

A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization

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Received 28 May 2004; returned to author for revision 1 July 2004; accepted 9 July 2004

Available online 21 August 2004

Abstract

We report the first discovery and genome sequence of a virus infecting the red imported fire ant, *Solenopsis invicta*. The 8026 nucleotide, polyadenylated, RNA genome encoded two large open reading frames (ORF1 and ORF2), flanked and separated by 27, 223, and 171 nucleotide untranslated regions, respectively. The predicted amino acid sequence of the 5'proximal ORF1 (nucleotides 28 to 4218) exhibited significant identity and possessed consensus sequences characteristic of the helicase, cysteine protease, and RNA-dependent RNA polymerase sequence motifs from picornaviruses, picorna-like viruses, comoviruses, caliciviruses, and sequiviruses. The predicted amino acid sequence of the 3'proximal ORF2 (nucleotides 4390–7803) showed similarity to structural proteins in picorna-like viruses, especially the acute bee paralysis virus. Electron microscopic examination of negatively stained samples from virus-infected fire ants revealed isometric particles with a diameter of 31 nm, consistent with *Picornaviridae*. A survey for the fire ant virus from areas around Florida revealed a pattern of fairly widespread distribution. Among 168 nests surveyed, 22.9% were infected. The virus was found to infect all fire ant caste members and developmental stages, including eggs, early (1st–2nd) and late (3rd–4th) instars, worker pupae, workers, sexual pupae, alates (♂ and ♀), and queens. The virus, tentatively named *S. invicta* virus (SINV-1), appears to belong to the picorna-like viruses. We did not observe any perceptible symptoms among infected nests in the field. However, in every case where an SINV-1-infected colony was excavated from the field with an inseminated queen and held in the laboratory, all of the brood in these colonies died within 3 months. Published by Elsevier Inc.

Keywords: *Solenopsis invicta*; Picorna-like virus; RNA virus; Genome sequence

Introduction

The red imported fire ant, *Solenopsis invicta* (Buren), was introduced into the United States in the early 1900s and currently infests more than 129 million hectares from North Carolina to California (Anonymous, 2001). Comparative surveys between fire ant populations in the United States (introduced range) and Brazil (native range) provide strong

evidence that *S. invicta* has escaped natural enemies; fire ant populations are significantly greater (5-fold), found in higher densities (5.7-fold), possess larger mound volumes (2-fold), and comprise a larger fraction of the ant community (7.5-fold) in the United States (Porter et al., 1992, 1997). This conclusion is further supported by the paucity of natural enemies found in North American fire ants. Currently, two species of endoparasitic fungus (Jouvenaz and Kimbrough, 1991; Pereira, 2004), a microsporidian obligate parasite (Knell et al., 1977; Williams et al., 1998), a neogregarine parasite (Pereira et al., 2002), a strepsipteran parasite (Kathirithamby and Johnston, 2001), and phorid flies in the genus *Pseudacteon* (Porter, 1998), which were intentionally

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introduced, comprise the known self-sustaining, biological control agents in North American *S. invicta*. Discovery and exploitation of additional biological control agents, from either South or North American populations, could aid the control and suppression of fire ants (Williams et al., 2003).

Although viruses can be important biological control agents against insect populations (Lacey et al., 2001), none have been shown to infect *S. invicta*. Indeed, the only report present in the literature concerned with virus infections of fire ants was the observation of “virus-like particles” in a *Solenopsis* species from Brazil (Avery et al., 1977). Recently, while sequencing an expression library from a monogynous *S. invicta* colony, we discovered several genes exhibiting significant homology to genes from single-stranded RNA viruses like the *Picornaviridae*. A number of picorna-like viruses have been shown to infect insects, including hymenoptera (Bailey, 1969; Bailey and Gibbs, 1964), and the *Picornaviridae* are considered one of the largest and most important groups of human and agricultural pathogens (Rueckert, 1991). We cloned and sequenced what we believe is the entire genome of a picorna-like virus from the red imported fire ant, and provide a description of the genome organization and an initial characterization of the virus.

