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Source: Florida Entomologist, 93(1) : 80-88

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.093.0111

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CHARACTERIZATION OF *SOLENOPSIS INVICTA* (HYMENOPTERA: FORMICIDAE) POPULATIONS IN VIRGINIA: SOCIAL FORM GENOTYPING AND PATHOGEN/PARASITOID DETECTION

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ABSTRACT

Red imported fire ant, *Solenopsis invicta* Buren, workers were sampled from 26 colonies in Virginia during the 2007-2008 time period. Polymerase chain reaction (PCR) assays were used to determine colony social form (monogyny or polygyny) by genotyping ants at the *Gp-9* locus. Twenty of the colonies (76.9%) were found to be polygyne. Multiplex PCR was also used to detect the presence of several organisms currently being used as biological control agents for fire ants in the U.S., including the microsporidian parasite *Kneallhazia solenopsae* and *Pseudacteon* spp. parasitioid decapitating phorid flies in the sampled colonies. *Kneallhazia solenopsae* was detected in 11 of 26 colonies (42%). In addition, *Pseudacteon* spp. flies were detected in 2 (7.7%) colonies. The sampled colonies were examined by reverse transcription PCR (RT-PCR) for the presence of *Solenopsis invicta* viruses -1 and -2. Results indicated that 5 colonies were infected with SINV-1 (19%) and none were infected with SINV-2. This study is the first to characterize the red imported fire ant infestation in Virginia and documents the presence of biological control agents in this area.

Key Words: monogyne, polygyne, *Kneallhazia solenopsae, Solenopsis invicta* virus-1, *Pseudacteon*

RESUMEN

Se realizó un muestreo de las obreras de la hormiga de fuego roja importada, *Solenopsis invicta* Buren, en 26 colonias en el estado de Virginia durante el período del 2007 al 2008. Se utilizaron ensayos de la reacción en cadena por la polimerasa (RCP) para determinar la forma social de la colonia (monoginia = una sola reina reproductiva) o poliginia = reinas multiples) por medio del genotipo de las hormigas en el locus *Gp-9*. Se encontró que veinte de las colonias (76.9%) fueron poliginias. Se usó RCP múltiplex para detectar la presencia de varios organismos que estan usando actualmente como agentes de control biológico para las hormigas de fuego en los Estados Unidos, incluyendo el parasito microesporidiano *Kneallhazia solenopsae,* y la mosca *Pseudacteon* spp. (Diptera: Phoridae) que son parasitoides que decapitan las hormigas en las colonias muestreadas. *Kneallhazia solenopsae* fue detectado en 11 de las 26 colonias (42%). Además, se detectaron las moscas *Pseudacteon* spp. en dos (7.7%) de las colonias. Se examinaron las colonias muestreadas por la transcripción reversa de RCP (TR-RCP) para la presencia de los virus 1 y 2 de *Solenopsis invicta*. Los resultados indican que las 5 colonias fueron infectadas con SINV-1 (19%) y ninguna fue infectada con SINV-2. Este es el primer studio que caractiza la infestación de la hormiga de fuego roja importada en Virginia y documenta la presencia de agentes de control biológico en esta área.

The spread and current range of the red imported fire ant, *Solenopsis invicta* (Buren,) in the United States has been well documented (George 1958; Lofgren 1986, Callcott & Collins 1996; Williams et al. 2001). Currently, *S. invicta* infests over 150 million hectares of land in Alabama, Arkansas, Arizona, California, Florida, Georgia, Louisiana, Mississippi, New Mexico, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, Virginia, and Puerto Rico (Kemp et al. 2000; Vander Meer et al. 2007).

Although fire ant infestations have been observed throughout the southeastern U.S. since the 1930s, these aggressive ants were not documented in Virginia until 1989 (Gina Goodwyn, Virginia Department of Agriculture and Consumer Services, personal communication).

