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First record of *Culex* (*Culex*) *bidens* (Diptera: Culicidae) in Colombia: Taxonomic and epidemiological implications

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ABSTRACT

Arbovirus transmission cycles must be studied locally since both vectors and hosts vary in different regions. Colombia has a highly diverse mosquito fauna. *Culex* (*Culex*) *bidens* is reported here for the first time in Colombia. Because *Cx. bidens* Dyar and Knab and *Cx. declarator* Dyar and Knab share a close taxonomic history and because it is difficult to differentiate between them, a morphological and molecular comparison was performed. The male genitalia of three specimens of *Cx. bidens* from Colombia were mounted on microscope slides and morphologically compared with the male genitalia of *Cx. declarator* also from Colombia. In *Cx. bidens*, the individual teeth of the lateral plate are long, straight, laterally directed and sharply pointed; in *Cx. declarator* these teeth are robust, curved, with convex margins and bluntly rounded. Moreover, DNA was extracted from the same specimens and a fragment of the cytochrome *c* oxidase subunit I mitochondrial gene was amplified and sequenced. Neither *Cx. bidens* nor *Cx. declarator* were clustered in the Neighbour-joining topology, with K2P interspecific divergence between 0.15–1.45%. The circulation of Eastern Equine Encephalitis Virus in Colombia was reported since 1957 and *Cx. bidens* was suspected to be the vector of this virus during an epizootic in Argentina in 1988. Hybridization between species of the subgenus *Culex* has been demonstrated, hence the degree of reproductive isolation between *Cx. bidens* and *Cx. declarator* should be investigated, as well as their taxonomic status, because they only can be discriminated by a single male genitalic feature and not by nuclear or mitochondrial markers.

1. Introduction

Mosquitoes in Colombia are responsible for the transmission of several pathogens such as nematodes (Sotomayor-Tribin, 2014), protozoa causing malaria (Naranjo-Díaz et al., 2014; Ahumada et al., 2016) and arboviruses of the genera *Alphavirus* (Venezuelan Equine Encephalitis, Eastern Equine Encephalitis (EEEV), Mayaro), *Flavivirus* (Dengue, Yellow fever, West Nile (WNV), Saint Louis Encephalitis (SLEV)) and *Orthobunyavirus* (Guaroa, Wyeomyia) (Groot et al., 1996; Mesa et al., 2005; Mattar et al., 2011).

Arbovirus transmission cycles must be studied locally since there is evidence that both vectors and vertebrate hosts vary in different geographical regions (Díaz et al., 2013). While in the United States the SLEV transmission is maintained mainly by the vectors Linnaeus (1758); Say (1823), *Cx. nigripalpus* Theobald (1901) and *Cx. tarsalis* Coquillett (1896) with house sparrow hosts (Reisen, 2003); in temperate region of Argentina, the vectors *Cx. quinquefasciatus* and *Cx. interfor* Dyar (1928) are implicated with doves as hosts (Díaz et al., 2006). It is important to note that both *Cx. pipiens* and house sparrows are found in Argentina, but there is no evidence of being involved in SLEV maintenance. Thus, a mosquito species cannot be incriminated in the transmission of an arbovirus in a certain location because it is involved in transmission in another region. Natural infection and vector competence have to be demonstrated regionally. The knowledge about mosquito species distribution in a country is of paramount importance in order to determine areas of potential risk of pathogen transmission.

Colombia, as a megadiverse country, has a highly diverse mosquito fauna. Rozo-Lopez and Mengual (2015a) updated the mosquito fauna to 324 species in 28 genera, of which 105 belong to the genus *Culex* and 26 to the subgenus *Culex*. *Culex* (*Culex*) *bidens* Dyar and Knab (1922) is being reported here for the first time in Colombia. *Culex bidens* was suspected to be the vector of EEEV during the epizootic in Chaco Province in northern Argentina in 1988 (Sabattini et al., 1998). *Culex bidens* and *Cx. declarator* Dyar and Knab (1906), the latter of which has

