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Identification of insect-specific flaviviruses in areas of Brazil and Paraguay experiencing endemic arbovirus transmission and the description of a novel flavivirus infecting *Sabethes belisarioi*

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ABSTRACT

Viral infection was examined with pan-flavivirus and pan-alphavirus sets of primers in mosquitoes collected in four South American regions with confirmed pathogenic arbovirus circulation. Positive pools for flavivirus infection were sequenced and screened for specific arboviruses, which were not detected. However, NS5 gene sequencing showed that most sequences corresponded to the insect-specific *Culex* flavivirus. One sequence retrieved from an *Aedes albopictus* pool grouped with the insect-specific *Aedes* flavivirus and two *Sabethes belisarioi* pools were infected by a previously unknown flavivirus, tentatively named *Sabethes* flavivirus (SbFV). Phylogenetic inference placed SbFV as ancestral to a clade formed by *Culiseta* flavivirus, Mercadeo, and Calbertado. SbFV polyprotein showed an average aminoacidic identity of 51% in comparison to these flaviviruses. *In vitro* studies suggest that SbFV infects insect cells, but not vertebrate cells, therefore, we propose it as a new insect-specific flavivirus. These results highlight the wide distribution of insect-specific flaviviruses concomitant with the circulation of emergent arboviruses.

1. Introduction

Arboviruses are defined as viruses maintained in the nature through biologic transmission between hematophagous arthropods and susceptible vertebrate hosts, while insect-specific viruses (ISVs) infect insects and replicate only in insect cell lines in vitro, but not vertebrates. Not-known vector viruses (NKVs) are flaviviruses that have been isolated in almost exclusively from vertebrates. The evolutionary relationship between arboviruses, ISVs, and NKVs represent both a challenge and an opportunity to understand the dynamics of arbovirus transmission. Also, the co-circulation of these groups of viruses is commonly observed due to the widespread geographic distribution of hosts around the world, but little is known about the interactions between them (Blitvich and Firth, 2015).

Single-stranded positive-sense RNA-arboviruses (arthropod-borne viruses) of public health concern have attracted attention in South America in the last several years. Among them are St. Louis encephalitis virus (SLEV), dengue virus (DENV1/2/3/4), Zika virus (ZIKV), yellow fever virus (YFV), and West Nile virus (WNV), belonging to the genus *Flavivirus*, family Flaviviridae, and chikungunya virus (CHIKV) and

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Mayaro virus (MAYV), belonging to the genus *Alphavirus*, family Togaviridae (reviewed in Mota et al., 2016). The flavivirus genome of ~11 kb in length encodes three structural proteins 5′-capsid (C), premembrane/membrane (prM/M), envelope (E), and seven nonstructural proteins NS1, NS2a, NS2B, NS3, NS4A, NS4B, and NS5-3′. The alphavirus genome, which ranges between 11 and 12 kb in length, encodes four nonstructural proteins 5′-nsPs (nsP1–nsP4) and five structural proteins sPs (Capsid, E3, E2, 6K, and E1) -3′ (reviewed in Hernandez et al., 2014).

Arboviruses have a wide number of hosts in nature, including mammals and birds, and are transmitted to humans by mosquitoes. Mosquitoes belong to the family Culicidae (order Diptera) and are divided into three tribes: Culicini (type genus *Culex*), Aedini (including the type genus *Aedes* and *Haemagogus*), and Sabethini (type genus *Sabethes*) (Forattini, 2002).

ISVs have also been found to infect a wide range of mosquito species, but the most striking is the large number of arbovirus vectors infected by insect-specific flaviviruses (ISFVs). *Culex* mosquitoes that transmit WNV and SLEV to humans (Kopp et al., 2013; Turell, 2012) may carry the insect-specific *Culex* flavivirus (CxFV), a virus initially identified in *Culex* (*Culex*) *pipiens* (Linnaeus, 1758) in Japan (Hoshino et al., 2007). DENV, ZIKV, and CHIKV are transmitted to humans by *Aedes* spp., which may carry the insect-specific *Aedes* flavivirus (AEFV) (Fernandes et al., 2016). *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) can also transmit YFV to humans in the urban cycle, while *Sabethes* spp. and *Haemagogus* spp. transmit YFV in the sylvatic cycle (reviewed in Barrett and Higgs, 2007).

Two ISFV clades are known: “Classical ISFVs” (cISFs or ISFV-I) is the most diverse group and present ubiquitous hosts and geographical distribution. Among them are the *Aedes* flavivirus (AEFV), cell-fusing agent (CFAV), Kamiti River (KRV), *Culex* flavivirus (CxFV), Hanko (HANKV), Nakiwogo (NAKV), Palm creek (PCV), *Culiseta* flavivirus (CsFV), Calbertado (CLBOV), and Mercadeo (MECDV). The second clade is the “Dual-Host Affiliated ISFVs” (dISFs). This clade is phylogenetically related to the arboviruses and is divided in two subgroups, according to the primarily association with Aedini (ISFV-II) or Culicini (ISFV-III) mosquitoes. Phylogenetic inferences suggest that the different groups of ISFVs may have emerged in independent evolutionary events (Blitvich and Firth, 2015; Moureau et al., 2015; Simón et al., 2017).

The major interest here was to explore and characterize the ISVs present in mosquitoes (mainly *Culex* spp., *Aedes* spp., and *Sabethes* spp.) collected in areas with arbovirus circulation in Paraguay (year 2013), during the ZIKV and CHIKV epidemics in Brazil (year 2016), and during the ongoing YFV outbreak in Brazil (year 2017).