The initial specimens were collected from Hampton, Virginia, located on the southeastern coast of the state. Virginia's Department of Agriculture and Consumer Services (VDACS) has been responsible for documenting and treating all

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fire ant mounds that have appeared within Virginia. Since 1989, the number of fire ant-infested areas and mounds documented by VDACS has increased annually. From 1989 to 1999, VDACS identified 136 sites that were infested with *S. invicta*. From 2000-2006 the number of infested sites increased to 541. The largest number of mounds identified and treated by VDACS has been located in the developing coastal cities of Chesapeake (3,000 mounds) Norfolk (1,700 mounds), and Virginia Beach (2,400 mounds) (Gina Goodwyn, Virginia Department of Agriculture and Consumer Services, personal communication), which are all cities located in the Hampton Roads area. The fact that *S. invicta* is so prevalent in the southeastern region of Virginia is most likely due to the fact that this area has experienced the most rapid urban development within the state. Tschinkel (1988) found a positive correlation between areas of development (building construction) and fire ant presence.

As of 2009, *S. invicta* populations in Virginia are not as widespread as infestations observed in other southern states such as Texas and Florida; however, several predictive models have suggested that fire ants can certainly become established within the Virginia cities and counties immediately outside of the Hampton Roads area (Korzukhin et al. 2001; Morrison et. al 2004). In 2009, the cities of Chesapeake, Hampton, Newport News, Norfolk, Poquoson, Portsmouth, Suffolk, Virginia Beach, and Williamsburg and the counties of James City and York were placed temporarily under the Federal Red Imported Fire Ant Quarantine which aims to restrict the movement of fire ants from the quarantined areas to non-quarantined areas in the state. As a result of the quarantine's implementation, VDACS is no longer responsible for treating fire ant mounds in the quarantined cities and counties. Based on the increases in *S. invicta* incidents reported to VDACS and the recent implementation of the quarantine by APHIS, it is apparent that there is need for baseline biological and ecological information regarding *S. invicta* populations in Virginia.

In response to the rapid spread of *S. invicta* in the southern states, many of the states have developed fire ant research programs to determine the impacts of *S. invicta* on other organisms, how the ants were able to spread, and to establish optimal control strategies. Indeed, colony social form has been reported to play a major role in the dispersal, control and environmental impacts of *S. invicta* (Lofgren & Williams 1984; Glancey et al. 1987; Porter et al. 1988; Porter et al. 1991; King et al. 2009).

Fire ant colonies exhibit 1 of 2 social forms. Colonies contain either 1 egg laying queen (monogyne) or multiple egg-laying queens (polygyne) (Glancey 1973). Monogyne and polygyne fire ant colonies differ in many aspects of their biology including their reproductive strategies, territoriality, and colony founding methods (Keller & Passera 1989; Keller & Ross 1999). For example, polygyne fire ant colonies have a higher mound density in a given area than monogyne colonies (Tschinkel 2006) and the multiple colonies have a greater influence on local ecology, impacts on human activities and strategies necessary for control (King & Tschinkel 2009).

Colony social form is associated with *general protein-9* (Gp-9) (Keller & Ross 1999). Monogyne ants are consistently homozygous for the B allele ($Gp\text{-}9^{BB}$) and polygyne ants are heterozygous possessing both alleles ($Gp\text{-}9^{Bb}$). Previous work conducted by Valles et al. (2003) has shown that these 2 alleles can be distinguished from one another by multiplex PCR.

Until recently, chemical control methods were the only means available for *S. invicta* control (Williams et al. 2001). However, as part of the USDA's fire ant areawide suppression program, the effectiveness of several biological control agents are currently being examined and/or released in the U.S. These agents include the microsporidian parasite, *Kneallhazia solenopsae,* and decapitating flies found in the genus *Pseudacteon* (Pereira 2003).

The purpose of this study was twofold. First, we assessed the colony social form of *S. invicta* colonies sampled from Virginia. Social form data are intended to help direct future quarantine and control efforts and may also aid researchers in predicting the potential rate of spread of *S. invicta* colonies in Virginia. The second goal was to determine if any biological control agents were present in Virginia *S. invicta* colonies. To date, no biological control agents have been released in Virginia for *S. invicta* control. Therefore, determining whether these agents are already present or not in Virginia would guide future control efforts and releases.

