Isolation and characterization of *Solenopsis invicta* virus 3, a new positive-strand RNA virus infecting the red imported fire ant, *Solenopsis invicta*

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ABSTRACT

We report the discovery of a new virus from the red imported fire ant, *Solenopsis invicta*. *Solenopsis invicta* virus 3 (SINV-3) represents the third virus discovered from this ant species using the metagenomics approach. The single (positive)-strand RNA, monopartite, bicistronic genome of SINV-3 was sequenced in entirety (GenBank accession number FJ528584), comprised of 10,386 nucleotides, and polyadenylated at the 3′ terminus. This genome size was confirmed by Northern analysis. The genome revealed 2 large open reading frames (ORFs) in the sense orientation with an untranslated region (UTR) at each end and between the two ORFs. The 5′ proximal ORF (ORF 1) encoded a predicted protein of 299.1 kDa (2580 amino acids). The 3′ proximal ORF (ORF 2) encoded a predicted protein of 73.2 kDa (651 amino acids). RNA-dependent RNA polymerase (RdRp), helicase, and protease domains were recognized in ORF 1. SDS-PAGE separation of purified SINV-3 particles yielded 2 bands (ostensibly capsid proteins) with a combined molecular mass of 77.3 kDa which was similar to the mass predicted by ORF 2 (73.2 kDa). Phylogenetic analysis of the conserved amino acid sequences containing domains I to VIII of the RdRp from dicistroviruses, iflaviruses, plant small RNA viruses, picornaviruses, and 4 unassigned positive-strand RNA viruses revealed a trichotomous phenogram with SINV-3 and Kelp fly virus comprising a unique cluster. Electron microscopic examination of negatively stained samples of SINV-3 revealed isometric particles with apparent projections and a diameter of 27.3 ± 1.3 nm. SINV-3 was successfully transmitted to uninfected workers by feeding. The minus (replicative) strand of SINV-3 was detected in worker ants indicating replication of the virus. The possibility of using SINV-3 as a microbial control agent for fire ants is discussed.

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Introduction

The red imported fire ant, *Solenopsis invicta* Buren, was introduced into the U.S. from South America in the 1930s (Tschinkel, 2006). It has since spread to infest more than 128 million hectares from Virginia, south to Florida, and west to California (Williams et al., 2001). Efforts to control, prevent, repair damage, and otherwise attempt to mitigate the effects of this pest ant are estimated to exceed 6 billion dollars (U.S.) annually (Pereira, 2003). Although a number of highly efficacious chemical insecticides are available for fire ant control, they must be used regularly to maintain control. A number of studies have provided evidence that the *S. invicta* U.S. founding event(s) occurred without many of the native pathogens and parasites (Porter et al., 1992, 1997). Therefore, many laboratories have devoted a great deal of effort to discovery and use of parasites and entomopathogens in an attempt to provide long-term, sustained control of *S. invicta* in the U.S.

Despite intensive searches over the last 3 or 4 decades for viral infections of *S. invicta*, only recently were the first viruses reported and characterized from this ant (Valles et al., 2004, 2007, 2008; Valles and Strong, 2005). *Solenopsis invicta* virus 1 (SINV-1) and *S. invicta* virus 2 (SINV-2) were discovered using metagenomic and gene sequence identity/homology approaches (Valles et al., 2008). SINV-1 was the first virus discovered. It is a positive-strand, RNA virus with characteristics consistent with viruses in the *Dicistroviridae* (Mayo, 2002). It possesses a monopartite, single-stranded, 3′-polyadenylated, RNA genome that encodes 2 polyproteins. The 5′-proximal polyprotein contains sequences with identity to RNA-dependent RNA polymerase (RdRp), helicase, and cysteine protease proteins characteristic of single-stranded RNA viruses (Koonin, 1991) and the 3′-proximal polyprotein contains sequences consistent with viral coat proteins which was confirmed by purification and N-terminal sequencing (Valles and Hashimoto, 2008). SINV-2 was the second virus discovered in *S. invicta*. It also is a positive-strand RNA virus but with an unusual genome organization (Valles et al., 2007). The SINV-2 genome is monopartite and polycistronic, with 4 open reading frames in the sense orientation (Valles et al., 2007). SINV-1 and -2 have been associated inconsistently with fire ant colony mortality (Valles et al., 2004, 2007; Hashimoto and Valles, 2008).

Continued efforts and re-examination of expressed sequenced tags derived from an expression library created from *S. invicta* (Valles et al., 2008) have resulted in the discovery of a third virus. Referred to as

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Table 1
Strategy used to acquire the genome of SINV-3.

Reaction	Region acquired[a] (nts, 5′→3′)	Size (nts)	Oligonucleotide primers for:	
			cDNA synthesis	PCR amplification
5′ RACE	1–1167	1168	p763	p767/AAP
5′ RACE	1038–1582	545	p722	p762/AAP
5′ RACE	1338–2697	1360	p734	p755/AAP
5′ RACE	2423–3789	1367	p735	p754/AAP
5′ RACE	3610–4773	1164	p707	p750/AAP
5′ RACE	4585–5784	1200	p710	p739/AAP
5′ RACE	5636–6333	698	p734	p731/AAP
5′ RACE	6292–6773	482	p722	p720/AAP
5′ RACE	6677–7194	518	p707	p706/AAP
EST 16A4	7118–7812	695	N/A	N/A
3′ RACE	7164–8869	1706	oligo dT	p705/3′ primer
3′ RACE	8743–10411	1669	oligo dT	p789/3′ primer

Successive 5′ and 3′ RACE reactions conducted, the corresponding portion of the genome acquired, and oligonucleotide primers used for cDNA synthesis and PCR amplification are indicated.

[a] Positional information is based on the polyadenylated SINV-3 genome sequence in Genbank (FJ528584) containing 10,411 nucleotides.

Solenopsis invicta virus 3 (SINV-3), this virus was associated with significant mortality among workers and larvae of infected *S. invicta* colonies. The initial discovery and characterization of this virus are presented.

