


ORIGINAL ARTICLE

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 cocirculation 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, that is, 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.

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

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 tem-

perate 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

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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, USA) 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} µg/L) (test). Preliminary experiments with 10× lower and 10× 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

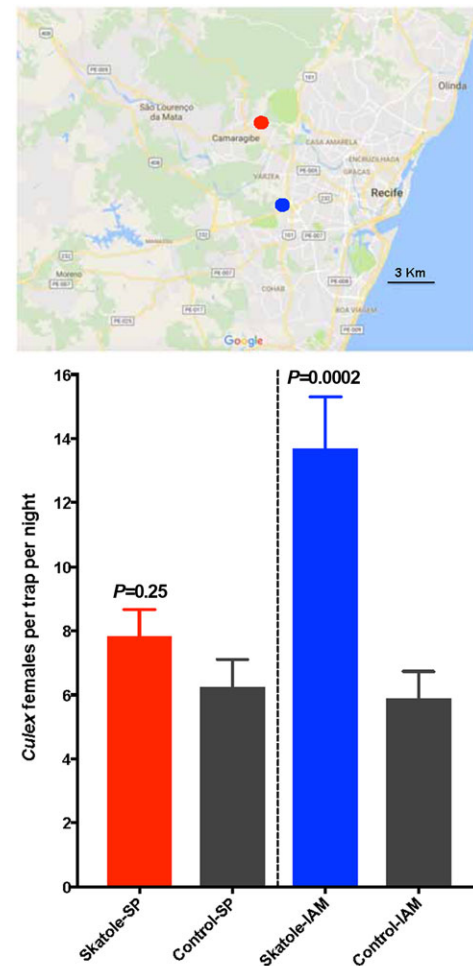


Fig. 1 Trapping of *Culex* mosquitoes in ovitraps baited with skatole in two areas of the metropolitan region of Recife, Brazil, Sítio dos Pintos (SP, red dot in the map) and Cidade Universitária near the Instituto Aggeu Magalhães (IAM, blue dot in the map). Whereas catches of mosquitoes in skatole-baited 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.

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 μ L 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, Cambridge, USA). 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' GGAGGRTTTG-GAAAYTGAYTAGTYCC 3') and COIR (5' GCW-GAWGTAAARTAAGCTCGWGTA 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 mmol/L dNTPs (Invitrogen), 0.4 μ mol/L of each primer, 1.5 mmol/L $MgCl_2$ 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 ethid-

ium bromide and visualized under a UV light. Amplified fragments were purified with GFX PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and sequenced in both directions with an ABI 3500xL sequencer (Applied Biosystems, Foster City, CA, USA). 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 10 000 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 Instituto 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

Table 1 Identification of *Culex* mosquitoes obtained by aspirations in Sítio dos Pintos, Recife-PE.