Materials and Methods

Fire Ant Sample Collection

Red imported fire ant workers, *Solenopsis invicta* Buren, were collected from 26 different colonies located in 7 different cities (Norfolk, Virginia Beach, Chesapeake, Portsmouth, Suffolk, Roanoke, and Richmond) within the state of Virginia from 2007 through 2008. Individual workers were collected by disturbing the mound and removing the emerging workers with a hand-held aspirator (BioQuip, Rancho Dominguez, CA). At least 10 worker ants were collected from each mound. All ant samples were placed in glass vials (Acme Glass and Vial Co., Paso Robles, CA) containing 90-95% ethanol. Vials were labeled with the date of collection and location information.

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DNA Preparation

Genomic DNA was extracted from the ants as described by Valles et al. (2002). Ten worker ants from each sample were removed from the collection vials and blotted dry with a clean paper towel. The dried ants (10 per sample) were placed into separate 1.5-mL microcentrifuge tubes (Eppendorf, Westbury, NY) containing 150 µL of lysis buffer (50 mM Tris-HCl, pH 8, 4% sodium dodecyl sulfate, and 5% 2-mercaptoethanol). Ants were homogenized by hand with a plastic pestle for 15-20 s. After homogenization, 200 µL of phenol:chloroform:isoamyl alcohol (Tris-HCl-saturated, pH 8) were added to the tube. The microcentrifuge tube was inverted 4-5 times until the solution became milky white. The samples were then centrifuged for 5 min at 20,817*g* in a model 5417C centrifuge (Eppendorf, Westbury, NY). Fifty microliters of the DNA-containing layer (supernatant) were removed from the microcentrifuge tube and transferred to a new 1.5- mL microcentrifuge tube (Eppendorf, Westbury, NY). DNA was precipitated by adding 900 µL of 100% isopropanol to the microcentrifuge tube. The mixture was inverted 5 times and centrifuged for 5 min at 20,817*g*. The isopropanol was decanted from the microcentrifuge tube, and the pellet was washed twice with 500 µL of 70% ethanol and centrifuged for 5 min at 20,817*g*. Ethanol was decanted from the microcentrifuge tubes and the tubes were centrifuged for an additional 4 s (Eppendorf, Westbury, NY), further separating the supernatant and the pellet. The remaining ethanol was removed by micropipette. Pellets were allowed to dry in an incubator set at 37°C for 5 min. Pellets were re-suspended and dissolved in 50 µL of TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0). The purified DNA was analyzed spectrophotometrically to determine the concentration in each sample. All samples were diluted with TE buffer to achieve concentrations in the range of 50 to 100 ng DNA/µL.

RNA Preparation

Total RNA was isolated from 10 fire ant workers from each colony sample. Fire ant workers were removed from collection vials and blotted dry with a paper towel. Dried ants were placed in a 1.5-mL microcentrifuge tube. The ants were homogenized with a plastic pestle in 0.5 mL of Trizol reagent (Invitrogen, Carlsbad, CA). Chloroform (0.2 mL) was added to the homogenate, which was vortexed briefly and centrifuged at 20,817 g for 5 min at room temperature. The supernatant was transferred to a clean 1.5- mL microcentrifuge tube and 0.5 mL of isopropanol was added. The RNA pellet was rinsed once with 70% ethanol, dried, and suspended in 20 µL of DEPC-treated water. The RNA concentration was measured spectrophotometrically and diluted with DEPC-treated water to a concentration between 10 and 50 ng RNA/µL.