Results

Molecular characterization

The genome of SINV-3 was constructed by compiling sequences from a series of nine successive 5′ rapid amplification of cDNA ends (RACE) reactions, two 3′ RACE reactions, and the sequence of EST 16A4 (accession number EH413252; Tables 1 and 2). The SINV-3 genome was found to be 10,386 nucleotides in length, excluding the poly(A) tail present on the 3′ end (GenBank accession number FJ528584). This genome size was consistent with Northern analysis results of total RNA extracted from purified SINV-3 (Fig. 1A). Northern blotting yielded a band at 10,968 ± 270 nucleotides. No hybridization was observed in RNA extracted from fire ants determined to be free of SINV-3 by reverse transcriptase polymerase chain reaction (RT-PCR).

The SINV-3 genome sequence was A/U rich (70.9% A/U; 29.1% G/C). Analysis of the genome revealed 2 large open reading frames (ORFs) in the sense orientation (out of frame with each other) with an untranslated region (UTR) at each end and between the two ORFs (Fig. 2). The 5′ proximal ORF (ORF 1) commenced at the first canonical (AUG) start codon present at nucleotide position 92 and ended at a UGA stop codon at nucleotide 7834 which encoded a predicted protein of 299.1 kDa (2580 amino acids). The 3′ proximal ORF (ORF 2), commenced at nucleotide position 8308 (canonical AUG start codon), terminated at nucleotide position 10,263 (UAA stop codon) and

Table 2
Genome sequencing strategy for SINV-3.

Clone designation	Oligonucleotide primers (orientation)	Genome region amplified[a]
12B/178/2	p791 (F)/p767 (R)	1–1167
12B/157/6	p764 (F)/p756 (R)	1039–2624
12B/157/5	p769 (F)/p752 (R)	2003–3696
12B/157/4	p770 (F)/p748 (R)	2938–4683
12B/157/3	p771 (F)/p738 (R)	4042–5746
12B/157/2	p772 (F)/p734 (R)	5038–6442
12B/157/8	p730 (F)/p708 (R)	6296–7271
12B/174/1	p705 (F)/p713 (R)	7164–8605
12B/180	p775 (F)/p787 (R)	8038–9730
12B/157/1	p789 (F)/3′-primer (R)	8743–10411

Oligonucleotide primers used to amplify overlapping regions of the SINV-3 genome. The clone designation and corresponding region of the genome sequence are indicated.

[a] Positional information is based on the polyadenylated SINV-3 genome sequence in Genbank (FJ528584) containing 10,411 nucleotides.

encoded a predicted protein of 73.2 kDa (651 amino acids). No large ORFs were found in the inverse orientation suggesting that SINV-3 is a positive-strand RNA virus. The 5′, 3′, and intergenic UTRs were comprised of 91, 123, and 473 nucleotides, respectively. Blastp analysis (Altschul et al., 1997) of the translated ORF 1 recognized a conserved domain (Genbank CD01699) for RdRp (Figs. 2 and 3) which is encoded in the genomes of all RNA viruses with no DNA stage (Koonin and Dolja, 1993). The most significant expectation scores (e-scores) from blastp analysis of the RdRp region of ORF 1 were to Kelp Fly Virus (KFV, 10^{-58}) and Nora virus (NV, 10^{-18}) with corresponding identities of 35.1 and 25.2%, respectively. Helicase and protease sequence motifs were also present in ORF 1 upstream of the RdRp (Fig. 2). Blastp analysis of ORF 2 did not yield any sequences with significant identity. SDS-PAGE separation and subsequent silver staining of purified SINV-3 particles yielded 2 bands (band 1: 41.0 kDa; band 2: 36.3 kDa) with a combined molecular mass of 77.3 kDa which was similar to the mass predicted by ORF 2 (73.2 kDa; Fig. 2).

Phylogenetic analysis of the conserved amino acid sequences containing domains I to VIII of the RdRp (Figs. 3 and 4) from dicistroviruses, iflaviruses, plant small RNA viruses, picornaviruses, and 4 unassigned positive-strand RNA viruses revealed a trichotomous phenogram with SINV-3 and KFV comprising a unique cluster (Fig. 4). The close relationship between SINV-3 and KFV is supported by a significant e-score and sequence identity when the SINV-3 ORF 1 and KFV ORF were compared.

Purification and electron microscopy

Particles purified from SINV-3-infected fire ants migrated to a density of 1.39 ± 0.02 g/ml CsCl. No corresponding particles were observed in samples prepared from uninfected fire ants. Electron microscopic examination of negatively stained samples from SINV-3-infected fire ants and particles purified from SINV-3-infected fire ants revealed isometric particles with apparent projections and a diameter of 27.3 ± 1.3 nm (Fig. 1B).

Tissue tropism, transmission, and virulence

SINV-3 genome was detected in all tissues of *S. invicta* queens, workers and larvae examined by quantitative PCR (QPCR, Table 3). The tissue comprising the largest percentage of SINV-3 genome equivalents was the carcass for queens (27.0%) and workers (50.4%), and midgut (34.6%) for the larvae. Based on these data, SINV-3 infection appears to be systemic. SINV-3 was detected in *S. invicta* eggs, workers, larvae, queens, and alate stages (Fig. 5).
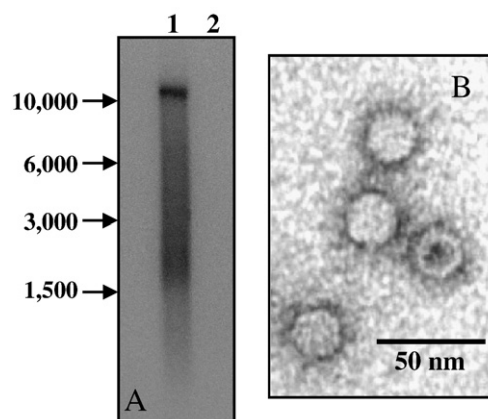

