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Solenopsis invicta virus-1A (SINV-1A): Distinct species or genotype of SINV-1?

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Abstract

We have cloned and sequenced a 2845 bp cDNA representing the 3′-end of either a new picorna-like virus species or genotype of *Solenopsis invicta* virus-1 (SINV-1). Analysis of the nucleotide sequence revealed 1 large open reading frame. The amino acid sequence of the translated open reading frame was most identical to structural proteins of SINV-1 (97%), followed by the Kashmir bee virus (KBV, 30%), and acute bee paralysis virus (ABPV, 29%). A PCR-based survey for SINV-1 and the new species or genotype (tentatively named *S. invicta* virus-1A, SINV-1A) using RNA extracts of *S. invicta* collected around Gainesville, Florida, revealed a mean colony infestation rate of 25% by SINV-1 and 55% by SINV-1A. Both SINV-1 and SINV-1A were found to co-infect 17.5% of the nests surveyed. Although the data preclude definitive species or genotype assignment, there is no doubt that SINV-1A is distinct from SINV-1, identifiable, and infects *S. invicta*. We provide a simple RT-PCR technique capable of discerning SINV-1 and SINV-1A infection of *S. invicta*.
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Keywords: *Solenopsis invicta*; *Dicistroviridae*; RNA virus; Genome sequence; SINV-1

1. Introduction

Red imported fire ant, *Solenopsis invicta* (Buren), was first detected in the United States near Mobile, Alabama, in the late 1920s (Loding, 1929). Since that time, it has spread to encompass more than 128 million hectares, primarily in the southeastern US (Williams et al., 2001). Although considerable research effort has been brought to bear against the red imported fire ant, it remains the primary pest ant species in infested areas; initial eradication trials failed, yielding to the wide distribution of pesticide-based control products and a federally imposed quarantine to prevent further spread. Recently, much of the research effort has focused on elucidating basic life processes in an attempt to develop unique control measures, and fostering the development of self-sustaining methods of control, including biocontrol organisms and microbes (Williams et al., 2003). Use of self-sustaining organisms (natural enemies) to control the fire ant is perhaps the most tenable approach because it could tip the ecological balance among the ant community favorably toward native ants, thus reducing red imported fire ant population density to non-problematic levels observed in South America (Porter et al., 1997).

Recently, a number of natural enemies have been discovered in the red imported fire ant in the United States, including a Neogregarine (Pereira et al., 2002), a fungus (Pereira, 2004), and a picorna-like insect virus tentatively named *S. invicta* virus-1, or SINV-1 (Valles et al., 2004). Although all of these organisms offer promise as self-sustaining biological control agents, the virus discovery represents the first infection of the red imported fire ant by this group of organisms. In the laboratory, SINV-1 was reported to be associated with brood death of the entire

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colony and infection of healthy colonies was accomplished (Valles et al., 2004). SINV-1 has an 8026 nucleotide, polyadenylated, RNA genome encoding two large open reading frames (ORFs) typical of picorna-like insect viruses. The predicted amino acid sequences of the ORFs exhibited significant identity to the helicase, cysteine protease, RNA-dependent RNA polymerase, and structural proteins of picorna-like insect viruses (Christian and Scotti, 1998; Govan et al., 2000). SINV-1 was found to infect all fire ant castes and developmental stages. Thus, SINV-1 potentially represents an exciting new control tool for use against the red imported fire ant.

During the elucidation of the genome of SINV-1, we serendipitously discovered nucleotide sequence similar to, but distinct from, SINV-1. The sequence is homologous to ORF 2 (i.e., structural proteins) of picorna-like insect viruses with highly significant identity to SINV-1. These data suggest two possibilities, (i) that a distinct, closely related species exists, or (ii) the sequence represents a genotype of SINV-1. We report the nucleotide sequence of the 3′-proximal ORF, compare this sequence with the corresponding sequence of SINV-1, conduct restriction fragment length polymorphism (RFLP) analyses on both sequences, and present data of field infection rates.

2. Materials and methods

2.1. cDNA synthesis, cloning, and sequencing

To distinguish the different genotypes/species, the original virus (Valles et al., 2004) is identified as *S. invicta* virus 1 (SINV-1) while the new species or genotype reported here is designated *S. invicta* virus 1A (SINV-1A).