Results

Genome

The genome of SINV-1 was constructed by compiling sequences from a series of six successive 5' RACE reactions, one 3' RACE reaction, and the sequences of three cDNA clones from a fire ant expression library (Fig. 1). The SINV-1 genome was found to be 8026-nucleotides long, excluding the poly(A) tail present on the 3' end (accession number AY634314). This genome size was consistent with the largest species (8.4 kb) produced by Northern analysis of RNA extracted from SINV-1-infected fire ants (data not shown). No hybridization was observed in RNA extracted from uninfected fire ants.

Typical of *Picornaviridae*, the genome sequence was A/U rich (32.9% A, 28.2% U, 18.3% C, and 20.5% G). Analysis of the genome revealed two large open reading frames (ORFs) in the sense orientation (within frame) with an untranslated region (UTR) at each end and between the two ORFs. The 5' proximal ORF (ORF1) commenced at the first start AUG codon present at nucleotide position 28 and ended at a UAA stop codon at nucleotide 4218, which encoded a predicted product of 160,327 Da. The 3' proximal ORF (ORF2), commenced at nucleotide position 4390 (AUG start codon), terminated at nucleotide position 7803 (UAA stop codon) and encoded a predicted product of 127,683 Da. No large ORFs were found in the inverse orientation, suggesting that the SINV-1 genome was a positive-strand RNA virus. The 5', 3', and intergenic UTRs were comprised of 27, 223, and 171 nucleotides, respectively. BLAST analysis (Altschul et al., 1997) of ORFs 1 and 2 revealed identity to nonstructural and structural proteins, respectively, from picorna-like viruses. ORF1 of the SINV-1 genome was found to exhibit the characteristic helicase, protease, and RNA-dependent RNA polymerase (RdRp) sequence motifs ascribed to *Picornaviridae* (Fig. 2; Koonin and Dolja, 1993). Although ORF2 exhibited homology to structural proteins in the *Picornaviridae*, the sequence identity was less well conserved as in the nonstructural proteins of ORF1.

Electron microscopic examination of negatively stained samples from SINV-1-infected fire ants revealed particles that were consistent with *Picornaviridae* (Fig. 3). Isometric particles with a diameter of 31 nm were observed exclusively in preparations from SINV-1-infected fire ants; no corresponding particles were observed in samples prepared from uninfected fire ants.

Field surveys, castes infected, transmission, and duration of infection

A PCR analytic survey for the SINV-1 from extracts of *S. invicta* collected around Florida revealed a pattern of fairly widespread distribution (Table 1). Among 168 nests surveyed, infection rates among different sites ranged from

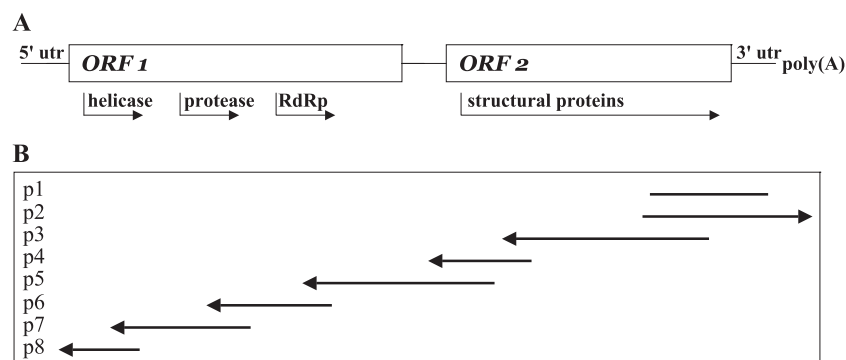


Fig. 1. (A) Schematic diagram of the SINV-1 genome. ORFs are shown in open boxes. Arrows represent approximate positions of nonstructural and structural proteins in ORF1 and 2, respectively. (B) Representation of the cloning strategy for the SINV-1 genome. Each line represents a cDNA fragment of the SINV-1 genome. The horizontal axis approximates corresponding positions in the genome diagram above. p1, contiguous fragment obtained from the fire ant expression library; p2, 3'RACE; p3–p8, successive 5'RACE reactions.

