Diversity and molecular characterization of Insect - specific Flavivirus in mosquitoes (Diptera: Culicidae) collected in Central and Northern Argentina.

Adrian Alejandro Farias (adrianalefarias@hotmail.com)
Instituto de Virología “Dr. J. M. Vanella” https://orcid.org/0000-0003-0497-0226
Victoria Laberdolive
Universidad Nacional de Córdoba Facultad de Ciencias Médicas: Universidad Nacional de Cordoba Facultad de Ciencias Medicas
Marina Stein
laboratorio de Entomologia Instituto de Medicina regional. Universidad Nacional del Noroeste
María Julia Dantur Juri
Universidad Nacional de Tucuman Facultad de Ciencias Naturales e Instituto Miguel Lillo
Andrés Visintin
Universidad Nacional de La Rioja
Walter R. Almirón
Universidad de Córdoba: Universidad de Cordoba
Marta S. Contigiani
Universidad Nacional de Cordoba Facultad de Ciencias Medicas
Viviana E. Re
Universidad Nacional de Córdoba Facultad de Ciencias Médicas: Universidad Nacional de Cordoba Facultad de Ciencias Medicas
Adrián Diaz
Universidad Nacional de Cordoba Facultad de Ciencias Medicas

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Abstract

In order to study the diversity and phylogenetic relationships among insect-specific flaviviruses circulating in the central and northern regions of Argentina we performed molecular detection and characterization of the NS5 protein gene in mosquitoes collected in Córdoba, Chaco and Tucumán provinces between 2001 and 2012, Sixty eight out of 1776 pools were positive. The KRV, CFA and CxFV viruses were detected by the generic PCR method, achieving the analysis of the existing phylogenetic relationships in the Flavivirus genus and the characterization of circulating CxFV in Argentina. It has also been possible to detect, for the first time, the CxFV virus in the mosquito species *Haemagogus spegazzini*.

Introduction

A group of viruses tentatively included in the *Flavivirus* genus and apparently exclusive of insects has been increasingly detected and isolated. These viruses were previously defined as mosquito-only flaviviruses or insect specific flaviviruses (ISFVs). Insect-specific flaviviruses (ISFVs), have been widely detected in mosquitoes from different countries in the last decades [2, 4, 11, 27](Blitvich et al., 2015; Calisher et al., 2018; Sang et al. 2003; Crabtree et al., 2003). The first virus characterized within the ISFV group was cell fusing agent virus (CFAV) isolated from an *Aedes aegypti* cell line in 1974 [11, 28](Stollar et al. 1975; Crabtree et al. 2003). Besides CFAV, many other cISFVs, including Culex flavivirus (CxFV), Kamiti River virus (KRV) and Aedes flavivirus (AEFV), have been isolated and characterized [2, 4](Blitvich et al., 2015; Calisher et al., 2018). CxFV is the most frequently reported cISFV to date [14](Fernandez et al., 2018). Based on their phylogenetic and antigenic relationships, the ISFVs can be separated into two distinct groups. The first and largest group consists of the classical insect-specific flaviviruses (cISFVs), such as CFA, CxFV, and KRV viruses. The cISFVs constitute a separate clade from the vertebrate pathogenic flaviviruses. The second ISFV group consists of the arbovirus related or dual host affiliated insect-specific flaviviruses (dISFVs). The dISFVs are phylogenetically more similar to the flavivirus vertebrate pathogens than to the cISFs. These similarities raise the possibility that some of the dISFs might modulate arbovirus infection and transmission in a dually infected mosquito host or that they could be useful in developing potential flavivirus vaccines or reagents [2, 18](Blitvich et al., 2015; Guzman et al., 2018). However, the evidence is scarce, unclear, and in some cases contradictory [3, 20](Kent et al., 2010; Bolling et al., 2011). Despite the search and detection of ISFV in different regions of the world, their knowledge for Argentina and its surroundings is very scarce. There is only one study carried out in Brazil in which CxFV was isolated in *Culex sp*. [24](Machado et al., 2012) and CFAV in mosquitoes from Amazon Region [14](Fernandez et al., 2018). The aim of the present study was to analyze the natural diversity and phylogenetic relationships among Insect specific Flaviviruses circulating in mosquitoes collected in the central and northern region of Argentina.

