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Discovery and effects of Texas *Solenopsis invicta* virus [SINV-1 (TX5)] on red imported fire ant populations

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ABSTRACT

Solenopsis invicta Buren (Hymenoptera: Formicidae), the red imported fire ant is native to South America but has invaded areas of the southeastern US, and parts of Southern California. The *S. invicta* virus-1 (SINV-1) is a positive sense, single-stranded RNA picorna-like virus that only affects *Solenopsis* species. The virus can infect all caste members and developmental stages. Infection of SINV-1 can result in colony collapse in less than 3 months under laboratory conditions. This study screened *S. invicta* colonies from Texas for the presence of SINV through Reverse Transcriptase PCR (RT-PCR). Positive samples were genetically characterized by direct sequencing and compared with known picorna-like viruses. SINV-1 was detected in ant colonies from Smith and Henderson TX counties. Amino acid sequence comparison of SINV-1 (TX5) ORF2 region showed homologies of 96% with SINV-1, 97% with SINV-1A, 17.6% with SINV-2, and 20.7% with SINV-3. In addition, SINV-1 (TX5) was compared to 18 other *Dicistroviridae* viruses. Ant-infecting viruses may provide new approaches to suppressing these important economic pests.

1. Introduction

The "picornavirus-like superfamily" includes *Comoviridae*, *Dicistroviridae*, *Picornaviridae*, *Potyviridae* and *Sequiviridae* which can be distinguished from one another by differences in the positioning of structural proteins and number of genomic segments present (Büchen-Osmond, 2006; Bonning, 2009). Picornaviruses and dicistroviruses are similar because they both possess unenveloped icosahedral virions which are tightly packed into four groups of equilateral triangles that make up the surface of the viral capsid protein (Rueckert, 1991) and differ in their various mechanisms for receptor attachment (Bonning, 2009).

The family *Dicistroviridae* is classified as Group IV insect-infecting viruses which have positive sense, single-stranded RNA genomes of 7.2–9.0 kb long (Mettenleiter and Sobrino, 2008). Currently, there are 12 dicistroviruses (according to the International Committee on Taxonomy of Viruses (ICTV), two additional viruses are awaiting approval (*Homalodisca coagulata* virus-1 (HoCV-1, Hunter et al., 2006) and Israeli acute paralysis virus (IAPV, Maori et al., 2007a); last updated 8/7/08). These viruses infect a variety of insect host orders including Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, and Decapoda (Crustacea) (Hunter et al., 2006; Bonning, 2009). This family consists of a single genus *Cripavirus*, named after the type species Cricket paralysis virus (CrPV) (Büchen-Osmond, 2006), and includes CrPV, Aphid lethal paralysis virus (ALPV), Black queen cell virus (BQCV), *Drosophila* C virus (DCV), Himetobi P virus (HiPV), *Plautia stali* intestine virus (PSIV), *Rhopalosiphum padi* virus (RhPV), Triatoma virus (TrV), and possibly HoCV-1 (Hunnicutt et al., 2006). A proposed new genus *Aparavirus*, named after Acute bee paralysis virus (ABPV), would separate out ABPV, Taura syndrome virus (TSV), Kashmir bee virus (KBV), *Solenopsis invicta* virus-1 (SINV-1), and possibly Israeli acute paralysis virus (IAPV).

Some dicistroviruses were discovered to be major contributors to honey bee mortality. ABPV, KBV, and IAPV are commonly detected in honey bee colonies associated with colony collapse disorder (CCD) (Anderson and Gibbs, 1988; Cox-Foster et al., 2007). Thus, a similar effect may exist in ants, and would be of economic importance if a viral causative agent could be determined for use as a biological control agent. Many dicistroviruses persist in hosts as asymptomatic, unapparent infection until some stressor (e.g. environmental, virus titer, malnutrition, other pathogens or parasites) or external vector causes the virus to transform to a lethal state (Christian and Scotti, 1998; de Miranda et al., 2004; Hashimoto and Valles, 2007; Maori et al., 2007a). In addition, Maori et al. (2007b) found that a small segment of IAPV RNA was able to

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integrate into the genome of honey bees through recombination and retrotransposition, which may aid in host viral resistance.