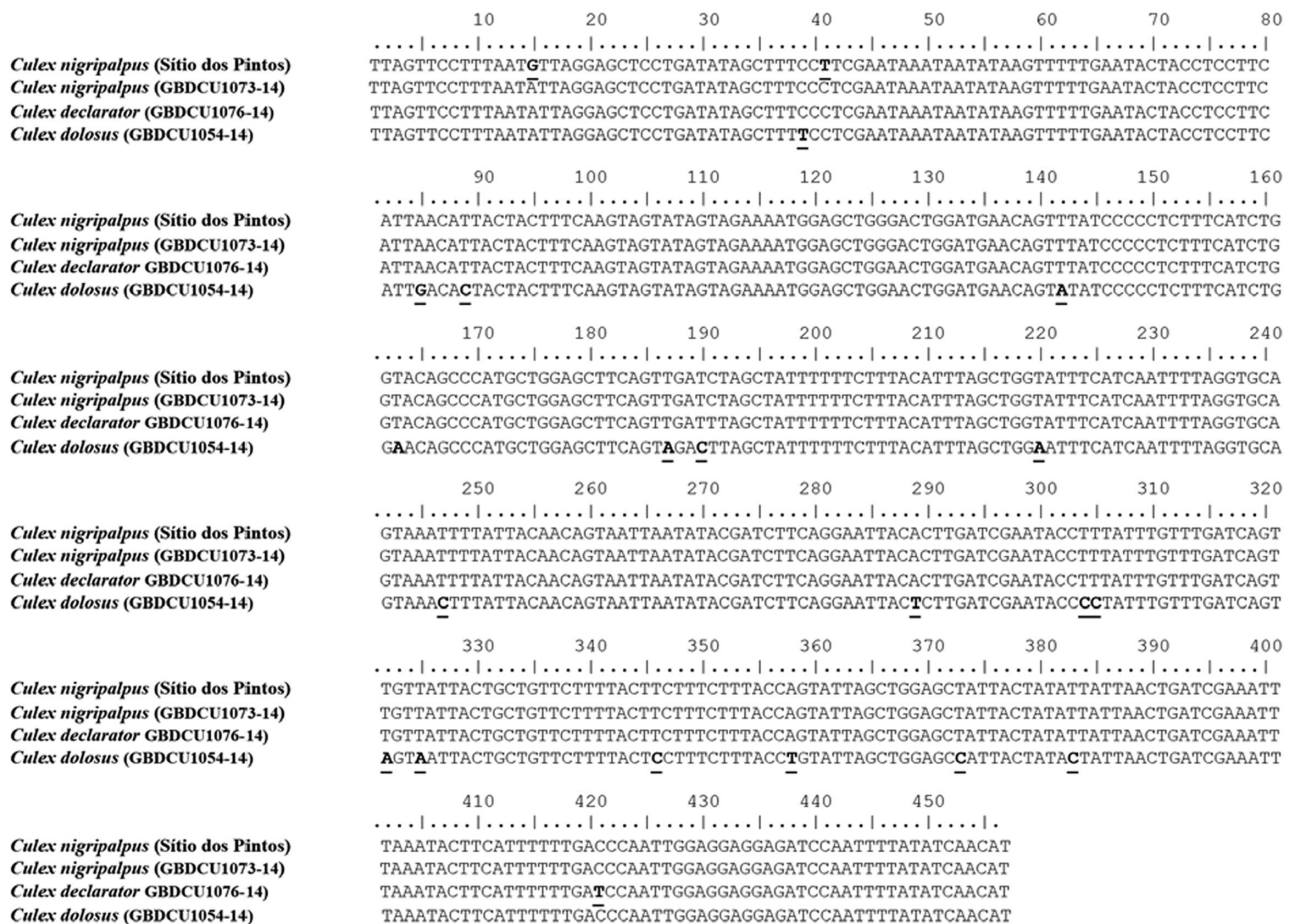
Area	First collection			Second collection		
	Total	Cq	Cn	Total	Cq	Cn
1	31	31	0	5	5	0
2	42	40	2	68	57	11
3	23	21	2	71	69	2
4	16	16	0	32	32	0
5	33	30	3	78	76	2
6	27	27	0	71	71	0

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

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 cocirculation 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