Materials And Methods

Mosquito samplings were carried out by means of CDC light traps supplied with CO2 in the provinces of Chaco (Resistencia, Pampa del Indio and Monte Alto), Córdoba (Córdoba, Mar Chiquita) and Tucumán (San Miguel de Tucumán) during 2001 to 2012 (Table 1). In the period between December 8, 2009 and March 18, 2010, 177 ovitraps were placed in different neighborhoods of the City of Córdoba, 202 pools of larvae were processed. Collected adult mosquitoes and larvae were transported in refrigerated conditions to the Laboratory of Arbovirus (Institute of Virology “Dr. JM Vanella”, Faculty of Medical Sciences, National University of Córdoba) where were taxonomically identified under chill table. Mosquitoes were sorted by species, sex, collection site, and date, with a maximum number of 50 individuals per pool. Pools were homogenated in sterile mortars and pestles, centrifuged and stored at -80 C until process for virological studies.

Table 1
Mosquito collection carried out in central and northern area of Argentina during 2001 and 2012.

Province	City of collection	Time period/Year	Pools Collected (n)	Total (n)
Córdoba	Córdoba	2001–2004	331	1259
		2006	36	
		2008–2013	696	
	Altos de Chipión	2008–2009	112	
		2004–2006	84	
Chaco	Resistencia	2001–2003	188	388
	Monte Alto	2001–2003	129	
	Pampa del Indio	2009	71	
Tucumán	San Miguel de Tucumán	2005	129	129

The molecular screening was performed by a generic flavivirus RT-Nested PCR that amplify a 143 base pair (bp) fragment of the flavivirus NS5 region [26](Sánchez Seco et al. 2005). To test whether the positive pools were the results of genomic RNA amplification or DNA forms, nucleic acid extracts were treated with RNAsa before amplification. Then, positive pools were analyzed by a generic RT-Nested PCR technique for the amplification of a 860 bp fragment of the NS5 region of *Flavivirus* genome in order to deepen in phylogenetic analysis [30](Vázquez et al. 2012). This region of the genome was chosen because it provides useful information for phylogenetic studies allowing a clear molecular identification of the members of the genus *Flavivirus*, and is one of the most representative regions in GenBank [10] (Cook et al., 2012). All the amplified and purified fragments were sequenced in both directions through automatic sequencers (MACROGEN, Korea).

Initial identification of the genomic sequences obtained was carried out by comparing them with all available sequences in the GenBank using BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were generated with the relevant ISFV genomes available at GenBank. Consequently, the construction of the maximum likelihood (ML) and Bayesian likelihood was completed under the GTR + I + G model for both the NS5 gene. The ML tree was performed by MEGA X software, with 1000 bootstrap replications using MEGA X software (Kumar et al., 2018).

Results

A total of 1272 mosquitoes pools and larvae were analyzed, comprising collections in the city of Córdoba (center of the province) between 2001 and 2012 (excepting 2005) (n = 800); in the town of La Para (Northeast of the province of Córdoba) in 2008, 2009 and 2010 (n = 129), in the city of Resistencia (province of Chaco) in the years 2003, 2004 and 2009 (n = 143), in the town of Pampa del Indio (province of Chaco) in the years 2009, 2010 and 2011 (n = 71); and in San Miguel de Tucumán (province of Tucumán) in 2005 and 2006 (n = 129). CxFV was detected in all the provinces studied except for Tucumán. In Córdoba city, the 3 ISF species were detected: CxFV, CFA and KRV, with CFA being the predominant one, while in Chaco the CFA and CxFV viruses were detected (Fig. 1). In the town of La Para, the only virus detected was CxFV and in Tucumán, only KRV. The presence of CxFV, KRV and CFA was not constant and was detected in an interrupted manner between the years of sample collection (2001–2012) in the three provinces (Fig. 1).