Anticipating the potential need to remove the virion protein, genome (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-1A-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. One-step reverse transcriptase polymerase chain reaction (RT-PCR, Invitrogen, Carlsbad, CA) was conducted with primer pairs p62,p63; p102,p191; p59,p221; p188,p222; p188,p189; p190,p191; and p137,p193 (Table 1) using the following temperature regime: reverse transcription (45 °C for 50 min), denaturation (94 °C for 2 min), 35 cycles of denaturation (94 °C for 15 s), annealing (for individual temperatures see Table 1, 15 s), and elongation (68 °C for 1.5 min) followed by a final elongation step of 68 °C for 5 min. Gel purified amplicons were ligated into the pCR4-TOPO vector and transformed into TOP10 competent cells (Invitrogen). Insert-positive clones were 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-1A-infected workers and brood using the GeneRacer Oligo(dT) primer. The cDNA was amplified by PCR with oligonucleotide primer p58 or p114 and the GeneRacer 3′ primer. Amplicons were cloned and sequenced as described above.

BLAST comparisons of the nucleotide sequence and predicted amino acid sequence of the 3′-proximal ORF

Table 1
Oligonucleotide primers and their annealing temperatures used throughout this study

Designation	Oligonucleotide (5′ > 3′)	Annealing temperature used (°C)
p58	GCGATAGGTTAGCTTTAAGTACAATTGGTG	56
p59	TCCCAATGTGCAATAAACACCTTCA	57
p62	GGAAGTCATTACGTGGTCGAAAACG	55
p63	CGTCCTGTATGAAAACCGGTCTTTACCACAGAAATCTTA	55
p102	CGCCTTAGGATTCGTTAGATACTCACCCG	59
p114	CTTGATCGGGCAGGACAAATTC	56
p116	GAACGCTGATAACCAATGAGCC	54
p117	CACTCCATACAACATTTGTAATAAAGATTTAATT	54
p118	CCAATACTGAAACAACTGAGACACG	54
p137	GTCACATCACGTCGGTGTCGT	57
p188	CTTAATTGTAATTTACTTGAATATGCGTTTGC	57
p189	GTATCTAACGAATCCTAAGGCGGATTG	57
p190	CAATCCGCCTTAGGATTCGTTAGATAC	57
p191	CGGATCTTATGAGTGAAGACACACCAG	57
p193	CAACCTCTGCTTCCCACGCAC	57
p221	GATGGTCTCGACCAAATGATATGGAG	57
p222	ATGAAGATATGAAGGTGTTTATTGCACATTG	57
p341	CACATAAGGGATATTGTCCCCATG	56
p343	TGGACGAGACGGATCTTATGAGTG	56
3′ Primer	GCTGTCAACGATACGCTACGTAACG	56

and ClustalW-based algorithm alignments were conducted using the Vector NTI alignment software (InforMax, Bethesda, MD).

2.2. Virus field surveys and RFLP analysis

A field survey was conducted to examine the extent of SINV-1 and SINV-1A infection and co-infection among *S. invicta* nests from 4 locations around Gainesville, Florida. Ten nests were sampled from 4 different areas in Gainesville ($n = 40$, Table 2). One-step RT-PCR with species/genotype-specific oligonucleotide primers was used to identify virus-infected *S. invicta* nests. 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). cDNA was synthesized and subsequently amplified using the One-Step RT-PCR kit (Invitrogen) with oligonucleotide primers p117 and p118 (SINV-1-specific) and p114 and p116 (SINV-1-specific) (Table 1). Samples were considered positive for each virus when a visible amplicon of anticipated size (646 nt for SINV-1 and 153 nt for SINV-1A) 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, 54 °C for 15 s, 68 °C for 30 s, followed by a final elongation step of 68 °C for 5 min.

In an attempt to gain additional insight into whether SINV-1A was a genotype or distinct species, oligonucleotide primers were designed to conserved areas (i.e., in common) of the 3′-end of the SINV-1 and SINV-1A sequences (p341 and p343, Table 1). These common primers were used for RT-PCR with representative ant colonies infected exclusively with either SINV-1 or SINV-1A ($n = 3$); the resulting amplicons were subjected to restriction fragment length polymorphism (RFLP) analysis. Amplicons generated with the common primers from SINV-1- and SINV-1A-infected ant colonies were digested separately with *Ava*I and *Bgl*II, separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

In addition, colonies identified as being negative (i.e., no amplification) for infection by either SINV-1 or SINV-1A, as determined previously using RT-PCR and virus-specific primers, were subjected to a second RT-PCR with the common primers (p341 and p343, Table 1) to possibly identify additional species or genotypes.

