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A new positive-strand RNA virus with unique genome characteristics from the red imported fire ant, *Solenopsis invicta*

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Abstract

We report the discovery of a new virus with unique genome characteristics from the red imported fire ant, *Solenopsis invicta*. This virus represents the second identified from this ant species. It is provisionally named *Solenopsis invicta* virus 2 (SINV-2). The SINV-2 genome was constructed by compiling sequences from successive 5′ RACE reactions, a 3′ RACE reaction, and expressed sequence tag, c246 (accession number EH413675), from a fire ant expression library. The SINV-2 genome structure was monopartite, polycistronic and RNA-based. The genome consensus sequence (EF428566) was 11,303 nucleotides in length, excluding the poly(A) tail present on the 3′ end. Analysis of the genome revealed 4 major open reading frames (ORFs; comprised of ≥ 100 codons) and 5 minor ORFs (comprised of 50–99 codons) in the sense orientation. No large ORFs were found in the inverse orientation suggesting that the SINV-2 genome was from a positive-strand RNA virus. Further evidence for this conclusion includes abolished RT–PCR amplification by RNase treatment of SINV-2 nucleic acid template, and failure to amplify without first conducting cDNA synthesis. Blastp analysis indicated that ORF 4 contained conserved domains of an RNA-dependent RNA polymerase, helicase, and protease, characteristic of positive-strand RNA viruses. However, the protease domain and putative structural proteins (ORFs 1, 2, and 3) were less well conserved. Phylogenetic analysis of the RdRp, helicase, and ORF 1 indicate unique placement of SINV-2 exclusive from the *Dicistroviridae*, iflaviruses, *Picornaviridae*, and plant small RNA viruses.
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Keywords: *Solenopsis invicta*; RNA virus; Genome sequence; Iflavirus; *Dicistroviridae*

Introduction

Red imported fire ant, *Solenopsis invicta* Buren, is an invasive species introduced into the United States from its native South America between 1933 and 1945 (Lennartz, 1973). Since its initial introduction near Mobile, Alabama, *S. invicta* has spread to infest 128 million ha from Virginia, south to Florida, and west to California (Williams et al., 2001) causing damage in excess of 1 billion dollars annually in the U.S. (Thompson et al., 1995). Evidence indicates that during the U.S. founding, most *S. invicta* natural enemies were left behind in its native range (Porter et al., 1997). Indeed, nearly all of the approximately 30 fire ant natural enemies identified in South America are absent from U.S. populations (Jouvenaz et al., 1977, 1981; Jouvenaz, 1983; Wojcik et al., 1987; Porter et al., 1997; Williams et al., 2003). Therefore, discovery and utilization of natural enemies is widely considered the most sustainable method of controlling *S. invicta* (Williams et al., 2001).

Viruses can be important biological control agents against insect populations (Lacey et al., 2001). Methods employed for discovery of fire ant viruses and other pathogens have been limited to examination of large numbers of randomly-collected colonies by microscopy (Jouvenaz et al., 1977, 1981; Jouvenaz, 1983; Wojcik et al., 1987). However, Valles et al. (2004) employed a molecular-based approach with the intention of virus discovery through homologous gene identification. Among 2304 expressed sequence tags (ESTs) from *S. invicta*, they identified 6 sequences that exhibited significant homology with viral genes. Three of these sequences were found to be of viral origin and led to the discovery of the first virus known to

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infect *S. invicta*, *Solenopsis invicta* virus 1 (SINV-1), a positive-strand, RNA virus with a monopartite, dicistronic genome (Valles and Strong, 2005; Valles et al., 2004).

When the fire ant ESTs were re-examined with lower stringency, an additional sequence (EST c246/accession number EH413675) exhibited homology (*E*-score = 5.9) with the polyprotein of the sacbrood virus (Valles et al., 2004). Further preliminary examination revealed that this sequence did indeed correspond to a positive-strand RNA virus that was infecting *S. invicta*. We report the entire genome sequence and organization, and provide the initial characterization of this new virus, provisionally named *Solenopsis invicta* virus 2 (SINV-2).