Sixty eight out of 1776 pools were positive by shorter fragment generic RT Nested PCR (Sánchez Seco PCR). According to the BLASTn analysis, 14 pools were classified as CFA, 4 pools as KRV and 19 grouped in CxFV cluster. Only 27% (10/37) were able to amplify by longer fragment NS5 PCR (Vázquez PCR) and then sequenced. The 100% of these sequences agree with the identification of the viral species compared to the shorter fragment. CFA was found mainly in *Aedes aegypti* in 85.71% (12/14), also been found in a pool of *Ochlerotathus scapularis* and another from *Mansonia titillans*, while KRV was found in *Aedes aegypti* and *Culex interfor* in a 3: 1 ratio. On the other hand, the 94.73% (18/19) of CxFV was found in mosquitoes of the genus *Culex*, excepting one positive pool from *Haemagogus spegazzini* collected in Chaco Province. It should be noted that ISF were found in both adult male and females mosquito and in larvae, as well. Detailed information on species distribution, precedence, collection year, sex and stage of development of positive pools are shown in Table 2.

Table 2
Summary of the insect-specific flaviviruses detected in different mosquito pools captured in Central and Northern Region of Argentina, during 2001 to 2012.

Pool	Host	Collection date	Stage	Province	City of collection	Virus	GenBank ID	Sex
LP2_045	*Culex saltanensis*	04/2009	Adult	Córdoba	La Para	CxFV[2]	KF316946	Female
LP2_047	*Culex dolosus*	04/2009	Adult	Córdoba	La Para	CxFV[2]	KF316947	Male
CHPI 79	*Haemagogus* sp*egazzini*	12/2009	Adult	Chaco	Pampa del Indio	CxFV[2]	KF316948	Female
819	*Culex interfor*	12/2009	Adult	Córdoba	Córdoba	CxFV[2]	KF316939	Female
CA13	*Culex sp.*	12/2009	Adult	Córdoba	La Para	CxFV[2]	KF316941	Female
CA18	*Culex sp.*	12/2009	Adult	Córdoba	La Para	CxFV[2]	KF316942	Female
CA19	*Culex sp.*	12/2009	Adult	Córdoba	La Para	CxFV[2]	KF316943	Female
CA26	*Culex sp.*	12/2009	Adult	Córdoba	La Para	CxFV[2]	KF316944	Female
CA28	*Culex sp.*	12/2009	Adult	Córdoba	La Para	CxFV[2]	KF316945	Female
925	*Culex interfor*	01/2010	Adult	Córdoba	Córdoba	CxFV[2]	KF316940	Female
3_95	*Aedes aegypti*	02/2004	Adult	Córdoba	Córdoba	CFA[1]		Female
3_86	*Culex interfor*	02/2004	Adult	Córdoba	Córdoba	KRV[1]		Female
3_76	*Culex quinquefasciatus*	03/2004	Adult	Córdoba	Córdoba	CxFV[1]		Female
3_75	*Ochlerotatus scapularis*	03/2004	Adult	Córdoba	Córdoba	CFA[1]		Female
AR374	*Aedes aegypti*	03/2004	Adult	Córdoba	Córdoba	CFA[1]	DQ335466	Male
AR336	*Aedes aegypti*	03/2004	Adult	Córdoba	Córdoba	KRV[1]	DQ335465	Female
AR349	*Mansonia titillans*	04/2004	Adult	Córdoba	Córdoba	CFA[1]	DQ335467	Female
AR310	*Aedes aegypti*	04/2004	Adult	Córdoba	Córdoba	CFA[1]	DQ431718	Female
ChRS7	*Culex quinquefasciatus*	03/2009	Adult	Chaco	Resistencia	CxFV[1]		Female
ChRS20	*Aedes aegypti*	03/2009	Adult	Chaco	Resistencia	CFA[1]		Female
LP2_046	*Culex bidens*	04/2009	Adult	Córdoba	La Para	CxFV[1]		Female
CA21	*Culex spp.*	12/2009	Adult	Córdoba	La Para	CxFV[1]		Female