Finally, we conducted a separate survey of monogyne and polygyne ants to determine if there was a social form-specific virus/genotype. Ant samples were taken from suspected monogyne- and polygyne-predominant areas and evaluated for infection with SINV-1 and SINV-1A as described above. These samples were concomitantly evaluated by PCR to determine the social form of the nest. Social form was determined with PCR by exploiting nucleotide differences between the 2 *Gp-9* alleles ($Gp\text{-}9^{B}$, $Gp\text{-}9^{b}$) found in North American *S. invicta* (Krieger and Ross, 2002) by the method described by Valles and Porter (2003).

3. Results

3.1. Nucleotide sequence of the 3′-end of the SINV-1A genome

The 3′-end of the genome of SINV-1A was constructed by compiling sequences from a series of RT-PCRs and a 3′RACE reaction. The sequence was 2845 nucleotides in length, excluding the poly(A) tail present on the 3′-end (Accession No. AY831776). The nucleotide sequence was comprised of 31.7% A, 28.6% U, 17.6% C, and 22.1% G. Analysis of the nucleotide sequence revealed 1 large ORF in the sense orientation with untranslated regions (UTRs) of 160 and 225 nucleotides at the 5′ and 3′ ends, respectively. Translation of the ORF commenced at nucleotide position 161 (AUG start codon), terminated at nucleotide position 2620 (UAA stop codon), and encoded a predicted product of 92,076 Da. When the SINV-1 and SINV-1A sequences were compared, the start signal in SINV-1 was further upstream and the corresponding ORF larger compared with SINV-1A. Because the sequences of SINV-1 and SINV-1A were so similar, it is likely that the start site we indicated for SINV-1A could actually be an internal methionine and in reality the ORF start site begins somewhere further upstream. No large ORFs were

Table 2
Field survey results of SINV-1 and SINV-1A infection of *S. invicta* from locations in Gainesville, Florida

Location (latitude/longitude)	SINV-1 infection (%)	SINV-1A infection (%)	Co-infection[a] (%)
N29° 35.242′ W082° 20.332′	20	50	10
N29° 45.824′ W082° 24.352′	30	40	20
N29° 39.1′ W082° 15.6′	40	70	40
N29° 40.128′ W082° 31.395′	10	60	0

Each location represents a sample size of 10 nests from within 30 m of the indicated position.
[a] SINV-1 and SINV-1A found in the same fire ant nest. Co-infection of individual ants could not be ascertained.

Table 3
Comparative identities of SINV-1A amino acid sequences with corresponding sequences from other positive strand RNA viruses

Virus	Identity (%)	Accession No.
Solenopsis invicta virus 1	97.4	AY634314
Kashmir bee virus	30.0	NC004807
Acute bee paralysis virus	28.5	NC002548
Drosophila C virus	16.2	NC001834
Triatoma virus	14.8	NC003783
Black queen cell virus	14.5	NC003784
Sacbrood virus	12.1	NC002066
Hepatitis A virus	11.7	NC001489
Cow-pea mosaic virus	10.3	NC003550

found in the inverse orientation. BLAST analysis (Altschul et al., 1997) of the translated ORF revealed identity to structural proteins from picorna-like viruses. The amino acid sequence was most identical to SINV-1 (97%), followed by the Kashmir bee virus (KBV, 30%), and acute bee paralysis virus (ABPV, 29%) (Table 3).

3.2. Field surveys, RFLP analysis, social form, and infectivity

A PCR-based survey for SINV-1 and SINV-1A using RNA extracts of *S. invicta* collected around Gainesville, Florida, revealed a mean colony infestation rate of 25% by SINV-1 and 55% by SINV-1A (Table 2). Among 40 nests surveyed, infection rates among the four different sites ranged from 10 to 40% for SINV-1 and 40 to 70% for SINV-1A (Table 2). Interestingly, both SINV-1 and SINV-1A were found to co-infect 17.5% of the nests surveyed. However, because we used 20–50 ants as the RNA source, we could not determine whether individual ants were infected with both SINV-1 and SINV-1A.