Fig. 1. (A) Northern blot of RNA prepared from purified SINV-3 particles (lane 1) and SINV-3-uninfected fire ant workers (lane 2). Numbers to the left of the blot indicate the size (in nucleotides) of single-stranded RNA standards. (B) Electron micrograph of a negative stain of purified SINV-3 used for the Northern analysis.
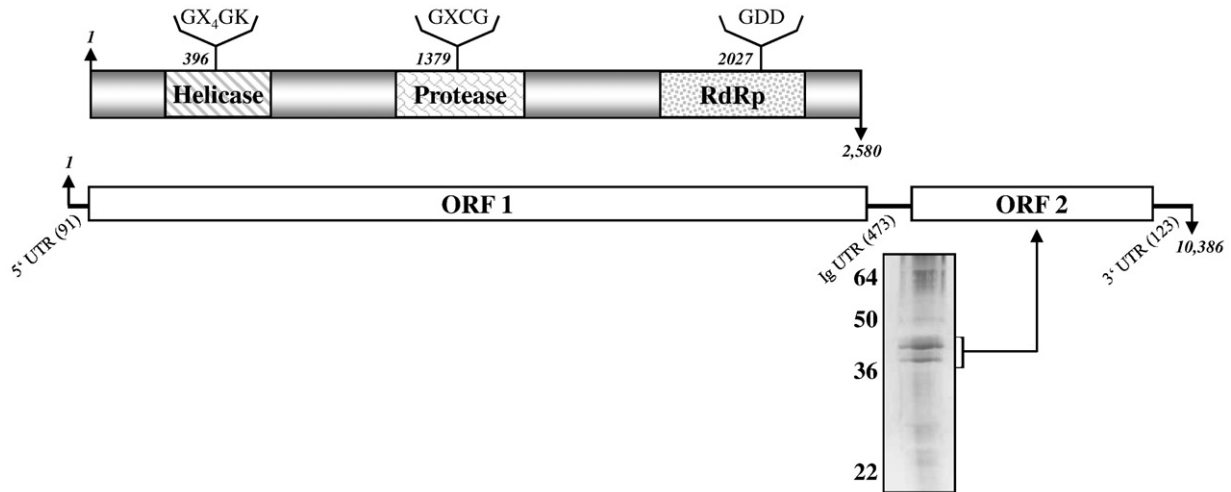
Fig. 2. Genome organization of SINV-3. Positions of sequence motifs for helicase, protease, and RNA-dependent RNA polymerase and their corresponding positions within the polyprotein of ORF 1 are indicated. The italicized numeral indicates the position of the first residue of the sequence provided. The lower right shows results of SDS-PAGE separation of proteins from a purified preparation of SINV-3 particles. Two bands were consistently observed with a combined molecular mass of 77.3 kDa. The predicted molecular weight of the translated ORF 2 was 73.2 kDa. UTR = untranslated region, Ig UTR = intergenic untranslated region.

SINV-3 was successfully transmitted to uninfected workers by feeding (Fig. 6). SINV-3 genome was detected within 7 days of providing uninfected *S. invicta* ants a sucrose solution containing partially purified SINV-3. SINV-3 was detectable for at least 21 days after treatment indicating transmission and sustained infection among recipient colonies. Furthermore, the minus (replicative) strand of SINV-3 was detected in worker ants indicating replication of the virus.

SINV-3 was consistently associated with significant mortality among *S. invicta* laboratory colonies. Whether fire ant colonies were initiated from newly mated queens or as whole colonies collected from the field directly, all invariably acquired the SINV-3 infection indicating a contamination of the laboratory rearing area. Early signs of infection were larger than normal midden piles of adult ants followed by near complete brood disappearance. Queens lost their physogastricity (no distension of the abdominal intersegmental membranes) and typically fell below 14 mg wet weight (11.8 ± 2.2 mg, mean $\pm$ SD). However, the queens continued to produce eggs during this period. Some workers typically remained alive for considerable periods after the initial brood die-off, and occasionally the colony would rebound exhibiting normal brood production.

Discussion

In 2003, blastx analysis of expressed sequence tags (ESTs) from a *S. invicta* expression library had indicated weak identity of EST 16A4 with the polyprotein of *Acyrthosiphum pisum* virus (Valles et al.,

2004). Subsequent re-analysis of this EST sequence revealed significant identity with a portion of the KFV genome (Hartely et al., 2005). EST 16A4 was therefore used as the primary sequence from which oligonucleotide primers were designed and RACE (3′ and 5′) reactions conducted to acquire additional sequence and confirm that this sequence was of viral origin. Indeed, subsequent sequence acquisition and analysis confirmed the presence of a new virus from *S. invicta* ants which has been named *Solenopsis invicta* virus 3 (SINV-3). The 10,386 nucleotide RNA genome was monopartite, dicistronic (non-overlapping), and single-stranded. The genome size was confirmed by Northern analysis in which a band was observed at $10,968 \pm 270$ nucleotides; no subgenomic RNA was evident. Blastp analysis of ORF 1 resulted in recognition of a conserved domain for RdRp, characteristic of positive-strand RNA viruses (Koonin and Dolja, 1993). These viruses invariably possess 8 common sequence motifs in the RdRp (Koonin, 1991); all eight of these motifs were present in the SINV-3 genome (Figs. 2 and 3). Further, sequence motifs IV, V and VI were reported to be unequivocally conserved throughout this class of viruses, exhibiting 6 invariant amino acid residues (Koonin and Dolja, 1993). These core RdRp motifs were shown by site-directed mutagenesis to be crucial to the activity of the enzyme (Sankar and Porter, 1992). SINV-3 possessed all 6 of these characteristic residues, D^{1920}, D^{1925} (motif IV), G^{1979}, T^{1983} (motif V), and D^{2028}, D^{2029} (motif VI).