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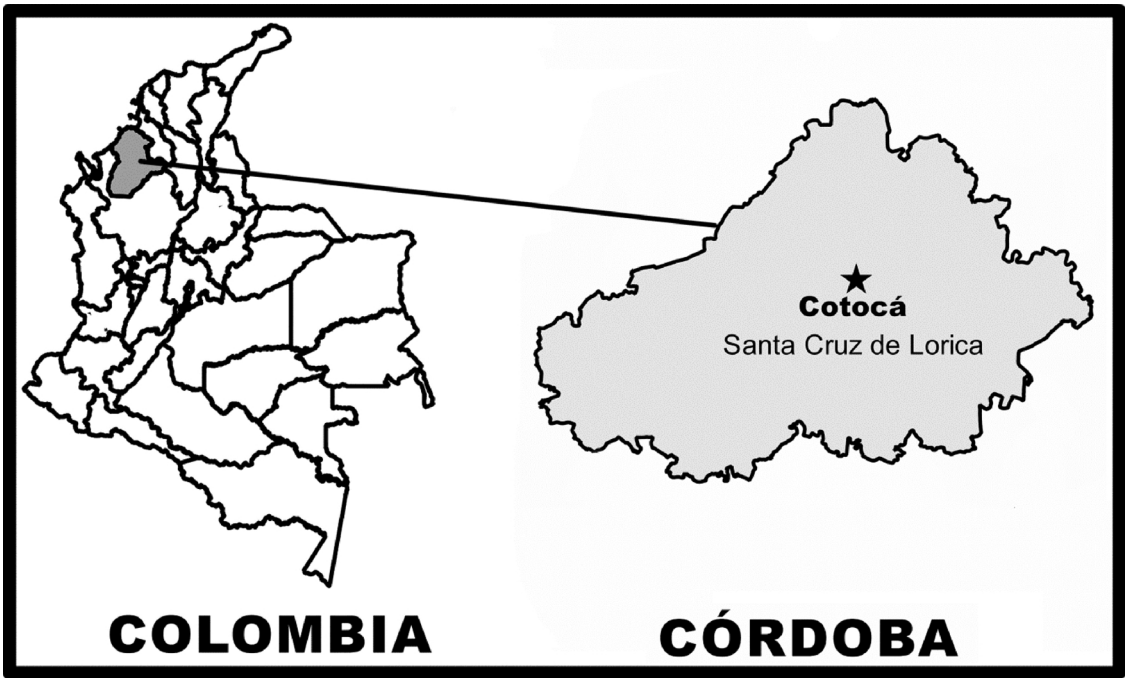

Fig. 1. Sampling site location in Córdoba Department, Colombia.

been recorded in Colombia, share a close taxonomic history even though their phylogenetic relationship has not been tested yet. Lane (1953) synonymized both species with *Cx. virgultus* Theobald (1901); Stone, (1956) realized that the genitalia of the specimen of *Cx. virgultus* was incorrectly associated with this specimen, resurrected *Cx. declarator* from the synonymy, and considered *Cx. virgultus* as an unrecognized species. Later, Belkin et al., (1968) established that *Cx. virgultus* should be considered a *nomen dubium*. Regarding *Cx. bidens*, Bram (1967) revalidated the species status of the synonymy of *Cx. declarator*, after the examination of the lectotype male genitalia of both species.

Culex bidens and *Cx. declarator* belong to the Tarsalis Subgroup of the Pipiens Group of the subgenus *Culex* (Harbach, 2011). Morphological identification of *Culex* (*Culex*) species, in general, and particularly the species considered here, is difficult because external anatomical characters of fourth-instar larvae and females are polymorphic and overlap among species. Morphological traits of the male genitalia provide a wealth of taxonomic characters that allow reliable identification of the species. Because diagnostic features in many species of the subgenus *Culex* are restricted to the male, and most of the surveillance studies focus on females, alternative tools such as molecular methods are currently used. A fragment of the cytochrome *c* oxidase subunit I (*COI*) mitochondrial gene has been largely employed for taxon barcoding (Hebert et al., 2003a) and as a tool to assess genetic divergence among closely related animal species (Hebert et al., 2003b). Particularly in mosquitoes, the effectiveness of *COI* as a marker for species identification has been tested in several studies in Canada (Cywinska et al., 2006), China (Wang et al., 2012), Ecuador (Linton et al., 2013), Argentina and Brazil (Laurito et al., 2013), Singapore (Chan et al., 2014), Colombia (Rozo-Lopez and Mengual, 2015b) and Australia (Batovska et al., 2015), among others. Even if the usefulness of *COI* as barcode for mosquito identification has been demonstrated and tested by its correspondence with morphology, its use can also be problematic in closely related species because of incomplete lineage sorting and introgression events (Beeble, 2018).

