Culex Flavivirus and West Nile Virus in *Culex quinquefasciatus* Populations in the Southeastern United States

REBEKAH KENT CROCKETT,[1,2] KRISTEN BURKHALTER,[1] DANIEL MEAD,[3] ROSMARIE KELLY,[4] JEFFREY BROWN,[5] WENDY VARNADO,[5] ALMA ROY,[6] KALANTHE HORIUCHI,[1] BRAD J. BIGGERSTAFF,[1] BARRY MILLER,[1] AND ROGER NASCI[1]

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ABSTRACT Little is known of the interactions between insect-only flaviviruses and other arboviruses in their mosquito hosts, or the potential public health significance of these associations. The specific aims of this study were to describe the geographic distribution, prevalence, and seasonal infection rates of Culex flavivirus (CxFV) and West Nile virus (WNV) in *Culex quinquefasciatus* Say in the Southeastern United States, investigate the potential association between CxFV and WNV prevalence in *Cx. quinquefasciatus* and describe the phylogenetic relationship among CxFV and WNV isolates from the Southeastern United States and around the world. Using ArboNET records, 11 locations were selected across Georgia, Mississippi, and Louisiana that represented a range of WNV human case incidence levels. *Cx. quinquefasciatus* were trapped weekly throughout the summer of 2009 and pools were screened for flavivirus RNA by reverse transcriptase polymerase chain reaction. *Cx. quinquefasciatus* from Georgia had significantly higher CxFV infection rates than either Mississippi or Louisiana. CxFV was not detected in Mississippi after July, and no CxFV was detected in *Cx. quinquefasciatus* in Louisiana. In Georgia, CxFV infection rates were variable between and within counties and over time. WNV infection rates were not significantly different across states or months, and WNV sequences from all three states were identical to each other in the envelope and NS5 gene regions. Phylogenetically, NS5 and E gene sequences from Georgia CxFV isolates clustered with CxFV from Japan, Iowa, and Texas. Multiple CxFV genetic variants were found circulating simultaneously in Georgia. No evidence was found supporting an association between WNV and CxFV infection prevalence in *Cx. quinquefasciatus*.

KEY WORDS Culex flavivirus, insect-only flavivirus, *Culex quinquefasciatus*, West Nile virus

Strains of Culex flavivirus (CxFV) and related insect-only flaviviruses have been discovered in mosquitoes worldwide (e.g., Hoshino et al. 2007, Morales-Betoulle et al. 2008, Kim et al. 2009, Blitvich et al. 2009). These insect-only flaviviruses appear to be transmitted solely among their invertebrate hosts, as replication does not occur in vertebrate cells (Sang et al. 2003, Hoshino et al. 2007). Circulation of insect-only flaviviruses in natural mosquito populations is likely maintained by vertical transmission (Sang et al. 2003, Lutomiah et al. 2007). While there has been extensive genetic and phenotypic characterization of insect-only flaviviruses, little is known of the interactions between these and other arboviruses within individual mosquito hosts or mosquito populations, or the potential public health significance of these associations.

Few data exist regarding the effect of insect-only flaviviruses on infection or transmission of a secondary-infecting arbovirus by the arthropod host. CxFV Izabal strain did not affect the vector competence of *Cx. quinquefasciatus* for transmitting West Nile virus (WNV) when mosquitoes were infected sequentially (Kent et al. 2010), however, more data are necessary as the effect of insect-only flaviviruses on vector competence may vary across virus species or strains and mosquito species or populations. The efficiency of transmission (vectorial capacity) also depends on variety of additional parameters including mosquito relative abundance, biting behavior, longevity, and the length of the extrinsic incubation period (Garrett-Jones and Shidrawi 1969). Previous studies have documented both positive and negative effects of arboviruses on various mosquito life history parameters and behaviors. Styer et al. (2007) found that WNV infection significantly decreased fecundity of *Cx. tar-*

[1] Centers for Disease Control and Prevention, Division of Vector-borne Diseases, Arbovirus Diseases Branch, Fort Collins, CO 80521.
[2] Corresponding author, e-mail: fxk7@cdc.gov.
[3] Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA.
[4] Georgia Division of Public Health Epidemiology Branch, Atlanta, GA.
[5] Environmental Services, Mississippi State Department of Health, Jackson, MS.
[6] Louisiana Veterinary Medical Diagnostic Laboratory, Louisiana State University, Baton Rouge, LA.

salis Coquillett, but increased blood feeding rate. Mahmood et al. (2004) found that *Cx. tarsalis* infected with Western equine encephalitis virus had significantly lower life expectancy, lower reproductive effort per generation, and shorter generation times compared with uninfected controls. Eastern equine encephalitis virus decreased the survival and reproduction of *Culiseta melanura* Coquillett (Scott and Lorenz 1998). *Aedes aegypti* L. infected with dengue three virus had significantly longer feeding times than uninfected controls. This behavior increased the potential for the blood meal to be interrupted, prompting infected mosquitoes to feed and probe more often on additional hosts (Platt et al. 1997). Adjusting parameters that affect mosquito abundance, rate of contact with susceptible vertebrate hosts, or vector competence results in linear changes to transmission efficiency, whereas changes in mosquito longevity affects vectorial capacity in an exponential manner (Garrett-Jones and Shidwari 1969).

The Southeastern United States has experienced a gradient in the prevalence of West Nile virus neuroinvasive disease (WNND) since the introduction of this virus (Lindsey et al. 2008). Georgia has experienced relatively little severe human disease attributable to WNV infection (1.0–4.9 cases per 100,000), as compared with Mississippi and Louisiana that have experienced relatively high levels of WNND (10.0–14.9 cases per 100,000) (Lindsey et al. 2008). *Cx. quinquefasciatus* is a major WNV vector throughout the Southeastern United States (e.g., Godsey et al. 2005, Molaei et al. 2007, Makay et al. 2010). To date there has been no exploration into whether CxFV or other insect-only flaviviruses are present in *Cx. quinquefasciatus* in the Southeastern United States, or if prevalence of insect-only flaviviruses may be one reason underlying the difference in the intensity of WNV disease observed in this geographic area.