Alignment of SINV-3 ORF 1 with nonstructural polyproteins of positive-strand RNA viruses revealed domains for a helicase and

Fig. 3. Alignment comparisons of predicted amino acid sequences of the RdRp of Kelp Fly virus (KFV), *Solenopsis invicta* viruses-1, -2, and -3, (SINV-1, SINV-2, and SINV-3) and Hepatitis A virus (HAV). The numbers on the left indicate the starting amino acids of aligned sequences. Identical residues in at least four of the five virus sequences are shown in reverse. Sequence motifs shown for the RdRp (I–VIII) correspond to those identified and reviewed by Koonin and Dolja (1993).
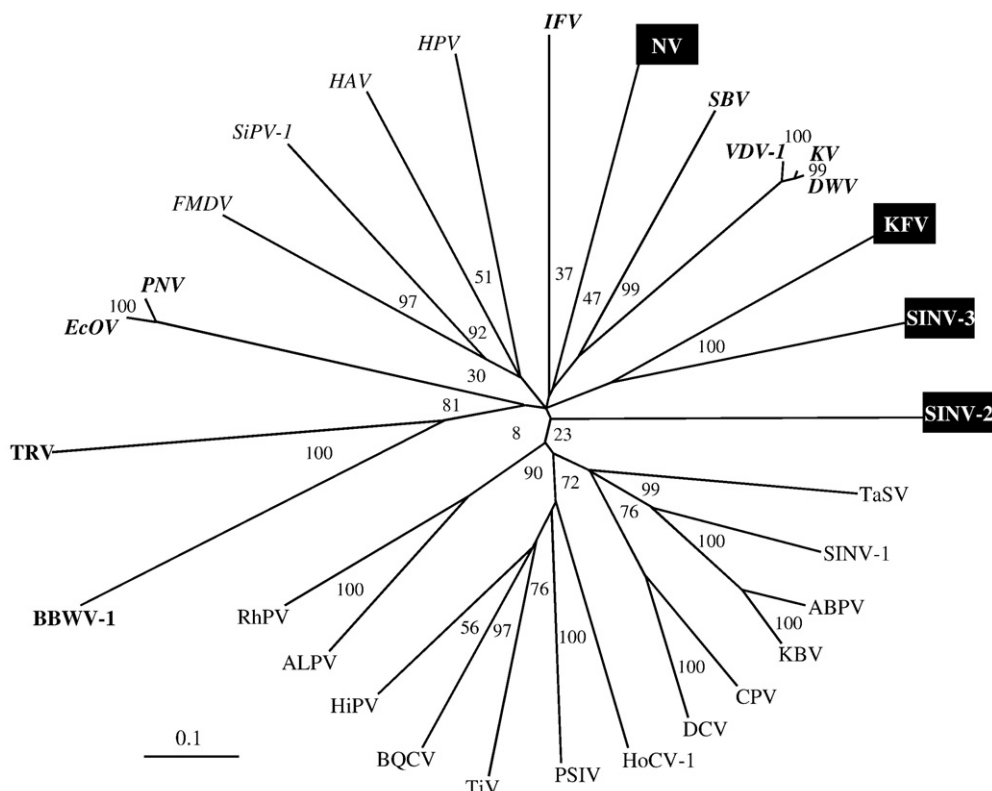
Fig. 4. Phylogenetic analysis of the conserved amino acid sequences containing domains I to VIII of the putative RdRp from thirteen dicistroviruses (unaltered text font), seven iflaviruses (bold, italic), two plant RNA viruses (bold), four picornaviruses (italic), and four unassigned viruses (white text). Virus abbreviation, accession number of the virus RNA or protein sequence, and amino acid residues of aligned sequences in a specific ORF (5′ proximal ORF of dicistroviruses, otherwise an ORF number is specified) include: Aphid lethal paralysis virus (ALPV) [AF536531], 1661–1955; Black queen cell virus (BQCV) [NP620564], 1317–1585; Cricket paralysis virus (CPV) [NP647481], 1423–1697; *Drosophila* C virus (DCV) [AF014388], 1415–1693; Himetobi P virus (HiPV) [AB017037], 1441–1710; *Plautia stali* intestine virus (PSIV) [NP620555], 1465–1739; *Rhopalosiphum padi* virus (RhPV) [AF022937], 1625–1916; *Triatoma* virus (TiV) [AF178440], 1446–1716; Acute bee paralysis virus (ABPV) [AAG13118], 1566–1837; *Homalodisca coagulata* virus-1 (HoCV-1) [DQ288865], 1446–1716; Kashmir bee virus (KBV) [AY275710], 1594–1864; *Solenopsis invicta* virus-1 (SINV-1) [AY634314], 1052–1327; Taura syndrome virus (TaSV) [AF277675], 1770–2036; Infectious flacherie virus (IFV) [AB000906], 2618–2888; *Perina nuda* virus (PNV) [AF323747], 2628–2899; Sacbrood virus (SBV) [NC002066], 2522–2790; Deformed wing virus (DWV) [AJ489744], 2556–2826; *Ectropis obliqua* picorna-like virus (EcOV) [AY365064], 2629–2900; Kakugo virus (KV) [AB070959], 2556–2826; *Varroa destructor* virus-1 (VDV-1) [AY251269], 2556–2826; Foot-and-mouth disease virus (FMDV) [AF308157], 2011–2264; Hepatitis A virus (HAV) [NC001489], 1904–2161; Human parechovirus (HPV) [AJ005695], 1871–2117; Simian picornavirus 1 (SiPV-1) [AY064708], 2119–2368; Broad bean wilt virus 1 (BBWV-1) [NP951030] RNA1, 405–657; Tomato ringspot virus (TRV) [ABG23688] RNA1, 406–672; Nora virus (NV) [DQ321720] ORF 2, 1763–2026; *Solenopsis invicta* virus 2 (SINV-2) ORF 4 [ABQ01575], 1814–2081; Kelp fly virus (KFV) [YP415507], 3015–3272; *Solenopsis invicta* virus 3 (SINV-3) ORF 1, 1848–2107.

protease. Three sequence motifs are conserved among positive-strand virus helicases, designated A, B and C (Gorbalenya et al., 1990). The consensus sequence for motif A, GX_4GK (Gorbalenya et al., 1990), thought to be responsible for nucleotide binding, was found in the translated ORF 1 of SINV-3 at amino acid position 396. Motifs B and C were also identified based on the presence of conserved residues (Q^{441}, D^{447}-motif B; and N^{497} preceded by a stretch of hydrophobic residues) (Gorbalenya et al., 1990).