A. Helicase

		Hel A	Hel B	Hel C
SINV-1	23	PRTPQPVVITLWFGESGVGKSG-42	QNVVVIYDDFG-27	HMAHLEDDKRRKTKFTSKILLMSTN
ABPV	53	PRTPQPVVITLWFGESGRGKSG-42	QNVVVCXDDFG-27	HMAHLEDDKRRKTKFTSKVIIMSTN
SBV	1369	VRYPFPVITCIEGPAGIGKSE-38	QPVVVYDDWA-26	EMAHLEEEK. IRGNPLIVILLCN
BQCV	441	VRNPVTLVLYGETGVGKST-49	QLVTVFDDFN-27	HMASTEEKANTVFQSKVILCSSN
CPMV	483	VRKMPFTLFFQGKSRTGKSL-35	QPPVLMDDFA-23	NMAGLEEKG. ICFDSQVFVSTN
HAV	1219	TRCEPVVCLVYGKRGGKSL-36	QLVCIIDDIG-22	NMASLEEKGR. HFSPPFIATSN

B. Cysteine protease

SINV-1	663	.LIPAHILGC-34-GESKEA-83-APT	TNGDCGAPLIINEPSVLR. K	IAGIHVA
ABPV	1166	MLAPGHILVGF-33-GESKEA-83-MPT	TNGDCGAPLVINETQVIR. K	IAGIHVA
SBV	2132	ALLPRHYVRA-34-SESTD-79-YS. Q	CCGSLCFLSR. SQRP. .	IVGMHFA
BQCV	904	AVAPGHYLR-46-LDSRDL-85-L	ETISGDCGAPLFTVNSKIGPKIIGHTA	
CPMV	982	FLACKHFFTH-29-IPDSEL-81-APT	IPEDCCSLVIAHIGGKHK. .	IVGVHV. .
HAV	1558	LLVPSHAYKF-31-VGFQDV-79-GE	GLPCCGALVSSNQSIQN. A	LLGTHVA

C. RNA-dependent RNA polymerase

		I	II	III	IV
SINV-1	1052	LKDERRPIEKV. DALKTRVFSNGPMD	FNLAFRKVF LGFTAHLMENRIDNEVA	IGINNVYSRDWT-15-GDFS	NFDGSLNA
ABPV	1566	LKDERRPIEKV. DQKTRVFSNGPMD	FSTIFRMVYLGFTAHLMENRITNEV	SIGINNVYSQDWN-15-GDFS	TFDGSINV
SBV	2522	LKDERKLPKVRKYGGTRVFCNPPID	YIVSMROYMHVAAFMEOQFKLMH	AVGINVOSTEWT-15-IDVSN	FGPGFNA
BQCV	1317	LKDERKPKHKA. . H. KSRMFSNGP	IDLVWSKMYFNPIVAVLSELKNVD	HISVGSNVYSTDWD-15-GDFE	CFDASEQS
CPMV	1357	.KDEKLPMRKVFDPKPKTRCF	TILPMEYNLVVRRKFLNVRVFIM	ANRHRSLCQVGINPYSMEWS-15-	CDVSSFDGLLSK
HAV	1904	PKDELRLPEKVLKES. KTR	AIDACPLDYSILCRMVWGP	PAISYFHLNPGFHTGVAIGID	PPDQWD-15-LDFSAFDASLSP
		V	VI	VII	VIII
SINV-1	1184	THS. QPSCNPATPPLNCLINSIGL-36-	LISYGDDNVI-41-TLEEVSFLKRG	FLFNEER. NCYDAPLDINTILEMI	
ABPV	1700	THS. QPSCNPATPPLNCFINSMGL-30-	IVSYGDDNVI-41-TIEDVQYLRK	KFRYDSKR. KVWEAPLCMDTILEMP	
SBV	2659	KCG. SPSCAPIIVVINLTVNII	LYI-25-LFCYGDLLIM-39-TLLNST	FLLKRGFHPHEVYPHLWQSA	LAWSSINDTT
BQCV	1453	WCKSLPSCHYLTAIINSVFN	NLVM-24-IVAYGDDHV-41-RLEDV	SYLKRNFVYDES. QRYIAPLSLDV	VLEMP
CPMV	1491	ECG. IPSGFPMIVVNSIFNEILI-26-	LVTYGDDNLI-40-RLECD	FLKRTFVQRSS. . TIWDAPEDK	ASLWSQL
HAV	2035	CGS. MPSCSPTALLNSIINNVNL-22-	. . CYGDDVLI-40-PVSELT	FLLKRSENLVED. IRPAISEKTI	WLSLIAW.