The first dicistrovirus to be isolated from the red imported fire ant, *S. invicta* Buren, has been characterized as the *S. invicta* virus-1 (SINV-1) (Valles et al., 2004). The RNA genome of SINV-1 is 8026 nucleotides long, has a polyadenylated tail, is single stranded, monopartite, and encodes two large non-overlapping open reading frames (ORF) separated by an intergenic region (IGR). The 5′ ORF1 contains genes that code for helicase, cysteine protease, and RNA-dependent RNA polymerase sequences (Valles et al., 2004; 2007), consistent with other dicistroviruses. SINV-1 also has a long untranslated region (UTR) at the 5′ end that is thought to be important for translation, virulence, and encapsidation (Mettenleiter and Sobrino, 2008). The 3′ ORF2 section of SINV-1 contains sequences similar to those of structural proteins, also known as capsid proteins (Valles and Hashimoto, 2008). The unaccompanied genetic material of most positive sense RNA genomes is infectious, becomes more virulent when contained in a viral particle, and subsequently increases in infectivity when transfected into cells (Büchen-Osmond, 2006).

Currently, three *S. invicta* viruses have been described: SINV-1 (Valles et al. 2004), SINV-2 (Hashimoto and Valles 2008), and SINV-3 (Valles and Hashimoto 2009) and are the first on record to infect all caste members and developmental stages of both monogyne and polygyne *S. invicta* colonies (Valles and Strong, 2005). SINV viruses are solely infectious to the *Solenopsis* genus (Valles et al., 2007). SINV-1A has been associated closely with SINV-1 and was determined to be a close genotype of SINV-1 (Valles and Strong, 2005). The SINV-1A partial genome contains 2845 nucleotides and encodes only one large ORF (Valles et al., 2004). SINV-1 and SINV-1A tend to co-infect only 17.5% of *Solenopsis* colonies; however, they are 97% genetically similar to each other (Valles and Strong, 2005). Virulence of these ssRNA viruses in fire ants have proven to be relatively benign or covertly infectious in field studies (Valles et al., 2004), however changes or mutations which alter amino acids could result in increased infectivity, pathogenicity, or transmissibility. Dicistroviruses can be transmitted horizontally (orally) from females to males (Gomirez-Zilber and Thomas-Orillard, 1993), and vertically by transovum (Reinganum et al., 1970) or transovarially (Hatfill et al., 1990). Additionally, virus particles are present in the feces of infected individuals, providing another mechanism of nestmate infect (Bonning, 2009). Hashimoto and Valles (2007) discovered that SINV-1 replicates primarily in midgut tissue and can be transmitted to other colony members by trophallaxis.

Small RNA insect viruses have previously been used to control pest populations, such as CrPV used to control populations of olive fruit fly (Manoussis and Moore, 1987) and *Helicoverpa armigera* stunt virus (HaSV) to control the moth *H. armigera* (Christian et al., 2005). Since *Solenopsis* species are known hosts of SINV (Valles et al., 2007), there is potential for this virus to be used as a biological control agent to suppress *S. invicta* populations. This study identified a variant of SINV-1 in Texas, evaluated the variant's affect on mortality in individual ants, and examined similarities of the variant to SINV-1, SINV-1A, and other dicistroviruses.

2. Methods and materials

2.1. Colony collection

S. invicta colonies were collected in February, March, and April, 2008 from Smith and Henderson counties, Texas. All colonies collected were the polygyne phenotype, with 20–30 queens. Colonies ($n \approx 10{,}000$) were excavated and placed into 18.14 kg collection containers with soil. Brood was collected along with queens. Prior to collection, INSECT-a-SLIP (BioQuip Products, Rancho Dominguez, CA) was administered to the top 7.5 cm of the collection containers to prevent the ants from escaping. Tap water was dripped (~10 ml/min) into the collection containers to separate the ants and their brood from the soil. Rafting ants were removed from the surface of the water and put into clear observation trays (57.5 cm × 41 cm × 14.5 cm) lined with 5 cm of INSECT-a SLIP (BioQuip Products, Rancho Dominguez, CA). Smaller (14 cm × 10 cm × 4 cm) clear boxes with a dried layer of plaster of paris on the bottom (0.64 cm) and a small hole in the lid to allow ants access was used as the ant's brood chamber. All colonies were maintained in the lab (~22.5 °C, 12 h L:D) and received half a Vienna sausage (Libby's, Chicago, IL) once a week with unlimited water.