The present study updates knowledge of the mosquito fauna of Colombia by incorporating a new record based on morphological and molecular characteristics and highlighting the relevance of the morphological features. Taxonomic and epidemiological implications are also taken into account.

2. Materials and methods

2.1. Mosquito sampling

Mosquitoes were collected as part of entomological and ecological surveillance in Cotocá; 9° 11´ 33.63´´ N, 75° 53´ 1.88´´ W; a rural area from the municipality of Santa Cruz de Lorica (Córdoba Department, Colombia), during December 2016 (Fig. 1). Mosquito larvae were found in several distinct phytotelmata and water bodies close to temporal ponds, swamps and small dams. These immature mosquitoes were raised until adult stage for taxonomic identification. Only male specimens were included in the study. Two legs from one side of each specimen were removed and preserved in 100% ethanol for molecular procedures while the rest were pin-mounted for morphological treatment. Until now, *Cx. declarator* has been reported in the rural area of Supia (mountain forest), Caldas Department, at 1150 masl and in La Pintada (tropical dry forest), Antoquia Department, at 660 masl (Rozo-Lopez and Mengual, 2015b).

2.2. Morphological treatment

Specimens were pin-mounted and the male gentitalia, on microscope slides in Canada balsam using standard protocols. Voucher specimens, including the entire specimen except the two legs and the associated genitalia preparations confirming this new record were deposited in the collection of the Universidad del Sinú, Colombia. Due to the close taxonomic history shared by *Cx. bidens* and *Cx. declarator*, a critical review of the following studies was carried out: Lane, (1953); Carpenter and Lacasse, (1955); Stone, (1956); Bram, 91967); Belkin et al., (1968); Harbach et al., 91986) and Forattini, (2002). Furthermore, a comparison between male genitalic features of *Cx. declarator* from Colombia and the specimens of *Cx. bidens* reported here, also from Colombia, was conducted. The photographs of the specimen of *Cx. declarator* belong to the specimen labeled as ZFMK-PR066 (GenBank accession number KM593051) in the study of Rozo-Lopez and Mengual (2015b), deposited in the collections of the Zoologisches Forschungsmuseum Alexander Koenig, Bonn (Germany), and were

Table 1
Information for sequences included in the study obtained from BenKank.

Species	Country	GenBank
Cx. bidens	Argentina	KY581209
		KY581211
		KY581213
		KY581213
		KY581215
		KY581215
		KY581217
		KY581218
		KY581221
		KY581222
		KY581235
		KY581237
		KY581238
		KY581239
Cx. coronator	Brazil	KJ812989
		KJ812990
	Ecuador	KF671025
		KF671026
Cx. declarator	Brazil	KF919211
		KF919212
		KF919193
	Colombia	KM592993
		KM592995
		KM593007
		KM593014
		KM593017
		KM593019
		KM593046
		KM593051
		KM593055
		KM593057
Cx. interfor	Argentina	KY581245
		KY581246
		KY581247
		KY581253
		KY581254
		KY581255
		KY581264
		KY581265
		KY581269
		KY581272
		KY581273
Cx. nigripalpus	Brazil	KF919227
		KF919228
		KF919229

generously taken and sent by X. Mengual. Morphological terminology follows Harbach (2018).

2.3. Molecular procedures

DNA extractions were obtained from the legs of three male specimens of *Cx. bidens* preserved in 100% ethanol. Genomic DNA was extracted using the protocol modified by Atencia et al. (2018), following the manufacturer's protocols. The DNA obtained was used as template to amplify the ~658 bp fragment of the *COI* barcode region. The amplification was carried out according to the protocol of Folmer et al. (1994). Each PCR mix contained final concentrations of the following reagents: 1X PCR Buffer, 0.2 mM of dNTP, 0.3 µM of each primer, 1.5 mM of $MgCl_2$, 2 U Taq DNA Polymerase and 2 u L DNA template. The mixture was increased to a total volume of 50 µL by adding ddH_20. The PCR products were amplified using the following parameters: 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 46 °C for 60 s and 72 °C for 60 s, followed by 72 °C for 1 min and a 4 °C hold. The PCR products were electrophoresed in 1% TBE agarose gels stained with bromure ethidium. All sequencing reactions were carried out in both directions using an ABI3730XL (Macrogen Inc., Corea) with the same set of PCR primers. The barcode sequences obtained in this study are deposited in GenBank under accession numbers MH931446 (LCA067), MH931447 (LCA088) and MH931448 (LCA087).

