Molecular characterization of mosquitoes (Diptera: Culicidae) from the Colombian rainforest

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ABSTRACT

A few studies have carried out the taxonomic and molecular characterization of sylvatic mosquito species in Latin America, where some species have been incriminated as vectors for arboviruses and parasites transmission. The present study reports the molecular characterization of mosquito species in the Sierra Nevada de Santa Marta, a natural ecosystem in the Northern coast of Colombia. Manual capture methods were used to collect mosquitoes, and the specimens were identified via classical taxonomy. The *COI* marker was used for species confirmation, and phylogenetic analysis was performed using the neighbor-joining method, with the Kimura-2-Parameters model. *Aedes serratus*, *Psorophora ferox*, *Johnbelkinia ulopus*, *Sabethes chloropterus*, *Sabethes cyaneus*, *Wyeomyia aporonoma*, *Wyeomyia pseudopecten*, *Wyeomyia ulocoma* and *Wyeomyia luteoventralis* were identified. We assessed the genetic variability of mosquitoes in this area and phylogenetic reconstructions allowed the identification at the species level. Classical and molecular taxonomy demonstrated to be useful and complementary when morphological characteristics are not well preserved, or the taxonomic group is not represented in public molecular databases.

KEYWORDS: Culicidae. DNA barcoding. COI, morphology. Taxonomy.

INTRODUCTION

The family *Culicidae* comprises 3,600 species, which are classified in the subfamilies Anophelinae and Culicinae. Anophelinae comprises the genera *Anopheles, Bironella,* and *Chagasia*, while the subfamily Culicinae includes the tribes Aedeomyiini, Aedini, Culicini, Culisetini, Ficalbiini, Hoggesiini, Mansoniini, Orthopodomyiini, Sabethini, Toxorhynchitini, and Uranotaeniini, comprising around 110 genera[1]. Different viruses have been associated with diseases, such as *Dengue virus* (DENV) that produces over 40,000 deaths every year[2] among others. The relationship between different taxonomic groups of viruses and mosquitoes is ancestral, with a recent description of a huge diversity of insect-specific viruses that may constitute the source for the future, leading to emerging viral diseases through species jumping from enzootic to epizootic and epidemic cycles[3].

Classical taxonomy has allowed us to obtain information about mosquito species through the definition of morphological characteristics used in dichotomic keys, leading to a clustering that may not always be monophyletic or similar in their distribution in ecosystems[4]. However, as the knowledge of biological species has become more detailed, the International Commission on Zoological Nomenclature (ICZN) explains what names in species are correct in a family, genus and at the

species level and uses a classification of specimens such as type series, name-bearing types and other specimens[5]. The presence of cryptic species cannot be separated by morphological characteristics[6] which also contribute to the problem. Molecular systematics is a complementary strategy to determine the evolutionary relationship between species that have been difficult to determine via morphological characteristics , development states, and sexual dimorphism[7]. DNA barcoding was established in 1993 as a strategy to unify the use of molecular markers for species identification and taxonomic allocation through phylogenetic inference based on genetic variability[8]. The cytochrome oxidase c subunit I (COI) gene has been widely used for molecular identification and, together with classical taxonomy, is a powerful tool for mosquito species demarcation[9].

In Colombia, some studies have used DNA barcoding through COI, internal transcribed spacer 2 (ITS2), and 16S subunit genes, which have allowed to characterize the phylogenetic relationship of several species belonging to the genera *Aedes*, *Anopheles*, and *Culex*[10-12]. Hematophagous mosquitoes involved in arbovirus transmission in the Sierra Nevada de Santa Marta (SNSM) have not been extensively studied[13], and there are no reports that include molecular taxonomy. In the present study, we investigated the diversity of rainforest mosquito species in a unique ecosystem in Colombia using DNA barcoding and classical taxonomy. The accurate identification of mosquito species is essential to determine the real and potential risk of arboviruses or parasites transmission and to implement vector surveillance and control programs.

MATERIALS AND METHODS

Study area

Mosquito specimens were collected following the technical and ethical approval (CEMIN-6-2017 from the Instituto Nacional de Salud, Bogota D.C., Colombia), in the SNSM foothills near Guachaca village, corresponding to the sylvatic area of Quebrada Valencia - La Piedra (11°14'22.6" N, 73°47'58.3" W) at an altitude of 80 meters above sea level (Figure 1). The region is characterized by bimodal type rains due to the variation in precipitation, which decreases in the Southeastern slope and increases in the Northern slope, leading to the formation of a hydrographic system. Temperatures during the year range from 28 °C to less than 0 °C in the highest altitude areas, and there is a distribution of eight biomes along the SNSM depending on altitude, climate, geographical and physicochemical conditions[14,15].