2.2. RNA extractions and sequencing

RNA was extracted using TRIzol reagent following the manufacture's protocol. Ten individuals were used in each extraction and two extractions were completed for each of the 35 colonies collected. A SuperScript One-Step Reverse Transcriptase PCR (RT-PCR) (Invitrogen, Carlsbad, CA) was performed to produce cDNA. Production of cDNA and RT-PCR was completed using specific primer sets for short segments of SINV-1 (Table 1). The RNA RT-PCR was performed under the following conditions: 45 °C for 50 min, 94 °C for 2 min; denatured 94 °C for 15 s, annealed 56 °C for 15 s, elongated 68 °C for 1:30 min, repeated 35 times; and an additional elongation step of 68 °C for 5 min, and then held at 4 °C (iCycler, BioRad, Hercules, CA).

Samples which resulted in correct band size (~260–650 bp depending on the primer set) were considered virus positive (1% agarose gels). Nucleic acid sequencing used the CEQ™8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Samples were not tested for the presence of SINV-2 or SINV-3. A minimum of 10 isolates were sequenced for each primer set and each infected colony was sequenced a minimum of 5 times. Consensus sequences were produced and predicted amino acid, AA, and

Table 1
List of oligonucleotide primers used to sequence SINV-1 and SINV-1 (TX5). Primers with (*) were designed in the Bextine Lab, others were acquired from Valles and Strong (2005).

Designation	Oligonucleotide (5′ → 3′)
p58	GCGATAGGTTAGCTTTAAGTACAATTGGTG
p59	TCCCAATGTGCAATAAACACCTTCA
p62	GGAAGTCATTACGTGGTCGAAAACG
p63	CGTCCTGTATGAAAACCGGTCTTTACCACAGAAATCTTA
p102	CGCCTTAGGATTCGTTAGATACTCACCCG
p114	CTTGATCGGGCAGGACAAATTC
p116	GAACGCTGATAACCAATGAGCC
p117	CACTCCATACAACATTTGTAATAAAGATTTAATT
p118	CCAATACTGAAACAACTGAGACACG
p137	GTCACATCACGTCGGTGTCGT
p188	CTTAATTGTAATTTACTTGAATATGCGTTTGC
p189	GTATCTAACGAATCCTAAGGCGGATTG
p190	CAATCCGCCTTAGGATTCGTTAGATAC
p191	CGGATCTTATGAGTGAAGACACACCAG
p193	CAACCTCTGCTTCCCACGCAC
p221	GATGGTCTCGACCAAATGATATGGAG
p222	ATGAAGATATGAAGGTGTTTATTGCACATTG
p341	CACATAAGGGATATTGTCCCCATG
p343	TGGACGAGACGGATCTTATGAGTG
SINV-1 F1*	CAACCTCTGCTTCCCACGCAC
SINV-1 R1*	AAGCTGGGTGGCAAGTTAGA
SINV-1 F2*	TGTTGTCCTCGATCAATTCG
SINV-1 R2*	CCATACAGGCAATTACCCCA
SINV-1 F3*	ACTCCCACCCCTGAAGATTT
SINV-1 R3*	CTGGACGAGACGGATCTTATG
SINV-1 F4*	CCTGGACGAGACGGATCTTA
SINV-1 R4*	GGTGTCAATGATGCATCTGGG

nucleotide sequences were analyzed using BioEdit Sequencing Alignment Editor (Ibis Biosciences, Carlsbad, CA). Sequences of SINV-1 and SINV-1A were obtained from Genbank.