RFLP analysis of a 1584 nucleotide amplicon at the 3′-end of the genomes produced with primers p341 and p343 from SINV-1- and SINV-1A-infected fire ants corroborated the sequence data assembled for each species/genotype (Fig. 1). Digestion of this amplicon from SINV-1-infected fire ants with *Ava*I and *Bgl*II resulted in a single cut by each restriction enzyme as anticipated from the sequence data; *Ava*I and *Bgl*II produced bands of approximately 550 and 1030, and 710 and 870 nucleotides in length, respectively. Conversely, the corresponding amplicon from SINV-1A-infected fire ants was not cut by either *Ava*I or *Bgl*II. All three replicates (from different colonies of fire ants) produced the same banding patterns and no amplicons were produced from uninfected ants.

RNA from colonies yielding no amplicon when utilizing SINV-1- and SINV-1A-specific primers (i.e., uninfected) was subsequently used with conserved primers (p341 and p343) in RT-PCR to possibly identify new viruses or genotypes related to SINV-1 and SINV-1A. In every instance ($n = 15$), no amplification was observed with the conserved primers.

SINV-1 and SINV-1A were found in monogyne and polygyne nests. Thus, infection by either virus does not appear to be limited to a specific social form (data not shown). The purpose of this experiment was to simply determine whether SINV-1 or SINV-1A infection was exclusive to either fire ant social form. Sampling was terminated after two nests of each social form were found to be infected with SINV-1 and SINV-1A.

4. Discussion

The sequence fragments comprising the 3′-end of SINV-1A were obtained serendipitously during cloning and sequencing of the SINV-1 genome (Valles et al., 2004). During the sequence assembly process for SINV-1, we noticed that several sequences were very similar, but

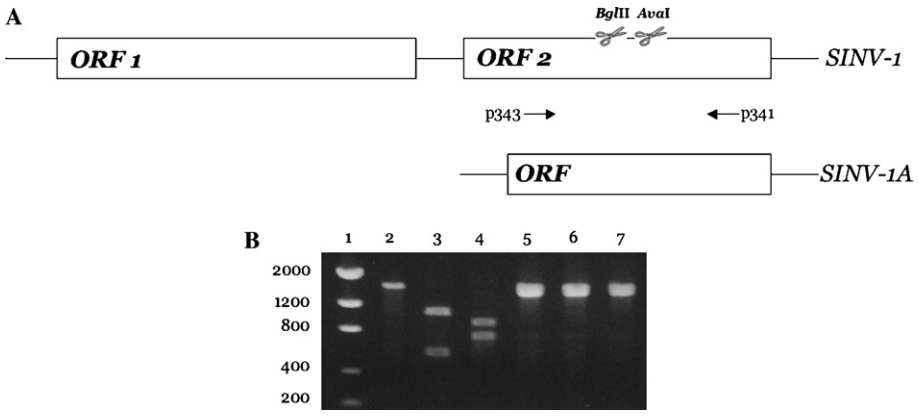

Fig. 1. (A) Schematic diagram of the SINV-1 and SINV-1A genomes. ORFs are shown in open boxes. Conserved oligonucleotide primer positions are indicated by p341 and p343. Restriction positions unique to SINV-1 are approximated with scissor symbols. (B) RFLP of a portion of the SINV-1 and SINV-1A genomes amplified with primers p341 and p343 and restriction digested with *Ava*I and *Bgl*II. Lane assignments are as follows: 1, molecular weight markers; 2, SINV-1 undigested; 3, SINV-1 *Ava*I-digested; 4, SINV-1 *Bgl*II-digested; 5, SINV-1A undigested; 3, SINV-1A *Ava*I-digested; SINV-1A *Bgl*II-digested.

distinct, from SINV-1. Often, simultaneous amplification of both genotypes/species occurred because the sequences were very similar and individual colonies of *S. invicta* were capable of being infected by both SINV-1 and SINV-1A simultaneously, unbeknownst to us at the time. Co-infection of nests was observed in our field survey in which 17.5% of field-collected colonies were found to be infected with both SINV-1 and SINV-1A. Although co-infection of honey bees with related viruses has been reported (Chen et al., 2004; Evans, 2001), we were unable to confirm whether individual fire ants were infected with both SINV-1 and SINV-1A. The infection rate for SINV-1 (25%) was consistent with the value reported previously (23%) by Valles et al. (2004). Interestingly, infection of fire ant colonies by SINV-1A was 2.2-fold higher (55%) than for SINV-1. Despite conducting RT-PCR and 3′RACE with oligonucleotide primers designed specifically to SINV-1, the genome sequence of SINV-1A was sufficiently similar to occasionally result in amplification—even in cases where oligonucleotide mismatches were present. Thus, SINV-1A is a compilation of contiguous fragments that did not match the SINV-1 sequence.