Polymerase Chain Reaction/Colony Social Form

Multiplex PCR was carried out in a PTC 100 thermal cycler (MJ Research, Waltham, MA) as described by Valles & Porter (2003). The *Gp-9*^{B} allele primers, 16BAS and 26BS were used to specifically identify samples from monogyne colonies and the *Gp-9*^{b} allele specific primers, 24bS and 25bAS were used to identify polygyne samples. The *Gp-9*^{B} primers corresponded to nucleotide positions 2167-2199 (16BAS) and 1683-1703 (26BS). The *Gp-9*^{b} allele primers correspond to positions 1307-1334 (24bS) and 1702-1729 (25bAS). PCR was conducted in a 50 µL volume containing 0.5 µL of prepared DNA (25 to 50 ng), 5 µL PCR buffer (10X), 2 µL of 50 mM $MgCl_2$, 1 µL of 10mM dNTP, 0.4 µL Platinum Taq polymerase (Invitrogen, Carlsbad, CA), 33.1 µL of H_20, and 2 µL of each primer (P16, P24, P25, and P26). The PCR process was conducted under the following temperature conditions: 1 cycle at 94°C for 2 min, then 35 cycles at 94°C for 15 s, 55°C for 15 s, and 68°C for 30 s, and a single elongation step at 68°C for 5 min. PCR products (17 µL) were separated on a 1% agarose gel and visualized by ethidium bromide staining. Positive and negative controls were run for both monogyne and polygyne samples.

Kneallhazia solenopsae and *Pseudacteon* spp. Detection

Oligonucleotide primers specific to the 16S rDNA gene (Moser et al., 1998; Moser et al., 2000; Valles et al. 2002; accession number: AF031538) of *K. solenopsae* (P1: 5'CGAAGCATGAAAGCGGAGC and P2: 5'CAGCATGTATATGCACTACTGGAGC) and the 18S rDNA gene of *Pseudacteon* flies (P800: 5'GTAGTACACCTATACATTGGGTTCGTACATTACTCTA and P801: 5'ATAAGTTTCAACGCTATAATCCTGAAAGCATC) were used in a multiplex PCR to detect the presence of *K. solenopsae* and *Pseudacteon* spp. genetic material within the Virginia ant samples (Valles et al. 2009). Multiplex PCR was initiated by the hot start method in a PTC 100 thermal cycler (MJ Research, Waltham, MA) under the following optimized temperature regime: 1 cycle at 94°C for 2 min, then 35 cycles at 94°C for 15 s, 55°C for 15 s, and 68°C for 50 s, followed by a final elongation step of 5 min at 68°C (Valles et al. 2009). The reactions were carried out in a 25-µLvolume containing 2 mM $MgCl_2$, 200 µM dNTP mix, 0.5 units of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.4 µM of primers P1 and P2, 0.05 µM of primers P800 and P801, and 25 to 50 ng of genomic DNA. PCR products were separated on a 1% agarose gel and visualized by

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ethidium bromide staining. For all experiments, positive and negative controls were conducted simultaneously.

Verification of *Pseudacteon* spp. Parasitization

To verify that the corresponding amplicon was produced from a *Pseudacteon* fly species by PCR, the produced amplicon was cloned and sequenced. The agarose gel-purified amplicon was ligated into pCR4-TOPO vector, transformed into TOP10 competent cells (Invitrogen, Carlsbad, CA), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). The sequences were subjected to BLASTn analysis (Altschul et al. 1997) then aligned with identified sequences with the Vector NTI software suite (Invitrogen, Carlsbad, CA).

Detection of *Solenopsis invicta* Viruses

One-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to identify *Solenopsis invicta* viruses 1 and 2 (SINV-1, -2) in *S. invicta* worker ants from Virginia. cDNA was synthesized and subsequently amplified by the One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) with oligonucleotide primers p517 (5'CAATAGGCACCAACGTATATAGTAGAGATTGGA) and p519 (5'GGAATGGGTCATCATATAGAAGAATTG) to detect SINV-1 (Hashimoto et al. 2007) and p64 (5'ATTTGTTTTGGCCACGGTCAACA) and p65 (5'GATGATACAAAGCATTAGCGTAGGTAAACG) to detect SINV-2 (Valles et al. 2007). RT-PCR was conducted in a PTC 100 thermal cycler (MJ Research, Waltham, MA) under the following optimized temperature regimen: 1 cycle at 45°C for 30 min, 1 cycle at 94°C for 2 min, 35 cycles of 94°C for 15 s, 56°C for 15 s, 68°C for 30 s, followed by a final elongation step of 68°C for 5 min. Amplicons were separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Results

