analyses, specimens of *Trichoprosopon* MOTU T1 probably correspond to a lineage or species close to *Tr. evansae*. In Venezuela and Ecuador, immatures of *Tr. evansae* have only been reported inhabiting bamboo internodes and fallen leaves of palm but not in a host plant of Araceae (Navarro *et al.*, 2007). However, a previous study conducted in a coffee-growing region of Colombia reported larvae of *Trichoprosopon* sp. in a *Xanthosoma* leaf axil (Suaza-Vasco *et al.*, 2015). Moreover, at least three species of *Trichoprosopon* are present in the Jardin and Jericó municipalities, including two species of the Pallidiventer Complex, *Tr. evanse* and *Tr.* sp. B (Barajas *et al.*, 2013; Suaza-Vasco *et al.*, 2015). Therefore, we hypothesize that the two *cox1* sequences from *Trichoprosopon* adult females collected in Jardín at 2,253 m are *Tr. evansae*, a species previously reported in this municipality. Three *cox1* sequences from *Trichoprosopon* larvae within MOTU T1 and collected from the axils of a *Xanthosoma* sp. in Jericó at 2,009 m are possibly *Tr.* sp. B. Further studies are necessary to determine the precise species identity of specimens assigned to *Trichoprosopon* MOTU T1.

In general, the tribe Aedini, with 1,261 species, is the largest in the family Culicidae (Harbach, 2017). In Colombia, specimens of this tribe have not been well studied in high altitude zones, thus additional and/or undiscovered species may exist. In the present study, combined analysis of *cox1* + ITS2 failed to show significant groupings of *Ae.* (*Howardina*) and *Ae.* (*Ochlerotatus*) MOTUs, five *Ae.* (*Ochlerotatus*) MOTUs (O1–O5) grouped with four *Ae.* (*Howardina*) MOTUs (H6–H9) in a strongly supported monophyletic group. In future studies, we suggest the use of sequences of some specimens from lowlands to test the monophyly and status of these subgenera. This, taking into account that the monophyly of groups within the tribe Aedini is only partially supported, making it reasonable, at the moment, to preserve the groups as subgenera or informal groups (Wilkerson *et al.*, 2015). Moreover, we consider that the number of MOTUs that were found for *Ae.* (*Howardina*) and *Ae.* (*Ochlerotatus*) is high and additional studies including other taxonomic and ecological characters are necessary to corroborate or modify that number.

In summary, our results provide 20 MOTUs of mosquitoes present in high Andean mountain ecosystems in Antioquia, Colombia. However, these 20 MOTUs may not necessarily correspond to 20 known or described species, but can be treated as such for measuring diversity and testing hypotheses, as has been previously suggested (Ryberg 2015). Further studies are necessary to establish the relationships between MOTUs and putative species, with a deeper analysis including detailed morphological study of newly collected mosquitoes. Finally, the multi-locus approach will be useful for less-experienced researchers in morphological identification who are interested in the MOTU identification of mosquito specimens.