Mosquito collections and identification

Mosquitoes were collected during two field expeditions
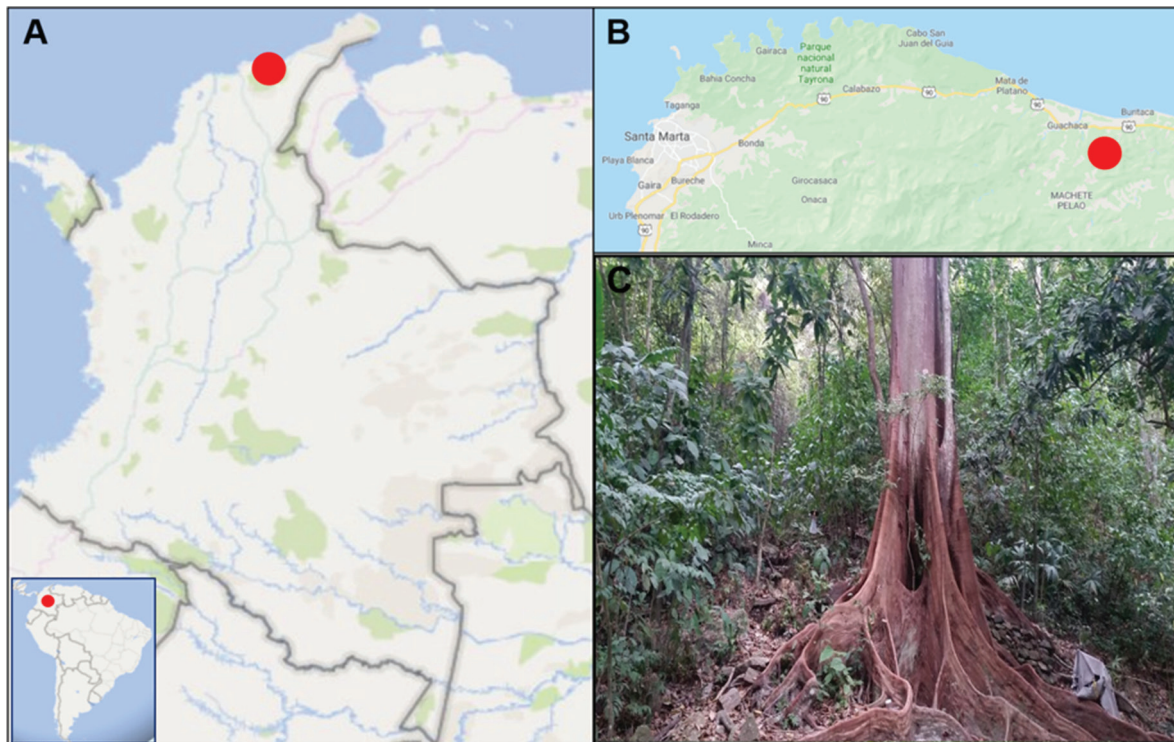
Figure 1 - Rainforest in Sierra Nevada de Santa Marta, Colombia: (A) Location of the SNSM rainforest area, (B) Guachaca locality, and (C) Quebrada Valencia-La Piedra forest.

during two days per month, between August to December 2018, the season with the highest rainfall incidence. Linear transects of 2 km were delimited inside the sampling area. The collection methods used were manual capture by entomological nets and aspirators, between 07:00-12:00 a.m. hours and 02:00-04:00 p.m. hours. Collected mosquitoes were transported in containers to the entomology laboratory at the Tropic Health Research Center (CIST) and sacrificed with ethyl ether. Subsequently, mosquitoes were identified using dichotomic keys[16-18]. The code structure CIST#### was used to register and deposit specimens into the entomological collection. Finally, the specimens were stored in vials with 1 mL of 96% ethanol for subsequent molecular characterization of female mosquitoes.

Molecular analysis

DNA extraction

Mosquito legs were removed using sterile tweezers. Homogenization was performed with nuclease-free zirconium beads, and DNA extraction was carried out using the DNeasy Blood & Tissue kit (QIAGEN Inc., Hilden, Germany). following the manufacturer's instructions.