Characteristic motifs for a 3C-like protease, but not a 2A protease, were also detected in the translated ORF 1 of SINV-3. Amino acids thought to form the catalytic triad of the 3C-like protease, H^{1258}, D^{1309}, and C^{1381} were present in SINV-3 ORF 1 (Koonin and Dolja, 1993; Ryan and Flint, 1997; Luke et al., 2008). Furthermore, the consensus GxCG sequence motif was present at amino acids 1379 to 1382.

Phylogenetic analysis of SINV-3 RdRp showed a close relationship with KFV (Fig. 4). Indeed, many of the unique features exhibited by KFV were also shared by SINV-3, including a smaller virion size (27.3 ± 1.3 nm; Fig. 1B) with apparent surface projections (Scotti et al., 1976; Hartely et al., 2005) and high buoyant density (1.39 ± 0.02 g/ml). Another similarity was the presence of only 2 major capsid proteins (VP1 and VP2) as opposed to 3 or 4 which is typical of iflaviruses or viruses in the *Dicistroviridae*. Although phylogenetic analysis of the RdRp showed a relationship between SINV-3 and KFV, a number of differences between these viruses were observed. Their genome structures were different; KFV genome is monocistronic and SINV-3

is apparently dicistronic. They also exhibited poor identity (<10%) among their structural proteins.

SINV-3 was found to infect all stages of *S. invicta*, including the eggs (Fig. 5). QPCR of different stages shows that immature ants contained significantly lower quantities of SINV-3 compared with adults (workers, alates and queens). However, the purpose of these

Table 3
Distribution of SINV-3 genome equivalents among different tissues and tagma of worker, larval (4th instars), and queen *S. invicta* ants.

Tissue/tagma	Percent of total SINV-3 genome equivalents for stage[a]		
	Queen	Worker	Larvae
Remaining carcass[b]	27.0 ± 3.7	50.4 ± 6.7	34.4 ± 6.8
Midgut	9.0 ± 7.5	33.6 ± 3.1	34.6 ± 6.7
Malpighian tubules	8.0 ± 5.5	3.0 ± 1.2	31.0 ± 9.9
Hindgut	4.2 ± 1.9	2.7 ± 0.7	
Poison sac	8.2 ± 4.1	2.0 ± 1.0	
Head	13.1 ± 11.1	4.5 ± 1.1	
Thorax	4.0 ± 1.6	2.0 ± 0.4	
Crop	7.3 ± 3.6	1.5 ± 0.3	
Ovary	12.6 ± 7.7		
Fat body	6.9 ± 5.3		

[a] Mean ($\pm$SE) number of SINV-3 genome equivalents was $1.46 \pm 2.83 \times 10^6$ for workers, $1.39 \pm 3.22 \times 10^9$ for larvae, and $2.0 \pm 2.67 \times 10^9$ for queens.
[b] For queens and workers the remaining carcass was comprised of tissues not removed from the abdomen; for larvae, tissues not removed from the entire body carcass.
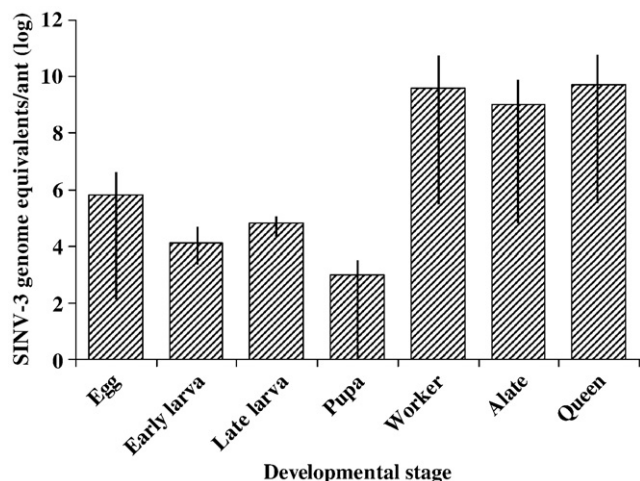
Fig. 5. SINV-3 genome equivalents determined for different developmental stages. Genome equivalents were interpolated from a standard curve generated simultaneously from a plasmid construct. Mean values are indicated by the bar and the internal line signifies the minimum and maximum value observed for each group.

experiments was to determine which developmental stages were infected, not to examine the progression of the infection. Therefore, SINV-3 titers may be higher in immature stages depending on the infection development.

SINV-3 represents the third virus to be discovered from *S. invicta* using the metagenomics approach (Valles et al., 2008) and it possesses features consistent with placement within the order *Picornavirales*: 1. non-enveloped particles with a diameter around 30 nm, 2. a positive-sense, single-stranded RNA genome, 3. no production of subgenomic RNA, and 4. a polyprotein containing helicase, protease, and RdRp domains (Le Gall et al., 2008). Although SINV-1, SINV-2, and SINV-3 are positive-strand RNA viruses infecting the same host (*S. invicta*), each virus is distinct phylogenetically (Fig. 4). Furthermore, they exhibit differences in genome organization; SINV-1 and SINV-3 are dicistronic, while SINV-2 is polycistronic. The most important difference between SINV-1, SINV-2, and SINV-3 is apparent pathogenicity. SINV-1 and -2 are similar to many positive-strand RNA viruses that infect honey bees; they are present as chronic, asymptomatic infections that may cause mortality under certain stressful conditions (Bailey, 1967; Chen and Siede, 2007; Oi and Valles, 2008). Transmission studies of SINV-1 and SINV-2 to uninfected ants were completed regularly by feeding. However, mortality among recipient colonies was an occasional event (Valles et al., 2004; Valles, unpublished). Conversely, SINV-3 transmission (Fig. 6) was associated consistently with ant mortality and a correspondingly high SINV-3 titer; dead ants typically contained greater than 10^9 viral particles. Further evidence of SINV-3 virulence was the presence of viral genome copies in all tissues of the ant (Table 3). These results indicated a systemic infection as opposed to SINV-1 and SINV-2 which were limited to the midgut epithelium (Hashimoto and Valles, 2007, 2008). According to Rivers (1937), viruses "associated with a disease with a degree of regularity" and "shown to occur in sick individuals" provides sufficient etiological evidence. Rivers argued that Koch's (1884) postulates required modification for diseases caused by viruses. Thus, following River's modified guidelines suggest that fire ant colony mortality is likely caused by SINV-3. Indeed, SINV-3 infection of *S. invicta* is also supported by the guidelines of Fredericks and Relman (1996) arguing that sequence-based identification may be used to support etiology. Specifically, SINV-3 nucleic acid was consistently present in declining *S. invicta* colonies and fewer or no copies of the SINV-3 genome are present in normal colonies. The remaining five guidelines for molecular based disease causation have not been established. However, Fredericks and Relman (1996)

