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Phenology, distribution, and host specificity of *Solenopsis invicta* virus-1

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Abstract

Studies were conducted to examine the phenology, geographic distribution, and host specificity of the *Solenopsis invicta* virus-1 (SINV-1). Two genotypes examined, SINV-1 and -1A, exhibited similar seasonal prevalence patterns. Infection rates among colonies of *S. invicta* in Gainesville, Florida, were lowest from early winter (December) to early spring (April) increasing rapidly in late spring (May) and remaining high through August before declining again in the fall (September/October). Correlation analysis revealed a significant relationship between mean monthly temperature and SINV-1 ($p < 0.0005$, $r = 0.82$) and SINV-1A ($p < 0.0001$, $r = 0.86$) infection rates in *S. invicta* colonies. SINV-1 was widely distributed among *S. invicta* populations. The virus was detected in *S. invicta* from Argentina and from all U.S. states examined, with the exception of New Mexico. SINV-1 and -1A were also detected in other *Solenopsis* species. SINV-1 was detected in *Solenopsis richteri* and the *S. invicta/richteri* hybrid collected from northern Alabama and *Solenopsis geminata* from Florida. SINV-1A was detected in *S. geminata* and *Solenopsis carolinensis* in Florida and the *S. invicta/richteri* hybrid in Alabama. Of the 1989 arthropods collected from 6 pitfall trap experiments from Gainesville and Williston, Florida, none except *S. invicta* tested positive for SINV-1 or SINV-1A. SINV-1 did not appear to infect or replicate within Sf9 or Dm-2 cells *in vitro*. The number of SINV-1 genome copies did not significantly increase over the course of the experiment, nor were any cytopathic effects observed. Phylogenetic analyses of SINV-1/-1A nucleotide sequences indicated significant divergence between viruses collected from Argentina and the U.S.
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1. Introduction

Escape from natural enemies during the founding bottleneck is considered the most likely reason why the red imported fire ant, *Solenopsis invicta* Buren, has become a major pest in the United States (Porter et al., 1997). Comparative ecological studies among populations in native (Argentina) and introduced (United States) regions have revealed that red imported fire ant populations are greater in number, have higher mound densities, possess larger mound volumes and comprise a larger fraction of the ant community in the U.S. (Porter et al., 1992, 1997). Therefore, it is not surprising that discovery, characterization, and utilization of fire ant pathogens and parasites are among the goals of U.S. fire ant researchers to achieve self-sustaining control of this aggressive, territorial ant pest.

Several years ago, the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, began to search for viruses infecting *S. invicta*. In pursuit of this goal, this laboratory created an expressed sequence tag (EST) library from *S. invicta*. Bioinformatics analyses of approximately 2000 ESTs revealed several sequences exhibiting strong homology to RNA viruses. Further investigation resulted in the discovery, characterization and complete genome sequence of a virus that has been assigned to the Dicistroviridae (Mayo, 2002) and named *Solenopsis invicta* virus-1 [SINV-1] (Valles et al., 2004; Valles and Strong, 2005). SINV-1 is a positive-strand RNA virus found in all stages of *S. invicta*. Its RNA genome is single-stranded, monopartite and comprised of 8026 nucleotides encoding two, non-overlapping open reading frames (ORFs). Genomic comparisons with other known dicistroviruses revealed that SINV-1 encodes 6 copies of the 5′, covalently-bound, genome-linked protein, *Vpg* (Nakashima and Shibuya, 2006), and a type II internal ribosomal entry site (Jan, 2006). These characteristics are hypothesized to facilitate efficient SINV-1 multiplication in host cells. Although SINV-1-infected fire ants or colonies did not exhibit any immediate, discernible symptoms in the field, brood die-off was often observed when infected colonies were reared under laboratory conditions (Valles et al., 2004). These characteristics are consistent with other insect-infecting positive-strand RNA viruses; they frequently persist as unapparent, asymptomatic infections that, under certain conditions, induce replication within the host resulting in observable symptoms and often death (Christian and Scotti, 1998). In an effort to learn more about the biology of SINV-1, we conducted experiments to examine its phenology, distribution and host specificity.

2. Materials and methods

2.1. Phenology

During initial experiments and genome sequencing, we noticed that the prevalence of the SINV-1 infection among *S. invicta* collected from Florida seemed to change during the course of the year. Because of these observations and previous reports of seasonal variation of related single-stranded, RNA viruses in other Hymenopteran insects (Bailey, 1967; Berenyi et al., 2006), we conducted a study to formally monitor the SINV-1 infection rate in *S. invicta* during the course of a year in Gainesville, Florida. Two roadside sites were chosen. The first site was located on State Route 441, north of Paynes Prairie Nature Preserve (N29° 35.242′; W82° 20.330′) and the second site at the crossroads of State Route 26 and East University Avenue (N29° 39.108′; W82° 15.650′). From December 2004 to December 2005, 10 fire ant colonies per site were sampled monthly and evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) for the presence of SINV-1. Ant collection was accomplished by plunging a scintillation vial (20 ml) into a nest for several minutes allowing fire ants to fall into the vial. Ant samples were taken to the laboratory and evaluated for SINV-1 infection. Total RNA was extracted from 20 to 30 worker ants by the Trizol method according to the manufacturer's instructions (Invitrogen, CA).