[1] Small segment secuenced is showed in Anexo 1, [2] Large Fragment secuenced, CFAV = Cell Fusing Agent virus, CxFV = Culex Flavivirus virus, KRV = Kamiti River virus,

Pool	Host	Collection date	Stage	Province	City of collection	Virus	GenBank ID	Sex
CA23	*Culex sp.*	12/2009	Adult	Córdoba	La Para	CxFV		Female
CHPI 158	*Culex sp.*	05/2011	Adult	Chaco	Pampa del Indio	CxFV[1]		Female
SMT105	*Aedes aegypti*	12/2005	Adult	Tucumán	San Miguel de Tucumán	KRV[1]		Female
SMT114	*Aedes aegypti*	11/2005	Adult	Tucumán	San Miguel de Tucumán	KRV[1]		Female
933	*Culex interfor*	01/2010	Adult	Córdoba	Córdoba	CxFV[1]		Female
942	*Culex interfor*	01/2010	Adult	Córdoba	Córdoba	CxFV[1]		Female
cba_52	*Aedes aegypti*	01/2011	Adult	Córdoba	Córdoba	CFA[1]		Female
cba_43	*Aedes aegypti*	01/2011	Adult	Córdoba	Córdoba	CFA[1]		Female
cba_103	*Aedes aegypti*	02/2011	Adult	Córdoba	Córdoba	CFA[1]		Female
cba_104	*Aedes aegypti*	02/2011	Adult	Córdoba	Córdoba	CFA[1]		Male
cba_86	*Aedes aegypti*	02/2011	Adult	Córdoba	Córdoba	CFA[1]		Female
cba_87	*Aedes aegypti*	02/2011	Adult	Córdoba	Córdoba	CFA[1]		Male
cba_132	*Culex saltanensis*	03/2011	Adult	Córdoba	Córdoba	CxFV[1]		Female
L897	*Aedes aegypti*	01/2010	Larvae	Córdoba	Córdoba	CFA[1]		Female
L497	*Aedes aegypti*	01/2010	Larvae	Córdoba	Córdoba	CFA[1]		Female

[1] Small segment secuenced is showed in Anexo 1, [2] Large Fragment secuenced, CFAV = Cell Fusing Agent virus, CxFV = Culex Flavivirus virus, KRV = Kamiti River virus,

In order to analyze phylogenetic relationships among CxFV detected and other ISF, we used the longer NS5 Flavivirus fragment. This analysis allowed us to distinguish two well-differentiated groups, one grouping viruses associated with *Aedes spp*. and *Ochlerotatus* spp. species, while the second group included viruses associated with *Culex* species. In this group we can distinguishe: CLBV, NKV, QBV, SCxFV and CxFV. The group associated with CxFV represents the most heterogeneous and diverse group where, at the host species level, there is a clear separation into two subgroups: one composed by *Culex pipiens, Culex quinquefasciatus* and *Culex tritaeniorhynchus* (subgroup 2) and another group that is not clearly defined due to the scarcity of samples and the lack of details when identifying the species (subgroup 1) (Fig. 2). CxFV strains detected in our study clustered with strains circulating in Santa Fe and Buenos Aires provinces (Argentina) and other countries such as Indonesia, Mexico, Uganda and Taiwan.

Discussion

Recently, several Insect Specific flaviviruses have been sequenced, characterized, and identified in arthropods [17] (Guarido et al., 2021). In this study we aimed to investigate the presence of ISF in mosquitoes and to analyze the natural diversity and phylogenetic relationships among them in the north-central region of Argentina.