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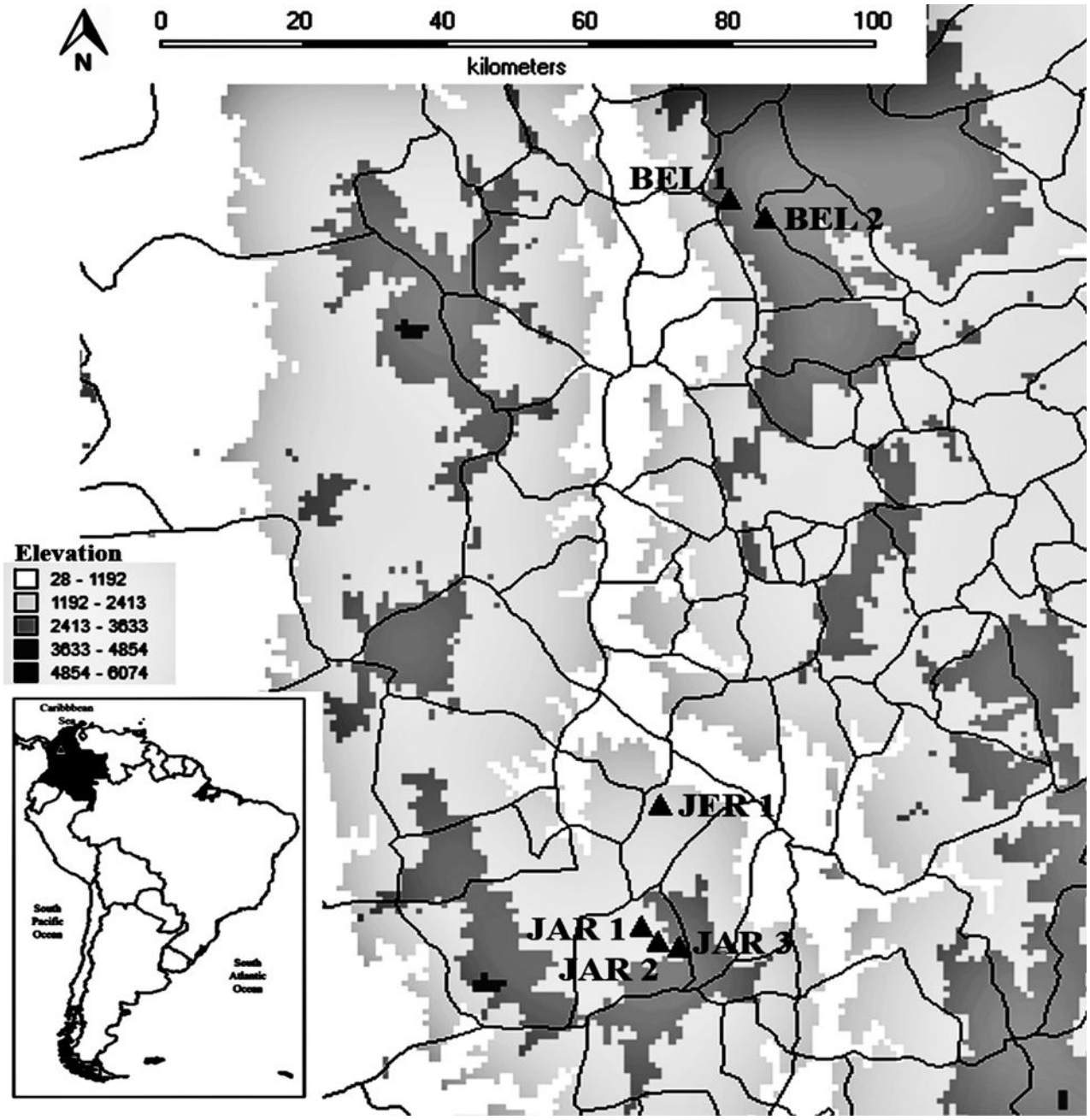

FIGURE 1.

Localities (BEL = Belmira, JAR = Jardín, JER = Jericó) for mosquitoes collected above 2,000 m.