COI gene amplification

The standard 658 base pairs (bp) barcode region of the mitochondrial *COI* gene was amplified using the primers LCO1490 and HCO2198[19]. The reaction mixture included 5 µL of extracted DNA, 0.4 µM of each primer, 1.25 U Go*Taq* DNA Polymerase (Promega), 0.2 mM of each dNTP, 1 X buffer with 1.5 mM MgCl$_2$, and nuclease free water for a final volume of 25 µL. The thermal profile consisted of an initial denaturation step at 94 °C for 10 min; followed by 35 cycles at 95 °C for 60 s for denaturation, 50 °C of 60 s for annealing, and 72 °C of 60 s for extension; and then a final extension at 72 °C of 5 min. A 5-µL aliquot of each PCR product was used to visualize the expected amplicon through an agarose gel electrophoresis. The quantification of amplicons was performed by using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a range between 26 to 431.5 ng/µL, which were subsequently purified by using the ExoSAP-IT™ PCR Product Cleanup Reagent enzyme (Thermo Fisher Scientific Inc. Waltham, MA, USA).

DNA Sequencing

The purified PCR products were sequenced via Sanger sequencing by Macrogen Inc, South Korea. Consensus sequences were obtained by assembling contings in Geneious Prime (version 2019.1, Biomatters, Inc., San Diego, CA, USA). The sequences were compared with those deposited in GenBank[20] database. A matrix with nucleotide sequences representative of the different genera and species included in the present study were subsequently created and aligned using the ClustalW tool, implemented in the MEGA software[21].

Phylogenetic analysis

The sequences did not show insertions/deletions (indels); therefore, no gap treatment was performed. The phylogenetic inference was made using the Neighbor-Joining method with the Kimura 2 parameter models (K2P) implemented in the MEGA software[21]. To assess the support of the phylogenetic tree topology, a resampling corresponding to 1,000 bootstrap replicates was performed. The consensus tree was visualized and edited in the MEGA software[21].

RESULTS

During our study, we collected and taxonomically identified 123 mosquitoes. The following genera were identified: *Aedes* (n = 2), *Anopheles* (n = 4), *Johnbelikinia* (n = 72), *Psorophora* (n = 25), *Sabethes* (n = 4), *Trichoprosopon* (n = 1), and *Wyeomyia* (n = 15). Seven species were identified via a combination of classical taxonomy and molecular analysis: *Aedes (Ochlerotatus) serratus* (Theobald, 1901), *Jonhbelkinia ulopus* (Dyar & Knab, 1906), *Psorophora (Janthinosoma) ferox* (Von Humboldt, 1819), *Sabethes (Sabethes) cyaneus* (Fabricius, 1805), *Wyeomyia (Triamyia) aporonoma* (Dyar & Knab, 1906), *Wyeomyia (Decamyia) pseudopecten*, and *Wyeomyia (Decamyia) ulocoma* (Theoblad, 1903). Two additional species, *Sabethes (Sabethoides) chloropterus* (von Humboldt, 1819) and *Wyeomyia (Dendromyia) luteoventralis* (Theobald, 1901) were identified through classical and molecular taxonomy, respectively. It was not possible to identify another morphotype belonging to the genus *Sabethes*, because of the poor preservation of some morphological structures preventing us from using taxonomic keys and the nucleotide sequence of COI that were distantly related to any other previously deposited sequence in publicly available databases. All the species reported in the present study have been previously recorded in some areas of Colombia (Supplementary Table S1).

Seventeen sequences of the *COI* gene (658 bp) were obtained and deposited in GenBank database corresponding to the mosquitoes in this study (Table 1). Eight species were confirmed through the GenBank database, with similarities varying from 96.19% to 99.54%. Only *Ae. serratus* and *Ps. ferox* had more than 15 sequences in open-access databases, and *Jo. ulopus*, *Sa. cyaneus*, *Wy. aporonoma*,

Table 1 - Molecular identification of species via database searching and availability of COI sequences[*].

