Virology 534 (2019) 72–79

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

An iflavirus found in stink bugs (Hemiptera: Pentatomidae) of four different species

Ethiane R. dos Santos[a,1], Luana B. Trentin[a,1], Assis Ecker[a], Leonardo A. Silva[b], Miguel Borges[c], Joseph D. Mowery[d], Bergmann M. Ribeiro[b], Robert L. Harrison[e], Daniel M.P. Ardisson-Araújo[a,*]

[a] Laboratory of Insect Virology, Department of Biochemistry and Molecular Biology, Federal University of Santa Maria, Santa Maria, RS, 97105-900, Brazil
[b] Laboratory of Baculovirus, Cell Biology Department, University of Brasilia, Brasilia, DF, 70910-900, Brazil
[c] Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, 70.770-917, Brazil
[d] Electron and Confocal Microscopy Unit, Beltsville Agricultural Research Center, USDA Agricultural Research Service, Beltsville, MD, 20705, USA
[e] Invasive Insect Biocontrol and Behavior Laboratory, Beltsville Agricultural Research Center, USDA Agricultural Research Service, Beltsville, MD, 20705, USA

ARTICLE INFO

Keywords:
Iflavirus
Iflaviridae
Covert infection
Stink bugs
Pentatomidae
Chinavia ubica
Dichelops melachantus
Euschistus heros
Halyomorpha halys

ABSTRACT

An analysis of transcriptomes from the antennae of the three South American stink bugs (*Euschistus heros, Chinavia ubica*, and *Dichelops melacanthus*) revealed the presence of picorna-like virus genome-length RNAs with high sequence identity to the genome of Halyomorpha halys virus (HhV), originally discovered in the transcriptome of the brown marmorated stink bug, *Halyomorpha halys*. Features of the genome, phylogenetic relationships to other viruses, and the appearances of virus-like particles isolated from host stink bugs all confirm that these viruses are iflaviruses and isolates of an undescribed species. Iflavirus RNAs were present at high levels (40%–90% of transcriptome reads) in the stink bug antennal transcriptomes. In whole-insect transcriptomes of *H. halys*, HhV reads were > 500-fold more abundant in adults than in nymphs. We identified from field population a subject of species *E. heros* infected by this iflavirus. The results of the analysis suggest that these iflaviruses are able to produce large quantities of their RNAs without causing any obvious pathology to their hosts.

1. Introduction

Iflaviruses belong to the picorna-like virus family *Iflaviridae* (order *Picornavirales*) and are classified within a single genus, *Iflavirus* (Valles et al., 2017). Members of *Iflavirus* infect invertebrates of phylum Arthropoda, including Lepidoptera, Hymenoptera, Diptera, Coleoptera, Acari, Araneae, Mesostigmata, and Decapoda (Aizawa et al., 1964; Liu et al., 2017). They are characterized by a positive ssRNA genome organized into a single ORF that codes for one polyprotein consisting of structural and non-structural peptides. The ORF is flanked by a long IRES-containing 5′-UTR and a short, conserved poly-A tail-containing 3′-UTR (Lanzi et al., 2006), which regulate both virus replication and translation (Belsham, 2009). A stable 30-nm non-enveloped ichosahedral capsid packages and shields the RNA genome (van Oers, 2010).

The iflavirus infection process is still not well understood and seems to differ depending on the host type. In some iflavirus hosts, such as the silkworm *Bombyx mori* (Himeno et al., 1974), the oil palm tree pest *Opsiphanes invirae* (Silva et al., 2015), and the honey bee *Apis mellifera* (Yue et al., 2007), signs of infection are quite evident depending on the virus species and host condition. For example, in silkworm larvae, the pathology of infectious flacherie virus (the type species of iflavirus) infection includes a brown color or other discoloration of the posterior and middle parts of the insect body and flaccidity (Inoue and Ayuzawa, 1972). In honeybees, iflaviruses may cause foreleg paralysis, wing deformities, a shortened abdomen and discoloration of the adult bees, as well as mortality (Bailey and Woods, 1974; Bailey and Ball, 1991). These viruses are usually associated and transmitted by the parasite mite *Varroa destructor* (Santillán-Galicia et al., 2010), and may be directly linked to colony collapse disorder (Bailey and Ball, 1991). However, the majority of iflavirus infections do not result in visible signs of infection or any apparent disease (van Oers, 2010).

The first picorna-like virus found infecting a stink bug was reported from the southern green stink bug, *Nezara viridula*, by Williamson and von Wechmar (1992, 1995). This virus (Nezara viridula virus 1; NVV1)

* Corresponding author.
E-mail addresses: ethi_rozo@hotmail.com (E.R. dos Santos), luanabelotrentin@gmail.com (L.B. Trentin), assisecker@hotmail.com (A. Ecker), leocbq@gmail.com (L.A. Silva), miguel.borges@embrapa.br (M. Borges), joseph.mowery@ars.usda.gov (J.D. Mowery), bergmann@unb.br (B.M. Ribeiro), robert.l.harrison@ars.usda.gov (R.L. Harrison), daniel.araujo@ufsm.br, daniel.araujo@ufsm.br (D.M.P. Ardisson-Araújo).
[1] Equal contribution.

https://doi.org/10.1016/j.virol.2019.06.002
Received 25 March 2019; Received in revised form 5 June 2019; Accepted 5 June 2019
Available online 06 June 2019

caused reduced insect longevity when associated with a toti-like virus with a monopartite dsRNA genome (NVV2). Another picorna-like virus (Plautia stali intestine virus, PSIV) causing disease in a stink bug was isolated from the brown-winged green bug, *Plautia stali*. PSIV was also shown to infect the brown marmorated stink bug, *Halyomorpha halys* (Nakashima et al., 1998). A covert picorna-like virus infection of a stink bug host was detected when a picorna-like virus genome was identified in a whole body transcriptome developed from a USDA colony of *Halyomorph halys* (Sparks et al., 2013). Analysis of the genome sequence indicated that this virus (termed Halymorpha halys iflavirus, or HhV) should be classified as an iflavirus. No further studies on the route of infection, pathology, or biological control potential of HhV have been published.