Colony Social Form

Both monogyne and polygyne fire ant colonies, were positively identified from the Virginia ant samples (Fig. 1, Table 1). Among the 26 fire ant colonies sampled, 20 (76.9%) were heterozygous at the *Gp-9* ($Gp\text{-}9^{Bb}$) locus indicating that the ants were of polygyne form (Table 1). The remaining 6 colonies were homozygous ($Gp\text{-}9^{BB}$) and considered monogyne.

Detection of *Kneallhazia solenopsae* and Decapitating Phorid Flies in *S. invicta* Colonies

Multiplexed PCR products for both *K. solenopsae* and *Pseudacteon* spp. phorid flies are shown in Fig. 2. Of the 26 sampled colonies, 11 (42%) were infected with *K. solenopsae* (Table 2) and

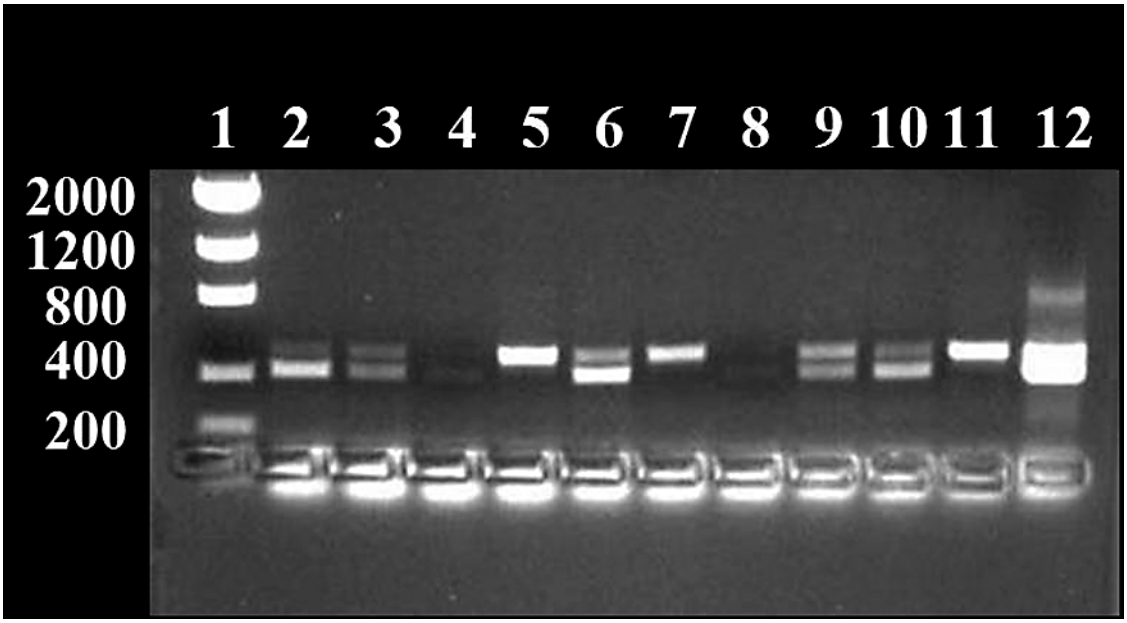

Fig. 1. Multiplex PCR banding patterns of Virginia monogyne and polygyne fire ant colonies separated on a 1% agarose gel. Lane 1, molecular weight marker expressed as base pairs; lane 2, polygyne band; lane 5, monogyne band; lane 11, positive monogyne control; lane 12, positive polygyne control.

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TABLE 1. DISTRIBUTION AND PERCENTAGE OF MONOGYNE AND POLYGYNE *S. INVICTA* COLONIES COLLECTED IN VIRGINIA CITIES.