Species	Obtained Sequences	Related Sequence from GenBank	Similarity (%) GenBank
Ae. serratus	MT418595	MF172270	99.06
Ps. ferox	MT418592	MG242536	99.06
	MT418593	MN997516	99.54
	MT418594	MN997519	99.39
Jo. ulopus	MT418581	MF172329	96.30
	MT418582	MF172329	96.35
	MT418583	MF172329	96.35
	MT418584	MF172329	96.35
	MT418585	MF172329	96.19
Sa. cyaneus	MT418579	GU908121	97.59
Sabethes sp.	MT418588	NC_044660	88.96
Wy. aporonoma	MT418589	MF172423	98.60
Wy. pseudopecten	MT418590	MF172493	99.53
	MT418591	MF172493	98.63
Wy. ulocoma	MT418586	KF671038	96.28
	MT418587	KF671038	96.35
Wy. luteoventralis	MT418580	MF172452	97.35

*Data accessed in October 2019.

Wy. pseudopecten, and *Wy. ulocoma* species had less than 15 accessible sequences. The *Sabethes* species was not confirmed through the database, and its identification was ambiguous; using GenBank, an identity value < 88.43% was observed when compared to *Sa.* (*Peytonulus*) *hadrognatus* (Harbach, 1995) and *Wy.* (*Dendromyia*) *ypsipola* (Dyar, 1922).

In a first phylogenetic reconstruction using a partial COI sequence (353 bp) for all the major taxa of interest, the tribes Aedini and Sabethini were identified as monophyletic clusters (data not shown). Taxonomic identification at the species level was confirmed for *Ae. serratus*, *Ps. ferox*, *Jo. ulopus*, *Sa. cyaneus*, *Wy. aporonoma*, *Wy. pseudopecten*, *Wy. ulocoma*, and *Wy. luteoventralis*. However, there was not clear clusters for the genera *Johnbelkinia*, *Wyeomyia*, *Sabethes*, *Trichoprosopon* and *Limatus*. Also, the species *Ae. aegypti* was clustered with species of the genus *Culex* such as: *Cx. corniger* (KP281757), *Cx. erythrotorax* (HM593011), *Cx. nigripalpus* (KP281764), and others.

Due to these inconsistencies, a more detailed phylogenetic reconstruction was performed independently for the Aedini (Figure 2) and Sabethini (Figure 3) tribes using more informative datasets of 530 and 447 bp, respectively. Within the Sabethini tribe, monophyletic clusters representing the different genera were not identified. The mosquito sample CIST0314 was identified through classical morphology as a member of the genus *Sabethes*. In the phylogenetic tree, the

corresponding sequence with accession number MT418588 was more closely related to the genera *Johnbelikinia* and *Wyeomyia*.

The sequence (KM593040) of *Wyeomyia* (*Dendromyia*) *luteoventralis* (Theobald, 1901) previously reported in Colombia, fell into the *Wy. pseudopecten* cluster in the current study (Figure 3). Also, the species *Ae. euiris* (MK592988) fell into the genus *Haemagogus* in the present analysis (Figure 2). The intra-species genetic variation was 0,007 for *Wy. aporonoma* and higher 0,0211 to 0,0511 for the other species in this study (Table 2). *Sabethes* sp. was not related to species previously reported in databases.

DISCUSSION

The present study allowed us to address mosquito diversity in the SNSM ecosystem and to describe the presence of potential arboviral vectors in this area of the country. This study is the first to report the identification of *Ae. serratus*, *Jo. ulopus*, *Wy. aporonoma*, *Wy. pseudopecten*, and *Wy. ulocoma* in the SNSM rainforest at the molecular and morphologic levels. The accurate identification of vector species is an essential factor in the study of arboviral diseases, allowing the local health authorities to target resources for vector control strategies[22].