D. Capsid protein

SINV-1	704	QLFOYWRATICFKISVVKTG	GFHTGRLETFDFG-27-YKY	LLDLTNDSEITIRVPFTSDR
ABPV	533	NMFSVWRATMCYRIAIVKTA	FHTGRLETFDFG-28-YKY	LLDLTNDSEITIRVPFVSNK
BQCV	425	SMFKYWTGSLVYTFKFKV	KDYHSGRVEISFHPF-12-	YRIIVDLREKSEFSVTIPFTSPV

Fig. 2. Comparisons of predicted amino acid sequences of nonstructural and structural proteins of SINV-1, picorna-like viruses (ABPV, SBV, BQCV), and viruses representative of the *Picornaviridae* (HAV) and *Comoviridae* (CPMV). Alignments are of the conserved regions of the putative helicase (A), cysteine protease (B), RdRp (C), and capsid protein (D). 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 above residues of the protease (B) correspond to the putative catalytic triad, which are considered essential for activity (Koonin and Dolja, 1993; Ryan and Flint, 1997). The last sequence shown (D) represents one of the conserved areas of the putative capsid protein region. The SINV-1 virus sequence exhibited greatest overall identity with acute bee paralysis virus.

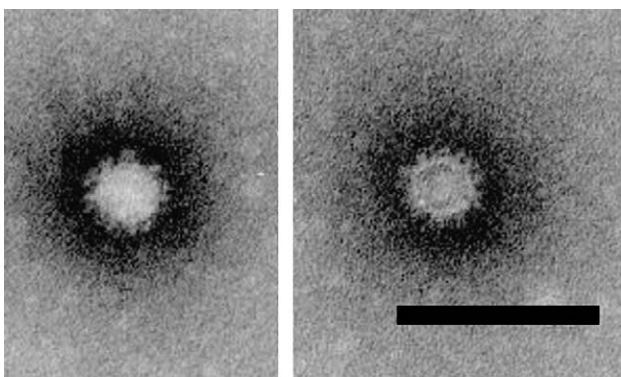


Fig. 3. Electron micrograph of a particle believed to be SINV-1. The preparation was isolated from SINV-1-infected fire ants. Scale bar represents 100 nm.

0% to 87.5% with a mean of 22.9% (SD = 26.3) infected. It appears that SINV-1 infects *S. invicta* year round in Florida because it was found from May to January. Although the rate of infection among individuals within SINV-1-infected nests was not determined, we did find that the infection was present in all caste members and developmental stages, including eggs, early (1st–2nd) and late (3rd–4th) instars, worker pupae, workers, sexual pupae, alates (♀ and ♂), and queens (data not shown).