The implementation of a screening by means of Sánchez Seco PCR for Flaviviruses [26](Sánchez Seco et al., 2005) made possible to identify the activity in the north-central region of Argentina of three ISF species: CFA, CxFV and KRV. Although only 10 pools belonging to CxFV were able to be amplified by Vázquez PCR technique [30] (Vazquez et al. 2012), our results indicate that the coincidence between both short and large fragments is 100%, making this screening technique PCR an appropriate tool for surveillance and identification of circulating flaviviruses. The use of NS5 gene as target for molecular characterization and identification of flaviviruses is wide used bringing useful information regarding phylogenetic relationships [13, 26, 29, 30](Sánchez-Seco et al., 2005; Vázquez et al., 2012; Fang et al., 2018; Talavera et al., 2018). These sequences strongly suggest the presence of other putative viruses not yet isolated, as in the case of OcFV, isolated after the detection of field sequences [5, 6](Calzolari et al., 2012; 2016).

Insect-specific flaviviruses is a highly divergent group within the genus *Flavivirus* that shares a common ancestor with all other members, including the disease-causing ones. A possible explanation of the origin of ISFVs, accounts for the existence of a co-divergence between vectors and viruses and this hypothesis is supported by the pattern of virus-vector association observed in this work (Fig. 2). In agreement with other studies [1, 19](Huanyu et al., 2012; Bittar et al., 2016), there is a clear divergence between Clade 1: ISFs associated with mosquitoes of the genus *Aedes / Stegomyia* (CFA, KRV, AeFV and SOcFV) and Clade 2: ISFVs associated with *Culex* (CLBV, QBV, CxFV, SCxFV). Despite the hypothesis that ISFV underwent multiple introductions with frequent vector changes and occasional genetic recombinations, particularly in CFAV, however, there are no studies to support that CxFV was generated by codivergence between these viruses and their vectors [1, 7, 9](Cook et al. 2009; Bittar et al., 2016; Chatterjee et al., 2021). As expected, based on previous evidence, CFAV (Clade 1) were found in mosquitoes of the genus *Mansonia* and *Ochlerotatus* which, according to the phylogeny of mosquitoes, are more related to the genus *Aedes / Stegomyia* than to *Culex* [10](Cook et al. 2012).

CxFV primarily infects globally distributed mosquito species of the genus *Culex*, which are vectors for pathogenic flaviviruses like WNV, SLEV, and Japanese encephalitis virus (JEV) [1](Bittar et al., 2016). According to other studies [14, 25](Fernandez et al., 2018; Miranda et al., 2018), CxFV has a greater diversity of reservoirs and its phylogeny could be influenced by these. It is the first time that CxFV has been detected in the mosquito species *Haemagogus spegazinii*, incorporating a greater diversity of reservoirs to this viral species. In Fig. 2, it is shown that this CxFV strain grouped within subgroup 1. The phylogenetic tree shows two clades: the first clade is associated with Asian and USA strains, where *Cx. pipiens* is the main reservoir. However, other mosquito species, such as *Culex tritaeniorhynchus* and *Anopheles sinensis* [22](Liang et al 2015), have been reported infected with CxFV belonging to the first clade. These isolates were classified in genotype I (Asian/USA). The second phylogenetic group of CxFV have been reported mostly in the Americas and Africa, with *Culex. quinquefasciatus* as the main reservoir [1, 15, 22](Goenaga et al., 2014; Bittar et al., 2016; Liang et al., 2015). The secuences detected in this study are more phylogenetically related to this group, described as genotype II, (Africa/Caribbean/Latin America; Fig. 2). Our findings agree with this phylogeographic separation demonstrated in other studies [15, 22](Goenaga et al., 2014; Liang et al., 2015). Our sequences clustered with previous detections made in Argentina (Buenos Aires, Córdoba, Chaco and Santa Fe provinces) [15](Goenaga et al., 2014). Moreover, all these Argentinian sequences are closed to that detected in Mexico and Africa. A wide circulation of CxFV was observed in the Central-North region of Argentina (Fig. 1). CxFV seems to have been introduced several times in the New World in association with the species of *Culex*, which could explain the distribution of these viruses detected in this work [3, 10, 14] (Bolling et al., 2011; Cook et al ., 2012; Fernandez et al., 2018).