2.3. Isolation of whole virus

Methods for isolation of pure virus generally utilize Cesium Chloride, high speed centrifugation, and dialysis (Maori et al., 2007a). This study utilized a crude virus preparation method (modified after Hunter et al., 2003) which proved effective in producing infective virus inoculums prepared from infected insects. Colonies that tested positive for SINV-1 were sacrificed to prepare whole virus inoculum. Approximately 2000–5000 infected ants were homogenized in 50 ml of 0.01 M phosphate buffer pH 7.0 containing 0.02% Diethyl Thio Carbamic Acid (DETCA) (Sigma–Aldrich, St. Louis, MO). The solution was centrifuged for 20 min at 1600 rpm. The supernatant was transferred and rotated 4 h at 37,000 rpm at 4 °C. The supernatant was discarded and the pellet was dissolved with 500 μl of 0.01 M phosphate buffer containing 0.4% sodium-deoxycholic acid (Sigma–Aldrich, St. Louis, MO) and 4% polyethylene glycol hexadecyl ether (Brij-52) (Sigma–Aldrich, St. Louis, MO) and the solution was centrifuged at 1600 rpm for 15 min. Samples were then pushed through 0.45μ filters, transferred to a dialysis membrane, and placed in phosphate buffer (pH 7.0) at 4 °C. The cloudy precipitate inside the dialysis tubing was collected; 5 μl of phosphate buffer pH 7.0 was added for each 500 μl of precipitate collected. Approximately 1 ml of whole virus was extracted from each colony. Virus concentrations were determined with a NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA). Positive samples of SINV-1 (TX5) were confirmed using RT-PCR and gel electrophoresis.

2.4. Delivery of virus

To deliver SINV-1 (TX5), ants were placed into Petri dishes with varying concentrations of whole virus extract. Whatman© filter paper was cut into 1/8 pieces and placed on the bottom of the Petri dish. The paper was wetted with 500 μl of autoclaved nanopure® water as the control ($n = 6$). Experimental plates: 100% whole virus extract ($n = 8$), or 1×10^{-1} dilution of the whole virus extract ($n = 8$), or 1×10^{-2} dilution of the whole virus extract ($n = 8$). Ten worker ants were added to each dish and observed daily for 20 consecutive days, and the number of dead ants/dish was recorded. All 300 ants used were obtained from the same non-infected colony. This experiment was replicated once with a freshly collected colony. Upon completion ants were collected, washed 5× in 10% bleach, washed 3× in 95% ethanol, and finally washed 2× in nanopure® autoclaved water. This ensured virus was not being detected from the exterior of the individuals. Ants from each dish were collected in 1.5 ml centrifuge tubes and subjected to Trizol RNA extraction (following manufacture's protocol). RT-PCR was performed in duplicate for each sample using specific primers for SINV-1 and tested on 1% agarose gels.

3. Results

3.1. Detection of SINV

RNA extractions and RT-PCR revealed that SINV-1 was present in several collected colonies. Of the 35 colonies collected, 16 were positive for SINV-1. Colony infection rate was 45.7%, higher than previous reports (~24% colony infection rate) (Valles et al., 2004; Valles and Strong, 2005). The fifth colony screened was positive; thus the isolate was named SINV-1 (TX5). RNA collected from all 16 infected colonies was used to generate ORF2 consensus sequences. Various primer sets were combined to produce overlapping segments of the genome and a minimum of 10 amplicons were completed for each primer set to confirm validity of the sequences. Structural proteins located in ORF2 of SINV-1 (GenBank accession No. AY634314) were sequenced and compared to other sequences in GenBank. The 3376 nucleotide consensus sequence was constructed from each of the 16 infected Texas colonies.

