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Scientific Note

Detection of *Culex flavivirus* (*Flaviviridae*) from a natural *Culex* (*Culex*) *chidesteri* Dyar, 1921 population, Caatinga Biome, Semiarid Scrubland, Brazil

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Abstract. The first detection of *Culex flavivirus* (CxFV) in mosquitoes was described in 2007 in Japan and subsequently in different areas of the Americas, Africa, and Asia. In this study, we report the identification of CxFV in *Culex* (*Culex*) *chidesteri* Dyar, 1921 mosquitoes collected in the Açu National Forest - FLONA/ AÇU-RN, a preserved area of Biome Caatinga, State of Rio Grande do Norte, Brazil. We performed nucleotide-sequencing analysis in order to compare with other previously identified CxFV collected from around the world. A total of 129 samples (pools of ≤30 female mosquitoes) were processed for generic reverse transcriptase PCR assay. CxFV infection was identified in only two pools from *Culex chidesteri*. Our phylogenetic analysis revealed that the AssuCxFV identified in this study belongs to the Africa/Caribbean/South America Subtype. Our study represents the first report of the CxFV from a natural *Culex chidesteri* population.

Keywords: Arbovirus; *Flaviviridae;* Mosquitoes, Açu National Forest, Culicidae.

According to the IXth Report of the International Committee on Taxonomy of Viruses, the genus *Flavivirus* (family *Flaviviridae*) contains 53 species of which 40 are known to cause human disease. Flaviviruses are small (~50 nm), enveloped, single-strand positive-sense RNA viruses. Phylogenetic analysis has classified the genus *Flavivirus* into mosquito-borne, tick-borne and viruses that are insect specific and do not infect vertebrates (Kuno & Chang 2005; Goenaga et al. 2014). This final group includes cell fusion agent virus (Stollar & Thomas 1975), *Culex flavivirus* (CxFV) (Hoshino et al. 2007), *Aedes flavivirus* (Hoshino et al. 2009), *Nakiwogo virus* (Cook et al. 2009), *Chaoyang virus* (Wang et al. 2009) and others.

CxFV was described and isolated for the first time from *Culex pipiens* s.l. Linnaeus, 1758 in 2007 in Japan and subsequently in different areas of the Americas (Hoshino et al. 2007; Kim et al. 2009; Machado et al. 2012; Goenaga et al. 2014; Moraes et al. 2019), Africa (Cook et al. 2009), and Asia (Chen et al. 2013). In this study, we report the identification of CxFV from *Culex* (*Culex*) *chidesteri* Dyar, 1921 mosquitoes collected in the Açu Nacional Forest - FLONA/ AÇÚ-RN, a semiarid scrubland preserved area of Caatinga Biome, State of Rio Grande do Norte, Brazil. Additionally, we perform the nucleotide sequencing analysis in order to compare with other previously identified CxFV collected worldwide.

This work was developed in FLONA/ AÇÚ-RN, located in the southwest of the urban site of Assu (05°34'20"S, 36°54'33"W), in the central region of the state of Rio Grande do Norte), an area of 215.25 hectares, with a perimeter of 6766.30 meters (Fig. 1). Mosquitoes were sampled monthly in the twilight and in the beginning of the night periods (17h-20h) between September 2011 to August 2013 using a Shannon trap, which consisted of a large central compartment and two smaller lateral ones with a central light. Mosquitoes were killed by CO_2 freezing, transferred to collection tubes, and stored at -70°C until identification and pooling. Adult mosquitoes were identified by their morphological characteristics and pooled according to species, sex, location, and date. The identifications were carried out with the morphological keys of Forattini (2002) and the classification adopted for Aedini tribe was proposed by Wilkerson et al. (2015). Pools of ≤ 30 female mosquitoes (for each species) were macerated by using plastic pistils in 500 µL of Leibowitz L15 medium (GIBCO-BRL, Gaithersburg, MD, USA) containing 2% fetal bovine serum, and centrifuged at 2500× g for 20 min at 4°C, to pellet the carcasses. The supernatant was split into 2 aliquots and stored at -70°C until use. Viral RNA was extracted from 140 µL original suspension of mosquitoes by the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA), in accordance with the manufacturer's suggested protocol. RNA was eluted in 60 µL of buffer AVE.