Results

Genome and SINV-2 characterization

The SINV-2 genome was constructed by compiling sequences from 15 successive 5′ RACE reactions, one 3′ RACE reaction, and EST c246 (accession number EH413675) from the fire ant expression library (Fig. 1A). The SINV-2 genome consensus sequence (EF428566) was 11,303 nucleotides in length, excluding the poly(A) tail present at the 3′ end. The genome sequence was A/U rich (27.9% A, 28.9% U, 20.1% C, 23.1% G). Analysis of the genome revealed 4 major ORFs (comprised of ≥100 codons) and 5 minor ORFs (comprised of 50–99 codons) in the sense orientation (Fig. 1B). Untranslated regions were present on the 5′ (nts 1–301) and 3′ ends (nts 10,917–11,303) of the genome. Intergenic regions were also indicated between ORFs 1 and 2 (nts 1079–1828), and ORFs 3 and 4 (nts 3793–4454). ORFs 2 and 3 overlap a stop and start codon, respectively, and are not, therefore, interrupted by an intergenic region. Among the major ORFs, ORF 3 was in the first reading frame, ORFs 1 and 2 were in the second reading frame, and ORF 4 was in the third reading frame. ORF 1 was followed directly by 2 additional minor ORFs. ORFs 1 through 4 encoded predicted proteins of 29,413; 31,160; 43,224; and 246,845 Da.

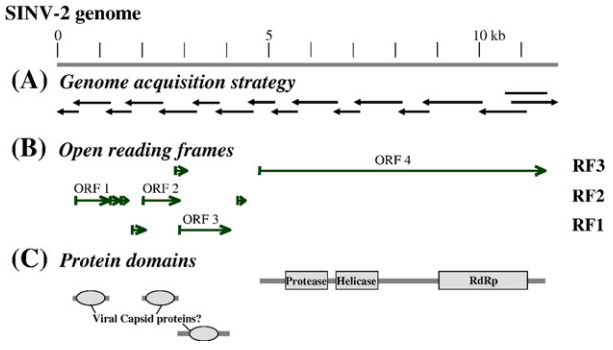

Fig. 1. Schematic diagram of the SINV-2 genome is indicated at the top (5′→3′ orientation). (A) Representation of the genome cloning strategy including expressed sequenced tags 18F8 and 1G9 (contig 246, accession number EH413675) indicated by a line without arrowheads, 3′ RACE reaction (line with arrow to the right), and 15 successive 5′ RACE reactions (lines with arrows to the left). (B) Open reading frames in the sense direction. Start and stop codons are represented by a vertical line and arrow, respectively. All three reading frames (RF1–3) are presented. Only ORFs comprised of at least 50 codons are presented. (C) Conserved protein domains illustrated in ORFs 1–4.

RT–PCR amplification with overlapping oligonucleotide primers and sequences generated from the 5′ RACE reactions provided at least 5-fold coverage of every region of the genome. Additional sequencing coverage of regions containing stop codons of ORFs 1–4 was conducted to validate these regions. Indeed, these regions of ORFs 1–4 were sequenced 10-, 12-, 13-, and 7-fold, respectively. No large ORFs were found in the inverse orientation suggesting that the SINV-2 genome was positive sense. Correspondingly, Fig. 2A shows that RNase A treatment of nucleic acid preparations from SINV-2-infected *S. invicta* failed to yield an amplicon by RT–PCR analysis. PCR also failed to yield an amplicon with oligonucleotide primers specific for SINV-2 indicating absence of a DNA stage or host genome integration. The *S. invicta* transferrin (*SiTF*) gene, an internal control, produced an amplicon by PCR in both RNase A-treated and -untreated samples.

Electron microscopic examination of negatively stained samples from SINV-2-infected fire ants revealed particles that were consistent with *Picornaviridae*, *Dicistroviridae* and related positive-strand RNA viruses (Fig. 2B). Isometric particles with a diameter of 33 nm were observed exclusively in preparations from SINV-2-infected fire ants; no corresponding particles were observed in samples prepared from uninfected fire ants.

Blastp analysis (Altschul et al., 1997) of SINV-2 ORF 4 revealed significant (*E*-score ≤ 10^{-5}) homology with RdRp and helicase conserved domains from positive-strand RNA viruses (Figs. 1C and 3). No significant homology was indicated after Blastp analysis of ORFs 1, 2 and 3 of the SINV-2 genome. However, capsid proteins of positive-strand RNA viruses were indicated (*E*-score > 1).