Therefore, we sought to determine if there was any evidence in the field for WNV infection rates in *Cx. quinquefasciatus* being affected by the prevalence of CxFV. Should CxFV infection prevalence affect mosquito life expectancy, reproduction, or behavior, these interactions may be reflected by an association between CxFV and WNV infection rates in wild-caught mosquitoes. The specific aims of this study were to (1) describe the geographic distribution, local prevalence, and seasonal distribution of CxFV and WNV in *Cx. quinquefasciatus* in the Southeastern United States, (2) investigate the potential association between CxFV prevalence and WNV infection rates in *Cx. quinquefasciatus*, (3) describe the phylogenetic relationship among CxFV and WNV isolates from the Southeastern United States and around the world. We hypothesized that CxFV was present in *Cx. quinquefasciatus* in the Southeastern United States and that spatial and temporal CxFV infection prevalence would be either positively or negatively associated with infection rates of WNV in the same mosquito collections.

Materials and Methods

Mosquito Collection. Using ArboNET records, 11 locations were selected across Georgia, Mississippi, and Louisiana that have programs routinely collecting *Cx. quinquefasciatus* for arbovirus surveillance and that represent a range of WNV human case incidence levels (Fig. 1) (Lindsey et al. 2008). Cumulative incidence of WNND per 100,000 population of WNV between 2002 and 2006 for each of the sampled counties in Georgia was: Clayton (<20), Chatham (<20), Fulton (<20), and DeKalb (<20); in Mississippi: Jackson (<20), Madison (<20), Hinds (20–39.9), and Rankin (20–39.9); in Louisiana: East Baton Rouge (20–39.9), St. Tammany (20–39.9), and Ouichita (20–39.9) (Lindsay et al. 2008). Mosquitoes were collected weekly by Centers for Disease Control (CDC) light traps and gravid traps from the same sites. Mosquitoes were trapped between July and October 2009 in Georgia, July and September 2009 in Mississippi, and June and September 2009 in Louisiana. Mosquitoes were kept on a cold chain, sorted to species (Darsie and Ward 2005), and pooled in groups of ≤25 individuals. Pools were homogenized in 1 ml BA-1 media (Hanks M-199 salts, 0.05M Tris pH 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/ml streptomycin, 1 μg/ml Fungizone). Aliquots of homogenized *Cx. quinquefasciatus* pools were shipped on dry ice to the CDC in Fort Collins, CO, where they were screened for flaviviruses by RT-PCR and subjected to virus isolation on *Aedes albopictus* C6/36 cells.

RNA Extraction. Total RNA was extracted from mosquito pool homogenates in a 96-well plate format using a Qiagen Biorobot 9604 (Qiagen, Valencia, CA) according to manufacturer's instructions. Nucleic acids were eluted in 100 μl AVE elution buffer supplied with the extraction kit, and stored at −20°C until use. Four wells consisting of tap water were included on each extraction plate as a control for contamination.

Virus Detection, Quantification, and Isolation. All pools were initially screened for flavivirus RNA by standard RT-PCR using broadly reacting NS5 gene primers FU2 and CFD3 (Kuno et al. 1998). Flavivirus RNA-positives were determined by visual examination of the amplicon following agarose gel electrophoresis. All flavivirus-positives were then screened for WNV RNA by quantitative RT-PCR (Lanciotti et al. 2000) and for CxFV RNA by standard RT-PCR using primers E92 F and E151R that target the E gene region (Kent et al. 2010). CxFV was isolated and quantified from mosquito pool homogenates by plaque titration on C6/36 cells (Miller et al. 1989). Plaque assays were performed on C6/36 cell monolayers in six-well plates using a double overlay method as previously described (Miller et al. 1989, Kent et al. 2010). A second overlay containing 1.6% neutral red was added at 7 d postinfection. Plaques were picked 9 d postinfection and diluted directly into 1 ml BA-1 media. Virus was then passaged in a T25 flask of C6/36 cells and titrated as above.

Sequencing and Phylogenetic Analysis. RNA was extracted from 140 μl of original and passage one virus