In this study, we achieved the identification of seven species that are mainly associated with rural environments.
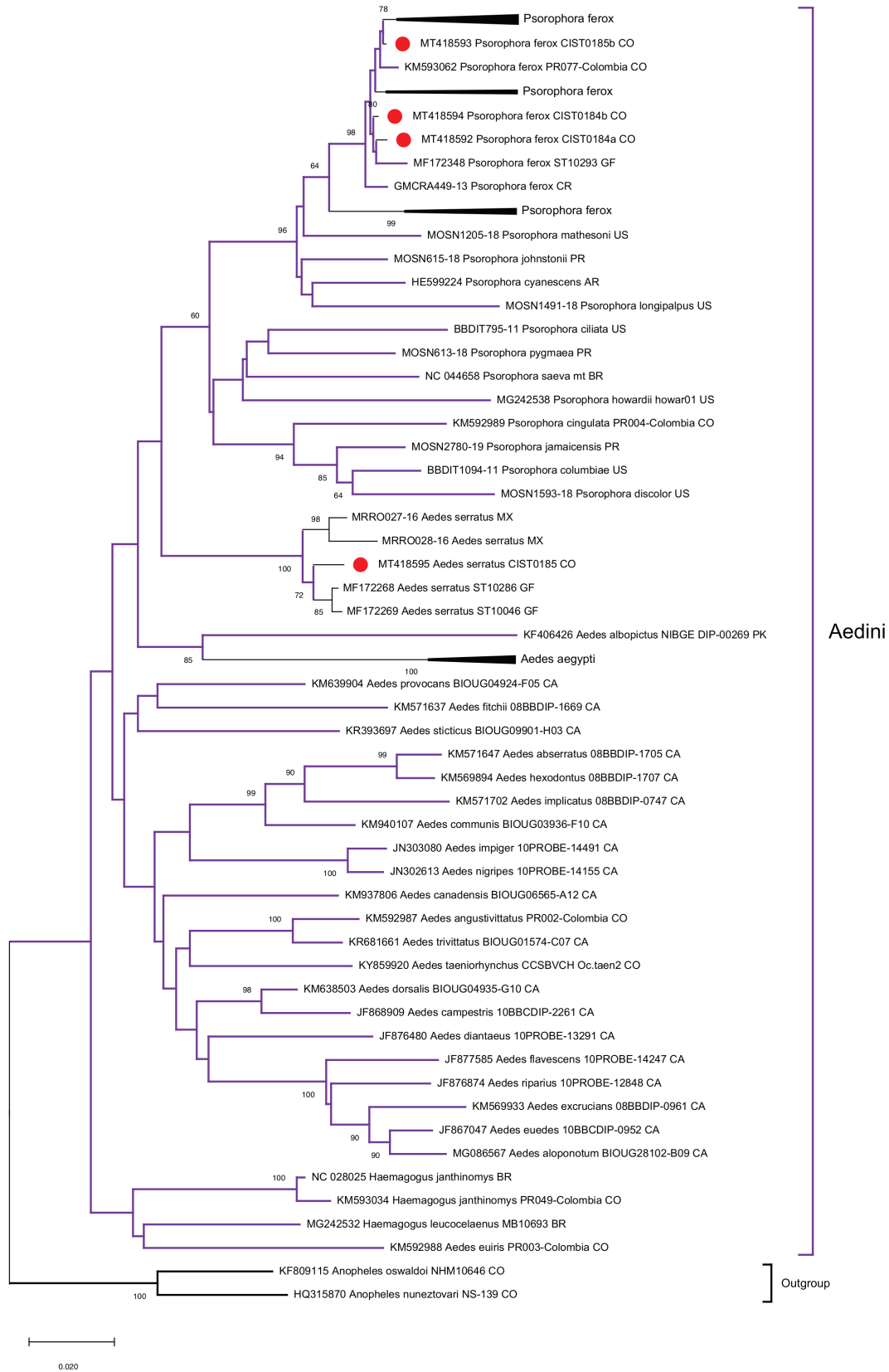
Figure 2 - Phylogenetic reconstruction of the Aedini tribe using the COI gene (530 bp). The Neighbor-Joining method was used, and the best nucleotide substitution model was K2P, with 1,000 bootstrap replicates. Sequences in the present study are highlighted in red circles. Bootstrap supports above 60 are shown. An external cluster included *An.* (*Nyssorhynchus*) *oswaldoi* (Peryassú, 1922) and *An.* (*Nyssorhynchus*) *nuneztovari* (Gabaldón, 1940).