2.4. DNA sequence analysis

The sequences were edited in BioEdit v. 7.2 (Hall, 1999). Primer regions were removed from sequences. Comparisons with available sequences using Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi) were carried out to check for sequence homology and species identification. Sequences from *Cx. bidens* and *Cx. interfor* from Argentina, *Cx. declarator* from Brazil and Colombia, *Cx. coronator* Dyar and Knab (1906) from Brazil and Ecuador and *Cx. nigripalpus* Theobald (1901) from Brazil were obtained from GenBank and included in the analysis (Table 1). DNA sequences were aligned by nucleotides using the Muscle algorithm (Edgar, 2004).

2.4.1. Barcode clusters

Kimura two-parameter (K2P) distance (Kimura, 1980) within *Cx. bidens* sequences and between *Cx. bidens* and the other included species were estimated with Mega v.7 (Kumar et al., 2016) and subsequently used to generate a neighbour-joining (NJ) tree to evaluate the clustering pattern between species, using the default parameters. Statistical support for the clusters was estimated using Bootstrap support value (BSV) obtained in 1000 bootstrap replicates.

The usefulness of the *COI* fragment for species identification was tested based on the criterion developed by Meier et al. (2006), known as best close match (BCM) and included

2.4.2. COI and species identification

in TaxonDNA (taxondna.sf.net/). The algorithm identifies the best sequence matches of a query and only assigns the species name of that sequence to the query if the sequence is sufficiently similar. Accordingly, a threshold similarity value was estimated for the dataset. Queries without sequence match below the threshold value remain unidentified. Those queries with match above the threshold value were considered a successful, ambiguous or incorrect identification. A correct identification was achieved if both names are identical. When at least two equally good best matches were found the identification remained ambiguous and when the names were mismatched, the identification was a failure.

3. Results

3.1. Morphological treatment

Three larvae of *Cx. bidens* were collected in several distinct phytotelmata and water bodies in sympatry with the following mosquito species (number of specimens): *Cx.* (*Ads.*) *amazonensis* (Lutz, 1905) (1), *Cx.* (*Cux.*) *chidesteri* Dyar (1921) (1), *Cx.* (*Cux.*) *quinquefasciautus* (4), *Cx.* (*Mel.*) *erraticus* (Dyar and Knab, 1906) (2), *Ma.* (*Man.*) *titillans* (Walker, 1848): (8), *Oc.* (*Cul.*) *taeniorhynchus* (Wiedemann, 1821) (12). The three male genitalia genitalia of *Cx. bidens* have been dissected and mounted on microscope slides (Fig. 2). Morphological identification was achieved following the diagnostic features of *Cx. bidens* given by Bram (1967) and Harbach et al. (1986). Since *Cx. declarator* had already been registered for Colombia, a comparison with this species was done based on Breland (1954); Carpenter and Lacasse (1955); Bram (1967) and photographs of specimens of *Cx. declarator* from Colombia (Fig. 3).

The gonocoxite (Fig. 2a) of *Cx. bidens* is about twice longer than basal width, uniformly covered with fine spicules. The subapical lobe (Fig. 2a) is prominent, undivided; setae *a–c* are stout, rodlike, and hooked at the tip, and *a* is shorter than *b* and *c*; seta *f* is flattened and broad on distal half with curved hook tip; seta *g* is foliform and asymmetrical and seta *h* is filiform and curved apically. The lateral plate of the phallosome (Fig. 2b-d) shows 1–3 (usually 2) large dorsolaterally directed and sharply pointed teeth, 0–3 minute conical denticles; dorsal process sharp at tip; ventral arm well developed as a spine