Two genotypes have been reported to infect *S. invicta*, SINV-1 (Valles et al., 2004) and SINV-1A (Valles and Strong, 2005). Therefore, cDNA was synthesized and subsequently amplified using the One-Step RT-PCR kit (Invitrogen) with genotype specific oligonucleotide primers (p117 and p118, SINV-1A-specific and p114 and p116, SINV-1-specific) (Valles and Strong, 2005). 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 thermal cycler (PTC 100, 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. Two controls were included in all assays, one with RNA from infected ants (positive control) and one devoid of RNA (non-template or negative control).

The percentage of colonies infected with SINV-1 (genotypes-1 and -1A) for each month was correlated with the average monthly temperature and rainfall using Pearson's correlation analysis (SAS Correlation Procedure [Cary, NC]). Meteorological data were obtained for the Gaines-

ville, FL, airport at the National Weather Service Forecast Office website: www.srh.noaa.gov/jax/climate.shtml.

2.2. Distribution

Several species within the *Solenopsis* genus were collected from across the infested U.S. range, as well as from Mexico, and Argentina. Sampling was conducted by arbitrarily choosing an ant-infested area and taking samples of worker ants from up to five colonies within a 50 m radius. Collected ants were either stored in 95% ethanol for later testing or tested immediately for virus. Samples were evaluated for the presence of SINV-1 and SINV-1A by RT-PCR as described above. SINV-1 was detectable from infected ants stored in ethanol for up to a year (Valles and Strong, unpublished data). Fire ant species were identified using taxonomic keys of Trager (1991) and Wojcik et al. (1976). Ant samples from Tennessee, Alabama, Mississippi, and Argentina were further evaluated by venom alkaloid and cuticular hydrocarbon analysis to discern *Solenopsis richteri*, *S. invicta*, and the *Solenopsis invicta*/*richteri* hybrid (Vander Meer et al., 1985; Ross et al., 1987; Vander Meer and Lofgren, 1990). Colony samples were obtained by collecting worker ants as described above using 20 ml scintillation vials plunged into mounds. Sample collection dates were as follows: *S. invicta*, Alabama (July 2005), California (April–May 2005), Florida (October 2005–May 2006), Georgia (June 2006), Louisiana (April–May 2005), New Mexico (September 2004, 2005), Oklahoma (May–June 2005), South Carolina (June 2005), Tennessee (July 2005), Texas (December 2004–January 2005), Argentina (October 2004–April 2005); *S. richteri*, Alabama (July 2005), Tennessee (August 2005), Argentina (November 2005); *S. invicta*/*richteri* hybrid, Alabama (August 2005), Mississippi (January 2005); *Solenopsis geminata*, Florida (December 2004–August 2005), Hawaii (September 2005), Mexico (October 2005); *Solenopsis xyloni*, California (January 2005), New Mexico (August–September 2004); *Solenopsis carolinensis*, Florida (July–September 2005); *Solenopsis pergandii*, Florida (September 2005).

2.3. Host specificity

To determine the host specificity of SINV-1, surveys of other arthropods were conducted by pitfall trapping. To increase our chances of identifying SINV-1 infections in other arthropods, site selections were biased toward areas with a high incidence of SINV-1 among *S. invicta* populations. SINV-1 prevalence was determined by sampling 10–20 colonies in an area and analyzing them for the presence of SINV-1 and -1A by RT-PCR. Sites with greater than 20% SINV-1 infection rates were chosen for inclusion in the study. Pitfall traps were constructed from 8.5 cm tall × 3 cm diameter plastic tubes. A 5 mm hole drilled into the side 4 cm from the bottom was covered with fine mesh stainless steel screen to prevent the tube from overflowing during rain storms (Porter, 2005). At each of six sites in Gainesville and Williston, FL, 15 pitfall traps were buried in the soil so the lip of the tube was flush with the surface. The traps were placed on a random transect approximately 1.5 m apart and filled with approximately 25 ml of ethanol. Traps were left in place for 48 h and their positions were marked with flags. After 24 h, additional ethanol was added to each trap. The 6 sites and collection dates were as follows: site 1 (9 May 2005), State Route 441 on the north side of Paynes Prairie Nature Preserve (N29° 35.242′; W82° 20.330′—Gainesville); site 2 (9 May 2005), intersection of Main and Depot Road (N29° 38.634′; W82° 19.499′—Gainesville); site 3 (13 September 2005), crossroads of State Route 26 and East University Avenue (N29° 39.108′; W82° 15.650′—Gainesville); site 4 (15 September 2005), west side of Waldo Road at the Gainesville airport (N29° 41.081′; W82° 17.388′—Gainesville); site 5 (20 September 2005), corner of SW 24th Avenue and SW 122 Street (N29° 37.574′; W82° 28.381′—Gainesville); and site 6 (20 September 2005), entrance to the Williston airport (N29° 21.732′; W82° 28.335′—Williston).

After 48 h, the traps were removed from soil, returned to the laboratory and the captured arthropods separated into two groups, ants and non-ants. Ants were classified to genus (Hölldobler and Wilson, 1990) and the non-ant arthropods were classified to family when possible (Borrer et al., 1989) under a dissecting microscope while submerged and maintained in 95% ethanol. Once the arthropods were separated, they were washed in ethanol to minimize cross-contamination and RNA was extracted by the Trizol method. One-step RT-PCR was conducted with total RNA and SINV-1 genotype-specific primers.