**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.

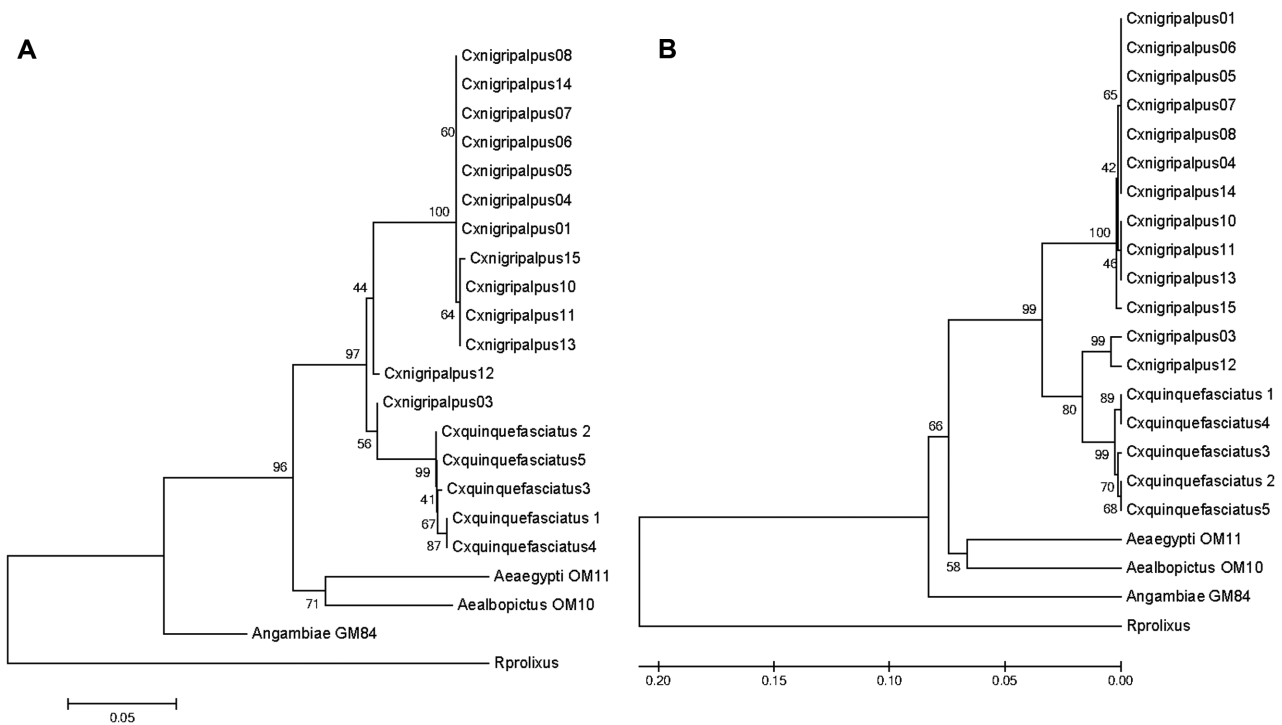


Fig. 3 Phylogenetic trees constructed with (A) Neighbor-Joining and (B) UPGMA based on COI sequences from different mosquitoes and a hemipteran insect, using the Neighbor-Joining method and tested by 10 000 replicates bootstrapping.

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 cocirculation 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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Disclosure

The authors declare that they have no conflict of interest.