2. Materials and methods

2.1. Mosquito collection and sample preparation

Mosquitoes were captured by the following collection techniques: aspiration and New Jersey/CDC light traps, for specimens collected in urban areas of Asunción/PY, Vitória and Curitiba/BR; human attraction at ground level and in the treetops in forest environments in Paraná State/BR, during the period between 10:00 a.m. and 6:00 p.m. All researchers involved in the human attraction technique used personal protective equipment and were vaccinated against yellow fever. In 2013, 2534 female *Culex* and *Aedes* mosquitoes (divided in 88 pools) were collected in November and December during a viral surveillance program carried out in the “Jardín Botánico y Zoológico de Asunción” (Paraguay). In 2016 (February–June), 311 *Culex* mosquitoes (divided into 86 pools) were collected in greenhouses, universities, schools, hospitals, and healthcare centers in Vitória (Espírito Santo (ES) State/ Southeast of Brazil). In 2016, 39 *Culex* (divided into 12 pools) and one *Aedes* (*Stegomyia*) *albopictus* female were collected in Curitiba Paraná (PR) State/ South of Brazil. In February 2017, 296 mosquitoes (divided into 100 pools) from different species were collected inside seasonal semideciduous forest remnant between Ribeirão Claro and Carlópolis municipalities, both cities of PR State (South of Brazil) (Fig. S1). Mosquitoes were classified according to dichotomous key systems of Darsie (in Asúncion), Consoli and Lourenço-de-Oliveira (in Vitória), and Lane and Forattini (in Curitiba and Ribeirão Claro/Carlópolis) (Consoli and Lourenço-de-Oliveira, 1994; Darsie, 1985; Forattini, 2002; Lane, 1953). Mosquitoes were divided by gender, genus/subgenus/species, date, place of collection, and pooled in samples up to 35 mosquitoes per pool (Appendix, and Table S2). Each pool was mechanically homogenized using a Precellys® homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) in 400–1000 µL nuclease-free phosphate-buffered saline (PBS), pH 7.4, centrifuged at 10,000 g/5 min at 4 °C and stored at −80 °C until RNA extraction.

2.2. Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 140 µL of mosquito lysate by using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and eluted in a final volume of 60 µL. Briefly, cDNA was synthesized by the incubation of 2.5 µL of random primers (Invitrogen, Carlsbad, USA) plus 5.6 µL of RNA at 70 °C/5 min; followed by an incubation with ImProm II Reverse Transcriptase (Promega, Madison, USA) and RNAse OUT Ribonuclease Inhibitor (Invitrogen, Carlsbad, USA) containing-mix at 42 °C/60 min.

2.3. Viral genome detection and molecular analysis

First, cDNA synthesis was confirmed by evaluating the presence of the mosquito's endogenous gene Actin-1 (Staley et al., 2010). Then, viral RT-PCRs were conducted with pan-flavivirus (Sánchez-Seco et al., 2005), pan-alphavirus (Pfeffer et al., 1997), SLEV (Ré et al., 2008), DENV1–4 (Lanciotti et al., 1992), ZIKV (Faye et al., 2008), and CxFV (Newman et al., 2011) primers. All PCR assays were performed using Recombinant Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA). In addition, real-time RT-PCRs were performed with the GoTaq® Probe 1-Step RT-qPCR System (Promega, Madison, USA) to detect ZIKV, CHIKV, and YFV (Drosten et al., 2002; Lanciotti et al., 2008, 2007). Primers sets and cycling protocols are described in Table S3. All protocols were validated in our laboratory, which is a reference laboratory designated by the Brazilian Health Ministry for ZIKV and YFV diagnostic and arboviral surveillance in South Brazil.

2.4. Mosquito authentication

Sabethes (*Sabethes*) *belisarioi* identity was confirmed by a DNA barcoding with universal primers that amplify a fragment of the mitochondrial cytochrome c oxidase subunit I gene (*COI*) from eleven invertebrate phyla, including Arthropoda (Table S3) (Folmer et al., 1994). PCR amplifications were performed with genomic DNA template extracted with the Genomic DNA Extraction Kit (Real Genomics, Taipei, Taiwan), and the PCR amplicons were purified with the High Pure PCR Product Purification Kit (Roche, Basel, Switzerland).

2.5. Nucleotide sequencing and phylogenetic analysis

RT-PCR amplicons were obtained using degenerate pan-flavivirus primers (Sánchez-Seco et al., 2005), which span 1385 (first reaction) and 143 (nested reaction) nucleotides from the gene that encodes the viral nonstructural protein 5 (NS5). In addition, specific primers for CxFV (Newman et al., 2011) were used to span a downstream 672 bp fragment into the NS5. The DNA fragments were purified from 1.2% agarose gels using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and were eluted in 30 µL ultrapure nuclease-free water. Positive samples were sequenced by Macrogen (South Korea). All high-quality sequences were primarily identified using the Nucleotide Basic Local Alignment Search Tool

(BLASTN, https://blast.ncbi.nlm.nih.gov/) and further edited with the BioEdit (v7.2.5) software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Alignments were constructed using Mafft 7.3 and Muscle softwares (Edgar, 2004; Katoh and Standley, 2013); the dataset included the here-reported sequences and representative sequences of the genus *Flavivirus* retrieved from GenBank. The ModelGenerator v0.85 software was used to estimate the most suitable model of nucleotide substitution (Keane et al., 2006). For NS5 sequences phylogenetic reconstruction was done under Bayesian analysis using the MrBayes v3.2 software (Ronquist et al., 2012). The analysis was conducted under the general time reversible + gamma + proportion invariant (GTR + Γ + I) model of nucleotide substitution. Two runs of 4 chains each (1 cold, 3 heated, temperature 0.20) were run for 2.5 million generations; trees were sampled every 100 generations. Convergence was assessed by using the average standard deviation in partition frequency values across independent analyses with a threshold value of 0.01; burn-in was set to 25%. Posterior probabilities (pp) were used as statistical support of the tree nodes. Phylogenetic reconstruction for the complete flavivirus sequences was done under the maximum likelihood criterion. To achieve a more accurate alignment, the deduced aminoacidic sequences of the polyprotein gene were used. Aminoacidic substitution model estimation and phylogenetic inference were done by the use of the PhyML v3.0 software (Guindon et al., 2010). The analysis was conducted under the LG model with a proportion of invariable sites of 0.081 and a gamma shape parameter on 1.379. Node supports were calculated by approximate likelihood ratio test (aLRT). All sequences were submitted to the NCBI database (GenBank accession numbers MG970151-MG970163 and MH899446).