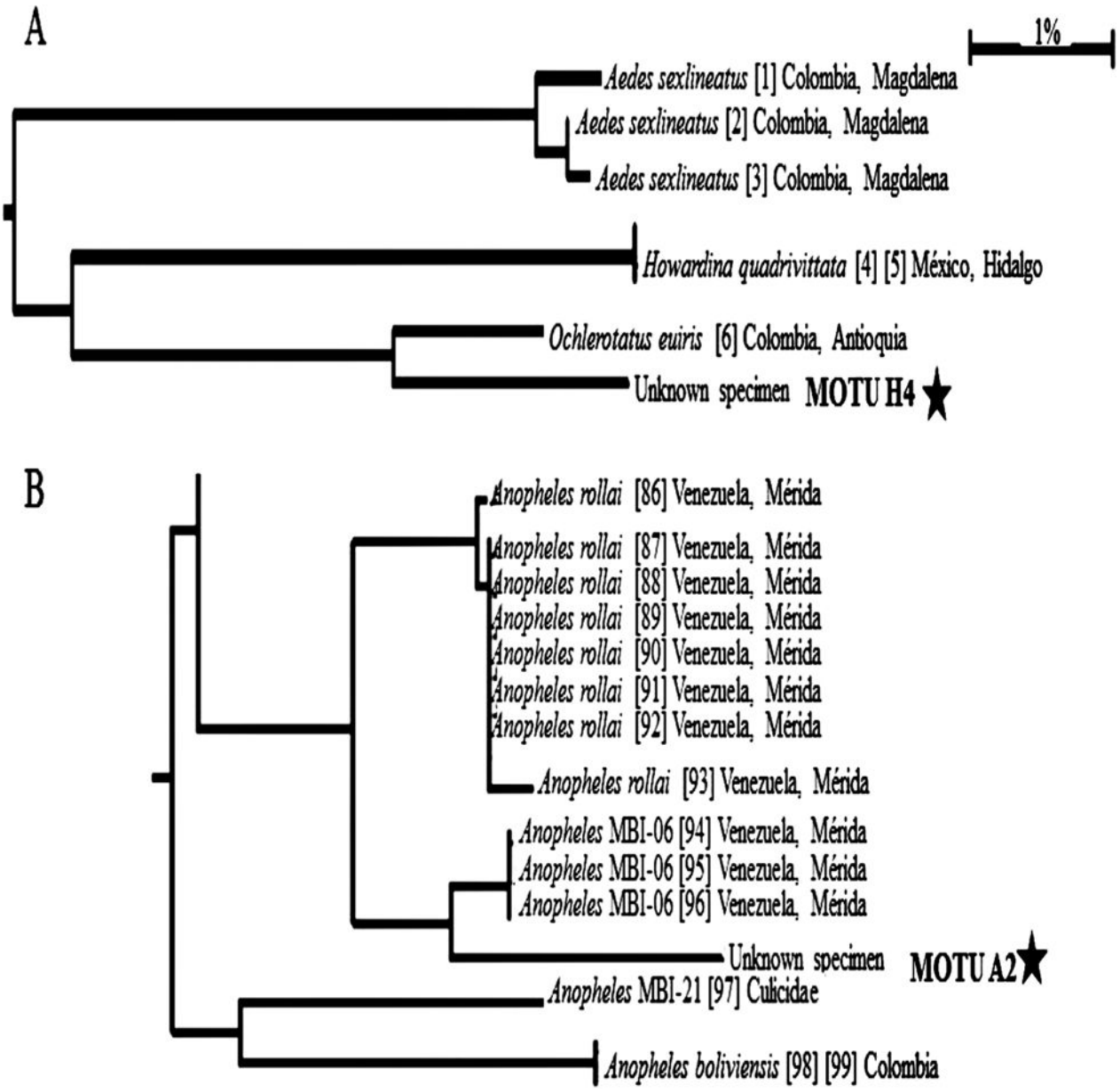

FIGURE 2.

Neighbour-Joining taxon identification dendograms based on *cox1* sequences. Original BOLD dendogram requested online for classification of an “unknown”. A. Sequences grouped with *Aedes sexlineatus* and *Aedes quadrivittatus*. B. Sequences grouped with *Anopheles* MBI-06 collected in Merida, Venezuela. There are no bootstrap support values shown on NJ tree because it is a BOLD systems tree.