In this study, we report the discovery of new isolates of HhV covertly infecting three other pentatomid hosts, including *Euschistus heros* (Fabricius, 1794), *Chinavia ubica* (Rolston, 1983), and *Dichelops melacanthus* (Dallas, 1851). *E. heros* is considered the major pest of soybean cultures in Neotropical regions (Panizzi et al., 2000). *C. ubica* and *D. melacanthus* are soybean secondary pests less abundant than *E. heros* and damage soybean crops as well (Panizzi et al., 2000, 2007). In general, stink bugs feed by inserting their needle-like stylets into stems, leaves, blooms, fruit or seeds (Velikova et al., 2010). By sucking on plant fluids and injecting toxic saliva into the host tissues, these organisms cause delayed maturation and deformities in seeds, which affects grain quality and production (Velikova et al., 2010). Several strategies to control the spread of stinkbug populations have been adopted, including the applications of broad-spectrum insecticides, the release of natural predators such as the Neotropical egg parasitoid *Telenomus podisi* (Ashmead, 1893) (Hymenoptera: Scelionidae), and the use of entomopathogenic biopesticides (Gouli et al., 2012) or pheromone lures (Weber et al., 2014). However, the intensive and indiscriminate use of chemical insecticides has resulted in the elimination of natural enemies and the selection of resistant insect populations (Baur et al., 2010). Because several hemipterans of family Pentatomidae may be invasive pests, the impact of covertly-infecting viruses on insect pest biology and ecology is of importance for the control of insect populations.

2. Materials and methods

2.1. SRA data, trimming, de novo assembly, and BLAST search

We obtained the raw transcriptome data from Sequence Read Archive (SRA) experiments (Table S1) assigned to the BioProject PRJNA246320 using the software CLC Genomics Workbench 11 (CLC bio, Aarhus, Denmark). The transcriptomes were generated from three pools containing 50 antenna pairs from males and females (1:1) of three Brazilian stink bug species *E. heros*, *C. ubica*, and *D. melacanthus* (for more details, see Farias et al., 2015) from a technical duplicate. The six raw SRAs were trimmed and *de novo* assembled using the same software. The generated contig lists were used as queries to search for similar viral sequences in a non-redundant local Viral protein Refseq database by means of the BLAST implemented in Geneious R10 (Kearse et al., 2012).

2.2. Viral genome assembly, annotation, and viral RNA quantification

Several contigs were found to have significant sequence similarity to the genome sequence of Halyomorpha halys virus (HhV), previously identified in the transcriptome of the brown marmorated stink bug *Halyomorpha halys* (Hemiptera: Pentatomidae; Sparks et al., 2013). In order to assemble complete viral genome sequences, we used the HhV genome (GenBank accession no. NC_022611) as a reference sequence and mapped individually trimmed reads from the six libraries that matched the HhV sequence with a pairwise identity of ≥80% in Geneious R10 (Kearse et al., 2012). After that, we extracted the consensus sequence for each mapped read and mapped back again the trimmed reads exhibiting a pairwise identity of ≥99% to confirm the results of the first mapping and the correct assembly of the viral genomes. Moreover, to quantify the viral RNA sequence reads in different transcriptomes, we mapped the total trimmed reads for each transcriptome against the HhV polyprotein CDS sequence. As an internal control, we used the CDS sequence of the highly expressed *actin* gene. The total number of mapped reads were divided by the total number of reads and the query size (*i.e.* the virus *polyprotein* CDS or the *actin* CDS) for each replicate and the mean ± standard deviation were plotted in graph bars as arbitrary values. In the case of *H. halys*, one single replicate was performed for transcriptomes generated from second and fourth instar nymphs, adult male, and adult female (Sparks et al., 2014). Imature and adults are graphed together with mean ± standard deviation.

2.3. Search for single nucleotide variants (SNVs) among isolates of HhV in the polyprotein CDS

To search for SNVs among the new virus isolate genomes, we conducted a MAFFT alignment between the HhV genome and each of the new identified genomes. After that, we set the HhV genome sequence as a reference and searched for variants within the polyprotein CDS. Effects of polymorphisms on conceptual translation were analyzed using the software Geneious R10 (Kearse et al., 2012).

2.4. Genome annotation and virus phylogeny

Iflaviruses encode a single polyprotein within a single ORF. Therefore, we used Geneious to identify a similar CDS occurring in each of the three genomes of the newly discovered HhV isolates. Moreover, we performed BLASTP analysis of the predicted polyprotein amino acid sequences to search in the non-redundant Genbank database for related iflaviruses and identify conserved regions (Altschul et al., 1997). Afterward, we carried out a phylogenetic analysis using the predicted amino acid sequence of the RNA-dependent RNA polymerase (RdRp) (Pfam entry, cd01699) domain found in several inflaviruses and other related picornaviruses, including dicistroviruses and secoviruses. The GenBank accession numbers of the taxa used are available in Table S1. The sequences were aligned by the MAFFT method (Katoh et al., 2002) and used to search for the best evolutionary model using the software MEGA 7 (Kumar et al., 2016). We inferred phylogenetic relationships among the RdRp sequences by maximum likelihood as implemented in PhyML (Guindon et al., 2010) using the model LG + G (0.81). The tree was rooted against the clade containing members of the *Secoviridae*.

2.5. Insects and iflavirus RNA prospection in field population

Stinkbugs of *E. heros*, *C. ubica*, and *D. melacanthus* (Hemiptera: Pentatomidae) used in this study were obtained from laboratory colonies previously established in the Semiochemicals Laboratory of EMBRAPA Genetic Resources and Biotechnology, Brasília, DF, Brazil (15° 47′ S and 47° 55′ W). The insects were kept in a climatized room, at 26 ± 1 °C, 65 ± 10% RH and a 14:10 (day:night) photoperiod. Individual stink bugs were reared in 8 L plastic cages separated by species. Insects were fed with a natural diet containing sunflower seed (*Heliantus annus* L. [Asteraceae]), peanut (*Arachis hypogaea* L. [Fabaceae]) and soybean (*Glycine max* L. [Fabaceae]), fresh bean pods (*Phaseolus vulgaris* L. [Fabaceae]) and water. Individuals of the brown marmorated stink bug (*H. halys*) were obtained from a colony initiated from adults collected in Pennsylvania, USA, maintained at the USDA-ARS Beltsville Agricultural Research Center in Beltsville, Maryland, USA, and supplemented with individuals collected locally in Beltsville (Sparks et al., 2014). Individuals of species *E. heros* were collected from soybean (G. max) fields in the Midwest Region of Brazil (Federal District). The whole body of ten field-collected adult individuals (picked randomly) of species *E. heros* and three pools (with five adult subjects

each) of laboratory-obtained individuals of species *D. melachantus*, *C. ubica*, and *E. heros* were macerated with Trizol™ Reagent (Thermo Fisher Scientific) and used for RNA extraction according to manufacturer's instructions. The extracted total RNAs were subjected to cDNA first-strand synthesis using GoScript™ Reverse Transcriptase (Promega) according to manufacturer's instructions. After cDNA synthesis, a dilution of 1:50 was individually used as template for PCR with 0.4 µM of the primers Penta-Iflavirus F (GAC CGA ACT CTT AAC GGA GGA GGA GC) and Penta-Iflavirus R (CTA TAA GAC ATA CCA GCA AAT TCT AT), 300 µM of dNTP mix (Fermentas), 1 U of GoTaq (Promega), and 1x of the supplied reaction buffer. The reactions were subjected to the following program: 95 °C/2 min, 35 cycles of 95 °C/30s, 53 °C/30 s and 72 °C/1 min with a final extension of 5 min at 72 °C.