Host specificity was also assessed by attempts to infect available lines of insect cells *in vitro*. *Drosophila melanogaster*-2 (Dm-2; Invitrogen) and *Spodoptera frugiperda*-9 (Sf9; from Dr. J. Maruniak, University of Florida) cells were used in these experiments. Multi-well cell culture plates were seeded with 7×10^5 Dm-2 cells in 2 ml of Schneider's insect medium (Sigma Chemical, St. Louis, MO) plus 10% fetal bovine serum (FBS) or 2×10^5 Sf9 cells in 2 ml of TNM-FH medium (Sigma) plus FBS. The cells were incubated at 28 °C for 2 h to allow for cell attachment. Unattached cells and medium were removed from each well by suction and replaced with 2 ml of fresh medium. SINV-1 inoculum was prepared by pooling 5–7 SINV-1-infected midguts from sexual larvae and homogenizing them in 500 μl of culture medium. The homogenate was centrifuged at 2000 rpm for 5 min. The supernatant was passed through a sterile syringe disc filter (0.2 μm) and brought up to 1 ml volume with culture medium. Cells were challenged with 200 μl of the filtered supernatant (inoculum). After incubating for 1 h at 28 °C to allow for viral adsorption, the culture medium was removed (spent inoculum) and the cells rinsed twice with 1 ml of fresh culture medium (rinsate). Fresh culture medium (3 ml) was added to each well and the SINV-1-challenged cells were incubated for 0, 1, 3, and 5 days. At the specified time, the cells were scraped from the well and cell density was determined by

Table 1
Taxa collected from pitfall traps from Gainesville (sites 1–5) and Williston (site 6), Florida

Taxa	Replicate (number of species: individuals) for					
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Malacostraca	—	1:2	—	—	—	—
Diplopoda	1:1	—	—	—	—	—
Arachnida	9:29	9:14	4:4	3:3	3:5	—
Hexapoda						
Collembola	1:15	—	1:30	1:3	—	1:4
Orthoptera						
Gryllidae	2:13	4:8	1:2	3:10	2:7	
Tettigoniidae	—	—	1:1	1:1	—	1:1
Acrididae	—	—	—	—	—	2:3
Coleoptera						
Carabidae	3:3	—	1:1	—	1:1	—
Scarabaeidae	—	2:4	—	2:6	3:3	—
Staphylinidae	1:1	2:2	—	1:1	3:4	—
Cephalobidae	1:1	—	—	—	—	—
Curculionidae	—	2:2	—	—	—	—
Elateridae	—	1:1	—	1:1	—	—
Scolytidae	—	1:2	2:7	1:1	—	1:1
Cucujidae	—	—	1:1		—	—
Tenebrionidae	—	—	—	1:1	—	2:2
Hemiptera						
Cydnidae	2:2	1:4	—	—	—	—
Miridae	—	2:4	—	—	—	—
Lygaeidae	—	1:2	—	—	—	—
Unknown	—	2:3	—	—	—	2:2
Homoptera						
Aphididae	1:1	—	—	—	—	—
Unknown	—	—	1:1	—	2:2	—
Dictyoptera						
Blattidae	1:1	—	—	—	—	—
Lepidoptera						
Noctuidae	—	1:3	—	—	—	—
Diptera						
Syrphidae	—	2:2	—	—	—	—
Dolichopodidae	—	1:5	—	—	—	—
Drosophilidae	—	—	—	1:2	1:1	—
Tachinidae	—	—	—	—	3:6	—
Muscoidea	—	1:1	—	—	—	—
Hymenoptera						
Ichneumonidae	—	—	—	2:8	—	2:3
Mutilidae	—	—	—	—	—	1:1
Neuroptera						
Chrysopidae	—	1:1	—	—	—	—
Formicidae						
Monomorium	1:28	—	2:80	—	—	—
Dorymyrmex	1:25	1:8	1:200	1:104	1:200	1:100
Cyphomyrmex	1:7	—	—	—	1:1	—
Camponotus	1:9	—	—	1:6	—	—
Hypoponera	1:2	—	—	—	—	—
Pheidole	1:49	—	2:60	4:120	3:150	2:50
Odontomachus	2:9	—	—	—	—	—
Cardiocondyla	1:12	—	1:2	—	—	—
Brachymyrmex	—	1:41	—	1:6	—	—
Neivamyrmex	—	1:1	—	—	—	—
Trachymyrmex	—	—	1:5	—	—	—
Pogonomyrmex	—	—	—	1:4	—	—
Paratrechina	—	—	—	1:32	1:10	1:50
Xenomyrmex	—	—	—	1:8	—	—

(*continued on next page*)

Table 1 (*continued*)

Taxa	Replicate (number of species: individuals) for					
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Formica	—	—	—	1:1	—	—
Linepithema	—	—	—	—	1:100	1:50
Solenopsis	1:73[a]	1:51[a]	1:20[a,b]	—	1:20[a,b]	1:20[a,b]
Total: non-ants	92	60	47	37	29	17
Total: ants	207	101	367	281	481	270

SINV-1 or -1A were not detected in any taxa, with the exception of *Solenopsis* ants.
[a] *Solenopsis* species were combined and 20 individuals were tested for SINV-1 or -1A.
[b] *S. invicta* tested positive for SINV-1 or SINV-1A.