The SINV-2 polyprotein encoded by ORF 4 was aligned with non-structural regions of the cricket paralysis virus (CrPV), *Plautia stali* intestine virus (PSIV), SINV-1, infectious flacherie virus (IFV), and sacbrood virus (SbV) using the Vector NTI alignment software with ClustalW algorithm (InforMax, Inc., Bethesda, MD). The alignments revealed sequence motifs for an RdRp, helicase, and protease, characteristic of *Picornaviridae*, *Dicistroviridae* and related positive-strand RNA viruses (Koonin and Dolja, 1993) (Fig. 3). Amino acid positions 694 to 805 exhibited similarity to helicase. The consensus sequence for the RNA helicase, Gx_4GK (Gorbalenya et al., 1990), thought to be responsible for nucleotide binding, was found in the polyprotein of the SINV-2 ORF 4 at amino acids 706 to 712 (Fig. 3A, motif Hel A). ORF 4 also encoded sequence with similarity to RdRp (amino acids 1690 to 2100; Fig. 3B). All positive-strand RNA viruses encode the RdRp (Koonin and Dolja, 1993) and comparative analysis revealed that they possess 8 common sequence motifs (Koonin, 1991). These core RdRp motifs were shown by site-directed mutagenesis to be crucial to the activity of the enzyme (Sankar and Porter, 1992). All eight of these motifs were present in SINV-2 ORF 4 (Fig. 3B). Furthermore, the 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,

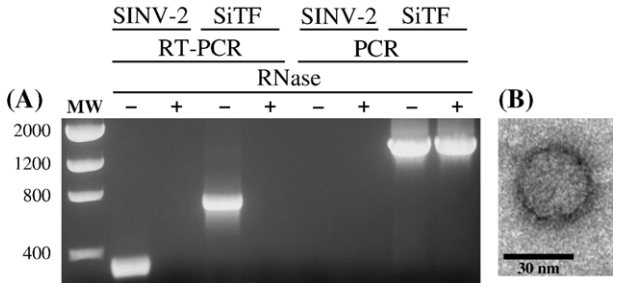

Fig. 2. Characterization of SINV-2. (A) Sensitivity of SINV-2 RNA to single-strand-specific RNase A. Control (−) or RNase A-digested (+) nucleic acids prepared from SINV-2-infected *S. invicta* worker ants. RT–PCR and PCR were conducted with SINV-2-specific (p64/p65) or *S. invicta* transferrin-specific (p297/p316) oligonucleotide primers. (B) Electron micrograph of a virus-like particle purified from SINV-2-infected *S. invicta* workers.

1993). SINV-2 ORF 4 possessed all 6 of these characteristic residues, D^{1893}, D^{1898} (motif IV), G^{1957}, T^{1961} (motif V), and D^{2003}, D^{2004} (motif VI). Thus, these data strongly support the conclusion that SINV-2 possesses an RdRp typically found in positive-strand RNA viruses.

Phylogenetic analysis

Phylogenetic analysis of the RdRp domain placed SINV-2, IFV, and the Nora virus each standing alone, separated from clusters comprised of dicistroviruses, iflaviruses, picornaviruses, and plant small RNA viruses (Fig. 4A). Bootstrap values between the major clusters and SINV-2 were relatively low, suggesting an uncertain common ancestor. A similar positioning of SINV-2 was observed when the conserved region of the helicase (domains A, B, and C) was analyzed (data not shown). When phylogenetic analysis was conducted using a conserved region identified from multiple alignments of SINV-2 ORF 1 and putative structural protein sequences of completely sequenced dicistroviruses and iflaviruses, both virus groups clustered independently, leaving SINV-2 isolated within the tree (Fig. 4B). Bootstrap values at nodes separating SINV-2 and neighboring clusters were greater than 70%. This independent placement of SINV-2 corresponds well with phylogenetic results from the RdRp and helicase which indicates a very early divergence of SINV-2 among the positive-strand RNA viruses.