2.6. Semi-purified virus negative staining

For extraction of virus particles from *E. heros*, *C. ubica*, and *D. melacanthus*, a total of 10 whole stink bug males and females from each species were homogenized with 25 mL of Phosphate Buffered Saline 1X (PBS, 137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO4, 2.0 mM KH_2PO_4, pH 7.4), and centrifuged at 4000 × *g* for 10 min for clarification. The supernatants were filtered through a syringe plugged with cotton, loaded in polyallomer ultracentrifuge tubes (Beckman) and centrifuged at 63,000 × *g* for 75 min. The pellets containing the virus particles were resuspended in PBS-1X and prepared for Transmission Electron Microscopy (TEM) by negative staining as described elsewhere (Brenner and Horne, 1959) and observed in a TEM Jeol 1011 at 100 kV.

For extraction of virus particles from *H. halys*, 20 adults were homogenized in 15 mL NT buffer (100 mM Tris-HCl, pH7.6, and 10 mM NaCl) with an Ultra-Turrax T-25. The homogenate was filtered through three layers of cheesecloth in a funnel with a steel strainer and further clarified by low-speed centrifugation (1467 × *g*) for 10 min. The supernatant was transferred to polyallomer tubes, underlaid with a 20% (w/v) sucrose pad, and centrifuged at 103,680 × *g* for 4 h. The pellet containing virus particles was resuspended in PBS. Virus particles were negatively stained with 2.5% phosphotungstic acid (w/v) for 5 s. After drying, grids with stained particles were viewed and imaged at 80 kV with a Hitachi HT-7700 transmission electron microscope (Hitachi High Technologies America, Inc., Dallas, TX, USA).

3. Results

3.1. Three virus isolates related to Halyomorpha halys virus (HhV) were found in the antenna transcriptomes of three Brazilian pentatomid species

We discovered complete viral genomes with a high degree of sequence identity to the iflavirus Halyomorpha halys virus (HhV) in the antennal transcriptomes of the stink bugs *E. heros*, *C. ubica*, and *D. melacanthus* (Hemiptera: Pentatomidae). Contigs of viral genomes were assembled from these transcriptomes with a high degree of sequencing coverage, ranging from 180,000 to 480,000 X. In Table 1, the characteristics of the viral genomes are compared to each other and to the previously sequenced HhV genome sequence. The viral isolates were named according to the host species: Euschistus heros iflavirus (EhIV), Chinavia ubica iflavirus (CuIV), and Dichelops melacanthus iflavirus (DmIV). DmIV was the isolate with lowest pairwise nucleotide identity when compared to the other isolates with about 83% identity, while EhIV and CuIV were almost identical to each other and exhibited about 98% nucleotide sequence identity with HhV.

Table 1
Genome characteristics of the pentatomid iflavirus isolates with their respective nucleotide identity (nt ID) percentages.

Virus	Host species	Genome Size (nt)	nt ID (%)			
			HhV	EhIV	CuIV	DmIV
HhV	*Halyomorpha halys*	9271	–	97.74	97.76	83.88
EhIV	*Euschistus heros*	9356	97.74	–	99.98	83.94
CuIV	*Chinavia ubica*	9351	97.75	99.98	–	83.96
DmIV	*Dichelops melacanthus*	9359	83.88	83.96	83.96	–

A search of seven pentatomid transcriptomes from *Nezara viridula*, *Plautia stali*, *Dolycoris baccarum*, *Murgantia histrionica*, *Acrosternum hilare*, *Chalcocoris rutilans*, and *Erthesina fullo* (Table S1) failed to identify reads mapping to the HhV genome sequence or any other isolate described in this work. We also failed to find HhV-related sequences in the genomic DNA sequence data of several pentatomids, including *E. heros*, *C. ubica*, *D. melacanthus*, *H. halys*, *Chinavia impicticornis*, *Stiretrus anchorago*, and *Piezodorus guildinii* (Table S1).

3.2. Phylogeny of Halyomorpha halys virus and the three new virus isolates

Features of the sequences of the three new isolates suggest they belong to the virus family *Iflaviridae*. This family is classified under order *Picornavirales* and is related to families *Dicistroviridae* and *Secoviridae* (Le Gall et al., 2008). In order to understand the relationship of the new isolates and HhV to other iflaviruses, we constructed a phylogenetic tree based on *rdrp* sequences retrieved from the non-redundant Genbank database. The four viruses clustered together, forming a highly supported monophyletic clade within a larger clade containing other iflaviruses. The most genetically distant isolate, DmIV, was the most basal taxon in the clade (Fig. 1).

The species demarcation criterion for members of genus *Iflavirus* is based on amino acid sequence identity in the capsid region of the polyprotein, with iflaviruses exhibiting > 90% capsid amino acid sequence identity with each other considered to belong to the same species (Valles et al., 2017). Amino acid sequence identities among the capsid sequences of HhV, CuIV, DmIV, and EhIV are all > 94%, indicating that they are isolates of the same species of genus *Iflavirus* (Fig. 2). Identities with capsid sequences of other iflaviruses are < 28.3%, suggesting that HhV and the stink bug viruses identified in this study represent a distinct iflavirus species.

3.3. Genetic variation among stink bug iflaviruses

Upon pairwise comparison of the HhV polyprotein CDS with those of the other stink bug iflaviruses (Table 2), DmIV was found to contain the most single nucleotide variants (SNV), with a total of 1175 SNVs. 82 of these SNVs constituted non-synonymous substitutions. In contrast, CuIV and EhIV each exhibited 199 SNVs with 20 and 19 non-synonymous substitutions, respectively. These sequence differences corresponded to polyprotein CDS nucleotide pairwise alignment identities was 83.7% for DmIV and 97.8% for CuIV and EhIV compared to HhV.

3.4. 5′- and 3′-Untranslated region analysis

Pairwise alignment of the 5′- and 3′-untranslated regions (UTRs) of the novel pentatomid iflavirus isolates and HhV revealed global sequence identities of 95.3 and 90.9%, respectively. Yet again, DmIV was found to possess the most divergent sequences for these two regions. As previously observed with the CDS sequence, the 5′-UTR sequences of CuIV and EhIV were identical to each other. CuIV and EhIV differed in a U-rich region of the 3′-UTR with a pairwise identity of 97.1%. Although DmIV was the most genetically different in sequence identity, the secondary structures of the 5′-UTRs predicted at 25 °C (Andronescu et al., 2007) were more closely related to each other than to the HhV 5′-UTR secondary structure (Fig. 3).