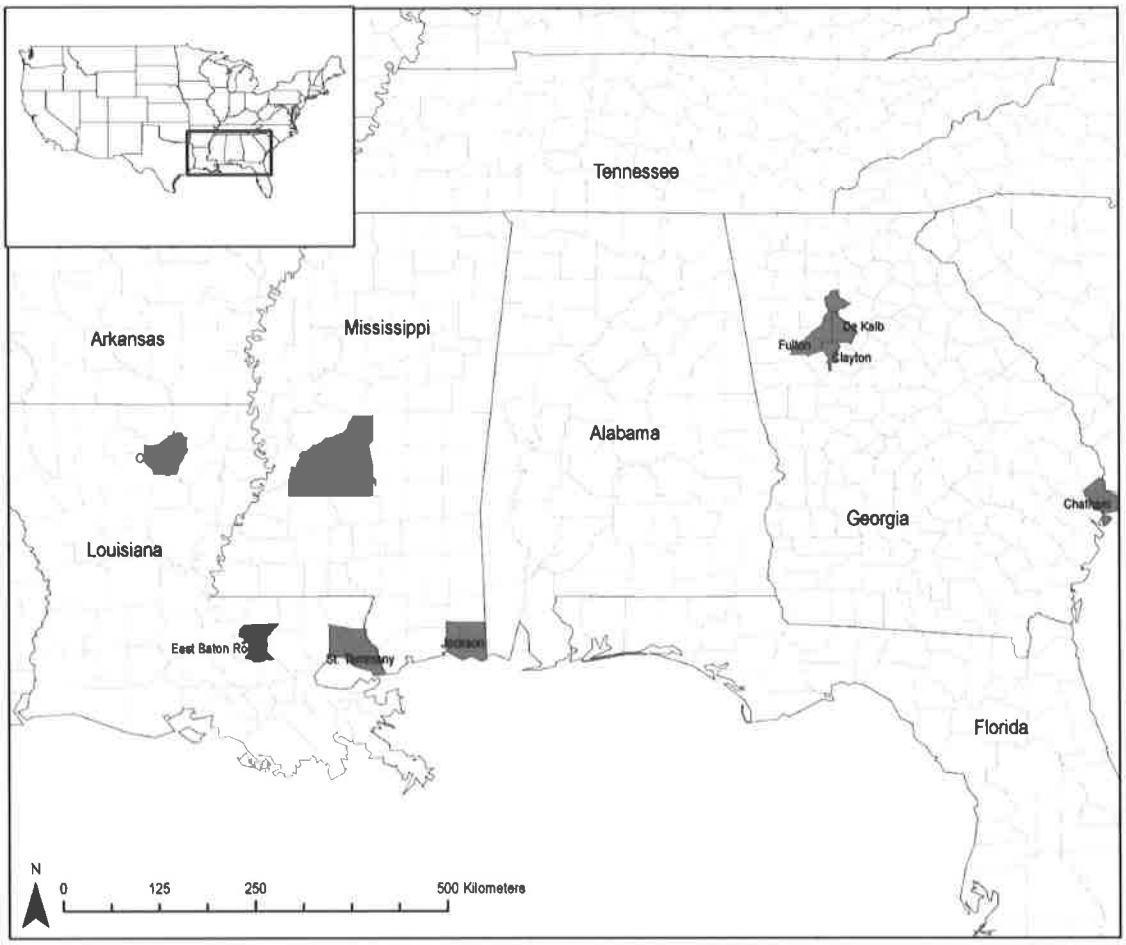

Fig. 1. Counties in the Southeastern United States where *Cx. quinquefasciatus* was collected, 2009.

isolates using the QIAamp Viral RNA Mini Kit (Qiagen). Fragments of the of the E and NS5 genome regions of CxFV were amplified using the Qiagen One-Step RT-PCR Kit (Qiagen) and previously published primers (Kuno et al. 1998, Hoshino et al. 2007). NS5 primers included FU1, FU2, cFD2, cFD3 (Kuno et al. 1998), NS5–2, NS5–3 (Hoshino et al. 2007), and E primers included E-1, CxFV E-1, CxFV E-2, and E-GSP-1 (Hoshino et al. 2007). Five microliters of RNA was used per 50 μl reaction. Homologous fragments of E and NS5 were amplified from WNV using WNV-specific primers (Lanciotti et al. 1999). All RT-PCR amplicons were electrophoresed on 2% agarose gels stained with ethidium bromide. Products of the expected size were gel-extracted and purified using a MinElute Gel Extraction Kit (Qiagen). Purified amplicons were bidirectionally sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, and analyzed on an ABI 3130 genetic analyzer (Applied Biosystems). Raw sequence files from each virus, gene, and pool were edited manually from multiple alignments of between 8 and 30 reads constructed in SeqMan (Lasergene 8.0). Consensus sequences were then aligned using the Clustal W method in Megalign (Lasergene 8.0), and percent identity of nucleotide and amino acid sequences determined manually from the multiple sequence alignment. Novel WNV E and NS5 consensus sequences were analyzed with previously published Lineage I WNV genome sequences from throughout the United States (AY712948, DQ164203, DQ164202, HQ671721, DQ164197, FJ527738, HQ596519, DQ164201, AF404753), Italy (AF404757), Argentina (GQ379161), Mexico (AY660002), China (AY490240), Nassau (DQ164195), Russia (AY278442), and Lineage II Sarafend strain (AY688948). Phylogenetic analysis of E and NS5 from CxFV and WNV was performed on NS5 and E nucleotide and amino acid sequence alignments using maximum parsimony with 500 bootstrap replicates in MEGA, version 4 (Tamura et al. 2007).

Statistical Analysis of Infection Rates. CxFV and WNV infection rates were calculated using bias-corrected maximum likelihood, and 95% confidence intervals (CI) for the true infection rates were computed using the skewness-corrected score interval (Hepworth 2005, Hepworth and Watson 2009). Data

Table 1. Summary of *Cx. quinquefasciatus* collections during 2009, including infection prevalence of WNV and CxFV in mosquito pools

Month	No. *Cx. quinquefasciatus*	No. *Cx. quinquefasciatus* pools	No. (%) CxFV-infected pools	No. (%) WNV-infected pools	No. (%) dually infected pools
Georgia					
June	—	—	—	—	—
July	5,044	269	13 (4.8)	4 (1.5)	1 (0.4)
Aug.	6,151	323	29 (9.0)	4 (1.2)	2 (0.6)
Sept.	3,368	198	18 (9.0)	2 (1.0)	1 (0.5)
Oct.	2,573	147	18 (12.2)	0 (0.0)	0 (0.0)
Mississippi					
June	—	—	—	—	—
July	2,035	52	1 (1.9)	5 (9.6)	1 (1.9)
Aug.	3,095	72	0 (0.0)	0 (0.0)	0 (0.0)
Sept.	332	15	0 (0.0)	0 (0.0)	0 (0.0)
Oct.	—	—	—	—	—
Louisiana					
June	1,568	35	0 (0.0)	4 (11.4)	0 (0.0)
July	11,874	223	0 (0.0)	8 (3.6)	0 (0.0)
Aug.	9,316	176	0 (0.0)	11 (6.3)	0 (0.0)
Sept.	9,049	176	0 (0.0)	4 (2.3)	0 (0.0)
Oct.	—	—	—	—	—

Em dashes indicate no sampling was performed during that month.

were stratified and infection rates calculated by state, month, county, and trap site. Differences in CxFV or WNV infection rates between groups (states, months, counties, etc.) were evaluated by constructing 95% CIs for the differences between the proportions of virus-infected mosquitoes. CxFV and WNV were analyzed separately, and unequal pool sizes were accounted for according to the methods of Biggerstaff (2008). Estimates are reported per 1,000 individuals. Confidence intervals were calculated for months and states, as well as for months and counties within each state. Comparisons were considered statistically different at 5% significance level if CIs did not contain zero.

