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An unsettling explanation for the failure of skatole-baited ovitraps to capture *Culex* mosquitoes

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Abstract

Culex mosquitoes are primarily found in temperate and tropical regions worldwide where they play a crucial role as main vectors of filarial worms and arboviruses. In Recife, a northeast city in Brazil, high densities of *Culex quinquefasciatus* are often found in association with human populated areas. In marked contrast to another part of the city, field tests conducted in the neighborhood of Sítio dos Pintos showed that trapping of mosquitoes in skatole-baited ovitraps did not differ significantly from captures in control (water) traps. Thus, classical and molecular taxonomic approaches were used to analyze the *Culex* species circulating in Sítio dos Pintos. Results obtained from both approaches agreed on the co-circulation of *Culex quinquefasciatus* and *Culex nigripalpus* in three different areas of this neighborhood. What was initially considered as an unexpected failure of this lure turned out to be a more unsettling problem, i.e., the first report in Recife of *Culex nigripalpus*, a vector of Venezuelan equine encephalitis virus and West Nile virus. Unplanned urbanization processes close to remnants of the Atlantic forest, such as observed in Sítio dos Pintos, may have contributed to the introduction of *Cx. nigripalpus* in urban areas.

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Disclosure

The authors declare that they have no conflict of interest.

Keywords

Culex quinquefasciatus; *Culex nigripalpus*; skatole; oviposition attractant; Zika; dengue; West Nile virus

Introduction

Species from the genus *Culex* are considered the main vectors of lymphatic filariasis and various arboviruses, such as Rift Valley Fever (RVF), Eastern Equine Encephalitis (EEE), Japanese encephalitis (JE), Saint Louis encephalitis (SLE), and West Nile Virus (WNV) (Komar et al., 2003, Fontes et al., 2005). *Culex* mosquitoes have a cosmopolitan distribution but are mostly found in temperate and tropical regions worldwide. These mosquitoes are found in a variety of habitats, although its highest density is often associated with human populated areas (Regis et al., 2000). In Recife, a northeastern city in Brazil, *Culex quinquefasciatus* is solely responsible for the endemic status of lymphatic filariasis (Regis et al., 1995, 2000, Fontes et al., 2005). Although entomological surveys conducted in urbanized areas of Recife show the predominance of *Cx. quinquefasciatus*, it is possible that other *Culex* mosquitoes may be circulating in areas of a tropical rain forest remnant.

Recife (8°3′S 34°54′W) has a diverse environment scattered over 218.4 km², with an ecosystem composed of islands, mangroves, beaches, and tropical rain forest fragments. Warm to hot temperatures, high humidity, intense rainfall, unplanned urbanization, and low sanitation conditions make Recife an appropriate place for mosquito breeding. Entomological surveys conducted in Recife showed a variety of potential vector species (Balbino et al., 2005, Aragão et al. 2010). Nonetheless, most studies conducted in this city are constrained to mosquitoes of economical and medical importance, such as *Aedes (Stegomyia) aegypti*, *Ae. Albopictus*, and *Cx. quinquefasciatus*.

Sítio dos Pintos (SP) is located in the northwestern region of Recife. This particular location has recently undergone a rapid and disorganized urbanization but is still surrounded by dense Atlantic forest. Captures in oviposition attractant-baited gravid traps (ovitrap) in peridomicile areas of residence in SP differed markedly from results obtained in another area in the same city despite using the same batch of lures and traps. In-depth taxonomic and barcode examination of captured mosquitoes led to the first record of *Culex nigripalpus* in this region.

Materials and methods

Field studies

Field tests using gravid mosquito traps (Catalog #2800, Bioquip®, Rancho Dominguez, CA) were initially conducted in two locations in the city of Recife (8°3′S/34°54′W), with one set of traps deployed closer to the Instituto Aggeu Magalhães (8°2′S/34°57′W) and the other in SP (8°0′S/34°57′W) (Fig. 1). SP is a neighborhood located in the northwestern part of Recife, the main endemic city for lymphatic filariasis and the epicenter of the Zika epidemic in Brazil. This district occupies an area of approximately 1.8 km², with a population of 7276 inhabitants distributed in 2132 domiciles. Most of SP is covered with remains of the Atlantic

Rain forest (Fig. 1), and has an inadequate city water supply and sewer networks. Gravid mosquito traps were filled either with 5 L of water (control) or 5 L of water and 100 μL of skatole (5×10^{-6} mg/mL; final concentration 10^{-4} $\mu\text{g/L}$) (test). Preliminary experiments with 10x lower and 10x higher concentrations of skatole did not show a significant difference between treatment and control. To minimize inconsistencies due to variations in battery power and fan speed, traps were modified to run on AC power. Traps were deployed for four weeks, inspected daily, mosquitoes were collected, traps were rotated, and at the end of each week both control and treatment were renewed. Data (N=12–16) were transformed to log (X +1), analyzed separately for each location using the Shapiro-Wilk normality test followed by paired, two-tailed *t* tests.

