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Short Communication

Detection and quantitation of *Solenopsis invicta* virus-2 genomic and intermediary replicating viral RNA in fire ant workers and larvae

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Abstract

Quantitative real-time PCR (QPCR) was used to quantify the genome of *Solenopsis invicta* virus-2 (SINV-2) from infected individual ants of *S. invicta*. Strand-specific cDNA synthesis oligonucleotide primers and RNase digestion after cDNA synthesis allowed quantification of plus (genomic) and minus (replicative) strands of the SINV-2 genome. Both strands were detected in adult workers and larval fire ants indicating that the virus was replicating within the ant. The differences between the genomic to replicative strand ranged from 199-fold in larvae to 479-fold in workers with an average ratio of 339:1.
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Keywords: Quantitative PCR; Fire ant; *Solenopsis invicta* virus-2; Positive-strand RNA virus

Solenopsis invicta virus 2 (SINV-2) was isolated and characterized recently from *S. invicta* (Valles et al., 2007) and represents only the second virus discovered from this species of ant (Valles et al., 2004). SINV-2 contains a single-stranded positive RNA genome 11,303 nucleotides in length which contains four major open reading frames (ORFs). ORF 4 (nearest the 3′ end, Fig. 1) is the largest and possesses protease, helicase, and RNA-dependent RNA polymerase (RdRp) domains, which are conserved among RNA viruses including picornaviruses, dicistroviruses, iflaviruses, and plant small RNA viruses. Possession of more than two ORFs per genome is unique among invertebrate positive-strand RNA viruses. With the exception of Nora virus in *Drosophila melanogaster* (Habayeb et al., 2006), SINV-2 is the only other positive-strand RNA virus with a monopartite, polycistronic genome. Phylogenetic analysis of the SINV-2 RdRp and helicase domains indicated unique placement of SINV-2 from other positive-strand RNA viruses (Valles et al., 2007). Although molecular characterization of SINV-2 has been reported and particles consistent with positive-strand RNA viruses found in *S. invicta*, replication of SINV-2 in the ant was assumed. Thus, we utilized real-time quantitative polymerase chain reaction (QPCR) to demonstrate that the replicative form of the genome is found in infected fire ants thus providing further evidence for SINV-2 replication within the fire ant.

SINV-2 infected *S. invicta* nests were identified in Gainesville, Florida, by one-step RT-PCR (Invitrogen, Carlsbad, CA) as described by Valles et al. (2007). Total RNA was isolated from individual fire ant workers and larvae with Trizol reagent (Invitrogen). Oligonucleotide primers, p511 (5′-CGGAGACACTGAGCCTTTCTGGACT CCATAG) and p515 (5′-GTATCGCGGAAATTACCC AACATCACAAC) were used for cDNA synthesis of plus (genomic) and minus (replicative) strands of SINV-2 RNA, respectively. cDNA was synthesized in a 0.5 ml, thin-walled PCR tube according to the manufacturer's recommendations except dithiothreitol was omitted from the reaction as it has been shown to interfere with down-stream SYBR dye-based reactions (Hashimoto et al., 2007). The RNA template was digested at 37 °C for 30 min in the presence of 5 U of RNase H and 0.5 U of RNase A. The digestion with RNases avoided the possibility that carryover RNA template and superscript reverse transcriptase (SsRT) into

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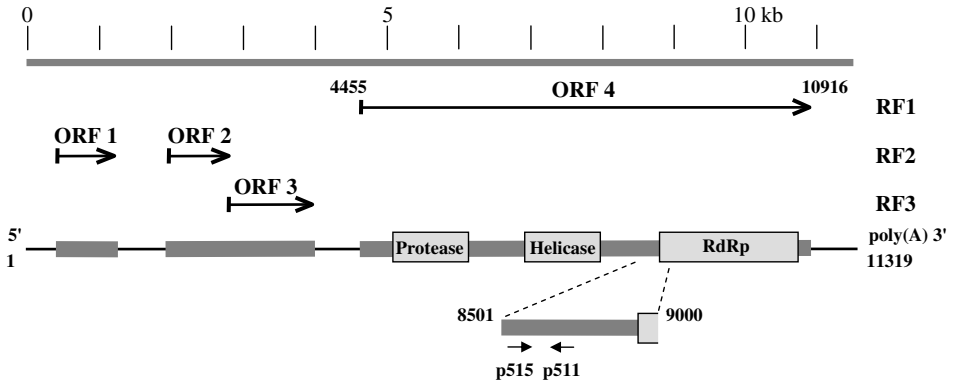

Fig. 1. Relative location of the RdRp domain (8501–9000 nt) within the SINV-2 genome (Accession No. EF428566). ORFs 1–3 putatively encode structural proteins and ORF4 encodes a helicase, protease, and RdRp, respectively, and are illustrated in the upper part of the figure. RF1–RF3 indicate the reading frame on which each ORF is encoded. The expanded area represents a portion of the upstream of RdRp conserved region used in the QPCR analysis. cDNA synthesis and QPCR were accomplished with primers p511 and p515, which correspond to genome sequences 8677–8707 and 8584–8613, respectively.

the QPCR would generate a complementary DNA strand as has been observed previously (Craggs et al., 2001).