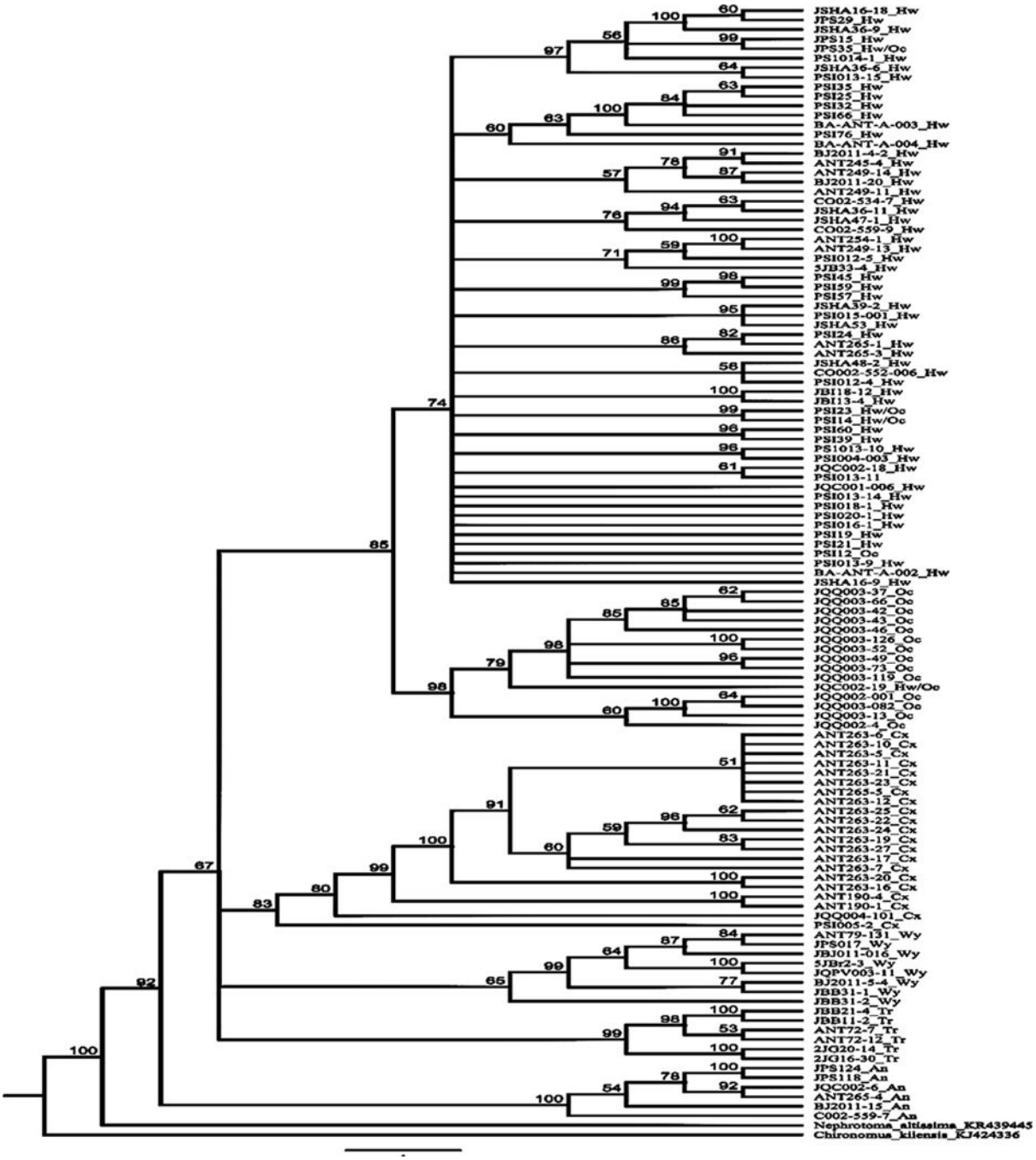

FIGURE 3.

Neighbour-Joining topology based on 117 *cox1* haplotypes of the mosquitoes collected above 2,000 m. Numbers on the branches represent bootstrap values (in percentages).

FIGURE 4.

Bayesian topology based on multi-locus (*cox1* + ITS2) for mosquitoes collected above 2,000 m. Numbers on the branches represent posterior probabilities and triangles denote the five single sequences.