2.6. Isolation and propagation of SbFV

A. albopictus cells (C6/36 ATCC® CRL-1660; Manassas, Virginia, USA) were grown in 25 cm^2 flasks containing Leibovitz's medium (L-15) (Gibco, Waltham, USA), 5% fetal bovine serum (FBS) (Gibco, Waltham, USA), 25 μg/ml gentamicin (Gibco, Waltham, USA), and 0.26% tryptose (Sigma-Aldrich, St. Louis, USA), then held at 28 °C. SbFV (samples Ms24 and Ms42) were isolated in a C6/36 cell culture by inoculating 50 μL of two mosquito pool homogenates onto sub-confluent cells. No cytopathic effects were observed up to 7 days post-inoculation (d.p.i). A subsequent passage (P1) of SbFV was performed in C6/36 cells, and morphological changes in the cell culture were observed after 5 d.p.i. Further, the SbFV isolate (Ms42) was titrated in the dilutions of 10^{-1} to 10^{-7} in three insect cell lineages: C6/36, *Ae. aegypti* (Aag-2 RRID:CVCL_Z617), and *Ae. pseudoscutellaris* (LSTM-AP-61 RRID:CVCL_Z362); and four vertebrate cell lineages: epithelial cells derived from *Cercopithecus aethiops* kidney (Vero E6 Sigma-Aldrich, 85020206; Salisbury, UK), human hepatocarcinoma (Huh-7.5 ATCC® PTA-8561™; Manassas, Virginia, USA), *Gallus gallus* embryo fibroblasts (UMNSAH/DF1 ATCC® CRL-12203™; Manassas, Virginia, USA), and baby *Mesocricetus auratus* kidney fibroblasts (BHK-21 ATCC® CCL-10™; Manassas, Virginia, USA). AP-61 cells were cultured in L-15 medium with 10% FBS, 25 μg/ml gentamicin, and 0.53% tryptose, while Aag-2 cells were cultivated in Schneider's Drosophila Medium (Gibco, Grand Island, NY, USA), 10% FBS, 25 μg/ml gentamicin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (P/S). Vertebrate cells were grown in Dulbecco's modified Eagle's medium/nutrient F-12 Ham (Gibco, Grand Island, NY, USA) with 10% FBS, and 100 U/ml/ 100 μg/ml of P/S. To test viral replication, the abovementioned cell lineages were incubated in parallel with SbFV for 1 h at two multiplicities of infection (MOI 1 and 10). After viral adsorption, cells were washed three times with PBS 1×, one aliquot of the supernatant was harvested for the time point of 0 d.p.i., and another aliquot was recovered 3 d.p.i. Virus infection was evaluated by pan-flavivirus RT-PCR and by indirect immunofluorescence (IFI) assay using the pan flaviviruses monoclonal antibody 4G2 (hybridoma D1–4G2–4–15, ATCC HB-112).

2.7. Cloning of RT-PCR fragments and Next-Generation Sequencing (NGS) of SbFV

To maximize the sequence information of the newfound *Sabethes* flavivirus, NS5 RT-PCR fragments (1385 bp) from the isolates Ms24 and Ms42 were cloned in the pGEM®T-Easy vector system (Promega, Madison, USA) following the manufacturer's guidelines. Plasmid DNA was purified with Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, USA). Cloned DNAs from SbFV were further sequenced with T7 and Sp6 primers at Macrogen (South Korea).

For NGS, total RNA from the Ms42 SbFV isolate was extracted from the supernatant of infected C6/36, following the manufacturer's protocol of the RNeasy®Mini Kit (Qiagen, Hilden, Germany). DNA contaminations were avoided using the RNase-Free DNase Set provided by the kit. The purified RNA was used to construct cDNA libraries using the TruSeq RNA library preparation kit (Illumina). The resulting libraries were sequenced on the Illumina MiSeq platform, in a paired-end configuration (2 × 75), generating around of 250,000 reads. The reads were trimmed for adapters, high quality (Phred > = 20) and shorter than 15 nucleotides using the CLC Genomics Workbench 10.5. The trimmed reads were assembled using the CLC *De novo* assembly tool in the default configuration.

3. Results

3.1. Prevalence of flavivirus infection in mosquitoes

The presence of viral genome in several species of *Culex* (2442 mosquitoes in 183 pools), *Aedes* (532 mosquitoes in 42 pools), *Sabethes* (83 mosquitoes in 19 pools), and others (124 mosquitoes in 43 pools) collected in four distinct areas of Brazil (BR) and Paraguay (PY) (Table 1 and Table S2) was investigated. The samplings in Asunción/PY (year 2013) (Fig. S1(A)), Vitória/BR (year 2016) (Fig. S1(B)), and Curitiba/BR (year 2016) (Fig. S1(C)) were performed in high-urbanized areas where clinical arboviral infections were confirmed. Ribeirão Claro and Carlópolis/BR (year 2017) (Fig. S1(D)) are non-populous cities at risk for YFV transmission. Remarkably, the most urbanized areas were the ones where mosquitoes presented the highest percentages of positivity for ISFVs (Fig. S1(A-C)). Of a total of 183 *Culex* pools, 139 were positive for flavivirus infection (76% infectivity). This number represented 90% (66 of 73) infectivity in Asunción, 76% (65 of 86) in Vitória, 58% (7 of 12) in Curitiba, and 8% (1 of 12) in Ribeirão Claro/Carlópolis. In addition, 13% (2 of 15) of the *Aedes* (*Ochlerotatus*) *scapularis* (Rondani, 1848) pools from Asunción/PY, one *A. albopictus* (Skuse, 1894) from Curitiba, and 11% (2 of 19) of the pools of *S. belisarioi* group from Ribeirão Claro were flavivirus positive (Table 1 and Table S2).