conclude that not all of the guidelines need be met for a compelling argument for causation.

Use of positive-strand RNA viruses as insect control agents has been proposed (Scotti et al., 1981; Christian et al., 1993) and successfully demonstrated for the olive fruit fly (Manousis and Moore, 1987) and *Helicoverpa armigera* (Christian et al., 2005). However, production of pure virus has been limited to *in vivo* or cell culture systems. A cell line is not available for *S. invicta* which has hampered development, study, and potential use of the *Solenopsis invicta* viruses as control agents against fire ants (Oi and Valles, 2008). Recently, successful *in vitro* expression of a positive-strand RNA virus was reported (Pal et al., 2007; Boyapalle et al., 2008). A clone of the *Rhopalosiphum padi* virus genome was expressed in a baculovirus expression system and found to be infectious and pathogenic to its aphid host (Pal et al., 2007; Boyapalle et al., 2008). This research permits further studies of viruses in which a cell line is unavailable (e.g., for *S. invicta*). More importantly, from a control aspect, this methodology provides potential large-scale production of pure virus that can be tested, formulated, and developed as a microbial control agent.

We are hopeful that SINV-3 can be exploited and developed as a microbial insecticide for *S. invicta*. Control efforts, prevention, and damage repair from this pest ant exceed 6 billion dollars (U.S.) annually in the U.S. and it has recently made incursions into Australia and China (Tschinkel, 2006). Comparative ecological studies have shown that *S. invicta* infestations are significantly higher in introduced areas which have been attributed to a lack of natural enemies (Porter et al., 1992, 1997). A biologically-based approach is arguably the most tenable at achieving widespread, sustained control of *S. invicta* in introduced areas.

Materials and methods

Molecular characterization

A series of nine 5′ RACE reactions were conducted to obtain the upstream sequence of the SINV-3 genome using the 5′ RACE system (Invitrogen, Carlsbad, CA) and primer walking. For each reaction, cDNA was synthesized for 50 min at 48 °C with 2.5 µg of total RNA extracted with Trizol from purified SINV-3 particles with a gene-specific oligonucleotide primer (GSP; Table 4), the RNA template was degraded with RNase H, and the cDNA purified. The 3′ end of the cDNA was polycytidylated with terminal deoxynucleotidyl transferase and dCTP. The tailed cDNA was then amplified with a nested GSP (3′ end) and an abridged anchor primer (AAP). Gel-purified amplicons were ligated into the pCR4-TOPO vector, transformed into TOP10 competent
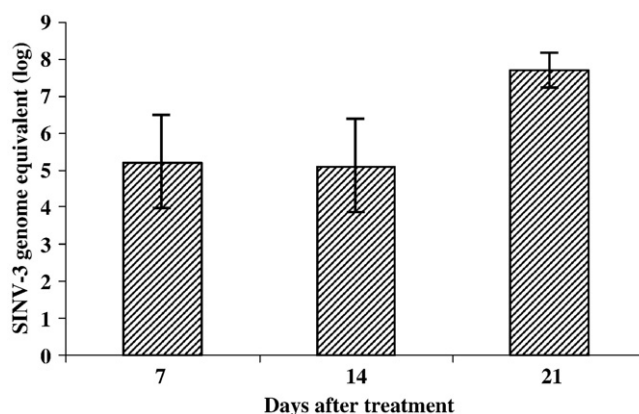

Fig. 6. SINV-3 transmission to uninfected *S. invicta* fragment colonies. Worker ants were sampled on the week specified from each colony and tested for the presence of SINV-3 by QPCR. SINV-3 was undetectable on day 0. Error bars represent the standard deviation.

Table 4
Oligonucleotide primers used throughout the study.