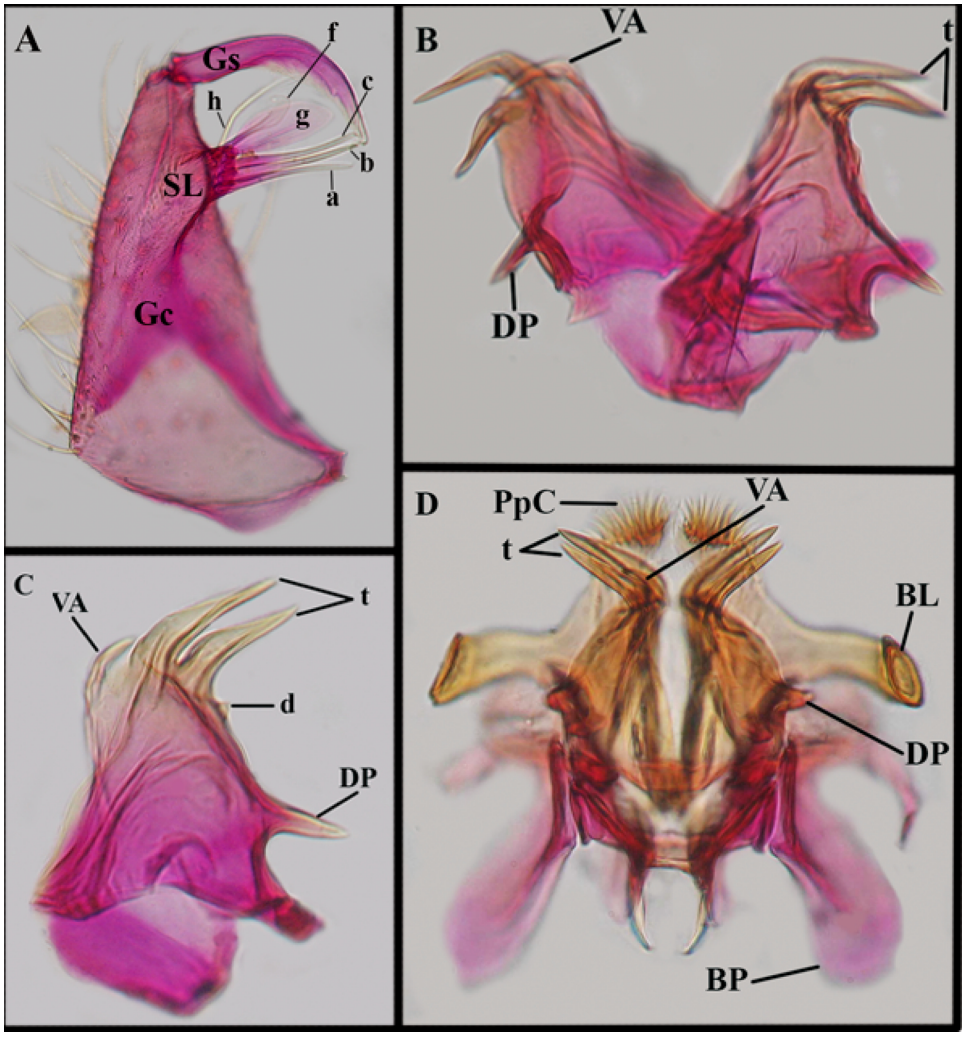

Fig. 2. Male genitalia structures of *Culex* (*Culex*) *bidens* Dyar and Knab from Colombia. A: gonocoxopodite of specimen sample ID LCA088; B: lateral plate of specimen sample ID LCA067; C: lateral plate of specimen sample ID LCA087; D: lateral plate of specimen sample ID LCA088. a, b, c, f, g, h = setae of subapical lobe; BL = basolateral arm; BP = basal piece; d = denticles; DP = dorsal process; Gc = gonocoxite; Gs = gonostylus; PpC = paraproct crown; SL = subapical lobe; t = teeth; VA = ventral arm.

bent dorsolaterally; dorsal arm absent. The proctiger (Fig. 2d) is crowned of pointed spicules covering the apex of paraproct, basolateral arm curved and compressed distally.