Following a method described by Ackey and Beck (1972), we successfully infected individuals from uninfected colonies. The infection appeared in uninfected colonies within 3 days of providing uninfected fire ants a food source mixed with a homogenate made from SINV-1-infected worker ants. SINV-1 did not appear to infect every

Table 1
Survey of fire ant nests for the presence of the fire ant virus (SINV-1)

Date	Location (city, state)	Nests surveyed	Nests with SINV-1 (%)
14 May 03	Gainesville, FL	10	20
12 June 03	Gainesville, FL	10	30
21 July 03	Gainesville, FL	16	87.5
18–30 September 03	Gainesville, FL	28	14.3
7 October 03	Newberry, FL	11	9.1
10 October 03	LaCrosse, FL	9	0
16 October 03	McIntosh, FL	9	44
23 December 03	Gainesville, FL	8	75
14 January 04	Fort Pierce, FL	6	0
14 January 04	Orlando, FL	4	0
14 January 04	Okahumpka, FL	4	25
14 January 04	Ocala, FL	4	50
14 January 04	Canoe Creek, FL	4	0
14 January 04	Fort Drum, FL	4	0
22 January 04	Cedar Key, FL	11	27
22 January 04	Otter Creek, FL	10	0
22 January 04	Bronson, FL	9	22
29 January 04	Perry, FL	11	9.1

individual within the recipient colonies; often, several samples had to be evaluated by RT-PCR to detect infection. The infection was detectable for at least 18 days after treatment, indicating sustained infection among recipient colonies. Additionally, the SINV-1 infection was detectable for at least 3 months among colonies excavated from the field and held in the laboratory.

Discussion

Here we report the first discovery of a virus infecting the red imported fire ant, *S. invicta*. While creating expressed sequence tags from a fire ant expression library, eight clones displayed homology to viruses (Table 2). Among the clone sequences with the most significant expectation values, three (3F6, 14D5, and 24C10) shared homology with a capsid protein from the acute bee paralysis virus (ABPV). This discovery provided the impetus for further investigation of this new virus, tentatively named *S. invicta* virus (SINV-1). Furthermore, based on the significant BLAST matches, *S. invicta* appears to harbor several additional RNA viruses that may find utility as control agents for this insect pest.

SINV-1 particles were isometric with a diameter of 31 nm. The monopartite, bicistronic, single-stranded RNA genome was composed of 8026 nucleotides. This genome size was confirmed by Northern analysis in which a band was observed at 8.4 kb. ORFs 1 and 2 were found to be homologous to nonstructural and structural proteins, respectively, of well-characterized picorna-like viruses (Ghosh et al., 1999; Govan et al., 2000; Leat et al., 2000). The genome was elucidated by designing oligonucleotide primers to the 1780 nt contiguous fragment from the fire ant expression library and subsequently conducting 5' RACE. We fully anticipated generating a near full-length cDNA of the genome using a gene-specific primer designed to the expression library fragment. However, six successive 5' RACE reactions, producing ~600- to 2000-nt fragments each, were required to clone the SINV-1 genome upstream of our sequence (Fig. 1). Several possibilities may have accounted for our failure to generate a continuous cDNA of the genome in a single reaction. First, despite heating the RNA sample before reverse transcription, the RNA could have assumed a secondary structure during reverse transcription that acted as a barrier to cDNA synthesis. Some picorna-like viral genomes assume secondary stem loop structures between the two ORFs that are involved in internal translation initiation (Sasaki and Nakashima, 1999; Wilson et al., 2000). Second, the extracted viral RNA may have been degraded by ribonucleases. Naked picornavirus RNA is known to be extremely sensitive (<0.01 µg/ml) to ribonuclease (Rueckert, 1991). And third, the SINV-1 source was infected fire ants; reverse transcription was conducted in the presence of both the viral and fire ant RNA. Typically, RNA is extracted from purified viral preparations to minimize interference from host nucleic acids.