After analysis of satellite images, the SP neighborhood was divided into six areas for further mosquito collection. Collected mosquitoes were separated into two groups (*Aedes* and *Culex*), individually placed in 1.5 mL microtubes and frozen at -80°C , until further molecular identification. Additionally, resting mosquitoes were collected by battery-powered aspirators (Horst[®], São Paulo, Brazil) from peri- and intradomicile areas of those six different areas in SP. The first set of collected *Culex* mosquitoes were identified by using DNA barcoding, and the second set of aspirated mosquitoes were identified by both taxonomy and DNA barcoding. All collections were performed with consent from residents and after approval by the Instituto Aggeu Magalhães Ethical Committee on Research (PlatBr Protocol number: 421.613).

Morphological Identification

Specimens obtained from the second set of aspirations were identified as *Culex* mosquitoes with an entomological magnifier, and individually separated into 1.5 mL microtubes with silica gel. These samples were sent to the University of São Paulo (USP), and taxonomic keys (Forattini, 2002) of the male genitalia were used to complement the identification. Briefly, the male genital was extracted from the VII abdominal segment, placed in a 200- μL microtube filled with 20% potassium hydroxide (KOH). After 12 h, the KOH solution was discarded and a 20% acetic acid-alcohol (a mixture of 8 parts of ethanol and 2 parts of glacial acetic acid) was added for 10 min. After this period, two drops of acetic acid-fuchsin (5 mg/mL of fuchsin plus 0.5% v/v of glacial acetic acid) were added and after 10 min, the material was washed with 80%, 90%, 95%, and absolute ethanol. After dehydration, clove oil (Vetec, Brazil) was added for an hour and then samples were assembled with Canada balsam (Vetec, Brazil) and observed under an optic microscope (Zeiss AX10). Once the identification was concluded, the remains of each mosquito were sent back to IAM/FIOCRUZ for DNA barcoding analysis.

DNA Barcoding

Genomic DNA was extracted from individual mosquitoes using a rapid alkaline protocol (Rudbeck & Dissing, 1998). Primers COIF (5' GGAGGRTTTGGAAAYTGAYTAGTYCC 3') and COIR (5' GCWGAWGTAAARTAAGCTCGWGTA 3') were manually designed, using multiple COI sequences from *Anopheles melas* (DQ792679), *An. gambiae* (L20934), *An. quadrimaculatus* (L04272), *Cx. pipiens* (HQ724615), *Cx. quinquefasciatus* (GU188856), *Ae.* (NC010241), and *Ae. albopictus* (AY072044) to amplify a 698 bp

fragment. PCR reactions contained 1U Taq polymerase (Invitrogen), 0.5 mM dNTPs (Invitrogen), 0.4 μ M of each primer, 1.5 mM MgCl₂ and 20 ng of genomic DNA. PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 10 min. PCR products were run on a 1% agarose gel, stained with ethidium bromide and visualized under a UV light. Amplified fragments were purified with GFX PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech) and sequenced in both directions with an ABI 3500xL sequencer (Applied Biosystems). After editing with CodonCode Aligner 3.7.1 software, sequences were blasted against two databases: NCBI (National Center for Biotechnology Information – <http://www.ncbi.nlm.nih.gov/blast>) and BoldSystems (The Barcode of Life Data Systems – <http://www.boldsystems.org>). Sequence alignments and phylogenetic analysis were performed using BioEdit v.7.2.5 (Hall, 1999) and MEGA6 software, respectively. DNA polymorphism analysis was performed with DNAsp v.5 (Rozas & Rozas, 1995). *Cx. nigripalpus* and *Cx. quinquefasciatus* sequences were submitted to the Genbank database. Phylogenetic trees were constructed by Neighbor-Joining (NJ) (Saitou & Nei, 1987) and Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) method (Sneath & Sokal, 1973), using 1000 bootstrap replicates, with 20 randomly selected COI sequences from *Cx. quinquefasciatus* and 13 *Cx. nigripalpus* sequences from mosquitoes collected in SP. COI sequences from *Ae. aegypti* (KP211399) and *Rhodnius prolixus* (AF449138.1) were retrieved from the GenBank and used as outgroups to both trees.