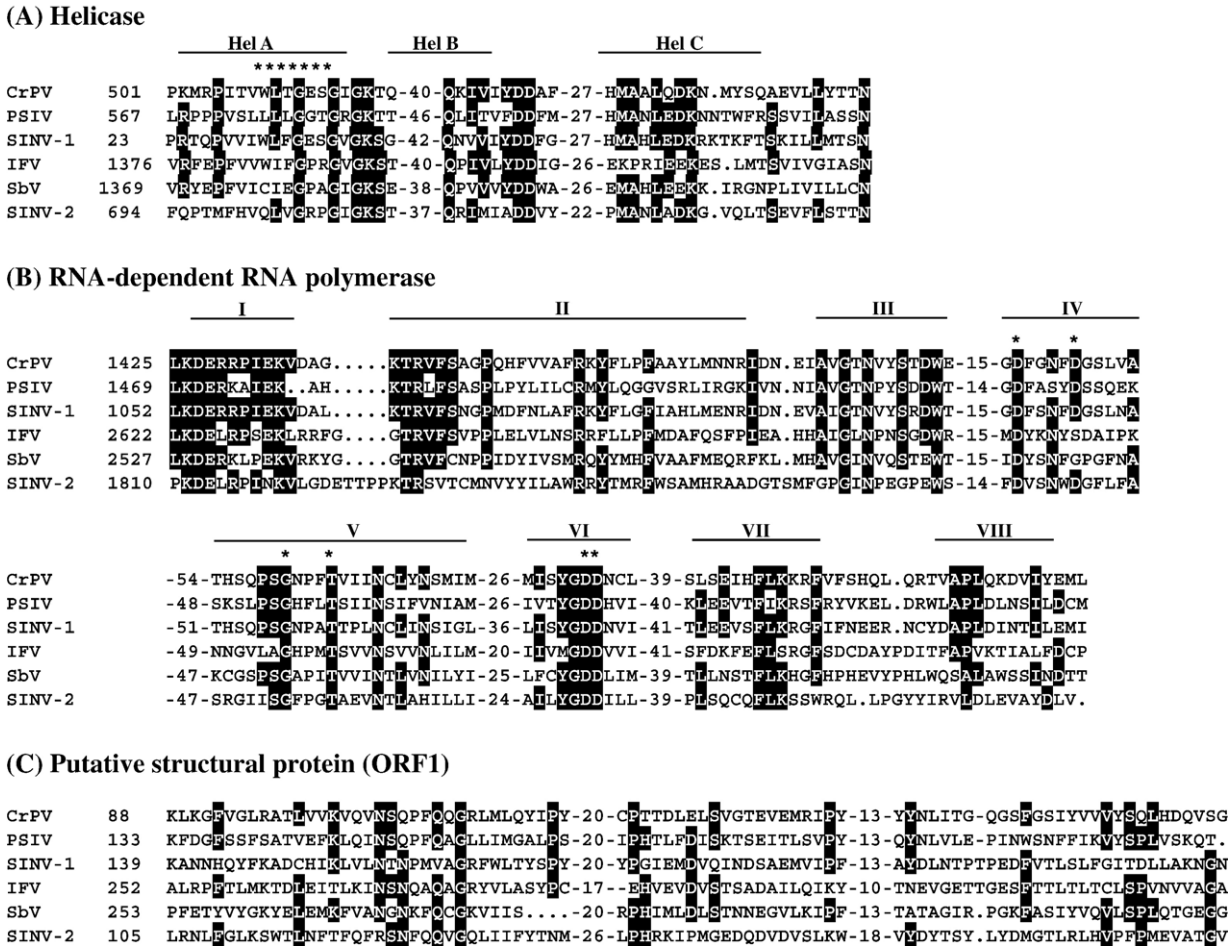

Fig. 3. Alignments of conserved regions of the putative helicase (A), RdRp (B), and ORF 1 (C) of SINV-2 with corresponding sequences from the CrPV, PSIV, SINV-1, IFV, and SbV. The numbers on the left indicate the starting amino acids of aligned sequences. Identical residues in at least four of the six virus sequences are shown in reverse. Sequence motifs shown for the helicase (hel A, hel B, and hel C) and RdRp (I–VIII) correspond to those identified and reviewed by Koonin and Dolja (1993). Asterisks indicate residues thought to be crucial to the activity of the protein.

Discussion

SINV-2 represents the second virus discovered that infects the red imported fire ant, *S. invicta*. SINV-2 appears to be a unique virus with a genome that differs considerably from currently described positive-strand RNA viruses. Isometric particles with a diameter of about 33 nm were found only in ants testing positive for SINV-2 by RT–PCR (Fig. 2B). This particle size is consistent with other positive-strand RNA viruses (Chen and Siede, in press). Colonies infected with SINV-2 did not exhibit any discernable symptoms in the field or consistently when reared in the laboratory. However, some infected colonies exhibited brood die-off during laboratory rearing. Whether SINV-2 was responsible for this pathology is undetermined. However, these characteristics are consistent with other insect-infecting positive-strand RNA viruses, including SINV-1 (Valles et al., 2004). They often persist as unapparent, asymptomatic infections that, under certain conditions, may induce replication within the host resulting in observable symptoms and often death (Christian and Scotti, 1998; Oldstone, 2006).

The SINV-2 genome structure was monopartite, polycistronic and RNA-based (Figs. 1B and 2A). It appears as though the non-structural proteins are found in the 3′-proximal ORF 4 and, ostensibly, structural, or capsid, proteins are encoded by ORFs 1, 2 and 3. This structure is in direct contrast to SINV-1 which exhibits a dicistronic genome with non-structural proteins encoded by the 5′ proximal ORF. Regions of the polyprotein encoded by SINV-2 ORF 4 exhibited significant homology to the RdRp and helicase of positive-strand RNA viruses (e.g. *Dicistroviridae*, iflaviruses, and *Picornaviridae*). However, only a partial domain for a protease/peptidase was recognized near the amino end of the ORF 4 polyprotein (amino acid residues 330 to 410). In addition, amino acids thought to form the catalytic triad of the protease (H, E, C) and the consensus GxCG sequence motif of positive-strand RNA viruses were absent in this region and across the entire ORF (Koonin and Dolja, 1993; Ryan and Flint, 1997). Nor was this sequence motif found in ORFs 1, 2, or 3. This poorly conserved protease domain characteristic was also reported for the Nora virus (Habayeb et al., 2006). Thus, SINV-2 shares a similarly