counting with a hemocytometer. The cell suspension was centrifuged (5000 rpm for 1 min) to separate the cells from the culture medium. RNA was extracted from representative aliquots of inoculum, spent inoculum, rinsate, growing medium, and cells. cDNA synthesis and quantitative PCR (QPCR) were subsequently conducted to determine the number of genome copies in each aliquot as described previously (Hashimoto et al., 2007). Briefly, cDNA was synthesized from total RNA corresponding to the SINV-1 RdRp region using SuperScript III Reverse Transcriptase (SsRT; Invitrogen) and a gene-specific primer. In a 0.5 ml, thin-walled PCR tube, 2 μl of an RdRp-specific primer (1 μM), p523 [5′CCTCATTGAAGATAAATCCT CTCTTGAGAAA], 1 μl (10 mM) of a dNTP mix, 9 μl of H_2O, and 100 ng of total RNA (1 μl) was heated to 65 °C for 5 min in a thermal cycler, followed by incubation on ice for 2 min. Then, 4 μl of 5× first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM $MgCl_2$), 2.5 μl of H_2O, and 0.5 μl of SsRT (200 U/μl) were added. The mixture was pulsed in a centrifuge and incubated at 55 °C for 1 h, followed by inactivation of the RT by heating to 70 °C for 15 min. QPCR was conducted on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) in a 25 μl reaction volume. The optimized reaction contained 12.5 μl of SYBR Green SuperMix (Invitrogen), 0.4 μl each of oligonucleotide primers, p517 (5′CAATAGGCACCAACGTATATAGTAGA GATTGGA) and p519 (5′GGAATGGGTCATCATATA GAAGAATTG) (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, 56 °C for 15 s; 72 °C for 1 min. For every QPCR run, non-template control reactions were included as negative controls and quantitative plasmid standards of the SINV-1 RdRp region (5 to 5×10^6 copies) were used to generate a standard curve.

2.4. Phylogenetic analysis

Because SINV-1 and -1A were found to infect *S. invicta* in North and South America, we were curious to know whether there was significant divergence in viral sequences from the two continents and, possibly, which sequence was the ancestral type. Therefore, gel-purified amplicons generated with SINV-1- and SINV-1A-specific oligonucleotide primers from virus-infected *S. invicta* 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). Regions of the genome amplified correspond to structural proteins (3′ proximal ORF). Sequences were generated from SINV-1- and SINV-1A-infected colonies from locations in the United States and Argentina (Table 2). Sequences were analyzed by the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) with the Vector NTI software suite (Invitrogen) without using an out-group or rooting the phylogram. Multiple alignments of the nucleo-

Table 2
SINV-1 genotype, collection location, and GenBank Accession No. used in the phylogenetic analysis

Virus genotype	Isolate designation	Collection location	Accession Nos.
SINV-1	Florida 1	Gainesville, FL	AY634314
SINV-1	Texas 1	Laredo, TX	DQ855654
SINV-1	Oklahoma 1	Lane, OK	DQ855653
SINV-1	Louisiana 1	Arcola, LA	DQ855652
SINV-1	Argentina 1	Chaco, Argentina	DQ855650
SINV-1	Argentina 2	Corrientes, Argentina	DQ855651
SINV-1	South Carolina 1	Clemson, SC	DQ855655
SINV-1A	Florida 2	Gainesville, FL	AY831776
SINV-1A	Texas 2	Millet, TX	DQ855660
SINV-1A	South Carolina 2	Orangeburg, SC	DQ855659
SINV-1A	Argentina 3	Corrientes, Argentina	DQ855656
SINV-1A	Argentina 4	Corrientes, Argentina	DQ855657
SINV-1A	Argentina 5	Corrientes, Argentina	DQ855658

tide sequences were completed with the CLUSTALW algorithm (Thompson et al., 1994). Phylogenetic trees were constructed by the NJ method followed by a reliability evaluation of the tree (topology at the branch) with the bootstrap test (1000 replications) (Felsenstein, 1985).

3. Results

3.1. Phenology

SINV-1 and -1A exhibited similar seasonal prevalence patterns (Fig. 1). Infection rates among colonies of *S. invicta* in Gainesville, FL, were lowest from early winter (December) to early spring (April). The infection rates increased rapidly in late spring (May) and remained high through August before declining again in fall (September/October). SINV-1A was consistently more prevalent compared with SINV-1; the SINV-1A infection rate reached a maximum of 60% (May, July, September) while SINV-1 peaked at 40% (August). Colony co-infections (colonies testing positive for both genotypes) were common in Florida.