The SINV-1 ORF 1 (amino acid sequence) was aligned with ABPV, sacbrood virus (SBV), black queen cell virus (BQCV), cow pea mosaic virus (CPMV), and hepatitis A virus (HAV) using the Vector NTI alignment software with ClustalW algorithm (InforMax, Inc., Bethesda, MD). Alignment of ORFs encoding nonstructural proteins with SINV-1 ORF1 showed identities ranging from 10% (SBV, CPMV, HAV) to 30% (ABPV). The alignments also revealed sequence motifs for a helicase, protease, and RdRp, characteristic of *Picornaviridae*, *Comoviridae*, *Sequiviridae*, and *Caliciviridae* (Koonin and Dolja, 1993). Amino

Table 2
Expression library clones exhibiting homology to viruses after BLAST analysis

Clone	BLAST match	Accession no.	Score
3B4	Finkel-Biskis-Reilly murine sarcoma virus	NP032016	3×10^{-22}
3F6	Capsid protein, acute bee paralysis virus	AAL05914	1×10^{-17}
11F1	Capsid polyprotein, <i>Drosophila C</i> virus	NP044946	4×10^{-16}
12G12	Noncapsid protein, <i>Urochloa hoja blanca</i> virus	AAB58302	5×10^{-12}
14D5	Capsid protein, acute bee paralysis virus	AAK15543	1×10^{-26}
16A4	Protein P1, <i>Acyrtosiphum pisum</i> virus	NP620557	5×10^{-4}
18F8	Polyprotein, sacbrood virus	NP049374	5.9
24C10	Capsid protein, acute bee paralysis virus	AAL05915	2×10^{-13}

acid positions 23 to 144 exhibited similarity to the helicase. The consensus sequence for the RNA helicase, Gx₄GK (Gorbalenya et al., 1990), ostensibly responsible for nucleotide binding, was found in the predicted ORF1 of SINV-1 at amino acids 34 to 40 (Fig. 2A, motif Hel A). Amino acids 663 to 823 showed similarity to the cysteine protease of picorna-, picorna-like-, sequi-, and comoviruses (Fig. 2B). Amino acids thought to form the catalytic triad of the protease, H⁶⁶⁷, E⁷¹⁰, and C⁸⁰² were present in this region of the SINV-1 (Koonin and Dolja, 1993; Ryan and Flint, 1997). Furthermore, the consensus GxCG sequence motif was present at amino acids 800 to 803. Lastly, ORF1 of SINV-1 contained sequence with similarity to RdRp (amino acids 1052 to 1327; Fig. 2C). According to Koonin and Dolja (1993), all positive-strand RNA viruses encode the RdRp and comparative analysis revealed that they possess eight common sequence motifs (Koonin, 1991). All eight of these motifs were present in SINV-1 (Fig. 2C). Further, sequence motifs IV, V, and VI were reported to be unequivocally conserved throughout this class of viruses, exhibiting six 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). The SINV-1 possessed all six of these characteristic residues, D¹¹³⁰, D¹¹³⁵ (motif IV), G¹¹⁹⁰, T¹¹⁹⁴ (motif V), and D¹²⁴⁸, D¹²⁴⁹ (motif VI). Thus, these data strongly support the conclusion that SINV-1 is a single-stranded positive RNA virus.

SINV-1 was found to infect all of the fire ant castes. The virus was transmissible by simply feeding uninfected ants a homogenate prepared from SINV-1-infected individuals. The virus was present in field populations of *S. invicta* from several locations in Florida. Nests from some areas were devoid of infection, but in some locations infection rates were as high as 88%. We did not observe any perceptible symptoms among infected nests in the field. However, in every case where an SINV-1-infected colony was excavated from the field with an inseminated queen and held in the laboratory ($n = 12$), all of the brood in these colonies died within 3 months. Uninfected colonies excavated as controls did not exhibit this brood die-off. Whether SINV-1 was responsible for this effect is not known. Some of these viruses have been shown to persist as inapparent infections (e.g., in the honeybee; Bailey, 1967) or require the presence of additional, unrelated pathogens to enable propagation (Bailey et al., 1983). The impact of SINV-1 on *S. invicta* and the conditions required for infectivity will have to be examined more closely.