Despite geographic and climatic differences (Table in Fig. S1), all cities present an abundance of mosquito species, which may act as potential vectors. In addition, sporadic outbreaks of arboviruses have been reported in these cities, justifying sustained epidemiological surveillance.

3.2. Prospection of DENV, ZIKV, CHIKV, SLEV, and YFV in flavivirus-positive mosquitoes

Mosquitoes collected in areas with potential arbovirus transmission were screened for viral RNA as a surveillance procedure (Fig. S1 and Table S2). As detailed in the Table S2, the Brazilian samples that yielded positive results using pan-flavivirus primer sets were further analyzed using RT-PCRs reactions with primer sets specific for DENV, ZIKV, and SLEV. We also performed real time RT-PCRs for CHIKV and YFV screening in samples from Paraná State and for a more sensitive ZIKV detection in inconclusive samples. The samples were negative for all arboviruses tested.

Table 1
Viral detection in mosquito pools.

City/state/country/year	Genus/species	Gender	♯ of Mosquitoes	♯ of Pools	Flavivirus detection			Alphavirus detection
					RT-PCR	Positivity of Infection (%)	♯ of High Quality Sequences	RT-PCR
Asunción PY/2013	*Aedes scapularis*	F	465	15	02 Positives	13	02 CxFV	Negative
	Culex.coronator	F	504	20	17 Positives	85	03 CxFV	Negative
	Culex declarator	F	25	01	01 Positives	100	01 CxFV	Negative
	Culex (*Melanoconiom*) spp.	F	70	02	02 Positives	100	02 CxFV	Negative
	Culex nigripalpus	F	585	17	17 Positives	100	03 CxFV	Negative
	Culex quinquefasciatus	F	885	33	29 Positives	88	04 CxFV	Negative
Vitória/ES BR/2016	*Culex* spp.	F	311	86	65 Positives	76	62 CxFV	NT
Curitiba/PR BR/2016	*Aedes albopictus*	F	01	01	01 Positive	100	01 AEFV	Negative
	Culex spp.	M	21	05	03 Positives	60	03 CxFV	Negative
	Culex spp.	F	18	07	04 Positives	57	04 CxFV	Negative
Ribeirão Claro/ Carlópolis/PR BR/ 2017	*Aedes* spp.	F	11	07	Negative	0	–	NT
	Aedes albopictus	F	06	03	Negative	0	–	NT
	Aedes hastatus/serratus/ oligopistus	F	30	04	Negative	0	–	NT
	Aedes scapularis	F	18	11	Negative	0	–	NT
	Aedes terrens	F	01	01	Negative	0	–	NT
	Culex spp.	M	12	05	Negative	0	–	NT
	Culex spp.	F	05	04	Negative	0	–	NT
	Culex (*Melanoconiom*) *pilosus* group	F	01	01	Negative	0	–	NT
	Culex (*Culex*) *coronator* group	F	01	01	01 Positive	100	01 CxFV	NT
	Culex quinquefasciatus	F	04	01	Negative	0	–	NT
	Coquellettidia venezuelensis	F	05	02	Negative	0	–	NT
	Haemagogus sp.	F	3	02	Negative	0	–	NT
	Haemagogus leucocelaenus	F	53	20	Negative	0	–	NT
	Psorophora ferox/ pseudomelanota	F	22	07	Negative	0	–	NT
	Psorophora lutzii/ amazonica	F	23	05	Negative	0	–	NT
	Sabethes sp.	F	02	02	Negative	0	–	NT
	Sabethes albiprivus	F	67	09	Negative	0	–	NT
	Sabethes belisarioi	M	02	01	Negative	0	–	NT
	Sabethes belisarioi	F	12	07	02 Positives	29	02 SbFV	NT
	Trichoprosopon compressum mogilasium	F	03	01	Negative	0	–	NT
	Wyeomyia spp.	F	07	03	Negative	0	–	NT
	Wyeomyia medioalbipes	F	08	03	Negative	0	–	NT
Total			**3181**	**287**	**144 Positives**		**88**	**0**

Mosquito pools were obtained in Asunción, Paraguay (PY) (from November 2013 to December 2013); Vitória/Espírito Santo (ES) State, Brazil (BR) (from February 2016 to March 2016); Curitiba/ Paraná (PR) State, BR (from April 2016 to June 2016); and Ribeirão Claro/Carlópolis/ PR, BR (February 2017). NT: Not tested.

3.3. Sequence comparison and phylogenetic analysis

Pan-flavivirus primer sets (Flavi1 and Flavi2) were used in nested-RT-PCR reactions to amplify and to sequence fragments of 1385 bp in the first round and 143 bp in the second round, both in the conserved flavivirus NS5 gene. This region is informative enough to identify diverse flaviviruses through sequencing and phylogenetic analysis (Sánchez-Seco et al., 2005). Among a total of 144 positive pools for pan-flavivirus, we were able to recover 88 high-quality nucleotide sequences: 15 (of 68) from Asunción, 62 (of 65) from Vitória, 8 (of 8) from Curitiba, and 3 (of 3) from Ribeirão Claro/Carlópolis. Alignment of sequences against the database at NCBI showed a high nucleotide identity (> 95%) of 85 sequences with CxFV and one with AEFV (*A. albopictus* sample PR11) (Table 1). However, two sequences retrieved from *S. belisarioi* (Ms24 and Ms42 pools) presented a lower nucleotide identity (< 68%) with other ISFVs. The nucleotide identity between Ms24 and Ms42 viruses was 95%. Sequencing of the mitochondrial cytochrome c oxidase subunit I gene (*COI*) confirmed the morphological identification of mosquitoes from these samples as *S. belisarioi*, as it shared 99% of identity with the mitochondrial genome of this species (Aragão et al., 2018).