Results

To select areas with high mosquito densities for an ongoing project to improve lures for ovitraps, gravid traps baited with the oviposition attractant skatole (Olagbemiro et al., 2004) were deployed in two neighborhoods of the metropolitan region of Recife, Brazil (Fig. 1). Although lures were from the same batch and the same type of traps were used, the results were surprising. As indicated by total trap captures, the occurrence of *Culex* mosquitoes in the two areas was high. Intriguingly, however, captures in skatole-baited traps in SP were not significantly different from the catches in control traps. By contrast, traps baited with skatole captured significantly more mosquitoes than control traps in another area near the Institute Aggeu Magalhães (Fig. 1). We then surmised whether other *Culex* species, which may not respond to skatole, were circulating in SP. Mosquitoes were then trapped and aspirated in six areas of the neighborhood for in-depth identification.

Mosquitoes obtained from the first set of aspirations in SP and submitted to COI barcoding showed that *Cx. quinquefasciatus* was the main species found in all areas of study (Table 1, first aspiration). However, some of our COI sequences were ambiguous with alignment scores similar to sequences for *Culex* species (*Cx. dolosus*, *Cx. declarator*, and *Cx. conspirator*). Three haplotypes derived from those species were retrieved from the BoldSystems database and aligned with one of our *Cx. nigripalpus* COI sequence (Fig. 2). From 456 bp aligned, our *Cx. nigripalpus* (Sítio dos Pintos) sequence differed in 2 bp from the *Cx. nigripalpus* (GBDCU1073–14), 3 bp from the *Cx. declarator* haplotype (GBDCU1076–14) and 18 bp from the *Cx. dolosus* sequence (GBDCU1054–14). We were unable to retrieve *Cx. conspirator* haplotypes, because these sequences were not public available at the BoldSystems database.

To clarify which *Culex* species, beyond *Cx. quinquefasciatus*, was also circulating in SP, adult mosquitoes were again collected by aspiration in each area and now underwent morphological identification and subsequent DNA barcoding. Traditional taxonomy and DNA barcoding agreed on the co-circulation of *Cx. nigripalpus* in areas 2, 3, and 5 (Table 1, second aspiration). Thirteen COI sequences from *Cx. nigripalpus* (from second aspiration) and five from *Cx. quinquefasciatus* were deposited in the Genbank. COI sequences from *Cx. nigripalpus* have the following accession numbers: from KP211381 to KP211393; *Cx. quinquefasciatus*: from KP211394 to KP211398.

Two different phylogenetic trees were constructed with COI sequences obtained from both *Culex* species (Fig. 3). Both Neighbor-Joining (NJ) and UPGMA trees had the same results, two different clades for *Culex* individuals, with separation between both species supported with high bootstrap values. Among those 13 COI sequences from *Cx. nigripalpus*, DNA polymorphism analyses showed a high number of polymorphic sites (25) and five different haplotypes circulating in SP. A high mtDNA haplotype diversity ($H_d = 0.692 \pm 0.119$) was observed within *Cx. nigripalpus* from SP. Differently, analyses of 161 COI sequences obtained from *Cx. quinquefasciatus* resulted in a low haplotype diversity ($H_d = 0.037 \pm 0.021$), with 3 polymorphic sites and 4 haplotypes.