3.5. Viral RNA quantification in the antenna transcriptome

In order to quantify the viral RNA in the transcriptomes, we plotted the percentage of reads in the transcriptome dataset that mapped to the

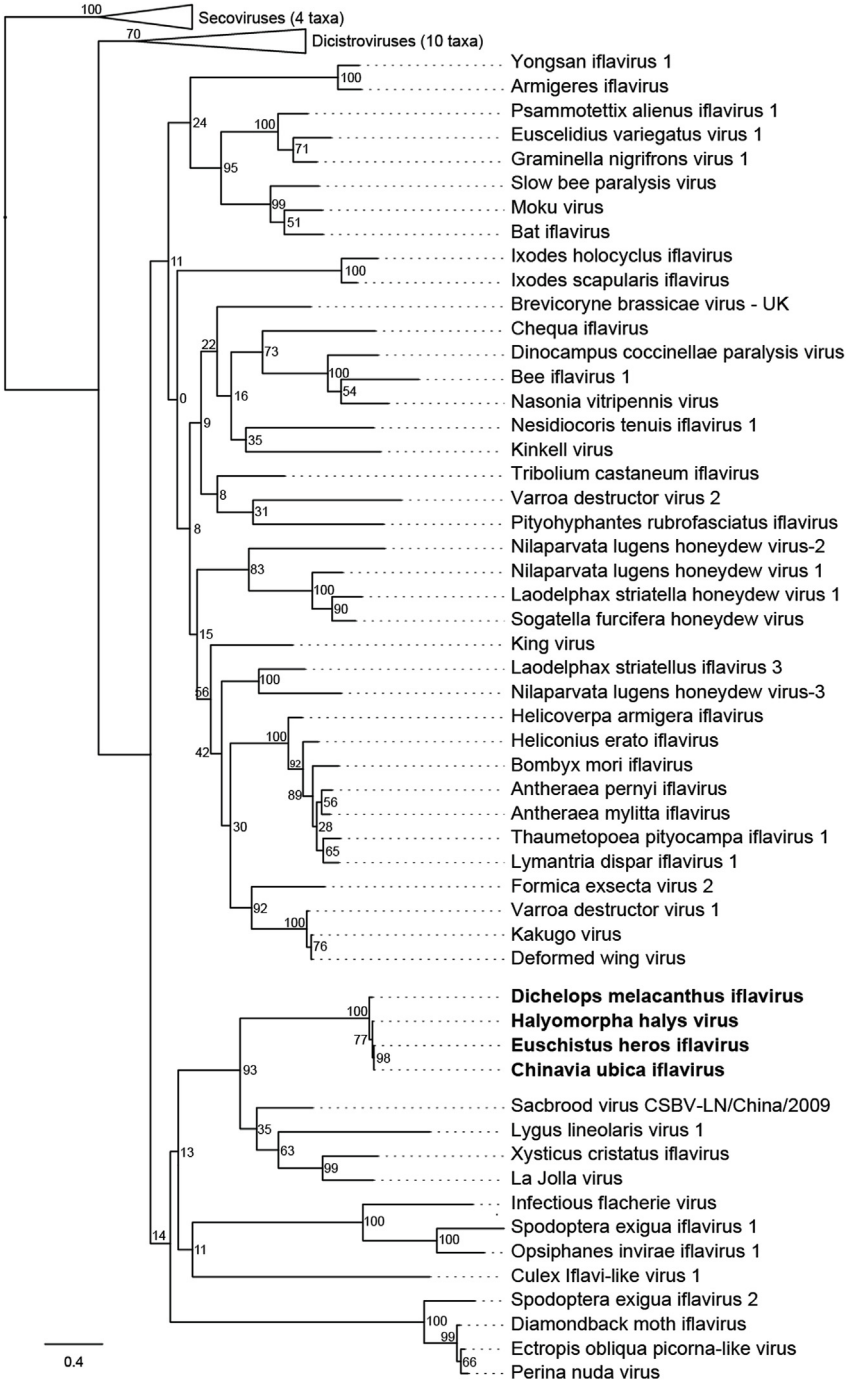
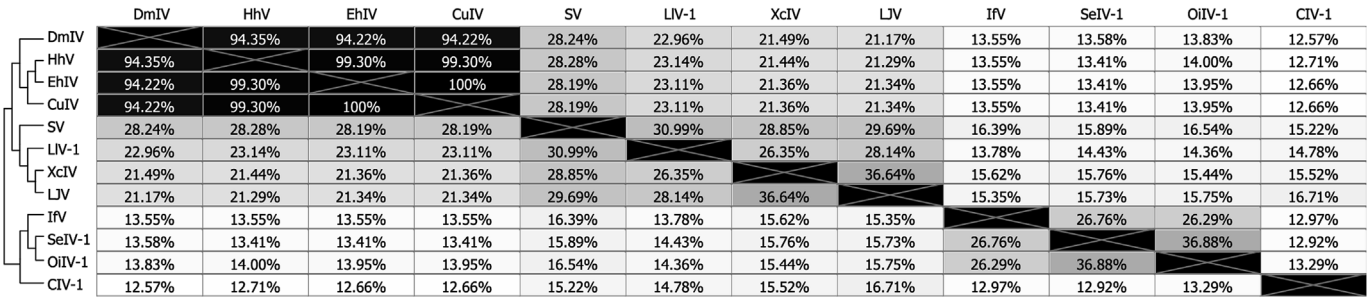


Fig. 1. ML phylogenetic inference of the relationships among Halyopmorpha halys virus and other related pentatomid iflaviruses to other iflaviruses, dicistroviruses, and secoviruses. Members of the *Secoviridae* were used to root the tree.

viral isolate genome sequences. For the antenna transcriptome of *C. ubica*, more than 40% of the total reads obtained mapped to the CuIV genome. Surprisingly, in the transcriptome of *D. melacanthus* and *E. heros*, the percentages of reads mapping to the iflavirus genome sequences were even higher, ranging from > 70% to almost 90% of the total number of reads, respectively (Fig. 4A). Therefore, we plotted the total number of reads for each transcriptome that mapped against either the viral isolate genomes or the *actin* mRNA as an internal control (Fig. 4B). The poly-A tails were subtracted for both the viral genome and the gene mRNA during mapping. The results suggested that the antennal tissues derived from the three stinkbugs contained a very high level of viral genomic RNA, much higher than *actin* mRNA (Fig. 4B). The *D. melacanthus* antenna transcriptome data set contained 2300-fold more reads mapping to the DmIV genome than to the *actin* mRNA. Viral genomic RNA levels for the three novel iflaviruses were similar when compared to each other.