3.2. Comparison of SINV-1 (TX5) and SINV-1

Alignment of the Texas SINV-1 (TX5) strain (GenBank accession No. FJ229495) with SINV-1 from Florida showed 62 nucleotide incongruence's in the ORF2 regions of the two sequences, resulting in a 98.1% identity match. The predicted AA sequence was constructed using the default algorithms in BioEdit to determine sequence differences between the two viruses (Fig. 1). A total of 48 AA dissimilarities were observed, between SINV-1 and SINV-1 (TX5). In 10 instances a polar AA was converted to another polar AA, and in 12 instances a nonpolar AA was converted to another nonpolar AA. However, in 13 instances a polar AA in the SINV-1 sequence was converted to a nonpolar amino acid in SINV-1 (TX5). Conversely, in 10 instances a nonpolar AA in the SINV-1 sequence was converted to a polar AA in SINV-1 (TX5). The remaining three dissimilarities were between unspecified or unknown AA conversions involving polar AA. RNA viruses typically have high transition frequencies partly because of strong G–U pairing during replication (de Miranda et al., 2004). Nucleotide identity showed SINV-1 (98.1%) was more closely related to SINV-1 (TX5), however, when comparing AA identity SINV-1A (97%) was more closely related to SINV-1 (TX5). A phylogenetic tree was constructed using amino acid sequences of the ORF2 region from 19 dicistroviruses. Hepatitis A virus, a picornavirus, was used as the outgroup (Fig. 2). Viruses SINV-1, 1A, and 1-TX5, all formed a separate clade. Within the tree, the closest sister clade was formed by the proposed new genus *Aparavirus,* ABPV, IAPV, and KBV. The previously described SINV-2 and SINV-3 grouped separately.

3.3. Viral isolation and delivery

Whole virus was extracted from colonies infected with SINV-1. High mortality in infected lab colonies prevented large amounts of virus inoculums from being produced (only 1–2 ml of extract from each colony was collected), thus viral extracts were combined from several colonies. Mortality was not affected for treatments of whole virus inoculum, dilutions, nor water (Fig. 3). Virus was detected in individuals from all three viral treatments. The non-diluted 100% virus extract was 75% effective at infecting individuals (six out of eight samples were positive for SINV-1), the first dilution was 71.4% effective (five out of eight samples were positive for SINV-1), and the second dilution was 12.5% effective (one out of eight samples was positive for SINV-1). Virus was not detected in any control samples. The virus was successfully delivered to *S. invicta* as 100% extract and as a diluted 1×10^{-1} concentration mixed with autoclaved nanopure® water. The amount of virus needed to infect an entire colony was not examined.

4. Discussion

4.1. Classification of SINV-TX5

The ICTV recognizes a new species in the *Dicistroviridae* family as possessing a nucleotide sequence identity of 90% or less (Christian et al., 2000). SINV-1 (TX5) was ~98% identical across ORF2, thus it is considered a variant of SINV-1. However, 62 significant