Site	No. of Samples	No. of Monogyne Colonies	% Monogyne	No. of Polygyne Colonies	% Polygyne
Chesapeake*	12	1	8.3	22	91.7
Norfolk*	1	0	0	1	100
Portsmouth*	1	0	0	1	100
Richmond	1	1	100	0	0
Roanoke	2	0	0	2	100
Suffolk*	3	2	66.7	1	33.3
Virginia Beach*	6	2	33.3	4	66.7
Total	26	6	23.1	20	76.9

*Indicates cities in Hampton Roads.

worker ants from 2 colonies were parasitized by phorid flies. Among the 11 colonies infected with *K. solenopsae*, 8 (73%) were polygyne and 3 (27%) were monogyne (Fig. 2). Phorid fly genetic material was found in ant samples collected from 2 different locations: Chesapeake and Virginia Beach (Table 2). One of the *Pseudacteon*-parasitized colonies was polygyne and 1 was monogyne. The small ribosomal subunit (18S) sequences from both of the ant samples (Chesapeake and Virginia Beach) revealed that the fly genes were identical. The *P. tricuspis* 18S gene was sequenced in an effort to determine which *Pseudacteon* species was discovered in Virginia. Unfortunately, definitive species identification could not be determined (Table 3) because the sequences for *P. litoralis* and *P. obtusus* are identical to *P. tricuspis* in the sequenced region. However, sequence differences were sufficient to exclude *P. cultellatus* and *P. curvatus*. Thus, the Virginia fire ant samples were likely *P. litoralis*, *P. obtusus*, or *P. tricuspus*—all species released in the United States as biological control agents.

Detection of *Solenopsis invicta* Viruses

SINV-1 was detected in 5 of the 26 (19%) sampled fire ant colonies (Table 4). SINV-2 was not detected in any of the colonies.

DISCUSSION

Polygyne and monogyne fire ant colonies are present in Virginia and appear to be established in this state. Although, both social forms were collected, sample data suggest that the majority of *S.*

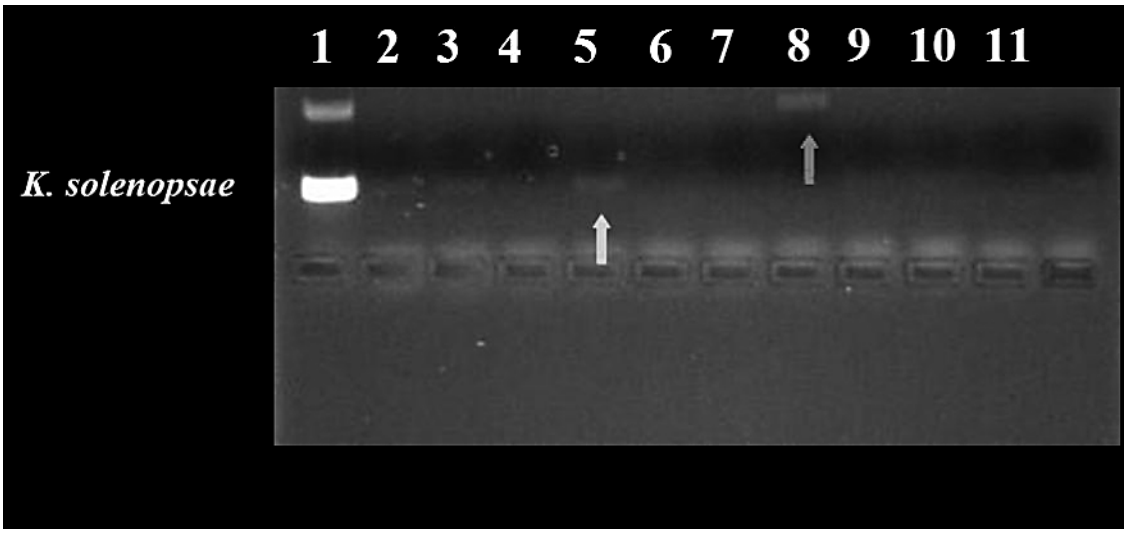

Fig. 2. Multiplex PCR banding patterns of *Pseudacteon* spp. phorid and *K. solenopsae* on a 1% agarose gel. Lane 1, top band positive *Pseudacteon* spp. control, bottom band positive *K. solenopsae* control; lane 5, *K. solenopsae* infected fire ant worker, lane 8 *Pseudacteon* spp. phorid fly parasitized fire ant worker.