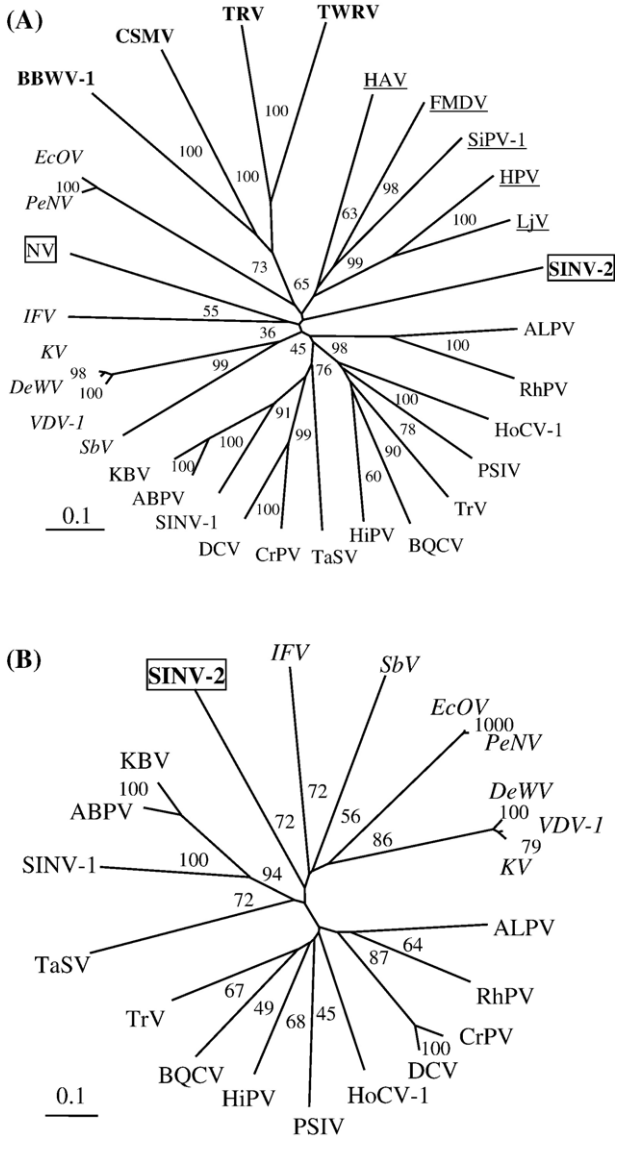

Fig. 4. Phylogenetic analysis of conserved amino acid sequences containing domains I to VIII of the putative RdRp from thirteen dicistroviruses, seven iflaviruses (italic), four plant RNA viruses (bold), five picornaviruses (underlined), Nora virus (boxed), and SINV-2 (bold and boxed) (A) and a domain of a putative structural protein from thirteen dicistroviruses, seven iflaviruses (italic), and SINV-2 (bold and boxed) (B). Abbreviation of virus, accession number of the virus RNA or protein sequences, and amino acid residue numbers of aligned sequences in a specific ORF (5′ proximal and 3′ proximal ORFs of dicitroviruses are used as non-structural and structural protein encoding sequences, respectively; otherwise an ORF number is specified) used to construct phylogenetic trees are: (A) Aphid lethal paralysis virus (ALPV) [AF536531], 1661–1955; Black queen cell virus (BQCV) [AF183905], 1313–1585; Cricket paralysis virus (CrPV) [AF218039], 1421–1699; *Drosophila* C virus (DCV) [AF014388], 1415–1693; Himetobi P virus (HiPV) [AB017037], 1441–1710; *Plautia stali* intestine virus (PSIV) [AB006531], 1465–1739; *Rhopalosiphum padi* virus (RhPV) [AF022937], 1625–1916; Triatoma virus (TrV) [AF178440], 1408–1682; Acute bee paralysis virus (ABPV) [AF150629], 1562–1837; Homalodisca coagulata virus-1 (HoCV-1) [DQ288865], 1446–1716; Kashmir bee virus (KBV) [AY275710], 1594–1864; *Solenopsis invicta* virus 1 (SINV-1) [AY634314], 1048–1327; Taura syndrome virus (TaSV) [AF277675], 1770–2036; Infectious flacherie virus (IFV) [AB000906], 2618–2888; *Perina nuda* virus (PeNV) [AF323747], 2628–2899; Sacbrood virus (SbV) [AF092924], 2518–2789; Deformed wing virus (DeWV) [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 O (FMDV) [AF308157], 2011–2264; Hepatitis A virus (HAV) [M14707], 1900–2159; Human parechovirus (HPV) [AJ005695], 1871–2117; Ljungan virus (LjV) [AF327920], 1945–2191; Simian picornavirus 1 (SiPV-1) [AY064708], 2119–2368; Broad bean wilt virus-1 (BVWV-1) [NP_951030] RNA1, 405–657; Cowpea severe mosaic virus (CSMV) RNA1 [M83830], 1553–1816; Tomato ringspot virus (TRV) [ABG23688] RNA1, 406–672; Tomato white ringspot virus (TWRV) [ABM65095], 1601–1872; Nora virus (NV) [DQ321720] ORF2, 1763–2026; SINV-2 ORF4 [EF428566], 1810–2079: (B) ALPV, 89–228; BQCV, 72–200; CrPV, 88–220; DCV, 67–199; HiPV, 8–138; PSIV, 133–263; RhPV, 5–141; TrV, 115–246; ABPV, 130–264; HoCV-1, 131–261; KBV, 86–220; SINV-1, 139–272; TaSV, 122–253; IFV, 252–388; PeNV, 416–553; SbV, 253–391; DeWV, 318–456; EcOV, 417–554; KV, 318–456; VDV-1, 318–456; SINV-2 ORF1, 105–258.

unique genomic structure as the recently identified Nora virus that persistently infects *Drosophila melanogaster* (Habayeb et al., 2006). The Nora virus genome was also monopartite and polycistronic, possessing 4 major ORFs. However, the RdRp and helicase were found in the second 5′-proximal ORF.