The cDNA was then amplified and quantified by QPCR with SYBR green dye and oligonucleotide primers p511 and p515. QPCR assays were performed on an ABI PRISM 7000 Sequence Detection System (SDS) interfaced to the ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA) in a 25 μl reaction volume (Hashimoto et al., 2007). The optimized reaction contained 12.5 μl of SYBR Green SuperMix (with UDG and ROX, Invitrogen), 0.4 μl each of oligonucleotide primers, p511 and p515 (10 μM), 3 mM $MgCl_2$, 1 μl of the cDNA synthesis reaction, and 10.7 μl of H_2O. The thermal conditions were as follows: one cycle of 50 °C for 2 min; 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 64 °C for 15 s; 72 °C for 1 min. Dissociation analysis and agarose gel electrophoresis were carried out for all amplifications to inspect for the formation of primer dimers and extraneous amplicons. For every QPCR run, non-template control reactions were included as negative controls and quantitative standards which were amended with 1 μl of an RT-PCR reaction prepared without RNA template so that comparisons with cDNA synthesized samples would be identical. A plasmid construct of the SINV-2 RdRp conserved region was generated and used as a quantitative standard. The ratio of genomic to replicative SINV-2 RNA was calculated for each sample.

Positive-strand RNA viruses lack proofreading machinery and, therefore, exhibit fairly high mutation rates (Domingo and Holland, 1997; Holland et al., 1982). For this reason, the SINV-2 RdRp region was targeted for cDNA synthesis and QPCR because it is relatively conserved within the genomes of single-stranded RNA viruses (Koonin and Dolja, 1993). A representative standard curve generated from the plasmid construct of the SINV-2 RdRp region showed a strong linear relationship [$r^2 = 0.99$; $y = (-3.38 \pm 0.11)x + (39.27 \pm 0.73)$] between C_T (cycle threshold) and starting SINV-2 RdRp copy number over a dynamic range of 5–5 × 10^7 copies. C_T variability was stable from experiment to experiment and the standard deviation across the dynamic range represented less than 3.0% of the mean value. The efficiency (±SD) of the QPCR reaction was 96.9 ± 2.6%.

Both genomic and replicative strands of SINV-2 were detected in adult workers and larvae of *S. invicta*, indicating that the virus was replicating within the ant (Ongus et al., 2004). The number of genomic equivalents (plus strand) observed was $(1.1 \pm 0.2) \times 10^9$ for worker ants and $(7.7 \pm 1.4) \times 10^9$ for larvae (Fig. 2). The number of replicative equivalents (minus strand) observed was $(2.3 \pm 0.4) \times 10^6$ for worker ants and $(3.9 \pm 0.2) \times 10^7$ for larvae. The average ratio of positive to negative strand was 339 ± 140:1.

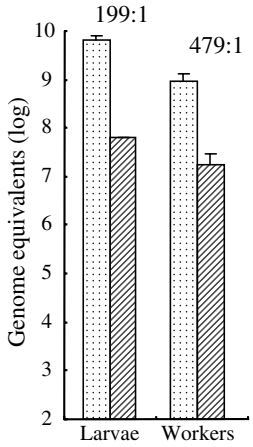

Fig. 2. SINV-2 RdRp copy number determined for individual ants. RdRp copy number was interpolated from a standard curve generated simultaneously from a plasmid construct. Samples were taken from field-collected colonies and included three groups of 10 larvae or 10 workers. Left bars represent the means for genomic RNA (plus strand) and right bars the replicative RNA (minus strand). Number above each bar group is the ratio of plus to minus strand for each stage.

SINV-2 genome copy numbers per individual larva and adult were relatively high and variable (3 log range/individual). RNA virus genome load can vary considerably depending on the insect and state of infection. For example, the chronic bee paralysis virus (CBPV) infection among honey bees exhibited an infection range of 13 orders of magnitude (Blanchard et al., 2007). Honeybees infected with CBPV showed a correlation between high CBPV genome load and expression of symptoms and an approximately 7 log range difference in CBPV copy number between asymptomatic and symptomatic/dead bees (Blanchard et al., 2007). Although to date, SINV-2 infection of *S. invicta* appears asymptomatic, virus genome load may need to be considerably higher to result in observable symptoms within the ant. QPCR should facilitate such studies. Furthermore, although no obvious symptoms were observed, changes in behavior and other associated alterations in the ant may occur and be imperceptible (Igbal and Mueller, 2007).

The ratio of genomic (plus) strand to replicative (minus) strand of the SINV-2 genome (339 ± 140:1) was in agreement with other strand-specific quantification studies of positive-strand RNA viruses. Among the published quantitative studies, viruses infecting plants (1- to 100-fold; Kielland-Brandt, 1974), mosquitoes (10- to 100-fold; Richardson et al., 2006), and humans (poliovirus, 30-fold and hepatitis C virus, 100- to 1000-fold; Girard et al., 2002; Komurian-Pradel et al., 2004) exhibited plus to minus strand ratios comparable to SINV-2. Consistent detection of the replicative (minus) genome strand of SINV-2 in larval and adult stages of the fire ant indicates that SINV-2 was replicating in both of these stages.

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