Sequences obtained from the 143 bp NS5 fragment of CxFV from Paraguay and Brazil showed a substantial genetic homogeneity. Despite this, we could recognize 7 haplotypes, which differed in 1–6 nucleotides. Both Paraguayan haplotypes (represented by the PY40 and PY126) differed in 5 or 6 nucleotides in relation to the main Brazilian haplotype (represented by ES01) (Fig. S2). Aiming to obtain additional sequence information for the final dataset, we selected five representative Brazilian samples (Ms93, ES01, ES29, PR01, and PR08) and performed a CxFV-specific amplification (Newman et al., 2011), which resulted in 672 bp amplicons. Previous phylogenetic studies showed that CxFV strains can be divided in two major clades: genotype 1, comprising strains from North America (USA - Iowa, Texas, and Chicago), and Asia (Japan, China, and Indonesia), and genotype 2, comprising strains from Latin America (Brazil, Argentina, Colombia, and Mexico) (Farfan-Ale et al., 2009; Goenaga et al., 2014), Caribbean (Guatemala and Trinidad) (Kim et al., 2009; Morales-Betoulle et al., 2008), Taiwan, and Africa (Uganda) (Bittar et al., 2016; Huanyu et al., 2012). Our phylogenetic analysis reinforced the Brazilian sequences as genotype 2. Viral sequences from Vitória and Curitiba were more similar, clustering together in a unique subclade, whereas the Ms93 viral sequence was more distantly related (Fig. 1). Interestingly, Ms93-CxFV came from a mosquito pool from a forest environment in Ribeirão Claro, whereas Vitória and Curitiba samples were trapped in urban

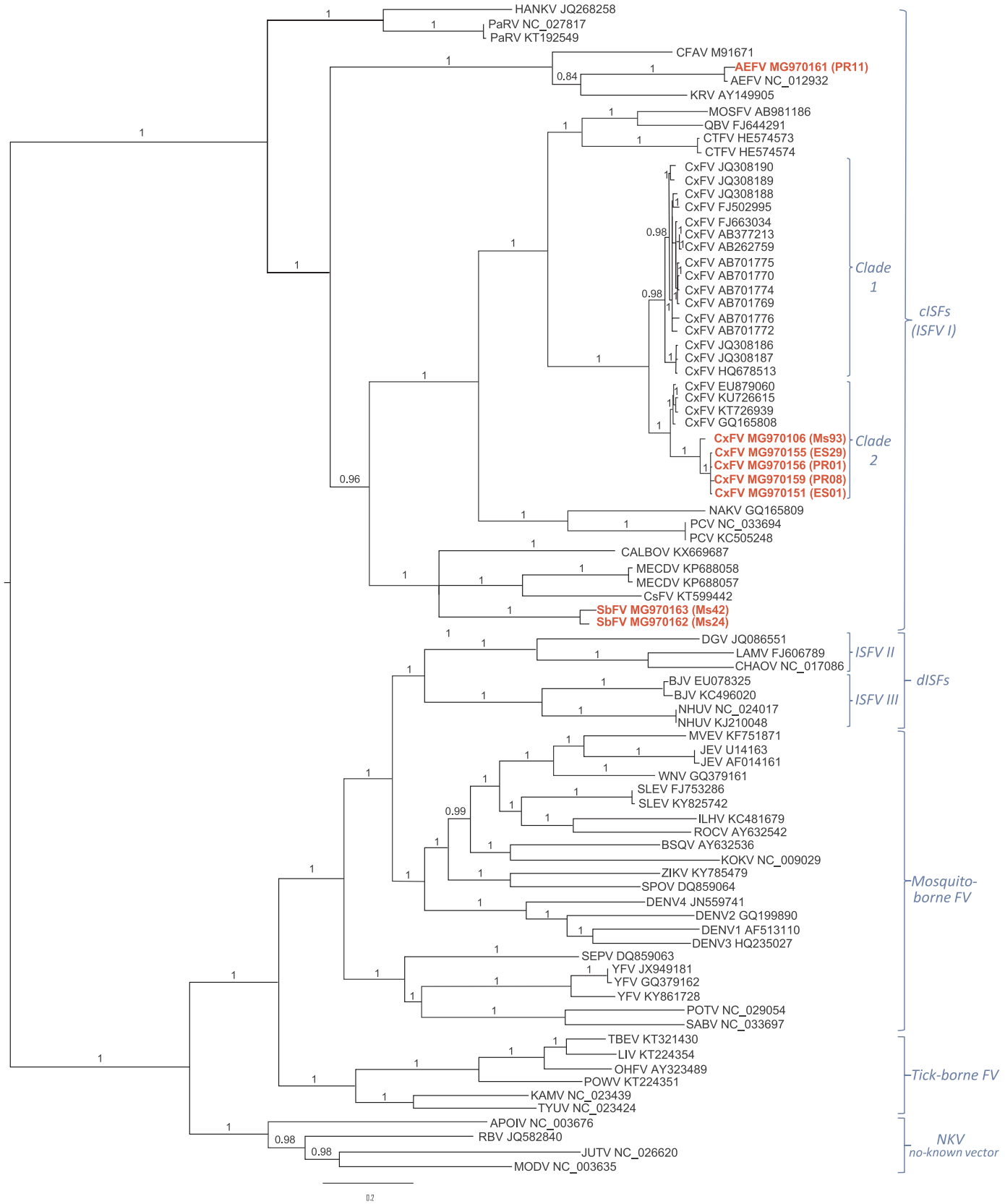

Fig. 1. Phylogenetic analyses. Alignment based on the NS5 gene from the ISFVs performed with the 1385 bp nucleotide sequences from the PR11, Ms24, and Ms42 samples, and the 672 bp sequences from the Ms93, ES01, ES29, PR01, and PR08 samples.