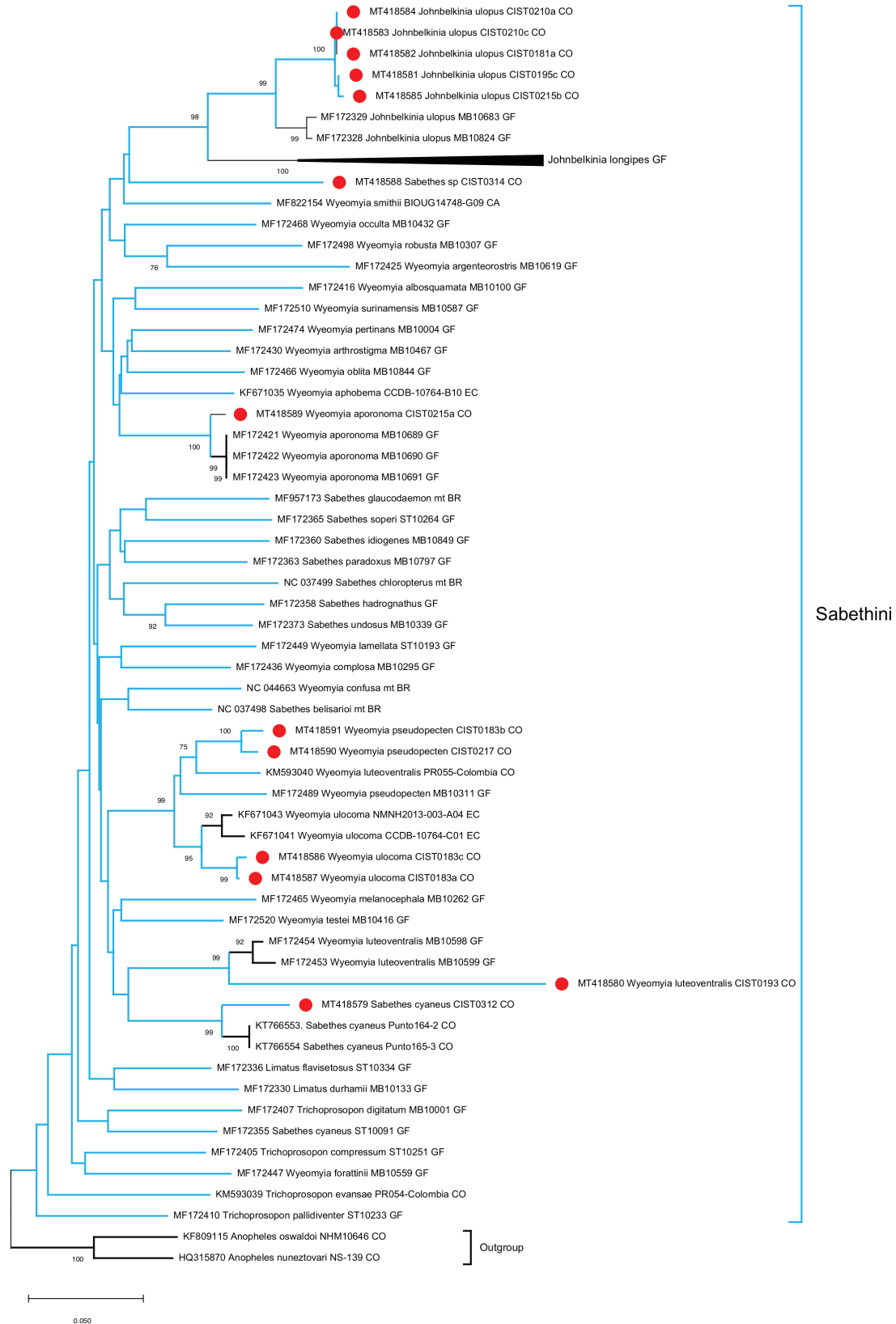
Figure 3 - Phylogenetic reconstruction of the Sabethini tribe using the COI gene (447 bp). The Neighbor-Joining method was used, and the best nucleotide substitution model was K2P, with 1,000 bootstrap replicates. Sequences in the present study are highlighted in red circles. Bootstrap supports above 60 are shown. An external cluster included *An.* (*Nyssorhynchus*) *oswaldoi* (Peryassú, 1922) and *An.* (*Nyssorhynchus*) *nuneztovari* (Gabaldón, 1940).