Although viruses in the genus *Flavivirus* share complex antigenic relationships, they can be divided into four phylogenetic/ecological groups. These divisions largely reflect the selective constraints imposed on the viruses by the

vertebrate hosts, the invertebrate vectors, and the associated ecologies. Some authors, describes the evolution and possible origins of individual flaviviruses, correlating ecological and epidemiological characteristics with their phylogenies and geographic dispersal [6, 12, 16](Gould et al., 2003; Calzolari et al., 2016; de Oliveira Ribeiro et al., 2020). Many of the phylogenetic lineages that define viral species diverged relatively recently, and the subsequent dispersal and epidemiology of these viruses have therefore been significantly influenced by increasing human population densities and activities such as recreation, urbanization, land reclamation, transportation, and deforestation [18] (Guzman et al., 2018).

Certain authors suggest that by not replicating in vertebrate cells, the main route of transmission and maintenance of these viruses would be the vertical and venereal route [3, 8, 23](Cook et al. 2006, Lutomiah et al. 2007; Bollling et al. 2012). However, in this study this virus was detected for the first time in another genus (*Haemagogus spegazzini*), raising questions about its mode of transmission. There would be other forms of transmission not discovered to date.

The temporal analysis of CxFV, KRV and CFA in the city of Córdoba gives evidence of an erratic maintenance with the presence of presence-absence events and with a permanence of the genetic identity of the circulating CxFV over time in the city of Córdoba. This is compatible with the seasonal fluctuations detected in the *Culex* mosquito populations for the temperate regions of our country. The explanation for the absence of this virus during certain periods could be due to the scarcity of mosquitoes collected / analyzed.

In conclusion, ISFVs, more specifically CxFV, KRV and CFAV, circulate in several mosquitoes species in the Central Region of Argentina. The presence of these viruses in mosquitoes could play an important role from the public health perspective, because it has been shown that previous CxFV infection can increase or block the infection of the mosquito by other pathogenic flaviviruses. Information on ISFV is very limited so far and, therefore, not much can be concluded about their frequency, distribution, and host range like in this work.

Declarations

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author Contributions

Adrian A. Farías: Writing – original draft - Reviewing-Data analyses **Victoria Laberdolive:** Data curation, Writing- Original draft preparation **Marina Stein:** Investigation, Writing - review & editing. **María Julia Dantur Juri:** Investigation, Writing - review & editing. **Andres Visintin:** Investigation, Writing - review & editing. **Walter Almirón:** Investigation, Funding acquisition, Writing - review & editing. **Marta S. Contigiani:** Conceptualization, Methodology, Funding acquisition, Writing

- review & editing. **Viviana E. Re:** Conceptualization, Methodology,Writing - review & editing. **Adrián Diaz:** Writing – original draft , Supervision and Editing Conceptualization, Methodology, Writing - review & editing.