The generic RT-PCR protocol for *Flavivirus* detection was used for testing these RNA extracts as described previously by Sánchez-Seco et al. (2005). Degenerated primers were designed based on conserved motifs in a region of the NS5 gene. Positive controls tested for dengue virus serotypes 1, 2, 3 and 4 (obtained from Laboratory of Molecular Biology for Infectious Diseases and Cancer, UFRN), and yellow fever virus (17D) (obtained from Biomanguinhos, Fiocruz, Brazil). Products from the second round of generic amplification were purified by using the PCR purification kit or gel extraction kit (Qiagen, US). The quantification of the purified DNA was performed by electrophoresis on a 2% agarose gel, using the kit "low mass DNA" (Invitrogen, Carlsbad, CA). Sequencing reactions on both strands were performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, US), and analyzed by using an ABI Prism 3730 Sequencer (Applied Biosystems, US). Electropherograms were visualized by Chromas

software version 1.45 (Technelysium Pty. Ltd., Queensland, Australia. Available at http://technelysium.com.au/wp/chromas/). Nucleotide sequences were aligned and analyzed by using the Clustal x program (Thompson et al. 1997) and later edited manually. All alignments are available on request. Representative sequences from CxFV were retrieved from Genbank (http://ww.ncbi.nlm.nih.gov), accession numbers MZ397994 and MZ397995. A sequence from the *Nakiwogo virus* (Accession No GQ165809) was used as outgroup. A phylogenetic tree was constructed using MEGA 6 software (Tamura et al. 2013), using the maximum-likelihood method and the K2+G+I model. The jModelTest software was used to choose the best evolutionary model (Posada 2008). A bootstrap of 1000 replications was used to estimate the reliability of the predicted tree.

A total of 129 samples (pools of ≤ 30 female mosquitoes) were processed for generic reverse transcriptase PCR assay: *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1894) (n=17), *Mansonia* (*Mansonia*) *wilsoni* (Barreto and Coutinho, 1944) (n=81), *Mansonia* (*Mansonia*) *indubitans* Dyar and Shannon, 1925 (n=1), *Aedeomyia* (*Aedeomyia*) *squamipennis* (Lynch Arribalzaga, 1878) (n=1), *Anopheles* (*Nyssorhynchus*) *albitarsis* s.l Lynch Arribalzaga, 1878 (n=1), *Aedes* (*Ochlerotatus*) *scapularis* (Rondoni, 1848) (n=4), *Haemagogus* (*Haemagogus*) *spegazzinii* Brèthes, 1912 (n=6), *Culex* spp. (n=14), *Culex chidesteri* (n=2), and *Mansonia* (*Mansonia*) *humeralis* Dyar and Knab, 1916 (n=2).

The CxFV infection was identified in only two pools from *Culex chidesteri*, the first pool with five females and the second pool with 10. All positive controls tested by generic RT-PCR (DENV1-4 and YFV) resulted in positive amplification. Double-stranded DNA products amplified in the generic RT-PCR (143bp from the NS5 gene) were directly sequenced. The obtained sequences were submitted to the "Standard Nucleotide BLAST" (BLASTN) program (http://blast.ncbi.nlm.nih.gov/) (Altschul et al. 1990), using the following parameters: database = others (nr etc.), optimize for = highly similar sequences (megablast). The BLASTN result revealed that the flavivirus identified in this study is a *Culex flavivirus*, with 95% identity with the CxFV identified in Uganda in 2008 (max score: 206; total score: 206; query cover: 88%; E value: 9e-50). The identities among the AssuCxFV and other Africa/Caribbean/Latin America subtype strains range from 91% to 98.6% for nucleic acid and 91.8% to 100% for amino acid sequences.

Partial NS5 gene nucleic acid and amino acid sequence identities among the Africa/Caribbean/Latin America subtype strains and the Asia/U.S. range from 88.3% to 95.8% and 91.8% to 100%, respectively (Tab. 1). The phylogenetic tree based on partial NS5 gene of AssuCxFV (143bp) was generated using the maximum-likelihood method (Fig. 2). Our phylogenetic analysis revealed that the AssuCxFV identified in this study belongs to the Africa/Caribbean/South America Subtype (Fig. 1). The minimum infection rate (MIR) was not calculated because <1,000 mosquitoes were collected. Additional studies are necessary to know the genetic diversity of CxFV in Brazil and in the world. Knowledge of the genetic diversity of CxFV may provide a basis for understanding the evolution of Flaviviruses and the development of biotechnological tools.