In contrast to *Dicistroviridae* (monopartite/dicistronic), iflaviruses, and *Picornaviridae* (monopartite/monocistronic), the SINV-2 genome appears to be unique with a monopartite poly (tetra)-cistronic genome organization. Analysis of the genome revealed 4 major ORFs (comprised of ≥100 codons) and 5 minor ORFs (comprised of 50–99 codons) in the sense orientation. The largest major ORF (# 4) contained domains consistent with the RdRp, helicase, and protease/peptidase of positive-strand RNA viruses. The remaining major ORFs (1, 2 and 3) putatively encode structural or capsid proteins. ORFs 2 and 3 overlapped a stop and start codon, which is unprecedented among arthropod-infecting positive-strand RNA viruses. However, the vertebrate-infecting *Caliciviridae* (monopartite, polycistronic) do exhibit overlapping ORFs within the polyprotein (Clarke and Lambden, 2000). Indeed, we were so concerned about the validity of the polycistronic nature of the genome, we increased the sequencing coverage of the stop codon regions of ORFs 1–4 to 10-, 12-, 13-, and 7-fold, respectively. Despite the use of multiple sources of SINV-2 from the field, these regions remained well conserved. The unique nature of the virus was corroborated by phylogenetic analysis of the RdRp, helicase and ORF 1 (Fig. 4). These analyses indicated a very early divergence of SINV-2 among the positive-strand RNA viruses and placed it exclusive from the *Dicistroviridae*, iflaviruses, *Picornaviridae*, and plant small RNA viruses. The SINV-2 genome organization is similar to that of the Nora virus which also encodes 4 major ORFs in a monopartite genome (Habayeb et al., 2006). However, the positional organization of the ORFs is different between the Nora virus and SINV-2. Because the SINV-2 genome is so unique, additional molecular characterization will be required to place this virus taxonomically.

Materials and methods

Virus detection, purification, and electron microscopy

One-step reverse transcriptase polymerase chain reaction (RT–PCR) was used to identify SINV-2-infected *S. invicta* ants. A 20-ml scintillation vial was plunged into a fire ant nest in the field for several minutes to collect a sample of worker ants. RNA was extracted from 10 to 20 ants using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized and subsequently amplified using the One-Step RT–PCR kit (Invitrogen, Carlsbad, CA) with oligonucleotide primers p64 and p65 (Table 1). RT–PCR was conducted in a PTC 100 thermal cycler (MJ Research, Waltham, MA) 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, 56 °C for 15 s, 68 °C for 30 s, followed by a final elongation step of 68 °C for 5 min. Samples were considered positive for the virus when a visible amplicon (319 nucleotides) was present after separation on an agarose gel (1.2%) stained with ethidium bromide.