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Figures

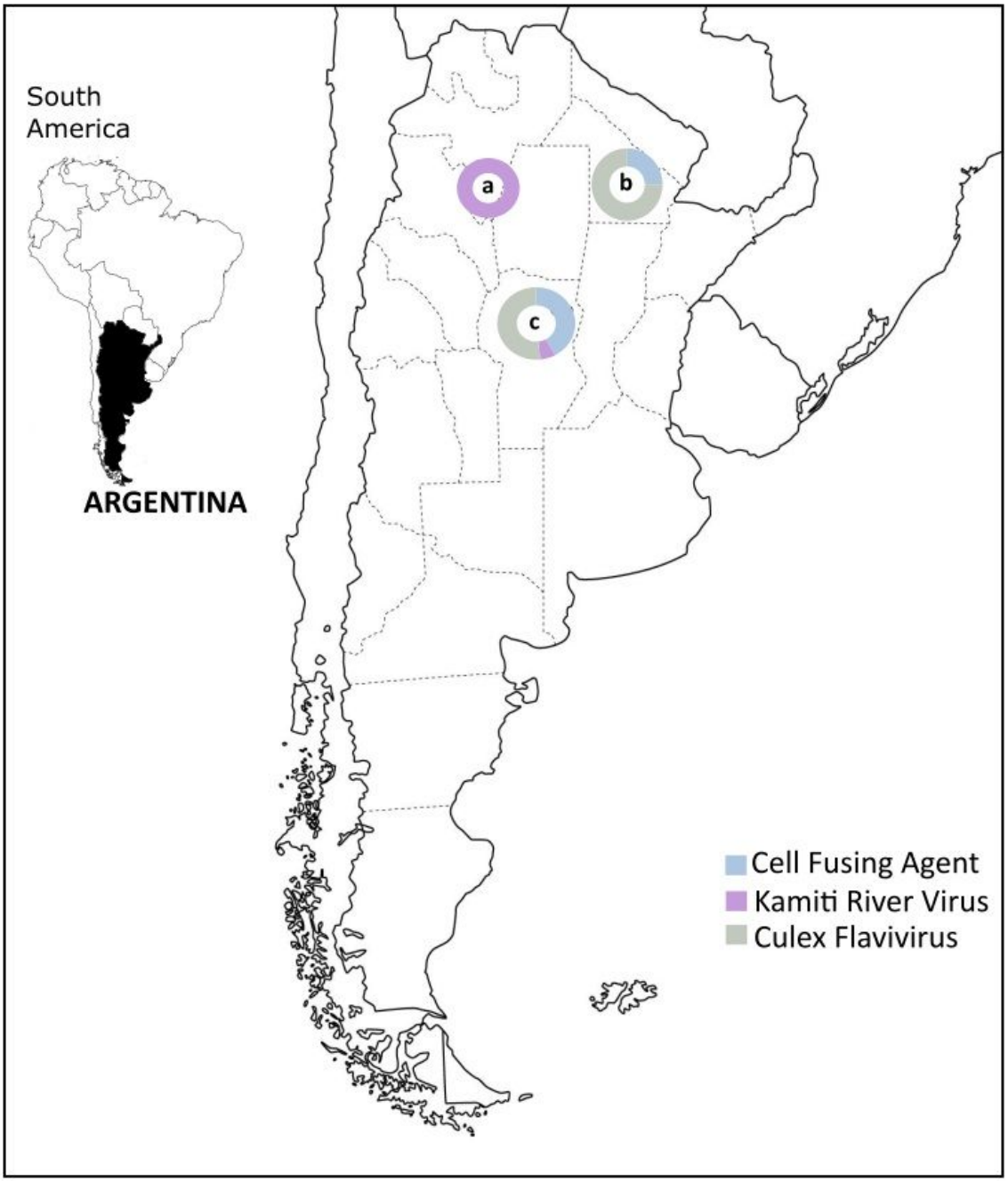

Figure 1

Geographical distribution and proportion of Insect specific flavivirus positive pools detected in 3 provinces of Argentina. a- Tucumán, b- Chaco, c- Córdoba.