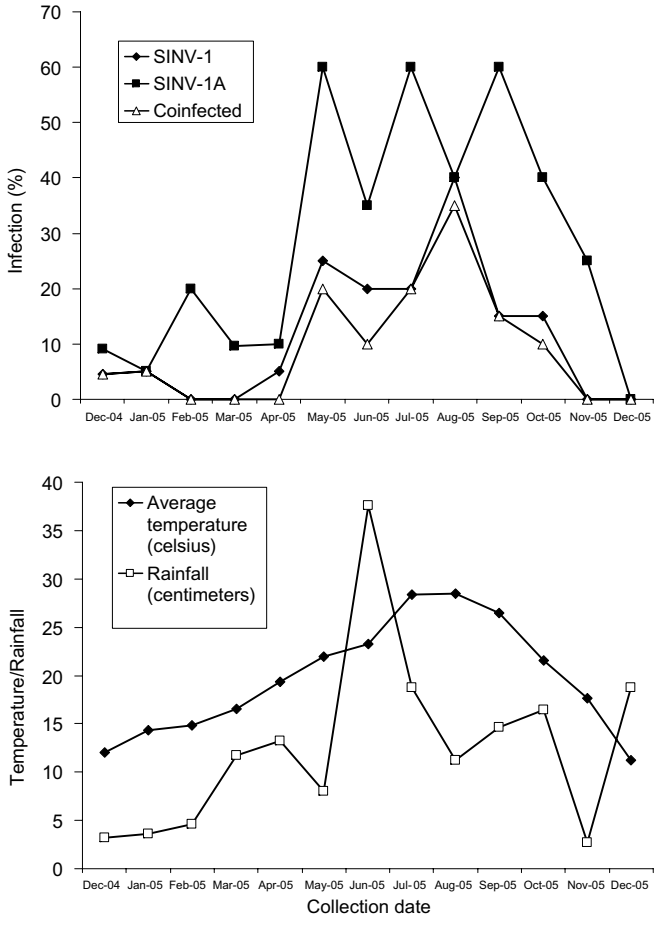

Fig. 1. Relationship between *S. invicta* infected with SINV-1 (genotypes-1 and 1-A) and collection date in Florida (top panel). Colonies were surveyed by analyzing a pool of 20–30 workers for the presence of SINV-1 or -1A by RT-PCR. A total of 20 colonies were evaluated from two different sites in Gainesville, Florida. Average temperature and rainfall measurements for the indicated month are presented in the lower panel.

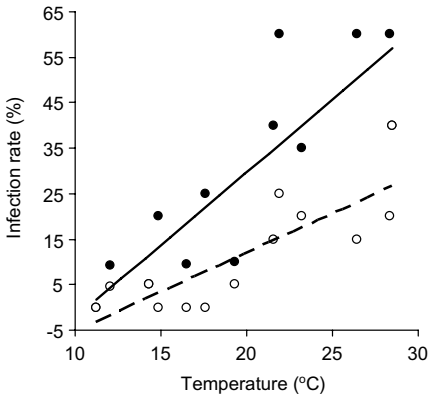

Fig. 2. Correlation between SINV-1 (○, dashed line) and SINV-1A (●, solid line) infection rate and average monthly temperature in Gainesville, Florida. (SINV-1, $r = 0.82$, $p < 0.0005$ and SINV-1A $r = 0.86$, $p < 0.0001$).

Pearson's correlation analysis revealed a significant positive relationship between mean monthly temperature and SINV-1 ($r = 0.82$, $p < 0.0005$) and SINV-1A ($r = 0.86$, $p < 0.0001$) infection rates in *S. invicta* colonies (Fig. 2). However, no significant relationship was observed between average monthly rainfall and SINV-1 ($r = 0.32$, $p < 0.28$) or SINV-1A ($r = 0.25$, $p < 0.41$) infection rates.

3.2. Geographic distribution and host specificity

SINV-1 was widely distributed among *S. invicta* populations (Figs. 3 and 4). The virus was detected in all U.S. states examined with the exception of New Mexico. SINV-1A appeared to be most common in eastern states and Texas, while SINV-1 was most prevalent in Oklahoma and Louisiana. Indeed, SINV-1A was not found in any ant sample examined from Oklahoma and Louisiana. Both virus genotypes were also detected in *S. invicta* colonies sampled from northern Argentina. Interestingly, the virus infection rates were lower among Argentinean *S. invicta* compared with U.S. *S. invicta*. The SINV-1 infection rate was significantly higher in *S. invicta* from the U.S. ($13.6 \pm 3.2\%$) compared with *S. invicta* in Argentina ($2.8 \pm 1.2\%$) ($t = -3.9$, $p < 0.001$). However, there was no significant difference in SINV-1A infection rate between *S. invicta* in the U.S. ($18.2 \pm 10.0\%$) and Argentina ($8.9 \pm 2.8\%$) ($t = -1.3$, $p < 0.2$).

SINV-1 and -1A were also detected in other *Solenopsis* species. SINV-1 was detected in *S. richteri* and the *S. invicta/richteri* hybrid collected from northern Alabama and *S. geminata* from Florida. SINV-1A was detected in *S. geminata* and *S. carolinensis* in Florida and the *S. invicta/richteri* hybrid in Alabama. Despite examination of 121 colonies from California and New Mexico, neither virus genotype was detected in *S. xyloni*. Similarly, we