SINV-2 was purified for electron microscopy by the method described by Ghosh et al. (1999). Briefly, 200 worker ants were homogenized in 5 ml of NT buffer (Tris–HCl, pH 7.4, 10 mM NaCl) using a Potter–Elvehjem Teflon pestle and glass mortar. The mixture was clarified by centrifugation at 1000×*g* for 10 min in an L8-70M ultracentrifuge (Beckman, Palo Alto, CA). The supernatant was extracted with an equal volume of 1,1,2-trichlorotrifluoroethane before the aqueous phase was layered onto a discontinuous CsCl gradient (1.2 g/ml and 1.5 g/ml) which was centrifuged at 270,000×*g* for 1 h in an SW60 rotor. A whitish band at the interface was removed by suction and desalted. The sample was submitted to the electron microscopy core at the University of Florida, Interdisciplinary Center for Biotechnology Research for examination. It was negatively stained with 2% phosphotungstic acid, pH 7.0, and examined with a Hitachi H-7000 transmission electron microscope (Hitachi, Pleasanton, CA) on a formvar film-coated grid. Uninfected workers ants were prepared and examined in the same manner and served as controls.

Rapid amplification of cDNA ends, cloning, genome sequencing, characterization

A contiguous fragment (634 nts) was assembled from ESTs 18F8 and 1G9 (contig c246, accession number EH413675) and used as the primary sequence from which oligonucleotide primers were designed and RACE (3′ and 5′) reactions were conducted (Fig. 1A). From this fragment, a series of 5′ RACE reactions were conducted to obtain the upstream sequence of the SINV-2 genome using the 5′ RACE system (Invitrogen, Carlsbad, CA) and primer walking. Briefly, cDNA was synthesized with a gene-specific oligonucleotide primer (GSP) from total RNA, 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, upstream GSP and an abridged anchor primer (AAP).

Fifteen successive 5′ RACE reactions were conducted to obtain the entire SINV-2 genome. Anticipating the potential need to remove the VPg often covalently attached to the 5′ end of insect picorna-like viruses (Christian and Scotti, 1998), 50 μg of total RNA prepared from SINV-2-infected ants was digested with proteinase K (600 μg/ml) for 1 h at 37 °C. The digested RNA was purified by acidic phenol/chloroform/isoamyl alcohol extraction. cDNA synthesis was conducted for 50 min at 45 °C with 2.5 μg of total RNA and a GSP for the 15 reactions (Fig. 1A and Table 1). After cDNA synthesis, PCR was conducted with AAP and a nested GSP (Table 1). Gel-purified amplicons were ligated into the pCR4-TOPO vector, transformed into TOP10 competent cells (Invitrogen, Carlsbad, CA) and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida).

A single 3′ RACE reaction was conducted with the GeneRacer kit (Invitrogen). cDNA was synthesized from total RNA (1 μg) purified from SINV-2-infected workers using the GeneRacer Oligo dT primer. The cDNA was amplified by PCR with oligonucleotide primer p64 and the GeneRacer 3′ primer.