Direct analysis of co-infection was hindered by pooling of individuals, as information in an individual was lost. To evaluate associations between CxFV and WNV infection rates in pools, Poisson and binomial regression methods were used. Neither model yielded statistically significant associations between the two viruses. The low number of positive pool counts in Mississippi and Louisiana resulted in unreliable model fits, and when analyzing Georgia alone, associations were not statistically significant, so these results are not further discussed.

Results

A total of 1,686 pools comprising 54,405 *Cx. quinquefasciatus* collected in Georgia, Mississippi, and Louisiana during the summer of 2009 were tested for flavivirus RNA (Table 1). These pools included 937 pools (17,136 mosquitoes) from Georgia, 139 pools (5,462 mosquitoes) from Mississippi, and 610 (31,807 mosquitoes) from Louisiana. CxFV RNA and virus was detected and/or isolated from multiple pools of *Cx. quinquefasciatus* from Georgia and a single pool from Mississippi (Table 1). CxFV-infection rates were significantly higher in Georgia than in either Louisiana or Mississippi (Table 2). Seasonally, CxFV infection rates were significantly higher in October as compared with June, July, August, or September (Table 2); however, this finding is likely because of small pool numbers and pool sizes obtained in October. WNV infection rates in *Cx. quinquefasciatus* across states or months were not significantly different (Table 3). No specific trends in CxFV- or WNV-infection rates or evidence

Table 2. Pairwise comparisons of CxFV infection rates across states and months, and within Georgia

Comparison	Rate difference	Lower	Upper
All states			
Georgia-Louisiana	**4.93**	**2.90**	**8.25**
Georgia-Mississippi	**4.76**	**3.33**	**6.23**
Louisiana-Mississippi	−0.18	−4.10	0.12
June–July	−0.79	−3.73	4.40
June–Aug.	−1.70	−5.21	3.53
June–Sept.	−1.42	−5.76	3.52
June–Oct.	**−7.39**	**−28.23**	**−0.72**
July–Aug.	−0.91	−2.33	0.36
July–Sept.	−0.63	−2.25	0.67
July–Oct.	**−6.60**	**−14.53**	**−2.01**
Aug.-Sept.	0.27	−1.47	1.87
Aug.-Oct.	**−5.69**	**−13.65**	**−0.98**
Sept.-Oct.	**−5.97**	**−13.93**	**−1.17**
Georgia			
July–Aug.	−2.50	−5.92	0.80
July–Sept.	−2.73	−7.31	1.03
July–Oct.	**−4.56**	**−10.47**	**−0.09**
Aug.-Sept.	−0.23	−5.01	3.89
Aug.-Oct.	−2.06	−8.12	2.72
Sept.-Oct.	−1.83	−8.18	3.84
Chatham-Clayton	−1.07	−5.62	0.62
Chatham-DeKalb	**−7.39**	**−14.9**	**−2.99**
Chatham-Fulton	**−13.34**	**−18.31**	**−9.40**
Clayton-DeKalb	**−6.31**	**−14.07**	**−0.01**
Clatyon-Fulton	**−12.26**	**−17.58**	**−6.51**
DeKalb-Fulton	−5.95	−12.77	2.46

Numbers reflect the value and 95% CIs for proportional differences in CxFV infection rates (see *Materials and Methods*). Statistically significant comparisons are shown in boldface type.

Table 3. Pairwise comparisons of WNV infection rates across states and months

Comparison	Rate difference	Lower	Upper
All states			
Georgia-Louisiana	−0.56	−1.85	0.81
Georgia-Mississippi	0.65	−0.75	4.78
Louisiana-Mississippi	1.22	−0.22	4.33
Months			
June–July	1.81	−0.71	8.03
June–Aug.	1.9	−0.62	8.11
June–Sept.	2.24	−0.27	8.46
June–Oct.	2.27	−0.59	17.89
July–Aug.	0.08	−0.83	0.99
July–Sept.	0.43	−0.5	1.31
July–Oct.	0.9	−1.25	3.1
Aug.–Sept.	0.35	−0.57	1.22
Aug.–Oct.	0.82	−1.33	3.06
Sept.–Oct.	0.47	−1.67	3.19

Numbers reflect the value and 95% CIs for proportional differences in CxFV infection rates (see *Materials and Methods*). No comparisons were statistically significant.

for an association between these two viruses were seen across states (Fig. 2).

CxFV prevalence and infection rates were highly variable seasonally and on multiple spatial scales. CxFV RNA was detected in mosquito pools throughout the summer in Georgia; however, only one CxFV-infected pool was detected in Mississippi and no CxFV-infected pools were recovered from Louisiana (Table 1). Within Georgia, CxFV was consistently observed in Fulton county throughout the summer, with DeKalb, Clayton, and Chatham counties each experiencing single peaks in CxFV infection rates in either August (Clayton) or October (DeKalb and Chatham) (Fig. 3). At the level of trap site within each county, CxFV prevalence and infection rates were highly focal, with CxFV detected over several consecutive months at some sites, appearing and disappearing at other sites, or not detected at all (Tables 4 and 5).