The nucleotide sequence of the 3′-end (structural proteins) of SINV-1 and SINV-1A exhibited 89.9% nucleotide identity and 97% amino acid identity (Table 3) of the translated 3′-proximal ORF. According to the International Committee on Taxonomy of Viruses (ICTV), nucleotide sequence identity of less than 90% can be used to demarcate virus isolates in the *Dicistroviridae* (formerly cricket paralysis-like viruses) as distinct species (Christian et al., 2000). Recently, cricket paralysis virus (CrPV) and acute bee paralysis virus (ABPV) have been assigned to the *Dicistroviridae* (Mayo, 2002a,b) and the SINV-1 genome most closely matches ABPV (Valles et al., 2004). At 89.9% identity, SINV-1A is on the cusp of the recommended level for consideration as a distinct species.

Single-stranded RNA viruses lack proofreading machinery, thus, they exhibit a high mutation rate [10^{-3}–10^{-4} per nucleotide, (Eggers and Tamm, 1965)]. These characteristics contribute to genomic variation and afford adaptability (Rueckert, 1991; Van Regenmortel, 2000). Thus, genotypic variability within a species can be quite high. Johnson and Christian (1999) reported 5 genotypes of *Drosophila* C virus (DCV) exhibiting nucleotide divergence of up to 10% based on restriction fragment analysis of a 1.2 kb region of the genome encoding part of the capsid proteins. They hypothesized that geographic isolation could be responsible for the different genotypes of DCV. A similar conclusion was proposed to explain genotypes of the CrPV (Johnson and Christian, 1996) and sacbrood virus (SBV, Grabensteiner et al., 2001). Grabensteiner et al. (2001) identified three distinct genotypes of SBV with nucleotide identities ranging from 83 to 99% depending on the area of the genome amplified. However, they concluded that simple geographic isolation was insufficient to explain the existence of the different genotypes because they found 2 of the 3 genotypes infecting *Apis* species to be sympatric in Nepal. However, it appeared that the 2 genotypes exhibited species specificity; one genotype infected *Apis mellifera*, while the other infected *Apis cerana*. In our case, SINV-1 and SINV-1A infect the same species, *S. invicta*, in the same geographic location. Thus definitive assignment of SINV-1A as a distinct species or genotype of SINV-1 is problematic. Interestingly, *S. invicta* exists in two distinct social forms, monogyne and polygyne, and these differences were shown recently to have a genetic basis (Krieger and Ross, 2002). Monogyne *S. invicta* are characterized as having a single fertile queen and polygyne *S. invicta* have multiple fertile queens. We were curious to know whether SINV-1 and SINV-1A had a preference for either social form because social form-specific pathogen infectivity has been reported previously in *S. invicta*. Oi et al. (2004) showed that infection of North American *S. invicta* with the microsporidian, *Thelohania solenopsae*, was restricted to the polygyne social form. We did not observe any distinction between SINV-1 and SINV-1A with regard to social form specificity; both viruses were found to infect both social forms. In fact, dual infections (SINV-1 and SINV-1A) were found in both monogyne and polygyne nests.

Our attempts to identify additional genotypes/species proved negative. RFLPs among all of the restriction enzyme-digested 1584 nucleotide amplicons were not different from SINV-1- and SINV-1A-infected fire ants.

The 90% nucleotide identity suggests that SINV-1A is a genotype of SINV-1, especially because the high level of identity was observed in the less conserved 3′-proximal ORF encoding the structural proteins (Johnson and Christian, 1998). However, co-infection and sympatry conflict with this conclusion. Furthermore, no particular level of genome sequence dissimilarity can be used to differentiate species (Van Regenmortel, 2000). Although the data preclude definitive species or genotype assignment, there is no doubt that SINV-1A is distinct from SINV-1, identifiable, and infects *S. invicta*. We provide a simple RT-PCR technique capable of discerning SINV-1 and SINV-1A infection of *S. invicta*. SINV-1 and SINV-1A are the first viruses shown to infect *S. invicta* (Valles et al., 2004); further evaluation, including geographic distribution studies, genome and protein features, and transmission mechanisms will be necessary to determine whether SINV-1A is a genotype or distinct species and to identify any additional genotypes/species.

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