Materials and methods

Virus detection, purification, and electron microscopy

One-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to identify SINV-1-infected *S. invicta*

ants. A 20-ml scintillation vial was plunged into a fire ant mound in the field for several minutes to collect a sample of the worker caste. The ants were returned to the laboratory and RNA was extracted from 20 to 50 workers using TRIZOL reagent according to the manufacturer's directions (Invitrogen, Carlsbad, CA). cDNA was synthesized and subsequently amplified using the One-Step RT-PCR kit (Invitrogen) with oligonucleotide primers p62 and p63 (Table 3). Samples were considered positive for the virus when a visible amplicon (327 nucleotides) was present after separation on a 1.2% agarose gel stained with ethidium bromide. 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, 55 °C for 15 s, 68 °C for 30 s, followed by a final elongation step of 68 °C for 5 min.

SINV-1 was purified for electron microscopy by the method described by Ghosh et al. (1999). Briefly, 0.5 g of a mixture of workers and brood 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 and 1.5 g/ml), which was centrifuged at 270,000 × *g* for 1 h in an SW60 rotor. Two whitish bands visible near the interface were removed by suction and desalted. 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. Uninfected worker ants were prepared and examined in the same manner and served as controls.

cDNA synthesis, cloning, sequencing, and Northern analysis

A portion of the SINV-1 genome was identified from an expression library produced from a monogyne *S. invicta* colony collected in Gainesville, Florida (Fig. 1B, p1). This contiguous 1780-nucleotide fragment exhibited significant identity with the acute bee paralysis virus and was comprised of clones 14D5, 3F6, and 24C10. From this fragment, a series of 5' RACE reactions were conducted to obtain the upstream sequence of the SINV-1 genome using the 5' RACE system (Invitrogen). Briefly, cDNA was synthesized with a gene-specific oligonucleotide primer (GSP) from total RNA, the RNA template was degraded with RNase, 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 second, upstream GSP and an abridged anchor primer (AAP; Table 3).

Table 3

Oligonucleotide primers used throughout the study

Oligonucleotide designation	Oligonucleotide (5'→3')
p62	GGAAGTCATTACGTGGTCGAAAACG
p63	CGTCCTGTATGAAAACCGGTCTTTACCACAGAAATCTTA
p113	GGAAGTCATTACGTGGTCGAAAAC
p134	CCAAGCTGCCCTTCATCTGCACCAGATC
p135	TTCATCTGCACCAGATCTCCAGGGCTC
p136	CAATGATTCAGCAGAAATGGTTATCC
p137	GTCACATCACGTCCGGTGTTCGT
p138	TCTGCCTTAAAGTATTGATG
p139	GTCTCCTGGCAAGGAATACTGTCTGATGGCTGG
p140	GGAAGAGCGACGCGAGGTTGTTCAACATC
p154	CGCATCAACTTTCTCAATGGGTCGCTCA
p157	CAGTGATACTAGCAATCTGAATA
p162	CTATCTAAATGTTGGGAATATC
p164	CACCGGATGTTGTGGCCTCCAGAATGAC
p165	AATGGAAGAAGACACTTCGATGTGGCAGACTC
p177	GAATCGTGCCACATCGAAGTGTCTTCTCCATTG
p180	CATTGGGTTGGTTAAATATG
p273	CACAACCTGGTGGGTTTCGAGGTTTG
p274	TGACTTACCTACGCCACTTTC
AAP	GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIIG

Six, 5RACE reactions were necessary to obtain the entire SINV-1 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-1-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 using oligonucleotide primers p134, p138, p138, p157, p162, and p274, for the six reactions (Fig. 1B, p3 to p8), respectively. After cDNA synthesis, PCR was conducted with AAP and p135, p139, p140, p154, p161, and p273, respectively. PCR was conducted using the following temperature regime: 1 cycle at 94 °C for 2 min, 35 cycles of 94 °C for 15 sec, 68 °C for 5 min, followed by a final elongation step of 68 °C for 5 min. Gel-purified amplicons were ligated into the pCR4-TOPO vector, transformed into TOP10 competent cells (Invitrogen), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida).