Our study represents the first report of the CxFV from a natural *Culex chidesteri* population. CxFV strains have been isolated from many species of mosquito including *Culex* (*Culex*) *maxi* Dyar, 1928, *Culex* (*Culex*) *nigripalpus* Theobald, 1901, *Culex* (*Culex*) *usquatus* Dyar, 1918, *Culex pipiens s.l* Linnaeus, 1758, *Culex* (*Culex*) *tarsalis* Coquillett, 1986, *Culex* (*Culex*) *quinquefasciatus* Say, 1983, *Culex* (*Culex*) *tritaeniorhynchus* Giles, 1901, *Culex* (*Culex*) *restuans* Theobald, 1901, and *Culex* (*Culex*) *interrogator* Dyar and Knab, 1906 (Kuno & Chang 2005; Hoshino et al. 2007; Blitvich et al. 2009; Kim et al. 2009; Saiyasombat et al. 2010). These findings indicate that CxFV occurs in numerous *Culex* species. In this manuscript we report the first identification of CxFV from a semiarid area in the Northeast Region, Brazil. CxFV was first identified in Brazil and in South America in 2008 in the city of São José do Rio Preto in female *Culex* sp. mosquitoes (Machado et al. 2012). Fernandes et al. (2016) reported the first identification of the CxFV in the city of São Paulo and Moraes et al. (2019) reported the first identification of the CxFV in naturally infected *Culex quinquefasciatus* females in Mato Grosso, Middle-West Brazil.

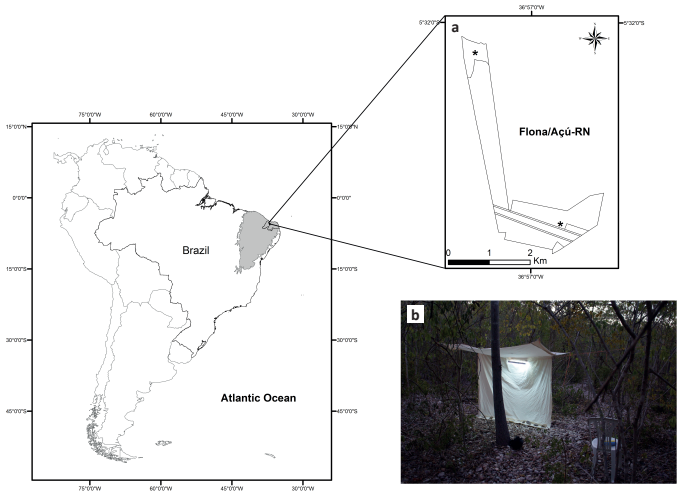

Figure 1. Location of the Açu National Forest (FLONA-Açu) in the Caatinga biome, Rio Grande do Norte, Brazil. a) FLONA-Açu (*collection points); b) view of the collection area and trap used.

Other insect-specific flaviviruses have been identified in Brazil. Kenney et al. (2014) isolated a novel flavivirus (designated '*Nhumirim virus*'; NHUV) that represents an example of a unique subset of apparently insect-specific viruses that phylogenetically affiliate with dual-host mosquito-borne flaviviruses despite appearing to be limited to replication in mosquito cells. In another study, Pauvolid-Corrêa et al. (2015) describe the isolation of NHUV, isolated from a pool of mosquitoes identified as *Culex* (*Culex*) *chidesteri* collected in 2010 from the Pantanal region of central west Brazil.

In this study, the phylogenetic tree based on partial NS5 gene sequences revealed that the AssuCxFV belongs to Africa/Caribbean/Latin America subtype (also called genotype 2), closely related to Uganda, México, and Taiwan isolates. In the same way, Chen et al. (2013) show that the Taiwan isolates are closely related to the Africa/Caribbean/Latin America subtype, although they form an independent

Table 1. The identities of nucleic acid and deduced amino acid (in bold) sequences of CxFV partial NS5 gene.