References

- Aguiar-Santos, A.M., Medeiros, Z., Bonfim, C., Rocha, A.C., Brandao, E., Miranda, T. *et al.* (2013) Epidemiological assessment of neglected diseases in children: lymphatic filariasis and soil-transmitted helminthiasis. *Jornal de Pediatria*, 89, 250–255.
- Albuquerque, C.M.R., Melo-Santos, M.A.V., Bezerra, M.A.S., Barbosa, R.M., Silva, D.F. and Silva, E. (2000) Primeiro registro de *Aedes albopictus* em área da Mata atlântica, Recife, PE, Brasil. *Revista de Saúde Pública*, 34, 314–315.
- Alencar, J., Lorosa, E.S., Silva, J.S., Lopes, C.M. and Guimarães, A.E. (2005) Observações sobre padrões alimentares de mosquitos (Diptera: Culicidae) no Pantanal Mato-Grossense. *Neotropical Entomology*, 34, 681–687.
- Anderson, R.A. and Brust, R.A. (1995) Field evidence for multiple host contacts during blood feeding by *Culex tarsalis*, *Cx. restuans*, and *Cx. nigripalpus* (Diptera: Culicidae). *Journal of Medical Entomology*, 32, 705–710.
- Aragão, N.C., Muller, G.A., Balbino, V.Q., Costa Junior, C.R., Figueiredo Junior, C.S., Alencar, J. *et al.* (2010) A list of mosquito species of the Brazilian State of Pernambuco, including the first report of *Haemagogus janthinomys* (Diptera: Culicidae), yellow fever vector and 14 other species (Diptera: Culicidae). *Revista da Sociedade Brasileira de Medicina Tropical*, 43, 458–459.
- Balbino, V.Q., Coutinho-Abreu, I.V., Sonoda, I.V., Marques da Silva, W. and Marcondes, C.B. (2005) Phlebotomine sandflies (Diptera: Phlebotomidae) of the Atlantic forest in Recife, Pernambuco state, Brazil: the species coming to human bait, and their seasonal and monthly variations over a 2-year period. *Annals of Tropical Medicine and Parasitology*, 99, 683–693.
- Barbosa, R.M. and Regis, L.N. (2011) Monitoring temporal fluctuations of *Culex quinquefasciatus* using oviposition traps containing attractant and larvicide in an urban environment in Recife, Brazil. *Memórias do Instituto Oswaldo Cruz*, 106, 451–455.
- Christensen, H.A., Vazquez, A.M. and Boreham, M.M. (1996) Hostfeeding patterns of mosquitoes (Diptera: Culicidae) from Central Panama. *American Journal of Tropical Medicine Hygiene*, 55, 202–208.
- Dantas-Torres, F., de Andrade, A.J., Tenorio, K.E., Andrade Filho, J.D., Balbino Vde, Q. and Brandao-Filho, S.P. (2010) Phlebotomine sand flies (Diptera: Phlebotomidae) in the State of Pernambuco. *Revista da Sociedade Brasileira de Medicina Tropical*, 43, 733–736.
- Demari-Silva, B., Vesgueiro, F.T., Sallum, M.A. and Marrelli, M.T. (2011) Taxonomic and phylogenetic relationships between species of the genus *Culex* (Diptera: culicidae) from Brazil inferred from the cytochrome c oxidase I mitochondrial gene. *Journal of Medical Entomology*, 48, 272–279.
- Fontes, G., Braun, R.F., Fraiha Neto, H., Vieira, J.B., Padilha, S.S., Rocha, R.C. *et al.* (2005) Lymphatic filariasis in Belem, Para State, North of Brazil and the perspective of elimination. *Revista da Sociedade Brasileira de Medicina Tropical*, 38, 131–136.
- Fontes, G., Leite, A.B., de Lima, A.R., Freitas, H., Ehrenberg, J.P. and da Rocha, E.M. (2012) Lymphatic filariasis in Brazil: epidemiological situation and outlook for elimination. *Parasite and Vectors*, 5, 272.
- Forattini, O.P. (2002) *Culicidologia Médica*. São Paulo, Brazil, EduSP.
- Freitas, H., Vieira, J.B., Braun, R., Medeiros, Z., Rocha, E.M., Aguiar-Santos, A. *et al.* (2008) Workshop to evaluate the epidemiologic situation of lymphatic filariasis in the Municipality of Belém, Pará, Northern Brazil. *Revista da Sociedade Brasileira de Medicina Tropical*, 41, 212–216.
- Gomes, A.C., Silva, N.N., Marques, G.R. and Brito, M. (2003) Host-feeding patterns of potential human disease vectors in the Paraíba Valley region, State of Sao Paulo, Brazil. *Journal of Vector Ecology*, 28, 74–78.
- Gomez, A., Kilpatrick, A.M., Kramer, L.D., Dupuis, A.P. 2nd, Maffei, J.G., Goetz, S.J. *et al.* (2008) Land use and west nile virus seroprevalence in wild mammals. *Emerging Infectious Diseases*, 14, 962–965.
- Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.
- Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D. *et al.* (2003) Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerging Infectious Diseases*, 9, 311–322.
- Laporta, G.Z., Crivelaro, T.B., Vicentin, E.C., Amaro, P., Brancquino, S.M. and Sallum, M.A.M. (2008) *Culex nigripalpus* Theobald (Diptera: Culicidae) feeding habitat at the Parque Ecológico do Tietê, São Paulo. Brazil. *Revista Brasileira de Entomologia*, 54, 663–668.
- Laurito, M., Oliveira, T.M., Almiron, W.R. and Sallum, M.A. (2013) COI barcode versus morphological identification of