The diagnostic character, found by Bram (1967) and used for resurrecting *Cx bidens* from synonymy from *Cx. declarator*, which allow to distinguish the former (Fig. 2b-d) from the latter (Fig. 3a,b) is related to the shape of the teeth of the lateral plate. Gonocoxite, including setae morphology and arrangement, are identical in both species (Figs. 2a, 3a). As previously noted, in *Cx. bidens* the individual teeth are long, straight, laterally directed and sharply pointed (Bram, 1967) (Fig. 2b-d). In *Cx. declarator* these teeth are robust, curved, with convex margins and bluntly rounded (Carpenter and Lacasse, 1955; Bram, 1967) (Fig. 3b). A mistake found in the male genitalia key of Forattini (2002), which could lead to misidentification of *Cx. bidens* and *Cx. declarator,* was warned by the authors. Forattini (2002) mentions that in *Cx. declarator*, the seta *f* of the subapical lobe is similar in appearance to the other setae of the subapical lobe and it is flattened and broad on distal half with curved hook tip (Fig. 3a), similar to seta *f* of *Cx. bidens* (Fig. 2a).

3.2. DNA sequence analysis

3.2.1. Barcode clusters

A pairwise K2P distance matrix was constructed for the 48 *COI* barcode sequences (Supplemental File). Only two well-supported clusters and one less supported were recovered in the NJ tree (Fig. 4), and they do not include target species (*Cx. bidens* and *Cx. declarator*). The clustered species were *Cx. coronator* (99% BSV), *Cx. interfor* (87% BSV) and *Cx. nigripalpus* (64% BSV). Neither *Cx. bidens* nor *Cx. declarator* were grouped together, not even in sequences of specimens from the same locations (Fig. 4). The mean genetic divergence within *Cx. bidens* was 0.38% (ranging from 0 to 0.96%) and in *Cx. declarator* was 0.21% (ranging from 0 to 0.8%) while the mean interspecific variation was 0.8% (ranging from 0.15 to 1.45%). The interspecific distance values were only 2.1 and 3.8 times higher than the variability within *Cx. bidens* and *Cx. declarator*, respectively.

3.2.2. COI and species identification

Forty-eight sequences were included in the analysis (Table 1),

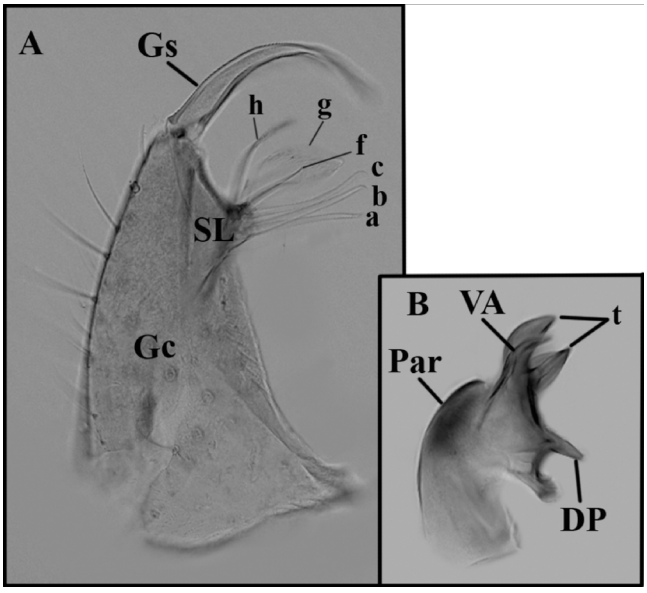

Fig. 3. Male genitalia structures of *Culex* (*Culex*) *declarator* Dyar and Knab from Colombia of specimen sample ZFMK-PR066 from Rozo-Lopez and Mengual (2015b). A: gonocoxopodite; B: lateral plate. a, b, c, f, g, h = setae of subapical lobe; DP = dorsal process; Gc = gonocoxite; Gs = gonostylus; Par = paramere; SL = subapical lobe; t = teeth; VA = ventral arm.

93.75% of which (45 sequences) was successfully identified in accordance with the BCM, whereas 4.16% (2 sequences) of all sequences were ambiguously identified, and 2.08% (1 sequence) were incorrectly identified. Two of three *Cx. bidens* from Colombia, all the specimens belonging to *Cx. bidens* from Argentina, all *Cx. declarator* from Colombia and one of three *Cx. declarator* from Brazil were successfully identified. The remaining two individuals of *Cx. declarator* from Brazil were ambiguously identified as *Cx. declarator* and *Cx. bidens*. Lastly, the remaining sequence of *Cx. bidens* from Colombia were incorrectly identified as *Cx. declarator*. It is important to note that of both sequences of *Cx. bidens* from Colombia successfully identified, the best close match was between them and the second best close match was a sequence of *Cx. declarator*.