Discussion

Recife and its metropolitan region remain as the only endemic region for lymphatic filariasis in Brazil (Freitas et al., 2008, Fontes et al., 2012, Aguiar-Santos, et al., 2013). Different factors contribute to this endemism, such as a combination of weather conditions and unplanned urbanization, which result in high population densities of *Cx. quinquefasciatus* all year long (Barbosa & Regis, 2011). This fact causes the need for monitoring the density of vector populations. While attempting to identify areas with high densities of *Cx. quinquefasciatus* for subsequent test of lures, we were surprised that captures in skatole-baited traps were not significantly different from catches in control traps. This was surprising given that skatole in the appropriate doses is indeed an oviposition attractant. To test whether other *Culex* species were present in the area, we collected mosquitoes and used COI sequencing as a marker. COI sequences have been used in taxonomy and phylogenetic studies of *Culex* species from Brazil (Demari-Silva et al., 2011, Laurito et al., 2013). The COI sequencing results of individuals from gravid traps demonstrated that besides *Cx. quinquefasciatus*, *Cx. nigripalpus* is circulating in SP. Because no report so far has been published of *Cx. nigripalpus* in the state, we followed up with a broader survey conducted in different parts of this area using aspirations, and the collected mosquitoes underwent COI barcoding. Results from the first aspiration demonstrated the co-circulation of *Cx. quinquefasciatus* and *Cx. nigripalpus*, in three of six areas studied in the district. Nevertheless, some *Cx. nigripalpus* COI sequences obtained in our study could not be distinguished from COI sequences from other *Culex* species, such as *Cx. dolosus* and *Cx. declarator*, thus resulting in similar alignment scores. This result is in agreement with other studies, which have pointed out limitations of the use of a unique molecular marker to resolve and identify species (Gomes et al., 2003, Alencar et al., 2005, Laporta et al., 2008). Wang et al. (2012) were able to successfully identify 122 species and subspecies of Chinese mosquitoes with a COI barcode approach. These authors concluded that even with

restrictions, DNA barcoding is more reliable than traditional taxonomic methods. However, Laurito et al. (2013), studying *Culex* species from Argentina and Brazil (no sample from northeast region) concluded that the use of the COI barcode itself could not provide sufficient information to distinguish *Culex* species, showing the importance of combined morphological and molecular identification in phylogenetic studies. According to Stockle (2003), the power of DNA barcoding depends on reliable reference sequences from specimens that were previously identified by traditional taxonomy.

To effectively identify this new species circulating in SP, mosquitoes from the second aspiration underwent classical taxonomy, based on the male genitalia, and then underwent COI barcoding. The combination of a classical and a molecular taxonomy approach enabled us to unambiguously identify *Cx. nigripalpus* mosquitoes for the first time in the State of Pernambuco, Brazil. The three areas of SP where *Cx. nigripalpus* was found are characterized by a mixture of dense preserved Atlantic forest segments and human habitations. The area description is in accordance with Forattini (2002), who identified a *Cx. nigripalpus* habitat in residual forests and man-altered environments. Contrary to *Cx. quinquefasciatus* mosquitoes, which have high anthropophilic feeding rates (Samuel et al., 2004), studies have demonstrated that *Cx. nigripalpus* have an opportunistic feeding behavior, with a variety of hosts, including amphibian, bird, mammal, and reptilian (Gomes et al., 2003, Alencar et al., 2005, Laporta et al., 2008).

In both statistical methods used to create the phylogenetic trees, NJ and UPGMA, all of *Cx. nigripalpus* individuals were clustered in a group, well separated from the *Cx. quinquefasciatus* clade. Albeit this is the first report of *Cx. nigripalpus* mosquitoes in the state, the high haplotype diversity found indicates that this species is well established in the area and through competition may be causing a decrease in size of the *Cx. quinquefasciatus* population.

Cx. nigripalpus is now added to the list of mosquito species reported in the State of Pernambuco, namely, *Ae. aegypti*, *Ae. albopictus*, *Cx. quinquefasciatus*, 15 *Anopheles* species, more than 30 *Lutzomyia* species, 2 *Coquillettidia* species, *Haemagogus janthinomys*, *Limatus durhamii*, *Manosonia wilsoni*, 2 *Ochlerotatus* species, *Sabethes tarsopus*, *Trichoprosopon lampropus*, and 6 *Wyeomyia* species (Albuquerque et al., 2000, Balbino et al., 2005, Silva & Vasconcelos 2005, Aragão et al., 2010, Dantas-Torres et al., 2010). The circulation of *Cx. nigripalpus* in Recife raises public health concerns, because it is implicated in the transmission of multiple arboviruses, such as Venezuelan Equine Encephalitis (VEEV), West Nile (WNV), and Saint Louis Encephalitis (SLEV) (Nayar et al., 1986, Mendez et al., 2001, Rutledge et al., 2003, Turell et al., 2005, Vitek et al., 2008). Considering that *Cx. nigripalpus* can be involved in pathogen transmission, is capable of feeding on a variety of hosts, from amphibians to humans (Anderson & Brust, 1995, Christensen et al., 1996, Gomez et al., 2008), and is well adapted to modified human environments (Lourenco-de-Oliveira, 1984), SP provides a suitable location for the establishment of this species due to a combination of human residences in a highly dense vegetation area. Studies performed in Florida have demonstrated that *Cx. nigripalpus* mosquitoes are the major vector of WNV and SLEV (Shroyer, 1991, Rutledge et al., 2003). In Brazil, a study conducted in Mato Grosso demonstrated that the local equine population