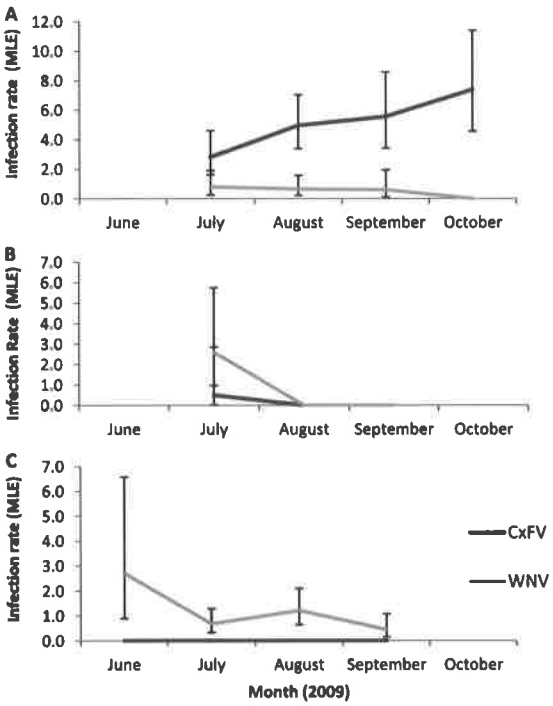

Fig. 2. Seasonal infection rates of WNV and CxFV in *Cx. quinquefasciatus* in (A) Georgia, (B) Mississippi, and (C) Louisiana in 2009. Infection rates were calculated by the maximum likelihood method (Biggerstaff 2006) and represent the most likely number of virus-infected mosquitoes per 1,000. Error bars indicate the upper and lower 95% CIs.

CxFV cDNA sequence from a 600-bp fragment of NS5 and 1,268-bp of E were obtained from five CxFV isolates from Georgia comprising pool numbers 2512, 2517, 2572, 2588, and 3028 (Accession numbers HQ634589 – HQ63598). All of these isolates were obtained from mosquito pools collected in July 2009. Pools 2512, 2517, and 2572 were collected in DeKalb County; Pools 2588 and 3028 were collected in Fulton County. Phylogenetically, CxFV isolates from Georgia clustered with those previously described from Houston, TX (Kim et al. 2009), Iowa, (Blitvich et al. 2009), and Japan (Hoshino et al. 2007) (Fig. 4). Georgia CxFV isolates shared 99% nucleotide sequence identity in E and NS5 with CxFV from Texas (Kim et al. 2009), 97–100% identity with CxFV from Iowa (Blitvich et al. 2009), and Japan (Hoshino et al. 2007), 89–90% identity with CxFV Izabal from Guatemala (Morales-Betoulle et al. 2008) and 89–91% with CxFV from Yucatan, Mexico (Farfan-Ale et al. 2009).

The CxFV NS5 gene fragment nucleotide sequence pairwise identity among the five Georgia isolates was >98.3% for all possible comparisons (data not shown). At the amino acid level, all five of these NS5 sequences were identical. The E gene fragment pairwise nucleotide sequence identity ranged from 97.7 to 99.9% (data not shown). However, amino acid differences were observed in the E gene fragment between the Georgia isolates. E glycoprotein position 252 in passage one isolates 3028, 2512, and 2517 was a phenyalanine whereas isolates 2588 and 2572 contained a tyrosine at this position. At position 362, isolates 3028, 2512, 2517, and 2572 contained lysine and 2588 contained arginine. At position 387, isolates 3028, 2512, and 2517 contained leucine whereas 2588 and 2572 contained phenylalanine. The differences in the E protein shared by 2588 and 2572 as compared with 2512, 2517, and 3028 result in the clustering of these groups in the topology of the phylogenetic tree constructed from the E amino acid sequence, however, bootstrap support for these clades was low (62%) (Fig. 4B). Despite these amino acid changes, the E gene fragment amino acid sequence identity among these isolates was >99%.

To determine whether or not these amino acid changes in the E protein could be because of passaging the virus in cell culture, CxFV E sequences from original mosquito pools were compared against those obtained from passage one virus. Mosquito pool isolate 2517 contained a tyrosine at amino acid position 252

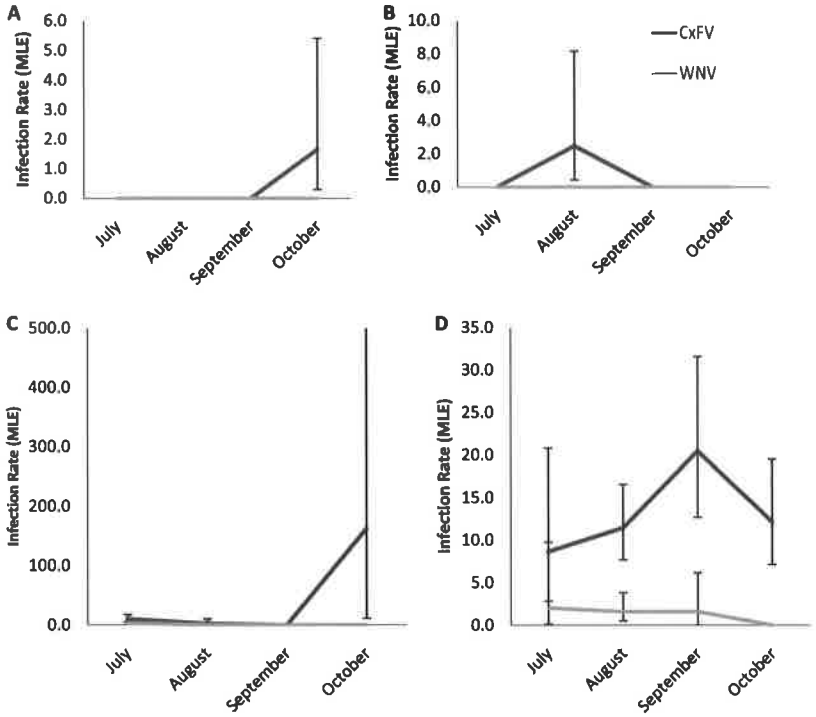

Fig. 3. Seasonal infection rates of WNV and CxFV in *Cx. quinquefasciatus* in Georgia, 2009: (A) Chatham County, (B) Clayton County, (C) DeKalb County, (D) Fulton County. Infection rates were calculated by the maximum likelihood method (Biggerstaff 2006) and represent the most likely number of virus-infected mosquitoes per 1000. Error bars indicate the upper and lower 95% CIs.

whereas the passage one stock of that same virus contained phenylalanine at that same position. For mosquito pool 3028, the original CxFV isolate contained a tyrosine at position 263 whereas the passage one stock of that same virus contained phenylalanine at that position. All other sequence comparisons between original and passage one virus stocks were identical at the amino acid level.