Table 1
Oligonucleotide primers used throughout the study

Oligonucleotide designation	Reaction	Oligonucleotide (5′→3′)	Genome position	Orientation
p64	3′ RACE/Ant infection	ATTTGTTTTGGCCACGGTCAACA	10758–10780	→
p65	Ant infection	GATGATACAAAGCATTAGCGTAGGTAAACG	11047–11076	←
p297	Transferrin	CCCAATCATTCCATTTGTCCCATGTGTTGTC	NA	←
p316	Transferrin	CTGTTATCAAGAGTGGCTCCTCCCTAAATGGATTG	NA	→
p479	Genome	CTTCGGAAAATTTCGTTAAAATCTGATTAACGGTGAGCT	10798–10836	←
p480	5′ RACE/Coverage	AGCGTAGGTAAACGCATTGCCCAACCGG	11033–11060	←
p482	cDNA synthesis	TGCGGAGGTTCTACGTCAA	11104–11122	←
p483	cDNA synthesis	TAGATCGGTGCGACATAGGTGTC	11158–11180	←
p500	5′ RACE/Coverage	GAAAGGGAATCTTCGAACTTGTAGTACCCTTGAAG	9810–9844	←
p502	cDNA synthesis	GTCTTTGGTGGTGTGATTTCATCA	9920–9943	←
p504	Coverage	CAGGGACTATATGGAGATTGATGAATTTGGTGAAG	9752–9786	→
p511	QPCR	CGGAGACACTGAGCCTTTCTGGACTCCATAG	8677–8707	←
p514	QPCR (RT)	TACACTTGGGTCTCAGGAACC	8816–8836	←
p515	Coverage/QPCR	TGTATCGCGGAAATTACCCAACATCACAAC	8584–8613	→
p525	5′ RACE	TTCCGCACTGCTGGGAATAGTCGCG	8872–8896	←
p527	cDNA synthesis	TTGAGGTCAAGGCGATCAAC	9018–9037	←
p541	Coverage	CACTTGTGCAATAACAGGTGATCCACTTCTTCC	9099–9131	←
p542	cDNA synthesis	GAGAACAGTGATGCAATTGATTT	8514–8536	←
p545	5′ RACE	TCAGTCCATTTGAGTAGACCTTTGCAACACATG	8158–8190	←
p546	cDNA synthesis	CTGCGGCAAATTCTCT	7217–7232	←
p548	5′ RACE/Coverage	TGCATACTCGTTGTAAACAATCTGCTCATCT	7112–7142	←
p549	Coverage	ACGGTCTCCGCAGCTCCTCCAAACACT	7073–7099	←
p551	cDNA synthesis	TAAAGTAGACTTACCAATTCCTG	6574–6596	←
p554	5′ RACE	TACATCTGACGATATTCAGGATTGTCACGGCA	6426–6457	←
p555	Coverage	TGCCGTGACAATCCTGAATATCGTCAGATGTA	6426–6457	→
p560	5′ RACE/Coverage	GACTTGTCCAATGTCCTTGGACTTCATAACCAGCT	5656–5690	←
p563	cDNA synthesis	TTCCTGAGAAACCTTCCAT	5787–5805	←
p573	5′ RACE/Coverage	AAAGGATTCCCTCAGATGTCAGGTTGGAA	5027–5055	←
p574	cDNA synthesis	TCCAGCGACTCCTCGAATGATAAGATGTAGAC	5062–5093	←
p579	Coverage	TGGAAGTTTCAACTCCGCGACGAAATTA	4494–4521	→
p580	5′ RACE	TAATTTCGTCGCGGAGTTGAAACTTCCA	4494–4521	←
p588	5′ RACE	CTTTCGCACTGCACAATATTCCGAGTGC	4029–4056	←
p599	5′ RACE/Coverage	GATGCTTTATATGAGTGATCCTGACGCTGGATTGC	3232–3266	←
p602	5′ RACE	ACTGAGTCACCAGAGAAGTCATCGTGTGGGTC	2261–2292	←
p610	5′ RACE	GCTCTGCTGTTCACGAATAAGATCTGCATTAAGACC	1603–1638	←
p615	5′ RACE	ACTGCTACTGCCAGGGACTCAATTTGCG	1167–1194	←
p617	Genome/Coverage	TTGGTACAGTGGGAACTTTGCTTCTTCTGGGAT	1113–1145	→
p619	Coverage	GGTGCCATGCAATACCGCATTCGTGTT	3109–3125	→
p621	Coverage	GAATGGAGACAGGAGCAGTACACACAAGATCAACTG	5118–5153	→
p623	Coverage	GAGAAGATGAGCAGATTGTTTACAACGAGTATGCAAA	7108–7144	→
p632	5′ RACE	CACTCCGGTTTTATCCACAGTGGTCGGGTCT	415–445	←

Amplicons were cloned and sequenced as described for 5′ RACE.

Experiments were conducted to confirm the RNA characteristics of the SINV-2 genome. Nucleic acids purified from SINV-2-positive ants were divided into two aliquots of 2 μg each. One aliquot was treated with 33 μg (1 μl) of RNase A (Sigma, St. Louis, MO) at 37 °C for 1 h in Tris–HCl, pH 8.0. RNase-treated and -untreated samples were then evaluated by PCR and RT–PCR using oligonucleotide primers specific to SINV-2 (p64 and p65) and to the *S. invicta* transferrin gene (p297 and p316) as an internal control (Valles and Pereira, 2005).

Multiple alignment and phylogenetic analysis of SINV-2 RdRp, helicase, and putative structural protein

Blastp analysis was conducted with conserved regions of SINV-2 RdRp, helicase and the N-terminal region of a putative structural protein (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 the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html) and used for comparative ORF analysis and multiple-alignment with SINV-2 using Vector NTI Advance software (Version 10.1.1, Invitrogen). Multiple alignments were carried out for the deduced amino acid sequences of the non-structural and structural polyproteins of viral genomes. Specifically, conserved regions of the RdRp (domains I to VIII), helicase (domains A, B, and C) and those corresponding to SINV-2 ORF 1 (a putative structural protein) 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).

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