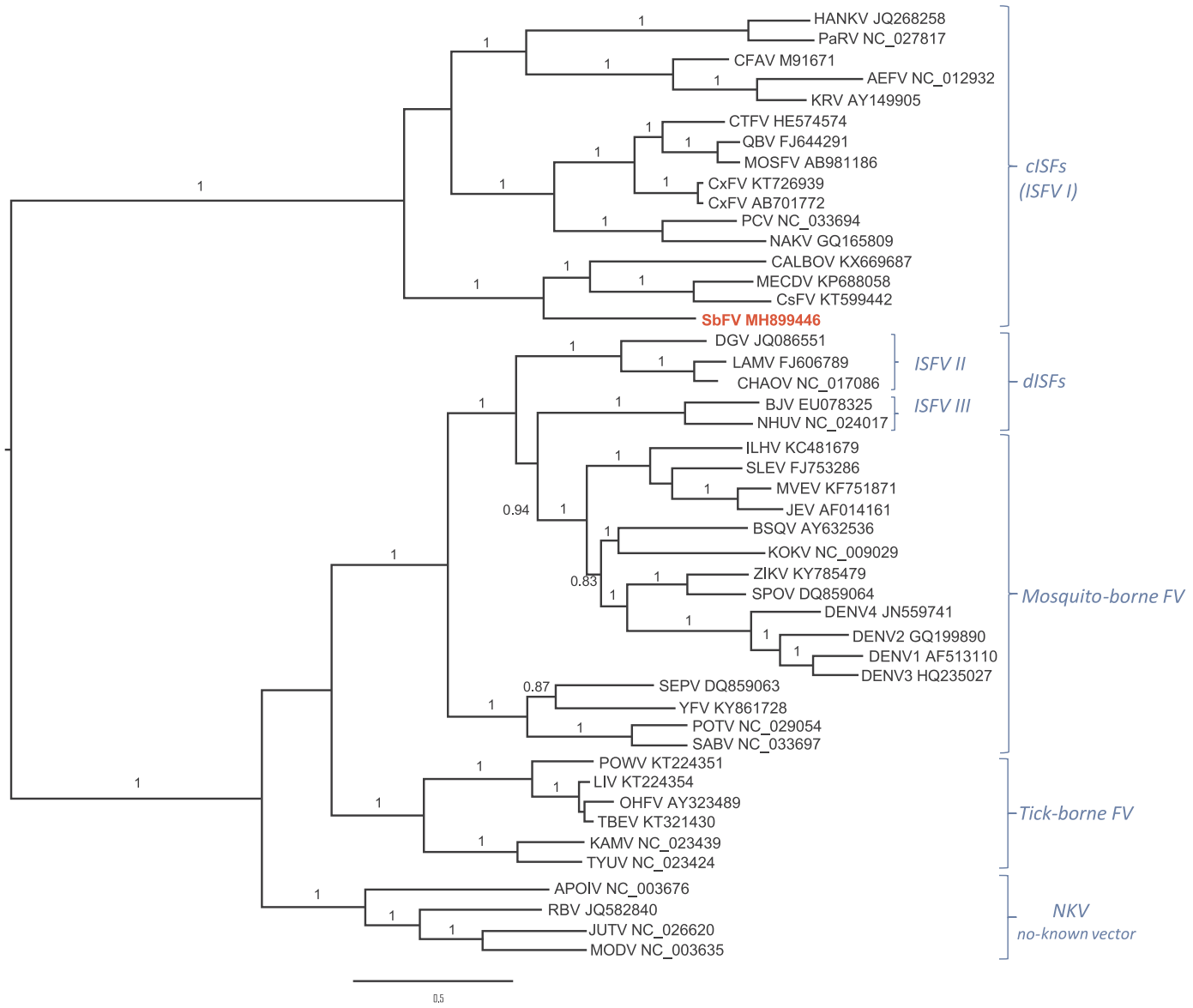

Fig. 2. Phylogenetic analyses. Alignment based on the complete genome from the isolate Ms42. The reference ISFVs are detailed in the Table S5.

settings.

The nucleotide sequences for the viruses from the samples Ms24 and Ms42 were retrieved either from viral isolation in C6/36, PCR-cloned samples, or NGS. Analysis performed on 1385 bp of the NS5 gene showed that the Ms24 and Ms42 viral sequences are related to the ISFVs CsFv, MECDV, and CBOV, displaying a nucleotide identity of 67% with CsFV, 66–67% with MECDV, and 65% with CLBOV and a deduced aminoacidic identity of 75% with CsFV, 74–75% with MECDV, and 70% with CLBOV (Fig. 1 and Table S4). The analysis of the sequence generated by NGS for the isolate Ms42 corroborated the phylogenetic reconstruction obtained on the NS5 gene. The virus genome was identified in a single contig containing, 10,585 bp with an average coverage of 1485×, through blastn comparison against the nr database. To build a more accurate phylogeny, the deduced aminoacidic sequence of the complete polyprotein gene was used. As result, Ms42 virus appears sharing a common ancestor, although distantly related, to a clade formed by MECDV, CsFV, and CLBOV with a high statistical support (aLRT = 1). Comparison of the aminoacidic sequences showed an average identity of 51% with the above-mentioned ISFVs (Fig. 2). CLBOV and MECDV are ISFVs first identified in *Culex* (*Culex*) *tarsalis* (Coquillett, 1896) in California/USA (2007) and in *Culex* sp. in Panama (2011), respectively (Bolling et al., 2011; Carrera et al., 2015). CsFV was first retrieved from *Culiseta* (*Climacura*) *melanura* (Coquillett, 1902) mosquitoes in the northeastern USA (2013) (Misencik et al., 2016).