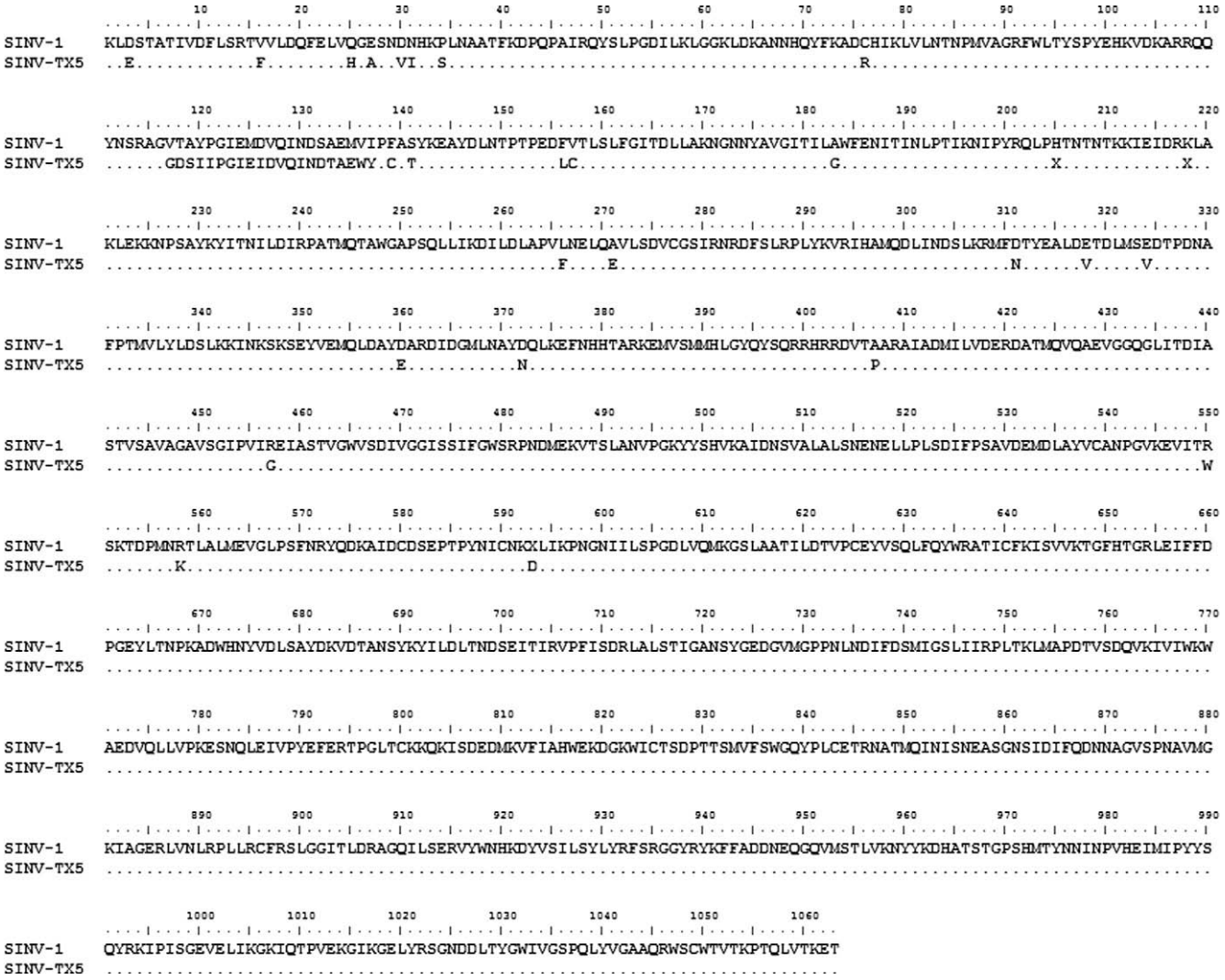

Fig. 1. Amino acid comparison of SINV-1 and SINV-1 (TX5) illustrating the 48 amino acid dissimilarities.

changes from SINV-1 genotype were noted. Accumulations due to high mutation rates, resulting from the lack of proofreading machinery in these types of viruses, is most likely occurring in the SINV-1 (TX5) strain to produce this genetic variation (Valles and Strong, 2005). RNA recombination could also be occurring within this virus resulting in protein divergence (Maori et al., 2007a; Palacios et al., 2008).

4.2. Delivery and mortality of SINV-1 (TX5)

A successful delivery method was developed which caused infection in *S. invicta* colonies with virus. Importantly the lack of increased mortality, which may be due to covert or latent infections, provides a virus which moves through the ant colony causing infection but with low selection pressure against it, thus providing a potential delivery system. Studies on other ssRNA viruses (e.g. ABPV, KBV, IAPV, *Drosophila* C virus, etc.) show comparable viral dormancy and asymptomatic infections when triggered by stressors resulting in increased mortality, such as in SINV-1 (Christian and Scotti, 1998; de Miranda et al., 2004; Hashimoto and Valles, 2007). Low pathogenic or latent viruses provide new tools as delivery mechanisms for RNAi strategies to reduce pest insects (Hunter et al., 2001, 2003; Hunter and Lapointe, 2003). Methods which can increase overall virus infection in ant populations are now needed to aid population suppression in these targeted pests. Increased suppression may also arise through: (i) genetic modification to increase virulence to ants or (ii) multiple infections with other pathogens to weaken the ant's immune system.

4.3. Future implications

Research by micro-encapsulating extracted whole virus was successful under lab conditions and now needs to be conducted to evaluate efficacy under field conditions. Colony collapse like conditions may be produced by SINV under lab colonies, but appears to have little effect in field colonies (personal observation; Valles et al., 2004). This may be due to differences in virus titers in infected individuals, and the reproductive differences in lab versus field colonies, which may replace infected individuals at a faster rate than virus infection rates. In addition, preliminary studies involving genome walking and inverse PCR (iPCR) to detect insertion of SINV-1 (TX5) RNA into the host genome are underway. Using a single primer set, a 330 bp section of SINV-1 was found integrated into the *S. invicta* genome of lab colonies (unpublished data). Integration may explain, in part, why infection with whole virus extract did not produce immediate mortality in individuals, as there was a 'natural' RNAi effect induced when ants were then infected with 'wild-type' virus, similar to the effect observed in