has serological evidence of arboviruses implicated in equine and human encephalitis (Pauvolid-Correa et al., 2010, 2014). In addition, the first WNV case in Brazil was reported in a rural area in Piauí, a northeastern state (SESAPI 2014). Knowledge of the mosquito fauna and arbovirus monitoring are key elements in well-organized surveillance programs, providing early warning signs to develop and execute appropriate measures.

Environmental changes resulted from the ongoing urbanization process taking place in SP may favor an increased density of species from sylvatic habitats, leading to colonization and potentially risking human to pathogen transmission. Although *Cx. nigripalpus* has not been implicated in lymphatic filariasis transmission, this mosquito might be epidemiologically important in Recife due to its association with arbovirus transmission, such as Saint Louis encephalitis and Venezuelan equine encephalitis. These viruses are maintained in enzootic and rural epizootic cycles, and environmental modifications observed in SP may provide a new link from a sylvatic to a domestic cycle of human infectious diseases.

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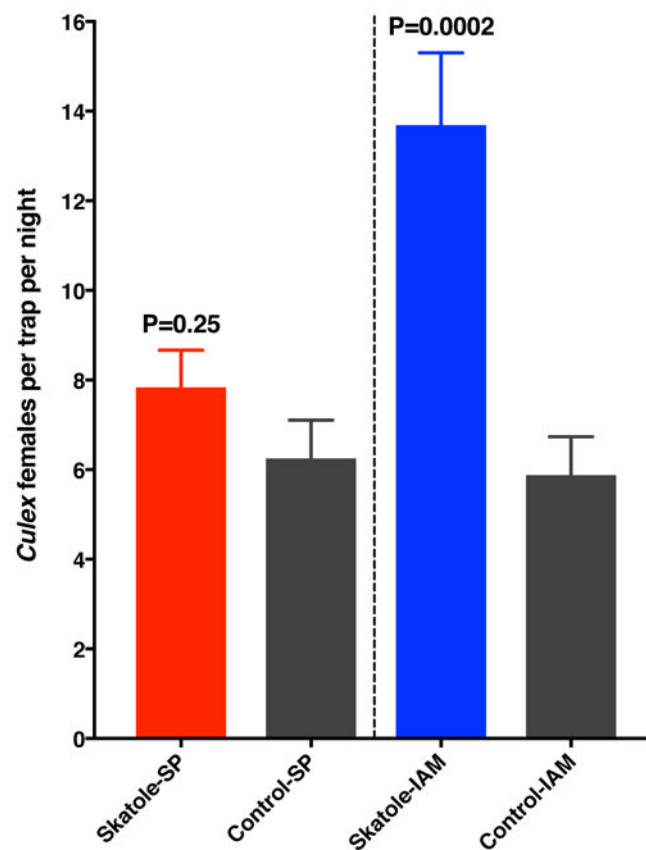
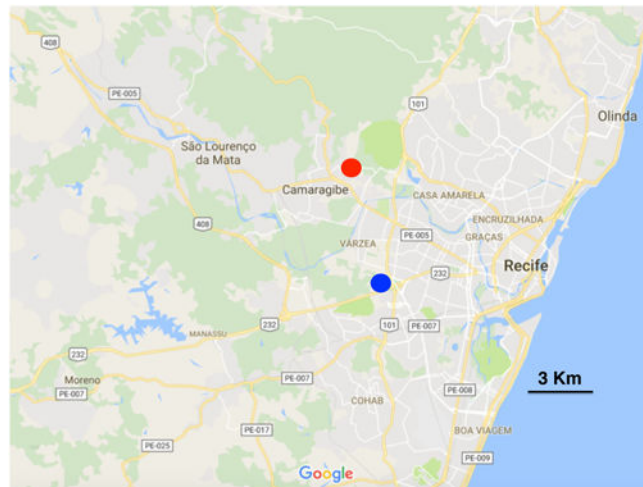


Fig. 1. Trapping of *Culex* mosquitoes in ovitraps baited with skatole in two areas of the metropolitan region of Recife, Brazil, Sítio dos Pintos (SP, red dot in the map) and Cidade Universitária near the Instituto Aggeu Magalhães (IAM, blue dot in the map). Whereas catches of mosquitoes in skatole-baited traps at the Cidade Universitária were significantly higher than in control traps, captures in treatment and control traps in the Sítio dos Pintos were not significantly different. Google Maps; accessed November 5, 2016.