3.6. Viral RNA quantification in the whole body transcriptomes of Halyomorpha halys in nymphs and adults

Separate sets of transcriptomic data were available for *H. halys* nymph (second and fourth instar) and adult (male and female) developmental stages. To quantify the viral RNA in these transcriptomes, we plotted the percentage of reads that mapped against the HhV genome



	DmIV	HhV	EhIV	CuIV	SV	LIV-1	XcIV	LJV	IfV	SeIV-1	OiIV-1	CIV-1
DmIV		94.35%	94.22%	94.22%	28.24%	22.96%	21.49%	21.17%	13.55%	13.58%	13.83%	12.57%
HhV	94.35%		99.30%	99.30%	28.28%	23.14%	21.44%	21.29%	13.55%	13.41%	14.00%	12.71%
EhIV	94.22%	99.30%		100%	28.19%	23.11%	21.36%	21.34%	13.55%	13.41%	13.95%	12.66%
CuIV	94.22%	99.30%	100%		28.19%	23.11%	21.36%	21.34%	13.55%	13.41%	13.95%	12.66%
SV	28.24%	28.28%	28.19%	28.19%		30.99%	28.85%	29.69%	16.39%	15.89%	16.54%	15.22%
LIV-1	22.96%	23.14%	23.11%	23.11%	30.99%		26.35%	28.14%	13.78%	14.43%	14.36%	14.78%
XcIV	21.49%	21.44%	21.36%	21.36%	28.85%	26.35%		36.64%	15.62%	15.76%	15.44%	15.52%
LJV	21.17%	21.29%	21.34%	21.34%	29.69%	28.14%	36.64%		15.35%	15.73%	15.75%	16.71%
IfV	13.55%	13.55%	13.55%	13.55%	16.39%	13.78%	15.62%	15.35%		26.76%	26.29%	12.97%
SeIV-1	13.58%	13.41%	13.41%	13.41%	15.89%	14.43%	15.76%	15.73%	26.76%		36.88%	12.92%
OiIV-1	13.83%	14.00%	13.95%	13.95%	16.54%	14.36%	15.44%	15.75%	26.29%	36.88%		13.29%
CIV-1	12.57%	12.71%	12.66%	12.66%	15.22%	14.78%	15.52%	16.71%	12.97%	12.92%	13.29%	

Fig. 2. Sequence identity at the amino acid level of the capsid proteins. We present the heat map (the darker the color, the higher is the protein identity) with their respective percentages. Moreover, the phylogenetic relationship addressed in the Fig. 1 is presented here as a cladogram. We compared capsid proteins of iflavirus closely related to Halyomorpha halys virus (HhV) that include Chinavia ubica iflavirus (CuIV), Culex Iflavi-like virus 1 (CIV-1), Dichelops melacanthus iflavirus (DmIV), Euschistus heros iflavirus (EhIV), Infectious flacherie virus (IfIV), La Jolla virus (LJV), Lygus lineolaris virus 1 (LlV-1), Opsiphanes invirae iflavirus 1 (OiIV-1), Sacbrood virus (SV), Spodoptera exigua iflavirus 1 (SeIV-1), and Xysticus cristatus iflavirus (XcIV).

Table 2
Number of Single Nucleotide Variants (SNVs) found in the polyprotein CDS of the new virus isolates in relation to the Halyomorpha halys virus.

Viruses	SNVs		
	Synonymous	Non-synonymous	Total
EhIV	180	19	199
DmIV	1093	82	1175
CuIV	179	20	199

for each group. The results indicated that the amount of HhV genome RNA was approximately 510-fold more abundant in adult compared to nymphs, suggesting that HhV RNA levels increase as the insect progresses through development (Fig. 5A). Moreover, the percentage of viral RNA obtained from the whole body of *H. halys* seemed to be less abundant when compared to the antenna transcriptome. When the total number of reads for the nymph and adult transcriptomes that mapped against either the HhV genome or the *actin* mRNA were compared, the results once again indicated that viral RNA was present at a higher level in *H. halys* than *actin* mRNA (Fig. 5B).

3.7. Iflavirus detection in field-collected adult individuals of species E. heros

To confirm the presence of iflavirus RNA in field-collected asymptomatic stink bugs, we carried out a RT-PCR screening of 10 individuals of species *E. heros* collected in soybean fields. Out of ten screened individuals, only one was found to be positive for the presence of the iflavirus RNA (Fig. 6). As positive controls, we used the cDNA produced from RNA pools of five adult individuals from the laboratory colony, including individuals of species *D. melacanthus*, *C. ubica*, and *E. heros*.

3.8. Microscopy of virus particles

To confirm the presence of virus particles in asymptomatic stink bugs, we carried out electron microscopy of semi-purified, negatively-stained virus-like particle preparations extracted from whole insects. We observed icosahedral particles resembling the virions of picorna-like viruses in preparations from the four stink bug species in this study (Fig. 7). Both empty capsids and intact capsids containing a core could be observed in the preparation from *H. halys*, similar in appearance to the Lygus lineolaris iflavirus (LyLV-1) particles reported from the lygus bug, *Lygus lineolaris* (Perera et al., 2012). Two visibly distinct sizes of particles were evident in the *H. halys* prep. The larger particles in the *H. halys* prep measured, on average, 41.2 ± 0.32 nm in diameter (n = 15), while the smaller particles were 31.9 ± 0.34 nm in diameter (n = 7). Most of the observed particles fell into the larger size class. The large particles were bigger than the typical 30-nm iflavirus virion, but appeared to be approximately the same size as the LyLV-1 particles, which were reported to be 38.7–40.2 nm in diameter (Perera et al., 2012).

4. Discussion

In this work, we described a very intriguing observation regarding virus covert infection found by a high throughput sequencing approach: isolates of the same virus species infecting healthy pentatomids of four different host species. Virus genomes of an iflavirus, HhV, originally discovered in the transcriptome of the brown marmorated stink bug, *H. halys* (Sparks et al., 2013), were found in the antennal transcriptomes of three Brazilian soybean stinkbug pests in a surprisingly high copy number. Features of the genome, sequence similarity, phylogenetic relationships, and the appearance of the presumptive virions produced by these viruses all indicate that these viruses represent a single species that should be classified in genus *Iflavirus* of family *Iflaviridae*. Moreover, we found a field collected individual of species *E. heros* positive