Oligonucleotide designation	Oligonucleotide (5′→3′)	Genome position	Orientation
p791	TTTTAAAATAGGAAATTAAAGTCCAGTAAGGTTACTG	1–37	→
p764	TGATAATTTCCTTGGTATTCCTAATTATAAATTTGCT	1039–1075	→
p767	ATTTCACTTTGTTTATCTTTAGGACAATCACGA	1135–1167	←
p762	ATGTTCAATATCTGCAGCAGCACAATTAAAATATGC	1502–1537	←
p763	ATTTACAGTTGCAAATACCAATTCAAATGGACATGGA	1546–1582	←
p769	GCAAATGCTACTAATCTTAAAATTGGATCAGAGG	2003–2036	→
p756	CCAATTCAAAAGGATTATTGAAAGAAACTCTATGAA	2589–2624	←
p755	GGTGAAAGATACATTTTCCAATCAAAATGCAAAAG	2663–2697	←
p770	TGATGGTGATTATGTTTATATTTCTGAGCATAAAATTC	2938–2975	→
p752	AATCCATTTACATCGGGTAAAGTAAGAACTTCCTGCT	3660–3696	←
p754	ATACAATTTTCATCATTAGCAATCATATAAATCTGA	3754–3789	←
p771	AGAAGAGCTTTATGATGCTGAAAATTGTAATACTGTTC	4042–4079	→
p748	GCAGTTGTAAGTTTCCAACCATTAGTAACCGTCAAT	4648–4683	←
p750	TTGAATATCGAATTGAATTGAACGTCAACAACTCTA	4738–4773	←
p772	AGTTGAAAATGAAACAGTTGAAATTTTAGGTATAACTCA	5038–5076	→
p738	ACGAGCTGTGAACTCACCAAGAATCCAACGTT	5715–5746	←
p739	TTACATCCTACAGCACAATTTTCATCCCAAGCA	5752–5784	←
p730	CCAAAATTTGAAACTATTGAAACATTATCTTTTATA	6296–6331	→
p731	TATAAAAGATAATGTTTCAATAGTTTCAAATTTTGG	6296–6331	←
p734	ATGTTCAGGAGTATCATCAGTTGCATAACAAA	6411–6442	←
p735	CTCTTCTTCCCATAATGCAGCTTCTTCTTGAATTG	6462–6496	←
p720	CTTTATTAAAATTGCCTTCAAGGGCAGCTT	6744–6773	←
p722	GATGAGGCCCTTTTGAAAGATTTCATTGAGGT	6816–6847	←
p705	CTGCTGGTATGATGGCAACAGATCCTTCTGT	7164–7194	→
p706	ACAGAAGGATCTGTTGCCATCATACCAGCAG	7164–7194	←
p707	AAGGAGTTTGTGTATTAGTTGCAATGCCAGAATCT	7201–7235	←
p708	ATGCTCTAGCTATGGGATTCAATACACGGGA	7241–7271	←
p710	CATCATTTCTTGAACATTAGTTATAGGATGTTCGAC	7718–7753	←
p775	CATAATATTACTGATGTTGTGGTTTCTTCAAAACC	8038–8072	→
p713	CGGGAACAAATGGATCAGAACGATATCCACCA	8574–8605	←
p789	ATGGTTAGACAGGGTTCTTCAACAACCCAATCTACTGATA	8743–8782	→
p787	CATGATTTTGTTGTTCAATAGGTTCATAAATATGTTCCTCAT	9689–9730	←

cells (Invitrogen, Carlsbad, CA) and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida).

Two 3′ RACE reactions were conducted with the GeneRacer kit (Invitrogen). cDNA was synthesized from total RNA (1 μg) using the GeneRacer appended Oligo dT primer. The cDNA was amplified by PCR with a GSP and GeneRacer 3′ primer. Amplicons were cloned and sequenced as described for 5′ RACE. Two 3′ RACE reactions were required because a region of high adenine density (nucleotides 8847–8874) was binding the oligo dT primer upstream of the 3′ poly A tail.

Northern analysis was conducted to determine the genome size and possibly whether subgenomic RNA was produced following the glyoxal denaturation procedure of Sambrook and Russell (2001). Total RNA (2 to 8 μg) prepared with Trizol from purified SINV-3 particles and SINV-3-uninfected fire ants was denatured with glyoxal and separated on a 1% agarose gel. Electrophoresis was conducted at 60 V for 1.5 h in BPTE buffer (30 mM Bis–Tris; 10 mM PIPES; 1 mM EDTA, pH 6.5). Gel-separated nucleic acids were transferred onto a Nytran Supercharge nylon membrane (Schleicher and Schuel Bioscience, Keene, NH) by downward blotting in 0.01 M NaOH and 3 M NaCl. After neutralization and blocking in SSC (0.9 M NaCl, 0.09 M sodium citrate) the blot was pre-hybridized in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA for 2 h. The 499 nucleotide probe was synthesized by PCR using oligonucleotide primers p762 and p764 (Table 4), ^{32}P-labeled dCTP, and a clone from the 3′ end of the SINV-3 genome as template. The probe was added to the hybridization chamber and incubated for 16 h under high stringency conditions at 68 °C. After hybridization, the blot was dried and exposed to X-ray film for 12 h. Molecular weight of the SINV-3 genome was extrapolated from a standard curve prepared from RNA molecular weight standards electrophoresed concurrently.

Blastp analysis was conducted with conserved regions of the SINV-3 RdRp of ORF 1. Representative viruses exhibiting significant e-scores ($<10^{-5}$) were included in the phylogenetic analysis. Viral nucleotide sequences were retrieved from the genome resource at NCBI (http:// www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html) and used for comparative analysis and multiple-alignment with SINV-3 using Vector NTI Advance software (Version 10.1.1, Invitrogen). Multiple alignments were carried out for the deduced amino acid sequences of the nonstructural polyproteins of viral genomes. Specifically, conserved regions of the RdRp (domains I to VIII) were aligned and subsequently used to construct an unrooted radial phylogenetic tree using the Neighbor-joining method (Saitou and Nei, 1987) in ClustalX (Thompson et al., 1994). The statistical significance of branch order was estimated by performing 1000 replications of bootstrap re-sampling of the original aligned amino acid sequences. Trees were generated with TreeView (Page, 1996).

Virus detection, purification, and electron microscopy

One-step RT-PCR was used to identify SINV-3-infected *S. invicta* ant colonies. To test field colonies, a 20 ml scintillation vial was plunged into a fire ant mound for several minutes to collect a sample of the worker caste. The ants were returned to the laboratory and RNA was extracted from 10 to 20 workers using Trizol reagent according to the manufacturer's directions. cDNA was synthesized and subsequently amplified using the One-Step RT-PCR kit (Invitrogen) with oligonucleotide primers p705 and p707 (Table 4). Samples were considered positive for the virus when a visible amplicon (72 nucleotides) was present after separation on a 1.2% agarose gel stained with ethidium bromide. RT-PCR was conducted under the following optimized temperature regime: 1 cycle at 45 °C for 30 min, 1 cycle at 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 63 °C for 15 s, 68 °C for 15 s, followed by a final elongation step of 68 °C for 5 min.