		Africa/Caribbean/Latin American					Asia/U.S.			
		1	2	3	4	5	6	7	8	9
1	R33 CxFVAssu RN Brazil 2013	-	**100**	**91.8**	**91.8**	**91.8**	**91.8**	**91.8**	**91.8**	**83.6**
2	R34 CxFVAssu RN Brazil 2013	100	-	**91.8**	**91.8**	**91.8**	**91.8**	**91.8**	**91.8**	**83.6**
3	GQ165808 CxFVUganda 2008	91	91	-	**100**	**100**	**100**	**100**	**100**	**89.7**
4	EU879060 CxFVMexico 2007	91	91	98.6	-	**100**	**100**	**100**	**100**	**89.7**
5	JX897904 CxFV Taiwan 2010	91	91	95.8	95.8	-	**100**	**100**	**100**	**89.7**
6	HQ634596 CxFV US 2009	89.7	89.7	94.5	94.5	95.8	-	**100**	**100**	**89.7**
7	AB377213 CxFV Japan_2003	89.7	89.7	94.5	94.5	95.8	100	-	**100**	**89.7**
8	AB701766 CxFVToyama 2004	88.3	88.3	93.1	93.1	95.8	98.6	98.6	-	**89.7**
9	GQ165809 Nakiwogovirus Uganda2008	76	76	80.8	80.8	78.7	80.8	80.8	81.5	-

cluster, based on full-length, E gene, NS3 gene, or NS5 gene sequences. Must flaviviruses can be classified into multiple genotypes/subtypes/lineage using the criteria of 6% nucleotide variation between genotypes (Kuno et al. 1998; Chen et al. 2013). In our study, the nucleotide distance between Africa/Caribbean/Latin America and Asia/U.S. subtypes range from 4.2% to 11.7%. Additional studies including those isolated from other parts of the world are essential for a better understanding of the evolutionary history of CxFV.

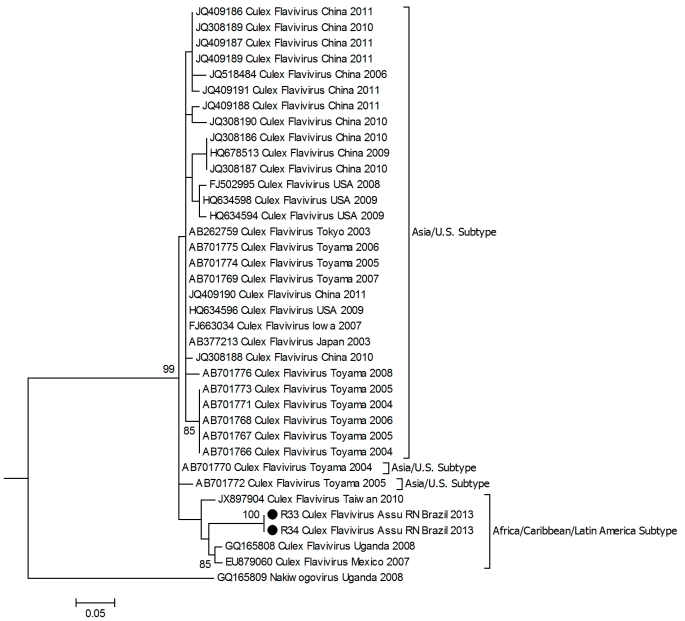

Figure 2. Maximum-likelihood (ML) phylogenetic tree of 37 flavivirus sequences (36 CxFV and 1 Nakiwogo virus - outgroup) using 143 nucleotides from the NS5 gene. Strains are denoted by accession numbers from Genbank or description, place and year of identification. Subtypes are indicated by brackets. Horizontal branch lengths are drawn to scale. Bootstrap values of statistical support for major branches are shown as percentage equivalents.

Finally, our findings suggest that CxFV may be widespread in *Culex* mosquitoes' populations in the Northeast Region of Brazil and could have a relevant role in the evolution of Flaviviruses. Thus, further collection and evaluation of CxFV samples are necessary to gain further insight into the evolutionary history of the virus and the host range.

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Authors' Contributions

Conceived and designed the project: RLC, RAG, JMGA. Collected field samples: RAG, RCMF, MSDB. Performed the lab work: JMGA, RCMF, DMPC, DMCS, DMCS, JDM, MSDB, KJSF. Analyzed the data: RLC, RAG, JMGA, TMB, JVF. Wrote the paper: JMGA. Critically revised manuscript: KJSF, RLC, JVF, TMB, RAG. All authors read and approved the final version of the manuscript.

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