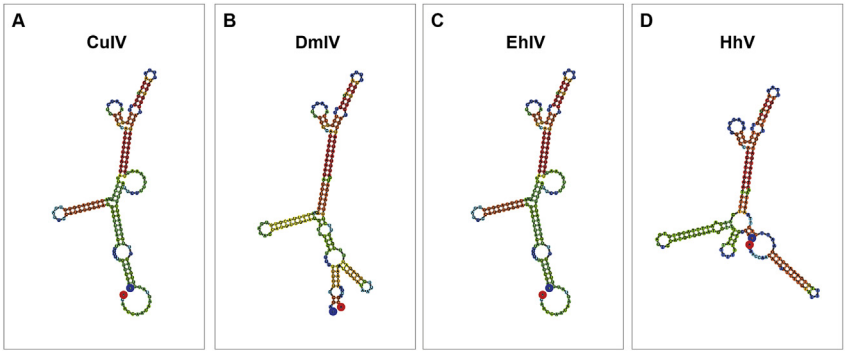


Fig. 3. Predicted RNA secondary structure of the 5′-untranslated region of pentatomid iflavirus genomes. (A) Chinavia ubica iflavirus (CuIV); (B) Dichelops melachanthus iflavirus (DmIV); (C) Euschistus heros virus (EhIV); and (D) Halyomorpha halys virus (HhV). The secondary structure of the RNA was predicted according to Andronescu et al. (2007). Ends are highlighted and the bases are colored to indicate a high (red), medium (green), or low (blue) probability of base pair formation.

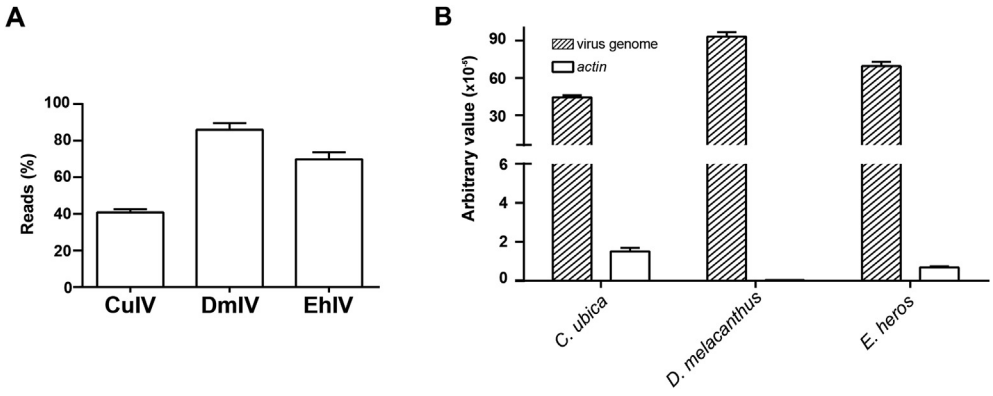


Fig. 4. Viral RNA quantification in the antennal transcriptome of three different Brazilian species of stink bug. (A) Mean percentage of the total number of antennal transcriptome reads that mapped against the complete genomes of Chinavia ubica iflavirus (CuIV), Dichelops melacanthus iflavirus (DmIV), and Euschistus heros iflavirus (EhIV). (B) Arbitrary value representing the total number of trimmed reads mapped against either the virus genome sequence (without the poly-A tail) or the *actin* mRNA used as reference sequences for each stinkbug antennal transcriptome. The total number of mapped reads were divided by both the total number of trimmed reads obtained for each transcriptome and the reference sequence sizes. Bar heights indicate the averages of two SRA trimmed datasets, and the error bars represent the standard deviations.

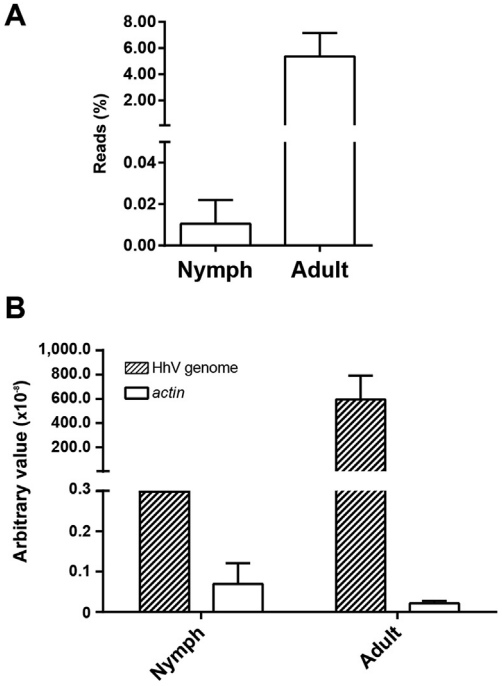


Fig. 5. Viral RNA quantification in the whole body transcriptome of nymphs and adults of the brown marmorated stink bug, *H. halys*. (A) Mean percentage of the total number of whole body transcriptome reads that mapped against the complete genome of HhV. (B) Arbitrary value representing the total number of trimmed reads mapped against either the HhV genome sequence or the *actin* mRNA used as reference sequences for each stinkbug antennal transcriptome (both without the poly-A tail). The total number of mapped reads were divided by both the total number of trimmed reads obtained for each transcriptome (nymphs and adults) and the reference sequence sizes. Bar heights indicate the averages of two trimmed datasets (male + female adults, and 2nd- + 4th-instar nymphs), and the error bars represent the standard deviations.

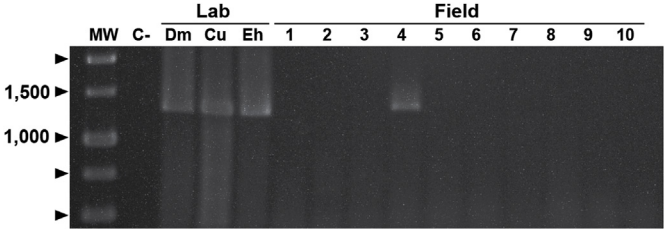


Fig. 6. RT-PCR screening in adult individuals of species *E. heros* collected from Brazilian soybean fields. As positive control, we used RNA extracted from laboratory reared insects of species *D. melachanthus* (Dm), *C. ubica* (Cu), and *E. heros* (Eh). The rows presented a positive amplified band of 1,304 bp. Ten randomly selected insects (numbered 1–10) from field-collected individuals were tested for the presence of the iflavirus RNA, and only one individual was found to be positive (row 4). Five-microliter samples of PCR products were loaded into a 0.8% agarose gel. MW, 1 kb Plus molecular marker.