A single 3RACE reaction was conducted with the GeneRacer kit (Invitrogen). cDNA was synthesized from total RNA (1 µg) purified from SINV-1-infected workers and brood using the GeneRacer Oligo dT primer. The cDNA was amplified by PCR with oligonucleotide primer p113 and the GeneRacer 3' primer. Amplicons were cloned and sequenced as described for 5RACE.

Northern analysis was conducted to determine the genome size following the general procedure of Sambrook and Russell (2001). Membranes were blotted with 6 µg of total RNA from SINV-1-infected and -uninfected fire ant colonies. The 327-nucleotide probe was synthesized using oligonucleotide primers p62 and p63 (Table 1) and a clone

from the 3' end of the genome as template (genome region 6246 to 6572).

Field surveys, castes infected, SINV-1 transmission, and duration of infection

A field survey was conducted to examine the extent of SINV-1 infection among *S. invicta* nests from locations around Florida. Nests were sampled from Gainesville ($n = 72$), Newberry ($n = 11$), LaCrosse ($n = 9$), McIntosh ($n = 9$), Fort Pierce ($n = 6$), Orlando ($n = 4$), Okahumpka ($n = 4$), Ocala ($n = 4$), Canoe Creek ($n = 4$), Fort Drum ($n = 4$), Cedar Key ($n = 11$), Otter Creek ($n = 10$), Bronson ($n = 9$), and Perry ($n = 11$). Samples of workers were retrieved from the field and treated as described above. Primer pairs p62/p63, p136/p137, or p164/p165 were used in an RT-PCR reaction to determine the presence of SINV-1 infection.

Experiments were conducted to determine if the virus was infecting all caste members. Samples of workers were taken from ant nests from areas in Gainesville, Florida, and examined for infection by the RT-PCR method using primer pairs p62/p63, p136/p137, or p164/p165. Nests determined to be infected were revisited on the same day, and samples of queens, workers, early instars (1st and 2nd), late instars (3rd and 4th), pupae, sexual pupae, and male and female alates were directly taken from the field. Queens were placed separately into 1.5-ml microcentrifuge tubes and held at 30 °C for 24 h to obtain a sample of eggs. All samples were analyzed for infection by the RT-PCR method.

To evaluate the transmissibility of the SINV-1, uninfected polygyne nests were identified by RT-PCR, excavated from the field, and parsed into two equivalent fragment colonies comprised of a queen, 0.25 g of brood and 0.5 g of workers. Colonies were infected by the method described by Ackey

and Beck (1972). Workers and brood (1–5 g) from an SINV-1-infected colony were homogenized in an equal volume of water and immediately placed onto a food source for the ants (boiled chicken egg yolks). A treated food source was placed into one of the fragment colonies for 3 days. The control was identical except uninfected ants were used. Samples of workers from treated and untreated paired fragment colonies were sampled 3, 11, and 18 days after introduction of the treated food sources and analyzed for the SINV-1 by the RT-PCR method.

To determine the duration of SINV-1 infection within a fire ant colony, infected colonies were identified in the field, excavated, and placed into rearing trays with a food source (cooked chicken egg yolks, frozen crickets, 10% sugar water), water, and a colony cell. Periodically, worker ants were removed and analyzed for infection by the RT-PCR method. Control colonies (without detectable SINV-1 infection) were removed from the field and treated in identical fashion as the SINV-1-infected colonies.

Acknowledgments

We thank J. Evans and J. Chen (USDA-ARS, Bee Research Laboratory, Beltsville, MD) for critical reviews of the manuscript. The use of trade, firm, or corporation names in this publication are for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

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