4. Discussion

The knowledge about mosquito species distribution in a country is of great importance in order to determine areas of potential risk of pathogen transmission. The current distribution of *Cx. bidens* includes Argentina, Bolivia, Brazil, Colombia (new record), Costa Rica, Mexico, Nicaragua, Paraguay, Uruguay and Venezuela.

Even though arbovirus transmission cycles must be studied locally since both vectors and vertebrate hosts vary in different geographical regions, the epidemiological relevance of the species should not be ignored. The new record of *Cx. bidens* in Colombia should be taken into account because the species was suspected to be the vector of EEEV during the 1988 epizootic in Argentina (Sabattini et al., 1998). EEEV has been isolated also in neotropical countries, including Brazil, Colombia, Guyana, Panama, Dominican Republic, Jamaica and Trinidad, all of which have had occasionally encephalitis outbreaks in horses (Sabattini et al., 1998). The circulation of EEEV in Colombia was reported for the first time as serologic evidence in 1957 (Groot et al., 1996) in Santander Department and isolated in 1969 in Nariño Department (Groot et al., 1996). Serologic evidence from monkeys in jungle areas of the Magdalena Medio region has also been recovered (pers. comm. Downs, 1961 cited by Groot, 1964). Succeeding studies have provided evidence of viral activity in other regions of the country such as an epizootic in 1992 with 13 fatal cases in equines in Antioquia Department (Mesa et al., 2005) and the virus isolation from horses and hamsters in Casanare and Boyaca departments and the Caribbean coast region (Mesa et al., 2005). More recent evidence has shown natural infection from EEEV in mosquitoes from La Pintada, Antioquia Department (Hoyos-López et al., 2015), but due to the difficulty of morphologically identifying several species from the genus *Culex*, the pool was identified as *Culex* spp (Hoyos-López et al., 2015). Thus, the vector of EEEV in Colombia remains unknown.

Original descriptions of several mosquito species have been carried out based on male specimens. The evolution of genital form is thought to be involved in the origin and diversification of species (Harbach et al., 2012). Hybridization between species of the subgenus *Culex* has been demonstrated based on genitalia structure, biotic sympatry and molecular biparental markers such as in the *Cx. pipiens* complex in North America, Argentina and Madagascar (Urbanelli et al., 1997; Humeres et al., 1998; Vinogradova, 2000) and between *Cx. bidens* and *Cx. interfor* in Argentina (Laurito et al., 2017). The last two, are similar species which can be separated by the male genitalia and some characters of the larva and pupa, not so by the adult female (Mureb-Sallum et al., 1996). It is important to note that *Cx. bidens* and *Cx. interfor* have shown evidence of hybridization (Laurito et al., 2017). These are two closely related species with only one diagnostic character of males allowing for differentiation between them in the adult stage, with sympatric distribution but also easily distinguishable by at least two molecular markers, and with 2.3% mean interspecific variation (Laurito et al., 2017). Even though, there is no evidence of intermediate male genitalia traits of sympatric populations of *Cx. bidens* and *Cx. declarator*, the degree of reproductive isolation between them should be investigated.

The morphological similarity, primarily of the female stage, with *Cx. declarator* carries another important epidemiological implication. *Culex declarator* has been incriminated in the transmission of several arboviruses outside of Colombia, such as SLEV in Brazil (Figueiredo, 2000), WNV in United States (Komar and Clark, 2006) and Oropuche in Brazil (LeDuc et al., 1981), among others. This highlights the importance of unequivocally identifying mosquito species with real or potential public health interest. Alternative tools such as molecular methods were considered to solve this problem. In the present study the two species of interest, *Cx. bidens* and *Cx. declarator*, could not be identified by the mitochondrial *COI* barcode region, which showed a negligible genetic divergence. This could be explained by the incomplete lineage sorting of *COI* sequences between recently diverged species, which can result in species being overlooked because shared *COI* sequences still exist in both populations as either ancestral haplotypes or through interspecies introgression events between related species (Surendran et al., 2015). Not only was the *COI* K2P interspecific distance was too low, but the BCM was also not able to correctly identify *Cx. bidens* from Colombia (if the three sequences of *Cx. bidens* from Colombia were removed from the analysis). Nevertheless, Vesgueiro et al. (2011) have shown similar results, based on a nuclear marker. The authors used ITS2 for elucidating problems associated with the identification of morphologically similar and recently diverged species of the genus *Culex*. Only an interspecific distance of 0.5% was established between *Cx. bidens* and *Cx. declarator*, the shortest found by Vesgueiro et al. (2011). Because these species cannot be identified by morphological characters of the fourth-instar larvae, females or molecular markers (nuclear and mitochondrial), and only by a single male genitalic feature, the taxonomic status of the species should be assessed in further studies since its synonymy is plausible.