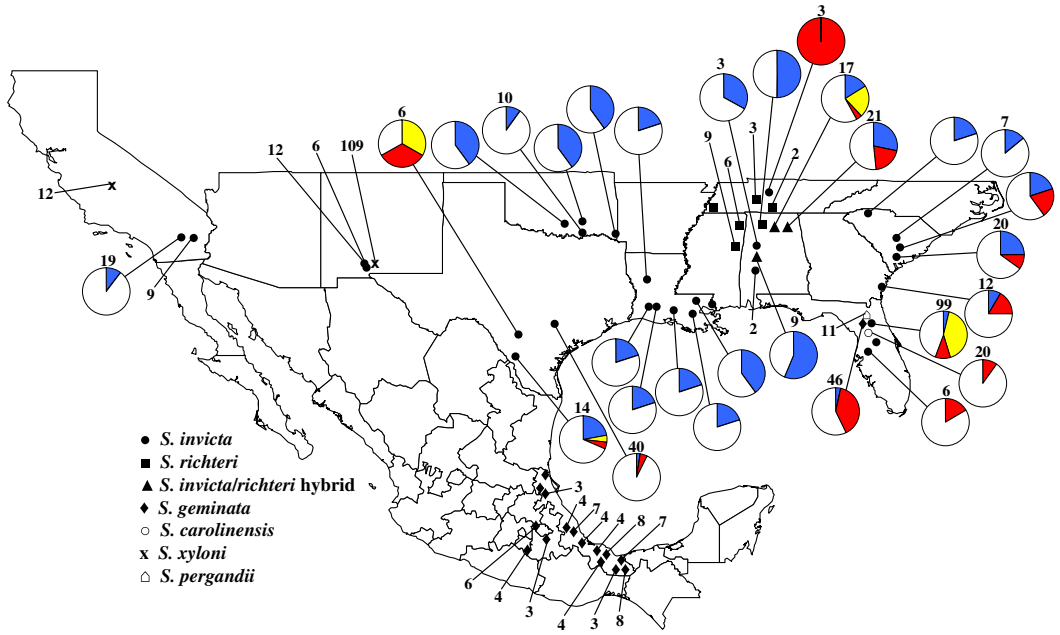

Fig. 3. Geographic distribution of SINV-1 and SINV-1A among *Solenopsis* species collected throughout the United States and Mexico. Species is indicated by the key at the lower left. Pie charts represent infection rate with SINV-1 (blue), SINV-1A (red), and those colonies infected with both genotypes (yellow). The number of nests sampled is provided at the top of each pie chart or at the end of each placement line in cases where virus was not detected. Numbers represent colonies sampled if sample size is different from five.

did not detect either virus genotype in *S. richteri* from Argentina or *S. geminata* from Mexico or Hawaii.

A total of 1989 arthropods was collected from the six pitfall trap experiments from Gainesville and Williston, FL (Table 1). The majority of the collections were comprised of insect species in the family Formicidae. Among all the arthropods collected in the pitfalls, none tested positive for SINV-1 or SINV-1A except for *S. invicta*. Indeed, five out of six of the pitfall collections included captured *S. invicta* testing positive for either SINV-1 or SINV-1A indicting that the virus was present in these areas. Excluding *S. invicta*, 1523 ants from 16 genera tested negative for SINV-1 and -1A. Likewise, 282 other arthropods in four classes and 10 families within the Hexapoda were negative for SINV-1 and -1A.

SINV-1 did not appear to infect or replicate within the Sf9 or Dm-2 cells *in vitro* (Fig. 5). The number of SINV-1 genome copies did not significantly increase over the course of the experiment (0–5 days). However, over the same period, the number of Sf9 and Dm-2 cells did increase significantly. Thus, SINV-1 did not appear to have any detrimental effect on these cell lines.

3.3. Phylogenetic analysis

Phylogenetic analysis of nucleotide sequences revealed similar phylograms for both SINV-1 genotypes; Argentinean and U.S. sequences formed distinct groups indicating divergence. North American sequences from SINV-1 and SINV-1A derive from a common branch from Argentinean sequences (Fig. 6).

4. Discussion

SINV-1 infection of *S. invicta* workers exhibited a seasonal variation that correlated well with temperature but not rainfall (Figs. 1 and 2). Prevalence was highest during months with the highest average monthly temperatures in Florida (May through September). Bailey et al. (1981, 1983) reported a similar increased incidence of black queen-cell virus (BQCV), sacbrood virus (SBV), bee virus Y (BVY) and acute bee paralysis virus (ABPV) in honey bees during the spring and summer months in Britain. They suggested that fecal depositions containing virus accumulate onto combs during winter which is subsequently spread throughout the colony by house-keeping bees in the spring, presumably by trophallaxis. Tentcheva et al. (2004) similarly reported a higher incidence of SBV and ABPV during the spring and summer in France. They postulated that seasonal variation may be caused by differences in bee susceptibility to the virus, changes in the environment (such as food quality), or the role of the parasitic mite, *Varroa destructor*. Although the growth rate of *S. invicta* colonies decreases during colder time periods (Porter, 1988), they actively forage all year long in north central Florida (Porter and Tschinkel, 1987) and are not confined to the nest as are honeybees. We also concur with Bailey et al.'s (1983) conclusion that viral prevalence is linked to increased growth rate of the colony. Many single-stranded RNA viruses are present in the gut and gut contents of their insect hosts (e.g. Gildow and D'Arcy, 1990; van den Heuvel et al., 1997; Nakashima et al., 1998; Fievet et al., 2006). Preliminary examinations have