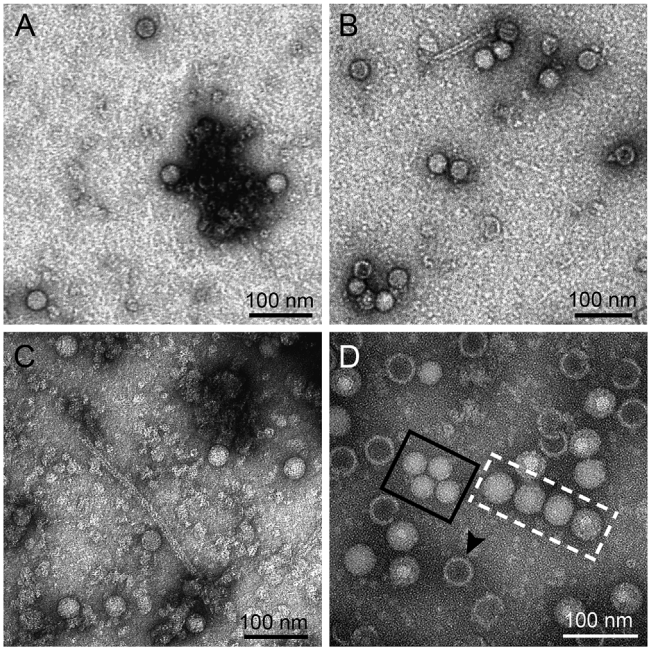


Fig. 7. Electron micrographs of semi-purified virus-like particle preparations from four stink bugs, including (A) *Chinavia ubica*, (B) *Euschistus heros*, (C) *Dichelops melacanthus*, and (D) *Halyomorpha halys*. We present representative fields with icosahedral particles that resemble iflaviruses. In (D), white arrows indicate empty capsids. Small virions in (D) are denoted by a black solid box, and large virions are denoted by a white dashed box.

for the presence of the iflavirus RNA.

While *H. halys* is an invasive pest in North America that is native to China, Japan, and Korea, the other three stink bug species examined in this study are native to South America. Thus, in addition to possessing a broad host range, isolates of this virus also exhibit a remarkably broad geographic distribution. The ability to propagate in different species and spread over long distances may be connected to the capacity of this virus, documented in this study, to produce large quantities of its RNA without causing any obvious pathology in its hosts. It is possible that the iflavirus reads discovered in the four pentatomid transcriptomes analyzed in this study derive from segments of the iflavirus genome sequences integrated into the DNA of the host, as previously described

for flock house virus in *Drosophila melanogaster* (Goic et al., 2013). However, no sequences with similarity to the HhV genome were identified in genomic sequence data for *E. heros*, *C. ubica*, or *H. halys*.

Sequence reads mapping to the HhV genome sequence do not appear to be present in the SRAs of a *H. halys* transcriptome published by Ioannidis et al. (2014). The differences in how *H. halys* was collected and maintained by the two groups of authors, and how transcriptomes were prepared from their respective *H. halys* colonies and analyzed, may have influenced the detection of HhV. It is possible that the insects collected by the Ioannidis et al. group to initiate their colony did not contain HhV, or that HhV was somehow purged from their colony during its rearing and maintenance. HhV was identified by the Sparks et al. group because of the sequence similarity of reads within their transcriptome to a partial iflavirus sequence discovered in the virome of a bat (Sparks et al., 2013), and it is possible that the Ioannidis et al. group may have discarded such reads as contaminants. More research is required to address the frequency and distribution of the iflaviruses described in this study among different populations and species of stink bugs.

The study of covert virus infections promises to add to our understanding of how different viruses interact within the same host, and to provide new insight into the use of insect pathogens for insect pest control. For example, the asymptomatic infection of *Helicoverpa armigera* with Helicoverpa armigera densovirus-1 (HaDNV-1) correlates with increased resistance to lethal infection of *H. armigera* larvae with its baculovirus, Helicoverpa armigera nucleopolyhedrovirus (HearNPV) and also to *Bacillus thuringiensis* (Bt) toxin. In contrast, co-infections of *Spodoptera exigua* larvae with both a baculovirus and an iflavirus reduced the dose of baculovirus occlusion bodies required to kill larvae relative to infections with baculovirus alone (Carballo et al., 2017). The presence of covert iflavirus infections of stink bugs may also have an impact on the deployment of pathogens for stink bug control.

Conflicts of interest

The authors declare that they have no competing interests.

Funding statements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant number 407908/2013-7 and 428799/2018-3), Coordenação de Aperfeiçoamento de Pessoal de nível Superior (CAPES/PROEX, grant number 23038.005848/2018–31) and Fundação de Apoio à Pesquisa do Distrito Federal (FAPDF, grant number 193.001532/2016).

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.06.002.

References

Aizawa, K., Furuta, Y., Kurata, K., Sato, F., 1964. On the etiologic agent of the infectious flacherie of the silkworm, Bombyx mori (Linnaeus). Bull. Seric. Exp. Stn. 19, 223–240.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Andronescu, M., Condon, A., Hoos, H.H., Mathews, D.H., Murphy, K.P., 2007. Efficient parameter estimation for RNA secondary structure prediction. Bioinformatics 23 (13), i19–i28. https://doi.org/10.1093/bioinformatics/btm223.

Baur, M.E., Sosa-Gomez, D.R., Ottea, J., et al., 2010. Susceptibility to insecticides used for control of Piezodorus guildinii (Heteroptera: Pentatomidae) in the United States and Brazil. J. Econ. Entomol. 103 (3), 869–876. https://doi.org/10.1603/EC09364.

Bailey, L., Woods, R.D., 1974. Three previously undescribed viruses from the honey bee. J. Gen. Virol. 25 (2), 175–186. https://doi.org/10.1099/0022-1317-25-2-175.

Bailey, L., Ball, B.V., 1991. Honey Bee Pathology, second ed. Academic Press, London, England.

Belsham, G.J., 2009. Divergent picornavirus IRES elements. Virus Res. 139 (2), 183–192. https://doi.org/10.1016/j.virusres.2008.07.001.

Brenner, S., Horne, R.W., 1959. A negative staining method for high resolution electron microscopy of viruses. Biochim. Biophys. Acta 34, 103–110. https://doi.org/10.1016/0006-3002(59)90237-9.

Carballo, A., Murillo, R., Jakubowska, A., Herrero, S., Williams, T., Caballero, P., 2017. Co-infection with iflaviruses influences the insecticidal properties of Spodoptera exigua multiple nucleopolyhedrovirus occlusion bodies: implications for the production and biosecurity of baculovirus insecticides. PLoS One 12 (5), e0177301. https://doi.org/10.1371/journal.pone.0177301.

Farias, L.R., Schimmelpfeng, P.H., Togawa, R.C., et al., 2015. Transcriptome-based identification of highly similar odorant-binding proteins among neotropical stink bugs and their egg parasitoid. PLoS One 10 (7), e0132286. https://doi.org/10.1371/journal.pone.0132286.

Goic, B., Vodovar, N., Mondotte, J.A., et al., 2013. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat. Immun. 14 (4), 396. https://doi.org/10.1038/ni.2542.