TABLE 1

Generic identification based on morphology and number of specimens (sequences) of each genus.

Genus (subgenus)	*cox*1	ITS2	Locality	Geographic coordinates′″	Altitude (m)
Anopheles	7F, 1F*, 1L	7F, 1F*, 1L	Jardín	05° 36′ 49″ N, 75° 48′ 57″ W	2,100–2,403
Anopheles	1F	1F	Belmira	06° 38′ 45″ N, 75° 42′ 14″ W	2,977
Culex	23L, 1F*	1L, 1F*	Jardín	05° 36′ 49″ N, 75° 48′ 57″ W	2,481
Culex	1F*	1F*	Belmira	06° 37′ 08″ N, 75° 39′ 25″ W	2,862
Aedes (Howardina)	17F, 7F*, 7M*	5F, 6F*, 4M*	Jardín	05° 35′ 53″ N, 75° 47′ 37″ W	2,100–2,521
Aedes (Howardina)	18F, 5L, 5F*, 3M*	15F, 1L, 4F*, 1M*	Belmira	06° 38′ 50″ N, 75° 42′ 35″ W	2,734–3,098
Aedes (Ochlerotatus)	2F, 1L,10F*, 5M*	1F, 7F*, 4M*	Jardín	05° 35′ 02″ N, 75° 46′ 04′ W	2,100
Aedes (Ochlerotatus)	1F	1F	Belmira	06° 38′ 45″ N, 75° 42′ 14″W	2,928
Aedes (How./Och.)	2F	2F	Jardín	05° 36′ 49″ N, 75° 48′ 57″ W	2,100–2,928
Aedes (How./Och.)	4F	4F	Belmira	06° 38′ 45″ N, 75° 42′ 14″ W	2,928
Trichoprosopon	1F,1F*, 1M*	0	Jardín	05° 36′ 50″ N, 75° 48′ 59″ W	2,025–2,253
Trichoprosopon?	1F	1F	Jardín	05° 36′ 50″ N, 75° 48′ 59″ W	2,253
Trichoprosopon	3L	3L	Jericó	05° 47′ 18″ N, 75° 47′ 26″ W	2,009
Wyeomyia	6F	5F	Jardín	05° 36′ 50″ N, 75° 48′ 59″ W	2,253–2,403
Wyeomyia	1F	1F	Belmira	06° 38′ 45″ N, 75° 42′ 14″ W	2,977
Wyeomyia	1L	0	Jericó	05° 47′ 18″ N, 75° 47′ 26″ W	2,044

F: female, M: male, L: larvae,

*reared in the laboratory.

TABLE 2

Comparison of Neighbour-Joining (NJ) and Bayesian Inference (BI) methods for MOTU generic clustering of mosquitoes collected above 2,000 m in Antioquia, Colombia.

Genus	Method and region							Mean uncorrected p-distances	
	NJ		*cox1* + ITS2		Similarity-based analyses				
	cox1	ITS2	NJ	BI	BINs	ABGD	jMOTU	Intraspecific	Interspecific
Anopheles	1	2	2	2	3	2	2	3.0–8.3%	13.7%
Culex	2	1	1	1	–	5	3	9.7%	21.7%
Howardina	14	9	13	10	2	9	4	0.5–8.8%	3.9–11.8%
Ochlerotatus	4	2	3	4	–	4	1	2.1–3.0%	3.8–5.4%
Trichoprosopon	2	1	1	1	–	2	1	3.2%	26.9%
Wyeomyia	2	3	2	2	–	1	0	3.0–3.3%	8.3%
Total	25	18	22	20	5	23	11	–	–

Table 3

Data for mosquitoes collected above 2,000 m in three localities of Antioquia, Colombia.