Table 2 - Average intra-species genetic distance for eight mosquito species identified in the present study*.

Species	Average intra-species distance (distance-p)**	Average intra-species distance (K2P)***
Ae. serratus	0.0443	0.0489
Ps. ferox	0.0211	0.0219
Jo. ulopus	0.0216	0.0227
Sa. cyaneus	0.0432	0.0458
Wy. aporonoma	0.0070	0.0071
Wy. pseudopecten	0.0374	0.0398
Wy. ulocoma	0.0256	0.0269
Wy. luteoventralis	0.0511	0.0547

*The p distances were calculated for each species included in this study by using representative sequences from GenBank accessed in October 2019; **Number of nucleotide changes between pairs of sequences per 100 nucleotides; ***Number of nucleotide changes (1 or more) between pairs of sequences per 100 nucleotides adjusted to the K2P evolutionary model, taking more frequent transitions than transversions.

Some of these species are under or not represented at all in the GenBank database according to an accurate identification (e.g., CIST0314 *Sabethes* sp.). In the case of other species such as *Wy. aporonoma*, *Wy. luteoventralis*, *Wy. pseudopecten*, *Wy. ulocoma*, *Jo. ulopus*, *Sa. chloropterus*, and *Sa. cyaneus* that are mainly associated with sylvatic ecosystems, the identification was also challenging because of the lack of information about their biology, ecology and genetics.

Sequences that led to the identification of *Ae. serratus* and *Ochlerotatus serratus* as the same species are found in the GenBank database due to taxonomic reclassification that has been proposed for the Aedini tribe; however, a recent analysis supported the use of the traditional classification[23]. In addition, there are difficulties in classifying the genus *Psorophora* due to similar male genitalia morphology among species[24].

In this study, some specimens were initially classified as *Trichoprosopon* sp.*,* using taxonomic keys that did not include the new genus *Johnbelkinia*. After the reclassification via the morphological characters of the absence of sows in the calypter and the iridescent yellow-green colors on the mosquito scutal[18], in which *Jo. ulopus* and *Jo. longipes* adult, morphology are well-described, we identified the morphotype as *Jo. ulopus* and the species designation was corroborated by DNA barcoding.

The Sabethini tribe has been classified as a monophyletic group using morphological characters, but when trying to show the phylogenetic relationships at the genus level, there were difficulties to find natural groups for several genera, including *Sabethes* and *Wyeomyia*[25,26]. In addition, *Wy. compta* (Senevet & Abonnenc, 1939) and *Wy. argenteorostris* (Bonne-Wepster & Bonne, 1920) are the same species that were initially named as two different species due to classification errors by the classical taxonomy[27]. It is mandatory to specify the taxonomic key used for species identification in order to consider the taxonomic classification updates.

Studies with *Ae. aegypti* showed a close phylogenetic relationship with *Hg. equinus* (Theobald. 1903)[28] and members of the genus *Psorophora*[29]. *Ae. serratus* from our study, collected in the sylvatic area showed a close relationship with sequences from the French Guyana and a more distant relationship with sequences from Mexico. Currently, the circulation of this species is mainly limited to sylvatic settings, although larvae and adults have been found at intra- and extra-domiciliary levels in low abundances, not significant to define mosquito circulation in urban areas[17].

The wide circulation of *Ps. ferox* has allowed the establishment of populations that begin to have between-population morphological differences, with intra-species variability in the egg and exochorion[30]. Genetic variability has also been observed in South, Central, and North American populations of this mosquito species identified in our study, suggesting that more in-depth morphological studies in these geographical regions should be conducted to identify changes in the life cycle stages.

Wy. aporonoma could have a wide distribution and circulation in the mountain ranges and rainforests in South American countries, although there are only reports in the French Guiana and Colombia at this time[31].

The species that have been previously reported as *Wy. luteoventralis* (KM593040) in the department of Antioquia, Colombia[11] resulted in similarities of 95.74% with *Wy. pseudopecten* when analyzed through BLAST-GenBank. In our study, this sequence has also shown a closer phylogenetic relationship with the *Wy. pseudopecten* cluster.

All the species initially characterized in this study may constitute potential arboviruses vectors with public health implications. *Ae. serratus*, *Sa. chloropterus*, and *Ps. ferox* could have acted as bridge vectors that led to the establishment of the YFV sylvatic cycle[13], responsible for the enzootic and epizootic transmission in this region of the country and a potential risk for human cases, mainly in rural areas. In the future, these mosquitoes may also serve as vectors for epidemic arboviruses to spillback in the Americas, such as DENV, CHIKV, and ZIKV[32].

The biogeographic features of the SNSM rainforest make it a rich area of speciation due to the isolation of populations as a consequence of the mountain ranges rising. In addition, it is close to the Serrania del Perija and other natural parks

in the same region that have been classified as speciation zones[33] due to their high diversity of fauna[34] and flora[35]. This study allowed us to corroborate the complementarity that exists between classical and molecular taxonomy. Further molecular taxonomy studies are required due to limitations of classical taxonomic keys. The keys used may be outdated regarding morphological characteristics of the species described; molecular identification tools have improved over time allowing the expansive characterization of sylvatic mosquitoes through genes[36] or complete mitochondrial genomes to identify species and reconstruct phylogenies[37]. However, the availability of sequences for sylvatic mosquito species is limited and more studies are needed to provide a greater support to the species identification.