SINV-3 was purified by discontinuous and isopycnic centrifugation. Briefly, 50 g of a mixture of workers and brood were homogenized in 150 ml of NT buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl) in a Waring blender on high speed for 2 min. The mixture was filtered through 8 layers of cheesecloth and then

extracted with an equal volume of chloroform for 10 min with constant mixing. The mixture was centrifuged for 5 min at 5000 g and the supernatant collected by pipette. The supernatant was layered onto a discontinuous CsCl gradient (1.2 g/ml and 1.5 g/ml) which was centrifuged at 190,000 g for 2 h in a Ti50.1 rotor. A whitish band visible near the interface was removed and brought to a density of 1.3 g/ml CsCl. This sample was then centrifuged at 330,000 g for 16 h in a Ti70.1 rotor. A whitish band at 1.39 ± 0.02 g/ml was collected. The sample was negatively stained with 2% phosphotungstic acid, pH 7, and examined with a Hitachi H-600 transmission electron microscope (Hitachi, Pleasanton, CA) at an accelerating voltage of 75 kV.

SINV-3 tissue and developmental stage tropism

Tissue tropism of SINV-3 was examined by dissecting different tissues or groups of tissues and quantifying the number of SINV-3 genome equivalents in each respective preparation by quantitative PCR (QPCR). The experiment was conducted for mated queens, workers, and fourth instar larvae. Ice-chilled fire ant workers and queens from SINV-3-positive colonies were placed on a glass microscope slide, and head, thorax, and abdomen were separated with a surgical blade under a dissecting microscope. The head and thorax were placed directly in Trizol and the abdomen was immersed in 10 mM Tris–HCl, pH 8.0, for further dissection. Tissues were isolated and removed from the abdomen with micro-dissecting forceps in the following order: crop, poison sac, midgut, hindgut, Malpighian tubules, fat body (queens only), ovaries (queens only), and the remaining tissues of the abdominal carcass. Fourth instar larvae from SINV-3-positive colonies were dissected to the following groups: Malpighian tubules, midgut, and remaining tissues of the carcass. Six replicates were conducted for each stage using individual queens per replicate and pooled groups of workers and larvae ($n = 10$). RNA was extracted with Trizol. cDNA was synthesized from the SINV-3 genome region corresponding to the RdRp with total RNA using SuperScript III Reverse Transcriptase (SsRT; Invitrogen) and a gene-specific primer (p707, Table 4). In a 0.5 ml PCR tube, 2 μl of primer p707 (1 μM), 1 μl of a dNTP mix (10 mM), and 10 μl of total RNA (50 ng) were mixed and heated to 65 °C for 5 min in a PTC 100 thermal cycler, followed by incubation on ice for 1 min. Then, 4 μl of first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM MgCl$_2$), 2.75 μl of DEPC-treated water, and 0.25 μl of SsRT (200 U/μl) were added. The mixture was incubated at 55 °C for 30 min, followed by inactivation of SsRT by heating to 70 °C for 15 min. This cDNA was used to perform QPCR.

QPCR was conducted on an ABI PRISM 7000 Sequence Detection System interfaced to the ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA) in a 25 μl reaction volume. The reaction contained 12.5 μl of SYBR Green SuperMix (with UDG and ROX, Invitrogen), 0.4 μl each of 10 μM SINV-3-specific primers (p705 and p707), 3 mM MgCl$_2$, 1 μl of the cDNA synthesis reaction, and 10.7 μl of DEPC-water. QPCR conditions consisted of one cycle at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 64 °C for 15 s, 72 °C for 1 min. The non-template control for QPCR included a complete cDNA synthesis reaction devoid of RNA template. A standard curve was constructed from a plasmid clone of the corresponding SINV-3 genome region using a copy number range of $5-5 \times 10^7$ copies. Reaction efficiencies were determined by regressing critical threshold (C_T) values against the template copy number (log) and calculated according to the formula $[E = (10^{-1/\text{slope}}) - 1]$ (Klein et al., 1999). Reaction efficiencies routinely exceeded 95%.

Experiments were conducted to quantify the SINV-3 infection in different developmental stages of S. invicta. Samples of eggs ($n = 20$), queens ($n = 11$), workers ($n = 18$), early (first and second instars) larvae ($n = 5$), and late (third and fourth instars) larvae ($n = 5$) were taken from SINV-3-positive colonies. Larval stages were differentiated by the method of Petralia and Vinson (1979). RNA was extracted from the specimens with Trizol reagent. The RNA concentration was

determined spectrophotometrically. The number of SINV-3 genome equivalents in different developmental stages and tissues was quantified by QPCR as described above.

SINV-3 genome strand-specificity

QPCR was also conducted to separately quantify the plus (genomic) and minus (replicative) RNA strands of SINV-3. cDNA was synthesized from the SINV-3 plus strand with oligonucleotide primer p707 and minus strand with oligonucleotide primer p705 as described above. After cDNA synthesis, the RNA templates were digested with RNase A and RNase H at 37 °C for 30 min. QPCR was subsequently conducted as described above with oligonucleotide primers p705 and p707.

SINV-3 transmission

SINV-3-uninfected laboratory-reared newly mated queen mono-gyne colonies were identified by RT-PCR. These colonies were at an early stage of establishment, comprised of approximately 1 ml of brood and 100 workers in addition to the queen. Three colonies were infected by feeding them a purified preparation (see above) of SINV-3 in 10% sucrose. QPCR was conducted on the purified preparation to determine the concentration of viral particles. The preparation was diluted in 10% sucrose to achieve a concentration of 1×10^6 SINV-3 particles per μl. This solution was placed into a small glass test tube (approximately 4 ml) with a cotton stopped end. The ant colonies were allowed to feed on the virus preparation for 3 days and then it was removed. Afterward, the ants were fed unadulterated 10% sucrose, water, frozen crickets (Acheta domesticus), and egg yolk (hard-boiled) ad libitum. Worker ants ($n = 10$) from each colony were examined for the presence of SINV-3 by extracting total RNA and conducting QPCR on 0, 7, 14, and 21 days after exposure to the SINV-3 preparation.

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