MOTU	Localities	Geographic coordinates	Altitude (m)	Habitat (larvae) collection method (adults)	Year	Stage, sex of adults	GenBank accession	
							cox1	ITS2
A1	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Shannon trap	2014	4F	KY117254-KY117257	KY117380-KY117382, KY117386
A1	Jardín 1	05°36′49″N, 75°48′57″W	2,481	Bromeliad	2012	1L	KY117253	KY117387
A2	Jardín 1	05°36′49″N, 75°48′57″W	2,403	Shannon trap	2014	3F	KY117248-KY117250	KY117378, KY117379, KY117384
C1	Jardín 3	05°35′02″N, 75°46′04′W	2,100	*Xanthosoma*	2014	1F*	KY117283	KY117377
C1	Jardín 1	05°36′49″N, 75°48′57″W	2,481	Bromeliad	2012	18L	KY117261-KY117280	KY117376
C1	Belmira 1	06°37′08″N, 75°39′25″W	2,862	Bromeliad	2013	1F*	KY117281	KY117375
H1	Jardín 2	05°35′53″N, 75°47′37″W	2,376	Shannon trap	2012	2F	KY117316, KY117317	KY117426
H1	Belmira 2	06°38′45″N, 75°42′14″W	2,905	Aspirator	2013	1F	KY117309	KY117403
H1	Belmira 2	06°38′45″N, 75°42′14″W	3,084	Bromeliad	2013	1F*	KY117313	KY117399
H1	Belmira 2	06°38′45″N, 75°42′14″W	3,098	Aspirator	2013	1F	KY117308	KY117402
H1	Belmira 2	06°38′45″N, 75°42′14″W	3,098	Bromeliad	2013	1F*, 1M*	KY117315, KY117310	KY117400
H2	Jardín 1	05°36′49″N, 75°48′57″W	2,245	Bromeliad	2012	2F*, 1M*	KY117303 -KY117305	KY117401, KY117420
H2	Jardín 2	05°35′53″N, 75°47′37″W	2,376	Shannon trap	2012	3F	KY117336, KY117359, KY117323	KY117423, KY117420
H3	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Shannon trap	2014	1F	KY117306	KY117430
H3	Jardín 2	05°35′53″N, 75°47′37″W	2,376	Shannon trap	2012	5F	KY117353-KY117357	KY117428, KY117421
H3	Jardín 1	05°36′49″N, 75°48′57″W	2,403	Shannon trap	2011	3F	KY117361, KY117362, KY117352	KY117421
H3	Belmira 2	06°38′45″N, 75°42′14″W	3,000	Bromeliad	2013	2L	KY117358, KY117314	KY117437
H4	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Aspirator	2014	1F	KY117367	KY117424
H4	Jardín 1	05°36′49″N, 75°48′57″W	2,492	Bromeliad	2012	1F*	KY117318	KY117398
H5	Jardín 1	05°36′49″N, 75°48′57″W	2,427	Bromeliad	2009	1M*	KY117365	KY117434
H5	Belmira 1	06°37′08″N, 75°39′25″W	2,734	Bromeliad	2013	1L	KY117366	KY117450
H6	Jardín 1	05°36′49″N, 75°48′57″W	2,481	Bromeliad	2012	1M*	KY117325	KY117431
H6	Jardín 1	05°36′49″N, 75°48′57″W	2,492	Bromeliad	2012	1F*	KY117327	KY117451
H6	Belmira 2	06°38′45″N, 75°42′14″W	2,977	Insect net	2011	2F	KY117326, KY117328	KY117452, KY117425
H7	Jardín 2	05°35′53″N, 75°47′37″W	2,376	Shannon trap	2012	2F	KY117320, KY117333	KY117432