Gouli, V., Gouli, S., Skinner, M., Hamilton, G., Kim, J.S., Parker, B.L., 2012. Virulence of select entomopathogenic fungi to the brown marmorated stink bug, Halyomorpha halys (Stål)(Heteroptera: Pentatomidae). Pest Manag. Sci. 68 (2), 155–157. https://doi.org/10.1002/ps.2310.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59 (3), 307–321. https://doi.org/10.1093/sysbio/syq010.

Himeno, M., Onodera, K., Tanami, Y., 1974. Properties of flacherie virus of the silkworm, Bombyx mori. J. Invertebr. Pathol. 23 (2), 164–171. https://doi.org/10.1016/0022-2011(74)90180-3.

Inoue, H., Ayuzawa, C., 1972. Fluorescent antibody study of an infectious flacherie of the silkworm, Bombyx mori. J. Invertebr. Pathol. 19 (2), 227–230. https://doi.org/10.1016/0022-2011(72)90213-3.

Ioannidis, P., Lu, Y., Kumar, N., et al., 2014. Rapid transcriptome sequencing of an invasive pest, the brown marmorated stink bug Halyomorpha halys. BMC Genomics 15 (1), 738. https://doi.org/10.1186/1471-2164-15-738.

Katoh, K., Misawa, K., Kuma, K.I., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30 (14), 3059–3066. https://doi.org/10.1093/nar/gkf436.

Kearse, M., Moir, R., Wilson, A., et al., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874. https://doi.org/10.1093/molbev/msw054.

Lanzi, G., de Miranda, J.R., Boniotti, M.B., et al., 2006. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). J. Virol. 80 (10), 4998–5009. https://doi.org/10.1128/JVI.80.10.4998-5009.2006.

Le Gall, O., Christian, P., Fauquet, C.M., et al., 2008. Picornavirales, a proposed order of positive-sense single-stranded RNA viruses with a pseudo-T= 3 virion architecture. Arch. Virol. 153 (4), 715. https://doi.org/10.1007/s00705-008-0041-x.

Liu, S., Chen, Y., Sappington, T.W., Bonning, B.C., 2017. Genome sequence of the first coleopteran iflavirus isolated from western corn rootworm, Diabrotica virgifera virgifera LeConte. Genome Announc. 5 (6), e01530 16. https://doi.org/10.1128/genomeA.01530-16.

Nakashima, N., Sasaki, J., Tsuda, K., Yasunaga, C., Noda, H., 1998. Properties of a new picorna-like virus of the brown-winged green bug, *Plautia stali*. J. Invertebr. Pathol. 71, 151–158. https://doi.org/10.1006/jipa.1997.4721.

Panizzi, A.R., McPherson, J.E., James, D.G., Javahery, M., McPherson, R.M., 2000. Stink Bugs (Pentatomidae). Heteroptera of Economic Importance. CRC Press, Boca Raton, Florida, USA, pp. 421–474.

Panizzi, A.R., Duo, L.J., Bortolato, N.M., Siqueira, F., 2007. Nymph developmental time and survivorship, adult longevity, reproduction and body weight of Dichelops melacanthus (Dallas) feeding on natural and artificial diets. Rev. Bras. Entomol. 51 (4), 484–488. https://doi.org/10.1590/S0085-56262007000400013.

Perera, O.P., Snodgrass, G.L., Allen, K.C., Jackson, R.E., Becnel, J.J., O'Leary, P.F., Luttrell, R.G., 2012. The complete genome sequence of a single-stranded RNA virus from the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). J. Invertebr. Pathol. 109, 11–19. https://doi.org/10.1016/j.jip.2011.08.004.

Santillán-Galicia, M.T., Ball, B.V., Clark, S.J., Alderson, P.G., 2010. Transmission of deformed wing virus and slow paralysis virus to adult bees (Apis mellifera L.) by Varroa destructor. J. Apic. Res. 49 (2), 141–148. https://doi.org/10.3896/IBRA.1.49.2.01.

Silva, L.A., Ardisson-Araujo, D.M.P., Tinoco, R.S., Fernandes, O.A., Melo, F.L., Ribeiro, B.M., 2015. Complete genome sequence and structural characterization of a novel iflavirus isolated from Opsiphanes invirae (Lepidoptera: nymphalidae). J. Invertebr. Pathol. 130, 136–140. https://doi.org/10.1016/j.jip.2015.08.001.

Sparks, M.E., Gundersen-Rindal, D.E., Harrison, R.L., 2013. Complete genome sequence of a novel iflavirus from the transcriptome of Halyomorpha halys, the brown marmorated stink bug. Genome Announc. 1 (6), e00910 13. https://doi.org/10.1128/genomeA.00910-13.

Sparks, M.E., Shelby, K.S., Kuhar, D., Gundersen-Rindal, D.E., 2014. Transcriptome of the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera:

Pentatomidae). PLoS One 9 (11), e111646. https://doi.org/10.1371/journal.pone.0111646.
van Oers, M.M., 2010. Genomics and biology of iflaviruses. In: Insect Virology. Caister Academic Press, Norfolk, pp. 231–250.
Valles, S.M., Chen, Y., Firth, A.E., et al., 2017. ICTV virus taxonomy profile: Iflaviridae. J. Gen. Virol. 98 (4), 527–528. https://doi.org/10.1099/jgv.0.000757.
Velikova, V., Salerno, G., Frati, F., Peri, E., Conti, E., Colazza, S., Loreto, F., 2010. Influence of feeding and oviposition by phytophagous pentatomids on photosynthesis of herbaceous plants. J. Chem. Ecol. 36 (6), 629–641. https://doi.org/10.1007/s10886-010-9801-7.
Weber, D.C., Leskey, T.C., Walsh, G.C., Khrimian, A., 2014. Synergy of aggregation pheromone with methyl (E, E, Z)-2, 4, 6-decatrienoate in attraction of Halyomorpha halys (Hemiptera: Pentatomidae). J. Econ. Entomol. 107 (3), 1061–1068. https://doi.org/10.1603/EC13502.
Williamson, C., von Wechmar, M.B., 1992. Two novel viruses associated with severe disease symptoms of the green stinkbug *Nezara viridula*. J. Gen. Virol. 73, 2467–2471. https://doi.org/10.1099/0022-1317-73-9-2467.
Williamson, C., von Wechmar, M.B., 1995. The effect of two viruses on the metamorphosis, fecundity, and longevity of the green stinkbug, *Nezara viridula*. J. Invertebr. Pathol. 65, 174–178. https://doi.org/10.1006/jipa.1995.1025.
Yue, C., Schröder, M., Gisder, S., Genersch, E., 2007. Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). J. Gen. Virol. 88 (8), 2329–2336. https://doi.org/10.1099/vir.0.83101-0.