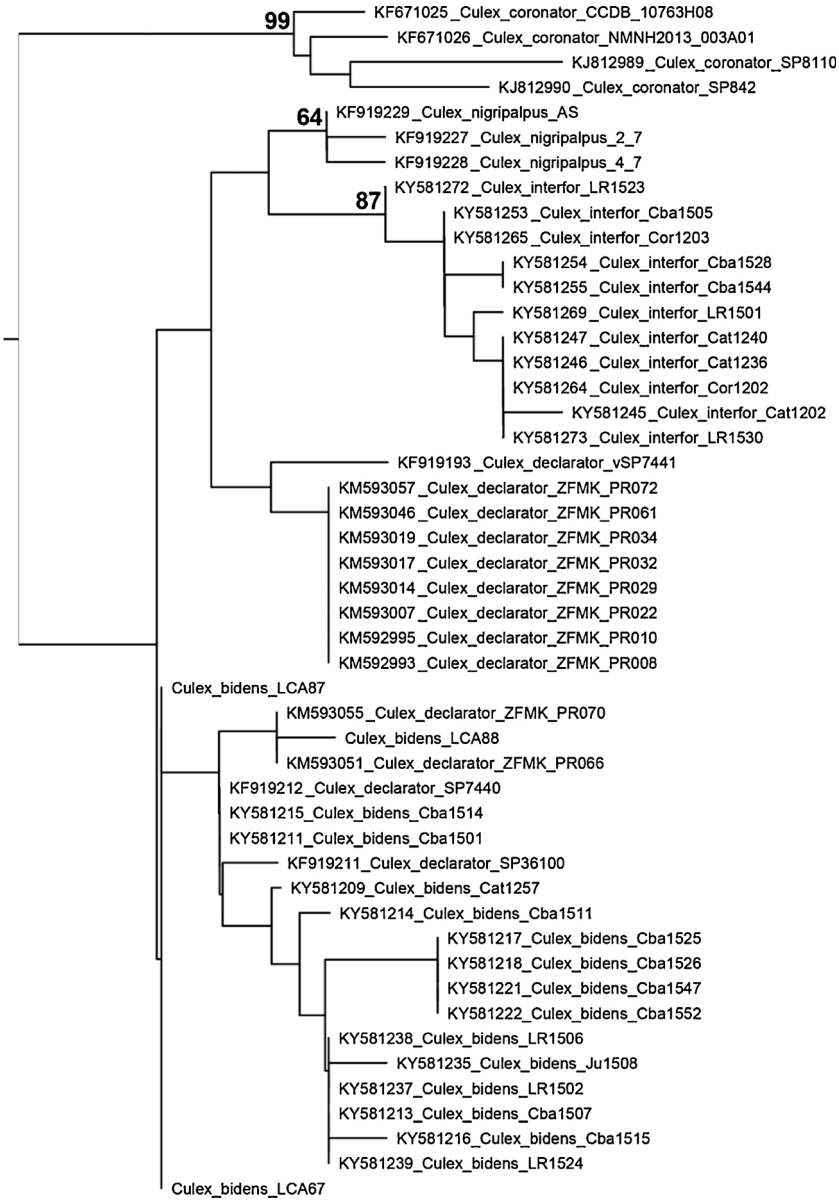

Fig. 4. Bootstrapped neighbour-joining tree of 48 cytochrome c oxidase subunit I (*COI*) sequences from specimens belonging to five *Culex* (*Culex*) species from Argentina, Brazil, Colombia and Ecuador based on the Kimura two-parameter distance algorithm and 1000 replicates. Bootstrap values less than 70% are not shown (except by *Cx. nigripalpus* cluster).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2018.09.010.

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