MOTU	Localities	Geographic coordinates	Altitude (m)	Habitat (larvae) collection method (adults)	Year	Stage, sex of adults	GenBank accession	
							cox1	ITS2
H7	Jardín 1	05°36′49″N, 75°48′57″W	2,521	Bromeliad	2013	2F*, 1M*	KY117321, KY117332 KY117319	KY117433
H7	Belmira 1	06°37′08″N, 75°39′25″W	2,734	Bromeliad	2013	2L, 2F*, 2M*	KY117331, KY117329 KY117307, KY117312 KY117324, KY117360	KY117449, KY117448
H7	Belmira 1	06°37′08″N, 75°39′25″W	2,862	Bromeliad	2013	2F*	KY117330	KY117427
H8	Belmira 2	06°38′45″N, 75°42′14″W	2,977	Insect net	2011	1F	KY117341	KY117446
H8	Belmira 2	06°38′45″N, 75°42′14″W	3,000	Aspirator	2013	2F	KY117342, KY117344	KY117438
H8	Belmira 2	06°38′45″N, 75°42′14″W	3,098	Aspirator	2013	2F	KY117343, KY117335	KY117436
H9	Jardín 1	05°36′49″N, 75°48′57″W	2,481	Bromeliad	2012	2M	KY117337, KY117338	KY117429
H9	Belmira 2	06°38′45″N, 75°42′14″W	2,905	Aspirator	2013	2F	KY117347, KY117350	KY117441, KY117435
H9	Belmira 2	06°38′45″N, 75°42′14″W	2,977	Insect net	2011	F	KY117345, KY117351, KY117334	KY117447, KY117442
H9	Belmira 2	06°38′45″N, 75°42′14″W	3,000	Aspirator	2013	3F	KY117339 KY117348 KY117346	KY117419 KY117443 KY117440
H9	Belmira 2	06°38′45″N, 75°42′14″W	3,098	Aspirator	2013	1F	KY117349	KY117444
O1	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Aspirator	2014	2F	KY117258, KY117284	KY117404
O1	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Rock hole	2014	1L, 7F*, 2M*	KY117259, KY117292-KY117299, KY117260	KY117410, KY117411 KY117413, KY117414
O2	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Rock hole	2014	2F*, 1M*	KY117290, KY117288, KY117291	KY117405, KY117406 KY117415
O3	Jardín 3	05°35′02″N, 75°46′04′W	2,100	Rock hole	2014	3M	KY117285, KY117289, KY117286	KY117409, KY117412
O4, O5	Belmira 2	06°38′45″N, 75°42′14″W	2,928	Aspirator	2013	5F	KY117300-KY117302, KY117311, KY117340	KY117416, KY117445, KY117439, KY117422
T1	Jardín 1	05°36′49″N, 75°48′57″W	2,253	Insect net	2012	2F	KY117243, KY117244	KY1173 97
T1	Jericó 1	05°47′18″N, 75°47′26″W	2,009	*Xamthosoma*	2011	3L	KY117240-KY117242	KY117394-KY117396
W1	Jardín 1	05°36′49″N, 75°48′57″W	2,253	Aspirator	2012	2F	KY117371, KY117282	KY1173 90, KY117392
W1	Jardín 1	05°36′49″N, 75°48′57″W	2,253	Insect net	2012	1F	KY117372	KY117392
W1	Jardín 1	05°36′49″N, 75°48′57″W	2,403	Shannon trap	2011	1F	KY117374	KY117393
W1	Belmira 2	06°38′45″N, 75°42′14″W	2,977	Insect net	2011	1F	KY117370	KY117391
W2	Jardín 1	05°36′49″N, 75°48′57″W	2,403	Aspirator	2014	1F	KY117369	KY117388
W2	Jardín 2	05°35′53″N, 75°47′37″W	2,317	Bromeliad	2009	1F	KY117366	KY117450

MOTU	Localities	Geographic coordinates	Altitude (m)	Habitat (larvae) collection method (adults)	Year	Stage, sex of adults	GenBank accession	
							cox1	ITS2
W2	Jericó 1	05°47′18″N, 75°47′26″W	2,044	Bromeliad	2011	1L	KY117368	KY117389

F: female, M: male, L: larvae,

* reared in the laboratory.