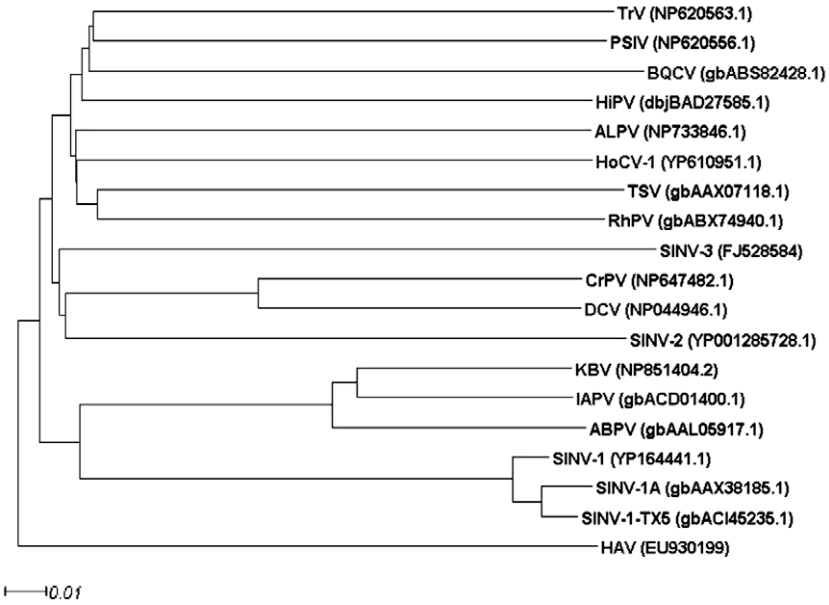

Fig. 2. Phylogenetic tree using amino acid sequences of the capsid protein, the second open reading frame (ORF2) region, of 19 dicistroviruses. Hepatitis A virus was used for the outgroup. TrV, Triatoma virus; PSIV, *Plautia stali* intestine virus; BQCV, Black queen cell virus; HiPV, Himetobi P virus; ALPV, Aphid lethal paralysis virus; HoCV-1, *Homalodisca coagulate* virus-1; TSV, Taura syndrome virus; RhPV, *Rhopalosiphum padi* virus; SINV-3, *Solenopsis invicta* virus-3; CrPV, Cricket paralysis virus; DCV, *Drosophila* C virus; SINV-2, *Solenopsis invicta* virus-2; KBV, Kashmir bee virus; IAPV, Israeli acute paralysis virus; ABPV, Acute bee paralysis virus; SINV-1, *Solenopsis invicta* virus-1; SINV-1A, *Solenopsis invicta* virus-1A; SINV-1 (TX5), *Solenopsis invicta* virus-1 (TX5); HAV, Hepatitis A virus. Genbank accession numbers are shown in parentheses.

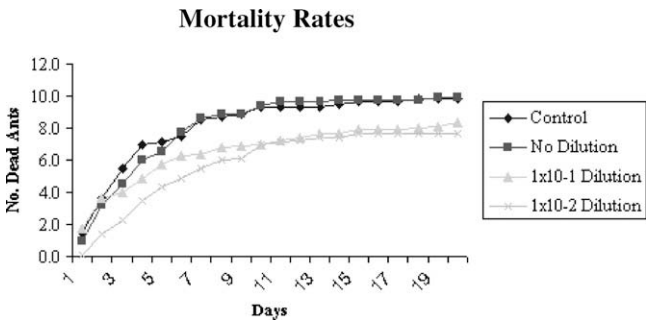

Fig. 3. Mortality rates of individual *Solenopsis invicta* exposed to a range of concentrations of whole virus extract SINV-1 (TX5) under laboratory conditions.

honey bees (Maori et al., 2007b). Efforts to increase infectivity of SINV may yet provide a viable delivery and expression system for the development of biological control strategies against *S. invicta* populations.

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