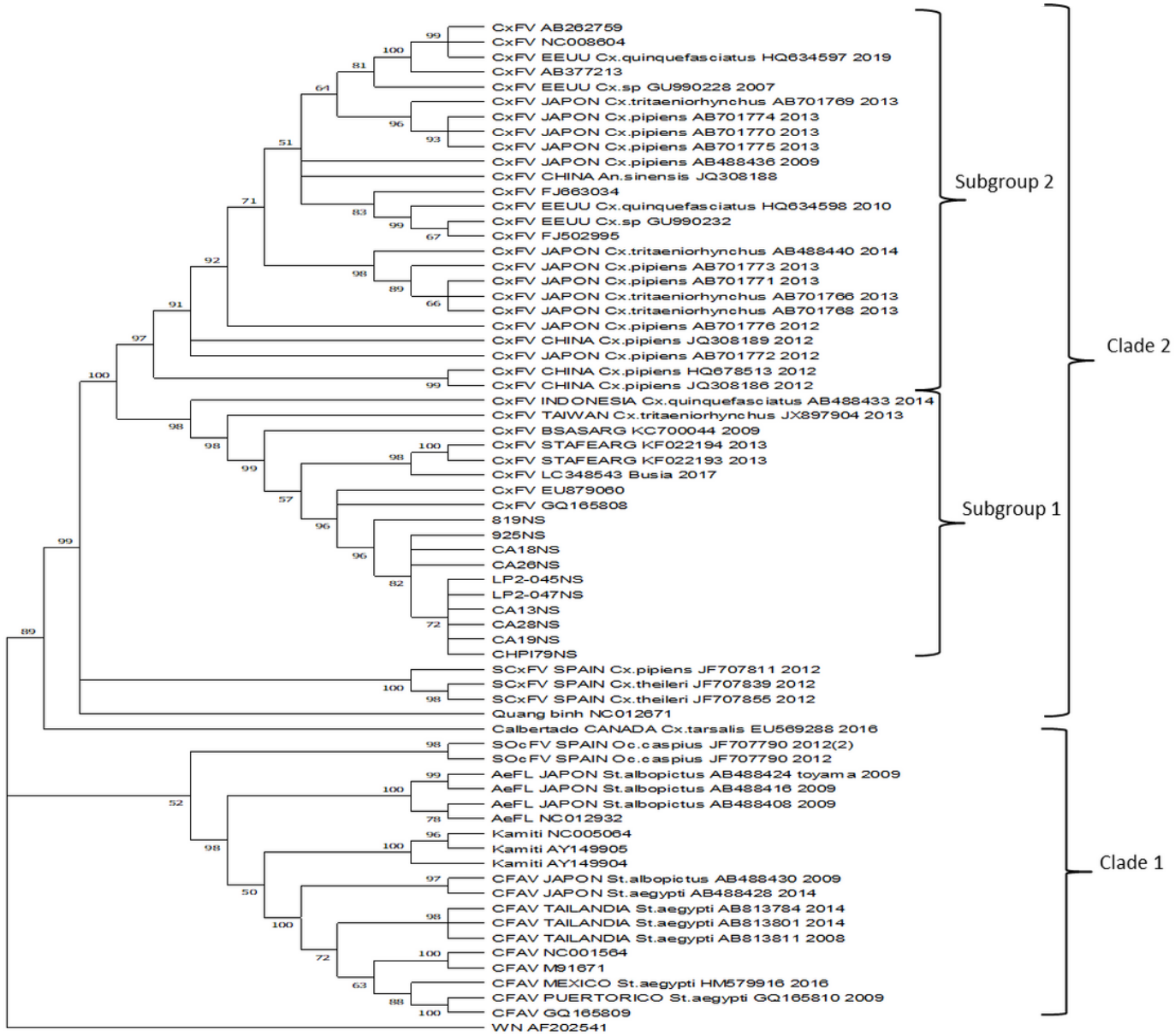

Figure 2

Phylogenetic tree estimated by using the Maximum Likelihood method and GTR +I+G model. This analysis involved 68 nucleotide sequences. There was a total of 824 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. The tree was constructed based on the NS5 target gene of mosquito samples positive for Culex flavivirus and ISFV sequences from GenBank. This figure represents the Maximum Likelihood cladogram with bootstrap values superimposed to indicate nodal support.

Supplementary Files

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- Supplementarydata.docx