3.4. Biological characterization of SbFV

To further characterize the virus Ms42 (SbFV), vertebrate cell lines as Huh7.5 (human), Vero E6 (monkey), UMNSAH/DF1 (chicken) and BHK-21 (hamster) were infected at MOIs of 1 and 10. Besides C6/36, other mosquito cell lines were included as positive controls for viral infection. As depicted in Fig. 3, only the mosquito cells were permissive to SbFV infection. All vertebrate cells were refractory to SbFV even at a MOI of 10 as confirmed by pan-flavivirus RT-PCR. Furthermore, pan-flavivirus mAb 4G2 did not detect SbFV in IFI assays. DENV-1 and YFV (MOI 1) were used as controls, showing the permissiveness of all cell lineages to flavivirus infection (Fig. 3).

4. Discussion

ISFVs (such as AEFV, CxFV, and SbFV) and arboviruses (such as DENV, ZIKV, CHIKV, SLEV, and YFV) share the same ecologic niches.

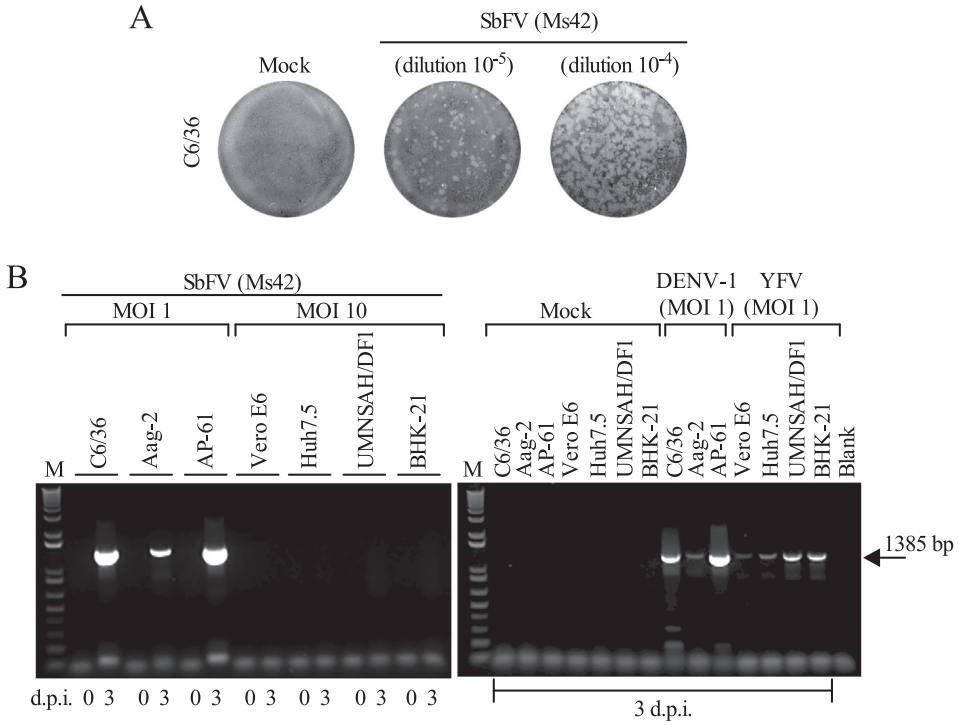

Fig. 3. Biological characterization of SbFV. (A) Titration and plaque morphology of SbFV (isolate Ms42) in C6/36 cell lineage. SbFV was inoculated in insect and vertebrate cell lineages in dilutions from 10^{-1} to 10^{-10}. (B) Pan-flavivirus RT-PCR reaction in the supernatants from insect and vertebrate cell lineages infected with SbFV, DENV–1, and YFV. The replication of SbFV was evaluated in the time points of 0 and 3 d.p.i.

Thus, circulation of ISFVs represents a challenge for interpreting epidemiological patterns of arboviruses, since the presence of ISFVs in mosquitoes may affect the secondary infection by arboviruses and their dynamics of transmission between vectors and hosts (Mosimann et al., 2011). Previous studies have revealed the phenomenon of super-infection exclusion. In these hypotheses, the virus infection acts as a barrier to the same or similar virus by eliciting an antiviral stage in insect cells (Blitvich and Firth, 2015; Kenney et al., 2014; Mosimann et al., 2011). Conversely, it has been proposed that certain ISFVs can facilitate a secondary infection, enhancing the virulence potential of other flaviviruses, as reported during WNV and CxFV co-infection (Kent et al., 2010; Newman et al., 2011). Both possibilities may directly impact arboviral dissemination. Thus, we are tempting to speculate that areas in which ISFVs are highly prevalent may present an augmented risk or resistance to a possible outbreak depending on the viruses involved. In this study we did not detect co-infections in the examined mosquitoes. Further experiments on co-infections in mosquitoes should help explore the interplay between ISVs and arboviruses, whether infected mosquitoes became refractory or more susceptible to a secondary infection.

A high prevalence of CxFV infection in a wide South American territory overlapped with areas of emergent arboviruses circulation, mainly in regions with a high human population density. Although CxFV is mostly found in *Culex* (*Culex*) *quinquefasciatus* Say, 1823, it was sporadically retrieved from other *Culex* species such as *Culex* (*Culex*) *nigripalpus* Theobald, 1901 or *Culex* (*Culex*) *maxi* Dyar, 1928 (Goenaga et al., 2014). Interestingly, we found CxFV in *Culex* (*Culex*) *coronator* group, *Culex* (*Culex*) *declarator* Dyar & Knab, 1906, *Culex* (*Melanoconion*) spp., *C. nigripalpus, C. quinquefasciatus,* and in *A. scapularis* species (Table 1). The findings of CxFV in five *Culex* species, and in other mosquito genus such as *Aedes* deserve further study. The predominant mosquito species evaluated in this work belong to the genus *Culex*. Most likely, the cosmopolitan behavior of the *Culex* mosquitoes, suggested by Batallán et al. (2015) together with the ISFV transmission between mosquitoes were determinant factors for the high prevalence of infection. In fact, vertical and venereal transmission are the main mechanisms by which ISFVs persist in mosquitoes in nature (Blitvich and Firth, 2015). A better understanding of ISFV transmission is another aspect that may be relevant to the knowledge on ISVs biology in nature.