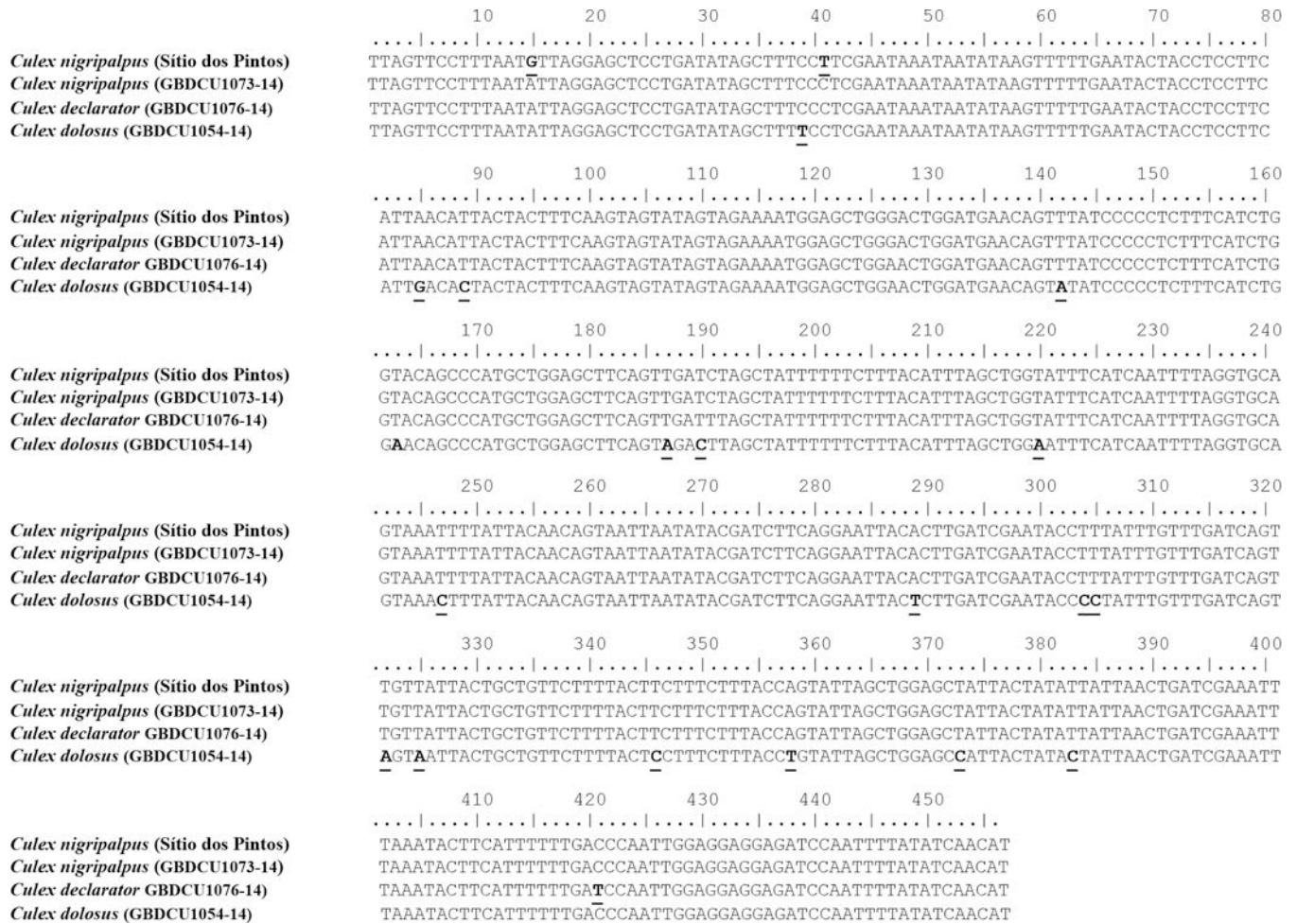


Fig. 2. Alignment of a partial Cytochrome Oxidase subunit I (COI) gene sequence obtained from a *Culex nigripalpus* individual from Sítio dos Pintos with COI sequences from other *Culex* species retrieved from GenBank.