WNV cDNA sequence from 1050-bp of NS5 and 960-bp of E were obtained in July 2009 from pool 2502 from DeKalb County, GA, pool 4515 from Hinds County, MS, and pool 5649 from St. Tammany Parish, LA (Accession numbers JF499910 – JF499915). WNV sequences from Georgia, MS and Louisiana shared >99% nucleotide sequence similarity across 1050 bp of NS5 and 960 bp of E, and were identical at the protein level for both genes (data not shown). The multiple sequence alignment of NS5 contained no parsimonious sites with any of the other Lineage I WNV genomes compared; NS5 amino acid sequences were identical except for one amino acid substitution each in previously published sequences from Argentina (DQ379161), China (AY490240), Louisiana (FJ527738), Ohio (DQ164202), and Colorado (DQ164203). Similarly, there were no differences in the E protein sequence between WNV in the Southeastern United States in this study and WNV isolates from Colorado (DQ164203), Georgia (DQ164197), Louisiana (FJ527738), Ohio (DQ164202), Maryland (AF404753), Texas (AY712948), New York (HQ596519, HQ671721), Arizona (DQ164201), Mexico (AY660002), Argentina (GQ379161), or Nassau (DQ164195). There was only one amino acid substitution in E between the WNV sequences from this study and those from China (AY490240), Italy (AF404757), and Russia (AY278442).

Discussion

This is the first report of the isolation CxFV from *Cx. quinquefasciatus* mosquitoes in the Southeastern United States, CxFV infection rates were focal and highly variable at multiple spatial scales. Georgia, which historically has had the lowest incidence of WNND of these three states (Lindsey et al. 2008), had significantly higher CxFV infection rates than Mississippi or Louisiana. Although this observation is superficially in support of our original hypothesis that there would be some relationship between CxFV prevalence in areas of high and low WNV transmission, WNV infection rates in *Cx. quinquefasciatus* did not vary significantly across states or months, and infection rates were relatively low for both WNV and CxFV (Tables 1 and 3; Fig. 2). Furthermore, looking across states, there was no evidence for an association between WNV and CxFV infection rates in pools of *Cx. quinquefasciatus* (Fig. 2). This conclusion is in contrast to that reported by Newman et al. (2011). In their case-control study analyzing the prevalence of CxFV in WNV-positive and WNV-negative pools of *Cx. pipi-*

Table 4. Seasonal infection rates of CxFV in *Cx. quinquefasciatus* at sites within Fulton County, GA, 2009

Collection site	CxFV infection rate (MLE)			
	July	Aug.	Sept.	Oct.
West Ave.	0.00	0.00	0.00	0.00
Stonewall Tell Rd.	NS	0.00	0.00	0.00
Abbotts Bridge Rd. and Bales	NS	47.95	200.00	113.19
Adamsville Health Center	0.00	13.35	19.49	162.28
Alpharetta Park	NS	35.98	29.05	19.40
Bobby Jones Golf Course	0.00	0.00	0.00	7.13
Brookfield West C.C	0.00	46.57	0.00	NS
Burdett Park	0.00	0.00	0.00	0.00
Cedar Grove Community Center	NS	NS	0.00	0.00
Duncan Memorial Park	NS	0.00	33.86	66.67
East Roswell Park	NS	6.83	71.43	20.74
Frankie Allen Park	9.77	6.63	0.00	0.00
Greens Ferry CSO	0.00	0.00	NS	0.00
Hapeville Police Dept.	142.86	0.00	12.71	0.00
Mounted Police	0.00	0.00	0.00	10.73
North Annex	0.00	11.47	0.00	0.00
North CSO	0.00	6.58	20.70	0.00
Ocee Park	NS	54.20	18.01	111.11
Rico Rd. Fire Station	NS	0.00	250	NS
Ronald Bridges Park	NS	0.00	0.00	200
Roswell Area Park	NS	12.38	0.00	0.00
Sandtown Park	NS	0.00	0.00	NS
Sandy Springs Health Center	0.00	0.00	15.50	19.03
Springvale Park	0.00	8.93	0.00	0.00
Tanyard Creek CSO	0.00	9.16	19.18	0.00
Truitt 4-H Center	0.00	0.00	0.00	0.00
White Column Golf Course	NS	0.00	NS	NS
Whittier Mills Park	20.80	16.64	22.74	0.00
Wilkerson Mill Farris Park	40	0.00	0.00	12.39
Wills Park	0.00	76.20	14.57	10.58

Infection rates were estimated using the bias-corrected max likelihood estimator (Biggerstaff et al. 2006) and reflect the most likely no. CxFV-infected mosquitoes per 1,000. NS, no sample.