As suggested by Bittar *et al.*, there is an association between CxFV phylogeny, climate, and host distribution (Bittar et al., 2016). Moreover, the most common ISFV-vector relationships known until now are between CxFV-genotype 1 and *C. pipiens*; CxFV-genotype 2 and *C. quinquefasciatus*; AEFV and *Aedes* sp. In Brazil, CxFV was previously reported in different regions where *Culex* spp. are present: the first Brazilian report was in the city of São José do Rio Preto/São Paulo State in 2012 (Machado et al., 2012) and, more recently, in the city of São Paulo/São Paulo State in 2016 (Fernandes et al., 2016). Additionally, CxFV was detected in mosquitoes from Cuiabá/Mato Grosso State in 2013 (Midwest of Brazil/GenBank: KP764779.1). CxFV has also been reported in several countries in South America, but there are no previous reports from Paraguay.

The presence of AEFV in the *A. albopictus* sample from Curitiba was the first mention of this ISFV in the Brazilian South region. AEFV was previously reported in Brazil only in the Southern São Paulo City in 2016 (Fernandes et al., 2016).

We have detected a new flavivirus (SbFV) infecting a Sabethini member, a tribe that possesses known relevance for arboviruses, such as YFV and SLEV. SbFV was detected in *S. belisarioi* mosquitoes captured in a region with suspicion for YFV sylvatic transmission (red arrows in Fig. S1(D)). Interestingly, this mosquito is the same species from which SLEV was first isolated in Brazil (Causey et al., 1964). Historically, the main mosquito species related to YFV transmission in South America are *Sabethes* (*Sabethoides*) *chloropterus* (von Humboldt, 1819), *Haemagogus* (*Haemagogus*) *janthinomys* Dyar, 1921, *Haemagogus* (*Conopostegus*) *leucocelaenus* (Dyar and Shannon, 1924), and *A. aegypti* (reviewed in Barrett and Higgs, 2007). However, the potential for other species from these genera to act as YFV vectors is yet poorly explored. *H. janthinomys* and *Sabethes* spp., including *S. belisarioi*, exhibit a canopy behavior, while *H. leucocelaenus* is most frequently observed at ground level (Pinto et al., 2009). This behavior is closely related to their feeding preferences on monkeys and birds, which are known hosts for YFV and SLEV, respectively. *S. belisarioi* has been found inside and in the vicinity of human dwellings in urban areas in the State of Paraná (Silva, 2003), enabling to potentially act as a “bridge-vector” of arboviruses between sylvatic and urban environments.

Kuno et al. (1998) suggested that a pairwise nucleotide sequence identity of > 84%, estimated on a 1000 bp NS5 sequence, correlates well with among-species antigenic characteristics for the flaviviruses (Kuno et al., 1998). NS5 sequence of SbFV presented a nucleotide identity ranging of 65–67% regarding the most related ISFVs. As expected, the analysis of the complete genome sequence showed a greater divergence when compared to the closest ISFVs, showing an average aminoacidic identity of 51%. Also, SbFV infected *S. belisarioi* and replicated only in insect cell lineages in vitro, but not vertebrates. Additionally, the phylogenetic inferences included this virus into the clade composed by CsFV, MECDV, and CLBOV. Altogether, these data confirmed the SbFV as an ISFV from the group I (ISFV-I or cISF).

Summing up, phylogenetic analysis and aminoacidic divergence of the entire polyprotein regarding the ISFV I group, together with the differences in geographical distribution and mosquito association, lead us to propose these viruses as novel insect-specific flaviviruses, tentatively named *Sabethes* flavivirus (SbFV).

Mosquitoes from distinct groups, such as Culicini, Aedini, and Sabethini, have different susceptibility for arbovirus and ISFV infection. As reviewed by Calzolari et al. (2016) a virus firstly identified in a mosquito species presents potential to infect other species, including mosquitoes from far distinct genera and in distinct countries, demonstrating the high dispersion of ISFVs. In accordance with this premise, CxFV was detected in five *Culex* species and *A. scapularis* mosquitoes. In addition, SbFV replicated in three cell lineages from distinct mosquito species but, induced cytopathic effect (CPE) and plaque formation only in C6/36 cells.

Thus, the ecological and behavioral relationships between mosquito species and the potential of viruses to break the species-to-species barriers of transmission are a matter to be exploit. This knowledge is relevant to understanding how the evolutionary divergences could determine the relationships between hosts and viruses, including the arboviruses.

5. Conclusion

In this study, we demonstrated the wide distribution and diversity of ISFVs in South American mosquitoes in areas overlapping the circulation of emergent arboviruses of public health importance. The impact of this sympatric distribution on arboviral ecology and transmission in nature deserves future study. Moreover, we described a new ISFV from *Sabethes belisarioi* thus contributing to the knowledge of the diversity and ubiquitous nature of insect-specific viruses.

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Availability of data and material

The GenBank/EMBL/DDBJ accession numbers for the NS5 gene sequences are MG970151-MG970160 (CxFV), MG970161 (AEFV), and MG970162/MG970163/MH899446 (SbFV). The GenBank/EMBL/DDBJ accession number for the *COI* gene sequence from *S. belisarioi* is MH248002. Please contact author for more data requests.

Competing interests

The authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.11.008.

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