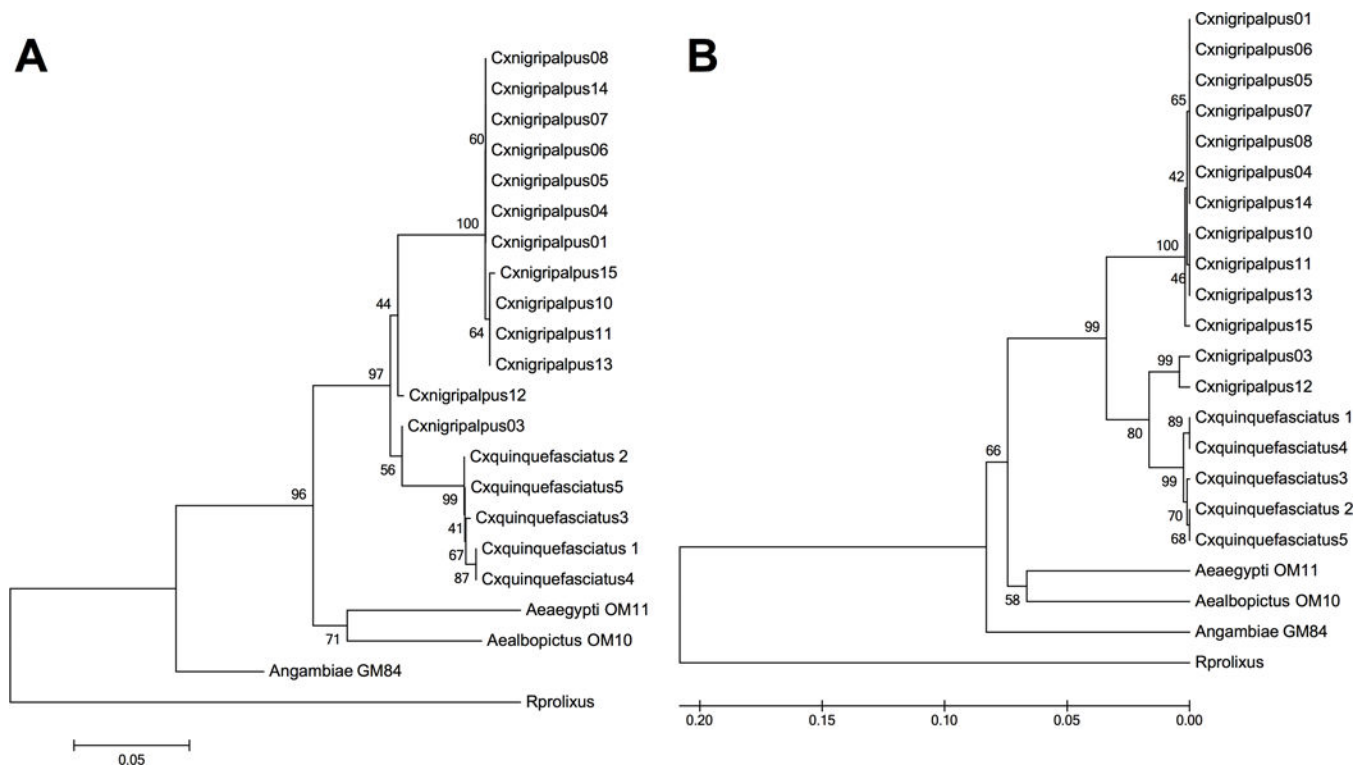


Fig. 3. Phylogenetic trees constructed with (A) Neighbor-Joining and (B) UPGMA based on COI sequences from different mosquitoes and a hemipteran insect, using the Neighbor-Joining method and tested by 10,000 replicates bootstrapping.

Table 1.

Identification of *Culex* mosquitoes obtained by aspirations in Sítio dos Pintos, Recife-PE.

Area	First Collection			Second Collection		
	Total	Cq	Cn	Total	Cq	Cn
1	31	31	0	5	5	0
2	42	40	2	68	57	11
3	23	21	2	71	69	2
4	16	16	0	32	32	0
5	33	30	3	78	76	2
6	27	27	0	71	71	0

Cq = *Cx. quinquefasciatus*; Cn = *Cx. nigripalpus*; Total = Total number of individuals

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