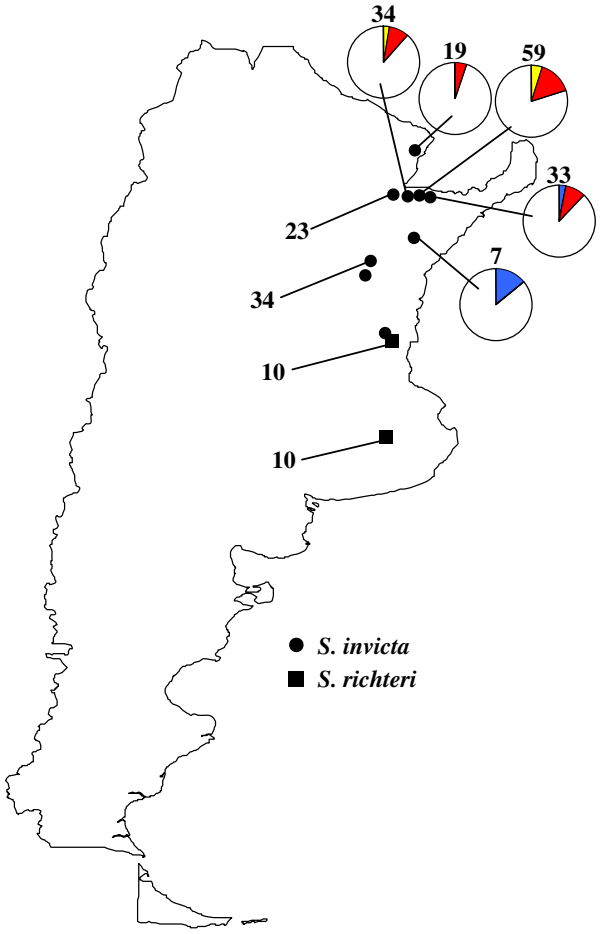

Fig. 4. Geographic distribution of SINV-1 and SINV-1A among *S.* invicta and *S. richteri* collected in central and northern Argentina. Pie charts represent infection rate with SINV-1 (blue), SINV-1A (red), and those colonies infected with both genotypes (yellow). The number of colonies sampled is provided at the top of each pie chart or at the end of each placement line in cases where SINV was not detected. In cases where no number is provided the sample size is 5.

localized SINV to the gut and gut contents of several stages of *S. invicta* (Valles and Hashimoto, unpublished data). Thus, it is possible that when fire ant colonies are growing rapidly during the spring and summer months, SINV-1 exchange via trophallaxis could be greatest. Alternatively, decreased SINV-1 prevalence during cooler time periods may simply be the result of colony death. It is widely considered that external stressors may induce active viral replication of single-stranded RNA viruses resulting in host death (Bailey, 1967; Fernandez et al., 2002; Tentcheva et al., 2004).

SINV-1 was found in *S. invicta* throughout the U.S. and Argentina (Figs. 3 and 4). The SINV-1A genotype was conspicuously absent from Oklahoma, Louisiana, and western Alabama. However, because SINV-1 and SINV-1A were found in *S. invicta* from Texas, Florida, South Carolina, Tennessee, and Georgia, it is most likely that both genotypes are found throughout the U.S. range and lack of detection in some areas was possibly due to low sample sizes. The overall mean (±SE) infection rates of SINV-1 and SINV-1A in the U.S. (13.6 ± 3.2 and 18.2 ± 9.9) were consistent with the previously reported values (Valles et al., 2004; Valles and Strong, 2005). Pattern of infection of *S. invicta* exhibited by SINV-1 is consistent with other insect-infecting single-stranded RNA viruses in that they are regularly present in the population, often at very high levels, but do not produce any overt symptoms in their hosts (e.g. Tentcheva et al., 2004; Berenyi et al., 2006). The widespread distribution exhibited by SINV-1 is also consistent with related viruses in honeybees in Europe (Berenyi et al., 2006) and other countries (Bailey, 1967). Similarly, *Drosophila* A, C, and P viruses (DAV, DCV, DPV) exhibit a worldwide distribution among *Drosophila* species (Plus et al., 1975; Brun and Plus, 1980; Christian and Scotti, 1998). Furthermore, virus infection in *Drosophila* species was skewed toward warm countries; higher infection rates were observed in tropical than in temperate countries (Plus et al., 1975). These observations agree with our data showing a correlation between temperature and SINV-1 infection rate in *S. invicta*.

With the exception of *S. invicta*, none of the arthropods captured in pitfall traps tested positive for SINV-1.

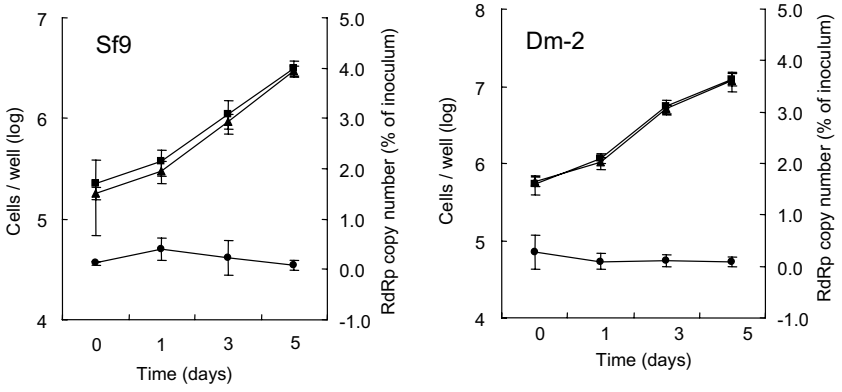

Fig. 5. *In vitro* exposure of Sf9 and Dm-2 cells with SINV-1. Cells exposed to SINV-1 (▲) and those treated as controls (■) correspond to the left axis. SINV genome copy number (●) was measured at various time intervals after exposure by amplifying part of the RdRp region (right axis).