- Culex* (*Culex*) (Diptera: Culicidae) species: a case study using samples from Argentina and Brazil. *Memórias do Instituto Oswaldo Cruz*, 108(Suppl 1), 110–122.
- Lourenco-de-Oliveira, R. (1984) Alguns aspectos da ecologia dos mosquitos (Diptera: Culicidae) de uma área de planície (Granjas Calábria), em Jacarepaguá, Rio de Janeiro. I. Frequência comparativa das espécies em diferentes ambientes e métodos de coleta. *Memórias do Instituto Oswaldo Cruz*, 79, 479–490.
- Mendez, W., Liria, J., Navarro, J.C., Garcia, C.Z., Freier, J.E., Salas, R. et al. (2001) Spatial dispersion of adult mosquitoes (Diptera: Culicidae) in a sylvatic focus of Venezuelan equine encephalitis virus. *Journal of Medical Entomology*, 38, 813–821.
- Nayar, J.K., Rosen, L. and Knight, J.W. (1986) Experimental vertical transmission of Saint Louis encephalitis virus by Florida mosquitoes. *American Journal of Tropical Medicine and Hygiene*, 35, 1296–1301.
- Olagbemiro, T.O., Birkett, M.A., Mordue Luntz, A.J. and Pickett, J.A. (2004) Laboratory and field responses of the mosquito, *Culex quinquefasciatus*, to plant-derived *Culex* spp. oviposition pheromone and the oviposition cue skatole. *Journal of Chemical Ecology*, 30, 965–976.
- Pauvolid-Correa, A., Campos, Z., Juliano, R., Velez, J., Nogueira, R.M. and Komar, N. (2014) Serological evidence of widespread circulation of West Nile virus and other flaviviruses in equines of the Pantanal, Brazil. *PLoS Neglected Tropical Diseases*, 8(2), e2706.
- Pauvolid-Correa, A., Tavares, F.N., Costa, E.V., Burlandy, F.M., Murta, M., Pellegrin, A.O. et al. (2010) Serologic evidence of the recent circulation of Saint Louis encephalitis virus and high prevalence of equine encephalitis viruses in horses in the Nhecolândia sub-region in South Pantanal, Central-West Brazil. *Memórias do Instituto Oswaldo Cruz*, 105, 829–833.
- Regis, L., Oliveira, C.M., Silva-Filha, M.H., Silva, S.B., Maciel, A. and Furtado, A.F. (2000) Efficacy of *Bacillus sphaericus* in control of the filariasis vector *Culex quinquefasciatus* in an urban area of Olinda, Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 94, 488–492.
- Regis, L., Silva-Filha, M.H., de Oliveira, C.M., Rios, E.M., da Silva, S.B. and Furtado, A.F. (1995) Integrated control measures against *Culex quinquefasciatus*, the vector of filariasis in Recife. *Memórias do Instituto Oswaldo Cruz*, 90, 115–119.
- Rozas, J. and Rozas, R. (1995) DnaSP, DNA sequence polymorphism: an interactive program for estimating population genetics parameters from DNA sequence data. *Computational and Applied Biosciences*, 11, 621–625.
- Rudbeck, L. and Dissing, J. (1998) Rapid, simple alkaline extraction of human genomic DNA from whole blood, buccal epithelial cells, semen and forensic stains for PCR. *Biotechniques*, 25, 588–590, 592.
- Rutledge, C.R., Day, J.F., Lord, C.C., Stark, L.M. and Tabachnick, W.J. (2003) West Nile virus infection rates in *Culex nigripalpus* (Diptera: Culicidae) do not reflect transmission rates in Florida. *Journal of Medical Entomology*, 40, 253–258.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406–425.
- Samuel, P.P., Arunachalam, N., Hiriyani, J., Thenmozhi, V., Gajanana, A. and Satyanarayana, K. (2004) Host-feeding pattern of *Culex quinquefasciatus* Say and *Mansonia annulifera* (Theobald) (Diptera: Culicidae), the major vectors of Filariasis in a rural area of South India. *Journal of Medical Entomology*, 41, 442–446.
- SESAPI (2014) Secretaria de Saúde de Piauí. Retrieved 18/12/2014, 2014, from <http://www.saude.pi.gov.br/noticias/2014-12-09/6102/coletiva-reune-imprensa-para-esclarecer-primeiro-caso-de-febre-do-nilo.html>.
- Shroyer, D.A. (1991) The 1990 Florida epidemic of St. Louis encephalitis: virus infection rates in *Culex nigripalpus*. *Journal of the American Mosquito Control Association*, 62, 69–71.
- Silva, D.F. and Vasconcelos, S.D. (2005) Flebotomíneo em fragmentos de Mata Atlântica na Região Metropolitana do Recife, PE. *Revista da Sociedade Brasileira de Medicina Tropical*, 38, 264–266.
- Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy: The Principles and Practice of Numerical Classification*. W H Freeman & Co., San Francisco.
- Stoeckle, M. (2003) Taxonomy, DNA, and the bar code of life. *BioScience*, 53, 796–797.
- Turell, M.J., Dohm, D.J., Sardelis, M.R., Oguinn, M.L., Andreadis, T.G. and Blow, J.A. (2005) An update on the potential of north American mosquitoes (Diptera: Culicidae) to transmit West Nile Virus. *Journal of Medical Entomology*, 42, 57–62.
- Vitek, C.J., Richards, S.L., Mores, C.N., Day, J.F. and Lord, C.C. (2008) Arbovirus transmission by *Culex nigripalpus* in Florida, 2005. *Journal of Medical Entomology*, 45, 483–493.
- Wang, G., Li, C.X., Guo, X.X., Xing, D., Dong, Y.D., Wang, Z.M. et al. (2012) Identifying the main mosquito species in China based on DNA barcoding. *PLoS ONE*, 7(10), e47051.

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