Species such as *Ps. ferox* have a long dispersal ability from fragment forest to open areas, enabling its wide distribution range and the potential dispersion of arboviruses to susceptible hosts as human population and domestic animals. Additionally, the changes in populations or communities of mosquitoes in rural environments, due to the habitat fragmentation and anthropogenic disturbances inside conserved ecosystems (rainforest) could accelerate outbreaks and potentially epidemic situations[38,39]. In this sense, the ecological settings in rural areas of the SNSM meet the requirements for the emergence of viruses and other pathogens.

CONCLUSION

In conclusion, a possible endemicity in the studied region reinforces the importance of developing regional DNA barcoding libraries for molecular species identification. The high diversity of mosquito species identified in the SNSM and the limitations for taxonomic assignment reinforces the need for a consensus in the classical taxonomy and the availability of curated sequences in the open-access databases for the proper use of DNA barcoding.

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AUTHORS' CONTRIBUTIONS

GJPH, JAUC and KLD conceived and designed the study. GJPH and ASMG carried out the taxonomic identification. JAUC, KLD and ASMG performed the DNA extraction and other experiments. JAUC, KLD and ASMG analyzed the molecular data. ASMG, JAUC, GJPH and KLD wrote the manuscript. All authors edited, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

Table S1 - Geographical records of mosquitoes in Colombia and associated arboviruses[1]

Species	Department	References[2]	Associated arbovirus	References[2]
Wy. aporonoma	Valle	Heinemann and Belkin[14] Barreto *et al.*[2]	--	--
Wy. luteoventralis	Antioquia	Rozo-López and Mengual[28]	--	--
Jo. ulopus	Boyacá Meta Nariño Norte de Santander Valle del Cauca Antioquia Caldas	Suaza-Vasco *et al.*[30]	--	--
Wy. pseudopecten	Valle del Cauca	Suaza-Vasco *et al.*[30]	--	--
Wy. ulocoma	Valle del Cauca	Suaza-Vasco *et al.*[30]	--	--
Sa. cyaneus	Meta	Bates[3]		
	Valle del Cauca	Suaza-Vasco *et al.*[30]		
	Caquetá	Molina *et al.*[20]		
	Córdoba	Hoyos-López *et al.*[16]		
Sa. chloropterus	Meta	Bates[3]	Primary vector: *Yellow fever virus* (YFV)	Galindo[10] Galindo *et al.*[11] Zsemlye *et al.*[31]
	Valle del Cauca	Suaza-Vasco *et al.*[30]		
	Caquetá	Molina *et al.*[20]		
	Córdoba	Hoyos-López *et al.*[16]		
Ae. serratus	Antioquia	López[19] Groot[12] Barreto *et al.*[2] Parra-Henao and Suarez[26]	Secondary vector: *Yellow fever virus* (YFV)	Cardoso *et al.*[5] Sick *et al.*[29] Pinheiro *et al.*[27]
	Meta	Antunes[1]		
	Caquetá	Molina *et al.*[20]	*Mayaro virus* (MAYV)	Muñoz and Navarro[23]
	Córdoba	Heinemann and Belkin[14] Morales and Vidales[22]	*Venezuelan equine encephalitis virus* (EEV)	Molina *et al.*[20]
	Valle del Cauca	Lee and Barreto[18]		
	Santander	Ferro *et al.*[8] Groot *et al.*[13]		
Ps. ferox	Antioquia	Rozo-López and Mengual[28] Hoyos-López[15] Parra-Henao and Suarez[26]	*West Nile virus* (WNV)	Christofferson *et al.*[6]
			Eastern equine encephalitis (EEEV)	Navia-Gine *et al.*[24] Oliver *et al.*[25]
	Valle del Cauca	Figueroa[9]	*St Louis encephalitis virus* (SLEV)	Beranek *et al.*[4]
	-	-	*Madariaga virus* (MADV)	Lednicky *et al.*[17]
	Caquetá	Molina *et al.*[20]	*Venezuelan equine encephalitis virus* (EEV)	Molina *et al.*[20]
	Guajira	Morales *et al.*[21]		
	Magdalena	Dickerman *et al.*[7]		

[1]data accessed in October 2019; [2]all references included in the table are listed below.

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