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TABLE 2. DISTRIBUTION AND PERCENTAGE OF VIRGINIA FIRE ANT COLONIES PARASITIZED BY *PSEUDACTEON* SPP. PHORID FLIES AND INFECTED WITH *T. SOLENOPSAE*.

Site	No. of Samples	No. of Colonies Parasitized by *Pseudacteon* spp. flies	Percentage of *Pseudacteon* spp. flies parasitization	No. of Colonies Infected with *T. solenopsae*	Percentage of *T. solenopsae* infection
Chesapeake	12	1	8.3	5	41.7
Norfolk	1	0	0	1	100
Portsmouth	1	0	0	0	0
Richmond	1	0	0	1	100
Roanoke	2	0	0	0	0
Suffolk	3	0	0	1	33.3
Virginia Beach	6	1	16.7	3	50
Total	26	2	7.7	11	42.3

TABLE 3. NUCLEOTIDE DIFFERENCES OBSERVED IN THE SMALL RIBOSOMAL SUBUNIT (18S) SEQUENCES OF *PSEUDACTEON* PHORID FLIES FROM VIRGINIA AND GENBANK DATABASE[A] SEQUENCES.

	Nucleotide position			
Sequence origin	266	630	796	887
Pseudacteon cultelatus	C	T	A	C
Pseudacteon littoralis	A	T	A	C
Pseudacteon obtusus	A	T	A	C
Pseudacteon curvatus	A	C	T	T
Pseudacteon (Chesapeake)	A	T	A	C
Pseudacteon (Virginia Beach)	A	T	A	C

[A]GenBank accession numbers are provided in the text.

TABLE 4. DISTRIBUTION AND PERCENTAGE OF VIRGINIA FIRE ANT COLONIES INFECTED WITH *SINV*.

Site	No. of Samples	No. of Colonies Infected with *SINV*	Percentage of *SINV* infection
Chesapeake	12	2	16.7
Norfolk	1	0	0
Portsmouth	1	0	0
Richmond	1	0	0
Roanoke	2	0	0
Suffolk	3	0	0
Virginia Beach	6	3	50
Total	26	5	19.2

invicta colonies in Virginia may be polygyne. This finding is particularly relevant to control efforts because polygyne colonies tend to be associated with higher mound and population densities (Macom & Porter 1996). The higher mound density is usually accomplished through colony budding, a process that can be initiated by the use of improper control techniques. During the budding process, workers from an established colony leave their original nest with 1 or more fecund queens, to found a new colony several meters away from the parent colony (Holldobler & Wilson 1990; Tschinkel 2006). Budding fire ant colonies are able to multiply quickly and 1 mound has the potential to split off into as many as 8 mounds in less than 5 months (Vargo & Porter 1989).

Twenty three of the 26 ant colony samples were collected from colonies located in the Hampton Roads area of Virginia (Table 1) and according to data provided by Virginia's Department of Agriculture and Consumer Services, population and mound densities in Hampton Roads are higher than those observed in other areas of the state. In the U.S., *S. invicta* ants were first identified in

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seaports located in Mobile, AL (Tschinkel 2006). Similarly, Hampton Roads is located on the southeastern coast of Virginia near the North Carolina border. The Hampton Roads area of Virginia is also a major port area and many of the more recent *S. invicta* infestations outside of the U.S. have occurred via human transport near port towns (King et al. 2008). Thus, the potential for repeated introductions of the ants into Virginia is high.