Table 5. Seasonal infection rates of CxFV in *Cx. quinquefasciatus* at sites within DeKalb County, GA, 2009

Collection site	CxFV infection rate (MLE)			
	July	Aug.	Sept.	Oct.
Bouldercrest Rd.	36.42	0.00	0.00	NS
Parkhill Dr.	0.00	0.00	0.00	NS
Clifton Rd.	12.18	0.00	0.00	NS
E. Davis St.	0.00	0.00	NS	NS
Bruce St.	0.00	NS	NS	NS
Skyland Dr.	0.00	0.00	0.00	NS
Clairmont Rd.	0.00	0.00	0.00	NS
Chicopee Dr.	15.73	0.00	0.00	NS
Da Vinci Crescent	0.00	NS	0.00	NS
Warren St.	0.00	NS	0.00	NS
Longview Dr.	0.00	NS	NS	NS
Klondike Rd.	65.02	NS	NS	NS
N. Druid Hills Rd.	0.00	0.00	0.00	NS
Norman Rd.	0.00	0.00	NS	NS
Gladney Dr.	0.00	0.00	NS	NS
Winn Way	0.00	0.00	NS	NS
Hathburn Ct.	10.18	0.00	0.00	NS
Covington Hwy.	0.00	0.00	NS	NS
Lakeshore Dr.	18.82	0.00	NS	NS
James B Rivers Memorial Dr.	0.00	0.00	0.00	NS
Camp Rd.	20.53	63.56	0.00	0.00
Spruce Dr. + Snapfinger Creek	0.00	0.00	NS	333.33[a]

Infection rates were estimating using the bias-corrected max likelihood estimator (Biggerstaff et al. 2006) and reflect the most likely no. CxFV-infected mosquitoes per 1,000. NS, no sample.

[a] Derived from only two pools of mosquitoes.

ens in Chicago, Newman et al. concluded that WNV-positive pools were four times more likely to also be infected with CxFV in spatiotemporally matched WNV-negative pools. While there may be geographical differences in CxFV and WNV infection rates between *Cx. quinquefasciatus* in the Southeastern United States and *Cx. pipiens* in Chicago, there are substantial differences in the study design used by Newman et al. (2011) making a direct comparison difficult. The CxFV minimum infection rates were not significantly different between WNV-positive and WNV-negative pools in Chicago (Newman et al. 2011).

The presence of CxFV was focal across states as well as within and between counties in Georgia. In the 750 pools comprised of 37,409 mosquitoes from Mississippi and Louisiana, only one pool was positive from these two states combined. Comparatively, there were 80/937 (8.5%) CxFV-positive pools from Georgia alone. Within Georgia, Fulton and DeKalb counties had significantly higher CxFV infection rates than either Chatham or Clayton counties (Table 2). Within Fulton and DeKalb counties, there were areas where CxFV was present, not detected, or infection rates fluctuated throughout the summer (Tables 4 and 5). Observationally, there were no patterns in the proximity of trap locations to each other, or in the type of habitat between these sites that would explain this distribution of CxFV-infected mosquito pools (Tables 4 and 5). Newman et al. (2011) noted that in their study CxFV-positive mosquito pools were more likely to be recovered from residential sites with dense housing than from urban green spaces. Previous reports of CxFV infection rates have also been highly variable. On the Yucatan Peninsula, CxFV infection rates in CxFV were 20.8 per 1,000 overall (26.0 at Tixkokob and 10.9 at Merida zoo), with 70% of pools infected with this virus (Farfan-Ale et al. 2009). Just across the border in the Department of Izabal, Guatemala, CxFV infection rates in *Cx. quinquefasciatus* were 4.7 mosquitoes per 1,000, or 3.8% of pools tested (Morales-Betoulle et al. 2008). In Iowa, CxFV minimal infection rates varied by county between zero and 67 per 1,000 (Blitvich et al. 2009). Therefore, while CxFV appears to have a cosmopolitan distribution, its prevalence is highly focal. Future studies should consider testing mosquitoes for CxFV over multiple years to examine potential yearly fluctuations in infection rates.

Also notable was the fluctuation of CxFV infection rates within a site over time (Tables 4 and 5). In particular, CxFV infection rates remained consistent in some sites like Whittier Mills Park in Fulton County, GA (Table 4); however, CxFV infection rates fluctuated by orders of magnitude from month to month at other sites such as Abbotts Bridge Road, Adamsville Health Center, Hapeville Police Department and Ocee Park, in Fulton County (Table 4). In DeKalb