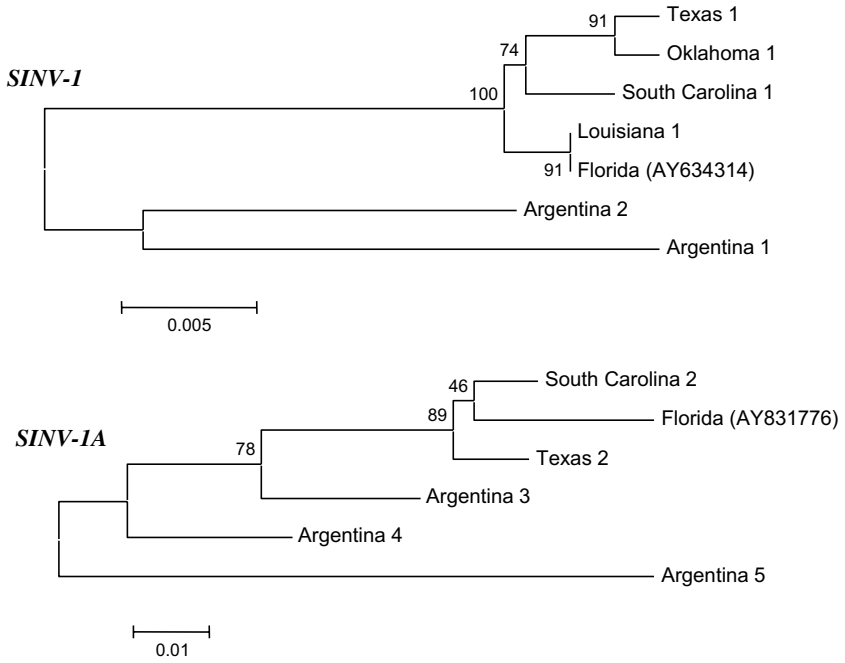

Fig. 6. Phylogenetic analysis of nucleotide sequences of structural protein regions generated with genotype-specific oligonucleotide primers for SINV-1 and SINV-1A infecting North American and South American *S. invicta*. Bars represent the number of nucleotide changes per site per replication.

SINV-1 or -1A infection was detected in *S. invicta* from five of the six (83%) pitfall collection sites indicating that virus was obviously present in the community. Among a total of 1989 arthropods captured, the majority (1707) were ant species (Formicidae). Interestingly, SINV-1 was found to infect other ant species within the *Solenopsis* genus. In addition to *S. invicta*, *S. geminata*, *S. richteri*, the *S. invicta*/*richteri* hybrid, and *S. carolinensis* also tested positive for at least one of the SINV-1 genotypes (Figs. 1 and 2). However, despite examination of 121 nests from California and New Mexico, SINV-1/-1A were not detected in *S. xyloni*. We did not detect SINV-1/-1A in *S. geminata* from Mexico or Hawaii either. Although some insect-infecting single-stranded RNA viruses have broad host ranges, many are confined to a single species or genus. For example, the cricket paralysis virus (CrPV) has been detected or isolated from at least 22 insect species in five insect orders (see citations in Christian and Scotti, 1998). Conversely, DCV is limited to *D. melanogaster*, *Drosophila simulans*, and *Drosophila* cultured cells (Brun and Plus, 1980; Christian and Scotti, 1998). Attempts to infect cultured cells (Dm-2 and Sf9) with SINV-1 also failed to yield production of virus or cytopathic effects. Thus, the SINV-1 infection appears to be limited to several species within the *Solenopsis* genus.

Phylogenetic analysis of nucleotide sequences of structural protein regions generated with SINV-1 genotype-specific oligonucleotide primers indicated divergence between viruses infecting North American and South American *S. invicta* (Fig. 6). RNA viruses are known to exhibit high mutation rates (Domingo and Holland, 1997) on the order of 10^{-4} to 10^{-3} per nucleotide site per replication (Holland et al., 1982). Thus, the data indicate a significant duration of separation among North and South American SINV-1/-1A. Furthermore, the phylograms (SINV-1 and -1A) indicate that the North American virus has diverged more recently compared with those from Argentina. Although unsubstantiated, these data suggest that SINV-1 was introduced into North America along with founding *S. invicta* or *S. richteri*. This notion is supported by the lack of infection among other *Solenopsis* species (*S. geminata* and *S. xyloni*) in areas completely devoid (Mexico and Hawaii) or with incipient infestations of *S. invicta* (New Mexico and California). We hypothesize that SINV-1 infection of *S. geminata* and *S. carolinensis* (in Florida) may have originated from introduced *S. invicta* or *S. richteri* to these native species. Additional phylogenetic study may provide a more conclusive determination of the origin of SINV-1 and may even provide additional insight into *Solenopsis* complex phylogenetic relationships. Rapidly-evolving RNA viruses can provide details about host population structure and demographic history that might not be possible from host genetic data alone (Biek et al., 2006).

We report studies examining the phenology, geographic distribution, and host specificity of the *S. invicta* virus-1. The virus is distributed widely among *S. invicta* in North and South America. The virus was not found to infect any arthropod examined outside the *Solenopsis* genus. However, it was detected in congeners, including *S. geminata*, *S. richteri*, the *S. invicta*/*richteri* hybrid, and *S. carolinensis*. Phylogenetic analysis of structural protein-encoding nucleic acid sequences indicated significant divergence among SINV-1 infecting North and South American *S. invicta*.

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