The discovery of polygyne colonies in Virginia has particular relevance to current, as well as, future *S. invicta* management practices (King et al. 2008). At present, some of the more successful fire ant control products are not labeled for use in Virginia and with the recent implementation of the Federal Fire Ant Quarantine, additional control measures will be needed. Consequently, we wanted to determine if any fire ant biological control organisms were present in the sampled colonies. We discovered that worker ants from several colonies were either parasitized by *Pseudacteon* decapitating phorid flies or infected by *K. solenopsae*. Five colonies were also infected with *Solenopsis invicta* virus 1, but none with SINV-2. None of these organisms have been intentionally released in Virginia, but both *K. solenopsae* and several *Pseudacteon* phorid fly species have been successfully released in the U.S. as part of an areawide fire ant suppression program being conducted by the United States Department of Agriculture (Vander Meer 2007).

Of the 11 Virginia colonies infected with *K. solenopsae*, 3 were monogyne. This finding is of particular interest because *K. solenopsae* infections of monogyne colonies are considered rare among North American colonies (Oi et al. 2004). However, Fuxa et al. (2005) did document a 63% monogyne colony infection rate in a multiple colony, monogyne *S. invicta* population in Louisiana. Milks et al. (2007) also examined the prevalence of *K. solenopsae* in Louisiana *S. invicta* colonies, and reported that monogyne colony infections accounted for about 20% of the total number of infected colonies.

Pseudacteon flies were detected in 2 of the worker ant samples. This discovery is interesting because the nearest release site for *Pseudacteon* flies was approximately 100 miles away from the sampled region on the east coast of North Carolina (S. D. Porter, USDA-CMAVE, Gainesville, Florida, personal communication). Identification of this fly in Virginia raises the possibility that parasitoid *Pseudacteon* flies may be spreading naturally in the U.S. Several different fly species including *P. curvatus*, *P. littoralis*, *P. obtusus*, and *P. tricuspus* have been released by the USDA. Attempts were made to differentiate between the 2 positive finds in the Virginia ant colonies with genetic sequencing. However, sequence data for the 18S rDNA gene could only exclude *P. cultellatus*, which has not been released as a biological control agent, and *P. curvatus*. Because *Pseudacteon* introductions were not specifically conducted in Virginia, it is a distinct possibility that previously infected ant colonies were brought into the state and are the source of the *Pseudacteon* introduction.

The findings presented in this report indicate that biological control organisms (*Pseudacteon* flies, *K. solenopsae*, and SINV-1) may likely be established in additional Virginia *S. invicta* colonies. Therefore, these organisms may be a practical addition to an integrated pest management program designed for *S. invicta* in Virginia, similar to the USDA Areawide Fire Ant Suppression Program. Preliminary results of the suppression program have demonstrated that *K. solenopsae* and *Pseudacteon* flies have become established in all of their release locations (Florida, Mississippi, Oklahoma, South Carolina, and Texas) and have reduced *S. invicta* populations by 85-99% (Vander Meer et al. 2007).

It is obvious that the small sample size (26) may not accurately represent the relative percentage of monogyne and polygyne colonies within the state of Virginia. The small sample size also limits our knowledge of the distribution and prevalence of biological control agents within the state. However, our results document that both social forms of *S. invicta* are present in Virginia and infected with *K. solenopsae*, *Pseudacteon* flies, and SINV-1. Many of the Virginia fire ant samples were collected from a single region, the Hampton Roads area. However, *S. invicta* infestations may be more widespread within the state, in areas that have not been sampled. Macom and Porter (1996) reported that polygyne colonies in the U.S. are distributed in a mosaic pattern separated from one another by monogyne colonies (Macom & Porter 1996, Mescher et al. 2003). This pattern may be present in the Virginia infestations because both social forms are documented to be present.

Although additional samples will be necessary to conclusively characterize the *S. invicta* populations in Virginia, this is the first report documenting the establishment, social form, and associated pathogens and parasites of *S. invicta* within the state. Undoubtedly, the *S. invicta* infestation is well established in Virginia. It is now evident that in order to extend the scope of knowledge on the expanding *S. invicta* population continuous statewide research efforts are necessary.

Acknowledgment

We thank Charles A. Strong for providing technical assistance, and the Plant and Pest Services division of Virginia's Department of Agriculture for providing fire ant incidence data.

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