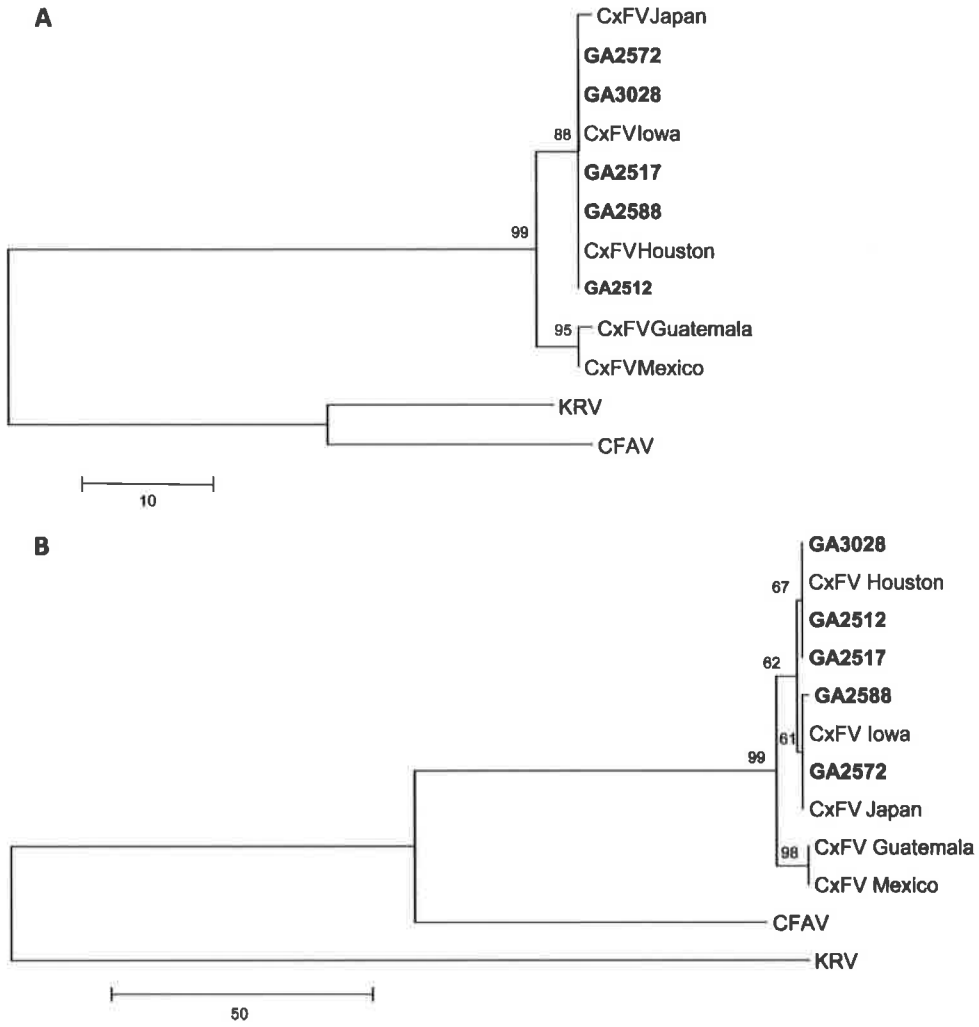

Fig. 4. Phylogenetic analysis of CxFV E and NS5 sequences included the five CxFV isolates from Georgia and previously published insect-only flavivirus genome sequences from Japan (AB262759) (Hoshino et al. 2007), Guatemala (EU805805, EU805806) (Morales-Betoulle et al. 2008), Mexico (EU879060) (Farfan-Ale et al. 2009), Texas (FJ502995) (Kim et al. 2009), Iowa (FJ663034) (Blitvich et al. 2009), Kamiti River virus from Kenya (NC 005064) (Crabtree et al. 2003), and Cell Fusing Agent Virus (NC 001564) (Camissa-Parks et al. 1992). Trees were constructed from amino acid sequences of (A) NS5 (200 aa) and (B) E (423 aa). Maximum parsimony analysis with 500 bootstrap replicates was performed using MEGA, version 4. Scale bar indicates branch length, and bootstrap values >50% are shown above branches (Tamura et al. 2007).

County, a seemingly high infection rate in October was the result of a sample of only two pools each containing three mosquitoes, in which one pool was infected with CxFV (Fig. 3C, Table 5). Variations in the seasonality of CxFV have been previously documented. In Houston, TX, all isolations of CxFV were made in February through March including isolations from pools comprised of only one to three mosquitoes (Kim et al. 2009). In contrast, no CxFV was detected in the same study area between April and August of that year (Kim et al. 2009). CxFV RNA was detected in *Cx. pipiens* in Iowa between July and October, with exception of May and June (Blitvich et al. 2009). Farfan-Ale et al. (2010) reported the presence of CxFV RNA in *Cx. quinquefasciatus* during each month between January and December 2008. Further study is required to investigate the nature of this temporal variation in CxFV infection rates. Possible explanations for these observed variations in CxFV infection rates and focality of detection include local movements of mosquitoes between sites of high and low infection rates, differential rates in the efficiency of vertical transmission within a mosquito population, or environmental/habitat influences.

Phylogenetically, the five CxFV isolates from Georgia clustered with CxFV isolates from the United States and Japan. The topology of the phylogenetic trees constructed from E and NS5 fragment sequences was similar, with only minor differences in the terminal clades. Latin American CxFV isolates grouped

together, separately from isolates from the United States and Japan (Fig. 4). This topological arrangement is consistent with that reported by Kim et al. (2009) and Blitvich et al. (2009).

Among the five CxFV isolates from Georgia, there were genetic variations identified in the E glycoprotein among these isolates. While passage of the virus in C6/36 cells can result a nonsynonymous point mutations, the variations reported at E glycoprotein at positions 252, 362, and 387 existed among CxFV strains amplified directly from mosquito pools. The topology of the phylogentic tree depicted in Fig. 4 was identical regardless of whether it was constructed from E sequence amplified from mosquito pools or CxFV passaged once in C6/36 cells. Both instances where passaged virus differed from the original virus isolate involved a single amino acid substitution from a tyrosine to a phenylalanine.

Functionally, it is unclear what effect, if any, these amino acid differences have. A potentially nonconservative difference observed at position 252 (phenyalanine/tyrosine) falls in the j strand of Domain II. This region is part of the dimerization domain but not involved in the fusogenic activity of the virus. The differences at positions 362 and 387 both fall in Domain III, the C-terminal domain of the E glycoprotein. Position 362 falls on the D_x strand of Domain III, part of the beta-pleated sheet comprised of strands A_x-C_xD_x. Position 387 falls between the F and G strands of the CFG sheet, in the FG loop. The CFG sheet forms the outer lateral surface of Domain III believed to be involved in receptor recognition. The FG loop is likely to be involved in host range determination, as mutations affecting virulence and cell tropism cluster in this region (Rey et al. 1995). Recently, it was demonstrated that the FG loop was critical for the infection of *Aedes aegypti* (L.) midguts and mammalian cells by dengue two virus, but was not important for infection of C6/36 cells (Erb et al. 2010). Additionally, a mutation at position 390 of Murray Valley encephalitis virus E glycoprotein resulted in attenuated virulence in mice (Lobigs et al. 1990). A more comprehensive survey may show if additional CxFV variants are circulating in this or other geographic areas, and more experimentation is needed to determine any functional significance of these amino acid substitutions.

In conclusion, here we report the detection and isolation of CxFV in the Southeastern United States, as well as describe the spatial and seasonal fluctuation in CxFV infection rates in *Cx. quinquefasciatus*. The presence and prevalence of CxFV is highly focal at multiple spatial scales as well as seasonally. CxFV from Georgia clusters phylogenetically with other CxFV isolates from North America and Japan, and there appear to be multiple genetic variants of CxFV circulating simultaneously in *Cx. quinquefasciatus*. There were no significant differences found among WNV E and NS5 sequences obtained in this study with previously published lineage one WNV sequences from around the world. Our results do not indicate any association between WNV and CxFV infection rates in *Cx. quinquefasciatus* across this study area.

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