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Authors: Valles, Steven M., Oi, David H., and Porter, Sanford D.

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KNEALHLAZIA (=THELOHANIA) SOLENOPSAE INFECTION RATE OF PSEUDACTEON CURVATUS FLIES DETERMINED BY MULTIPLEX PCR

STEVEN M. VALLES, DAVID H. OI AND SANFORD D. PORTER
Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, 1600 SW 23rd Drive, Gainesville, Florida, 32608, USA

ABSTRACT

A multiplex PCR method was developed and used to determine the infection rate of Kneallhazia solenopsae in individual Pseudacteon curvatus flies in north-central Florida. Among P. curvatus flies infected with K. solenopsae, 2 amplicons were produced, one of 800 nucleotides from the P. curvatus 18S rRNA gene, and one of 318 nucleotides from the K. solenopsae 16S rRNA gene. Multiplex PCR of DNA extracted from P. curvatus flies was capable of detecting 117.5 ± 82.7 K. solenopsae spore equivalents. The mean K. solenopsae infection rate of P. curvatus from 4 sites in Gainesville and Williston, Florida, was 12.3 ± 5.0%. The K. solenopsae infection rate for P. curvatus was independent of the K. solenopsae infection rate observed among S. invicta nests from where the fly collections took place. Not all P. curvatus flies that developed in K. solenopsae-infected fire ants were positive for K. solenopsae upon eclosion. Among 50 P. curvatus flies known to develop in K. solenopsae-infected S. invicta workers, 12 (24%) were positive for K. solenopsae at eclosion.

Key Words: Solenopsis invicta, red imported fire ant, phorid fly, biological control, parasite, entomopathogen, microsporidia

RESUMEN

Se desarrolló y utilizó un método de “RCP múltiplex” para determinar la tasa de infección de Kneallhazia solenopsae en individuos de la mosca Pseudacteon curvatus en el norte-central de la Florida. Entre las moscas P. curvatus infectadas con K. solenopsae, se produjeron 2 amplificaciones, uno de 800 nucleótidos del gene 18S rARN de P. curvatus y uno de nucleótidos del gene 16S rARN de K. solenopsae. El RCP múltiplex del ADN extraído de las moscas P. curvatus fue capaz de detectar 17.5 ± 82.7 de los equivalentes de las esporas de K. solenopsae. El promedio de la tasa de infección de K. solenopsae en las P. curvatus de 4 sitios en Gainesville y Williston, Florida, fue 12.3 ± 5.0%. La tasa de infección de K. solenopsae para P. curvatus fue independiente de la tasa de infección de K. solenopsae observado entre los nidos de Solenopsis invicta donde se hicieron las recolecciones de la mosca. No todas las moscas P. curvatus que se desarrollaron con las hormigas de fuego infectadas con K. solenopsae resultaron positivos para K. solenopsae al eclosionar. Entre las 50 moscas de P. curvatus que se desarrollaron con trabajadores de S. invicta infectadas con K. solenopsae, 12 (24%) fueron positivos para K. solenopsae al eclosionar.

Four species of Pseudacteon endoparasitoid flies (P. tricuspis Borgmeier, P. curvatus Borgmeier, P. litoralis Borgmeier, and P. obtusus Borgmeier) have been released and established in the USA for biological control of Solenopsis invicta and S. richteri (Graham et al. 2003; Williams et al. 2003; Porter et al. 2004; Vazquez et al. 2006; Gilbert et al. 2008). The parasitoid oviposits an egg into an adult fire ant worker and the maggot that hatches migrates to the head of the ant where it develops, pupates, and ultimately kills the host (Porter 1998).

Kneallhazia (=Thelohania) solenopsae is an intracellular microsporidian parasite that infects S. invicta and S. richteri (Sokolova & Fuxa 2008). Kneallhazia solenopsae is widespread among S. invicta in the USA and this infection causes decreased colony vigor and ultimately colony elimination (Oi & Williams 2003). The life cycle and natural transmission mechanisms of K. solenopsae are largely unknown (Sokolova & Fuxa 2008). Kneallhazia solenopsae appears limited to mostly polygyne S. invicta colonies in the USA, but is found in monogyne and polygyne colonies equally in South America where it is native (Oi et al. 2004; Valles & Briano 2004; Milks et al. 2008). Lack of an intermediate host has been proposed to explain the social form limitation (greater K. solenopsae infection in polygyne colonies) of K. solenopsae infections in North America (Oi et al. 2008). Recently Oi et al. (2008) reported the presence of K. solenopsae in several Pseudacteon decapitating fly species (obtusus, cultellatus and curvatus) suggesting that these endoparasitic flies may play a role in the life cycle or transmission of the microsporidian to fire ants.

The objectives of this study were to develop a multiplex PCR method capable of detecting K. so-
lenopsae in Pseudacteon flies and use the method to determine the infection rate of K. solenopsae among field-collected Pseudacteon curvatus. These experiments provide new descriptive data on the relationships between K. solenopsae and Pseudacteon flies as well as the Pseudacteon flies and their host, S. invicta.

**MATERIALS AND METHODS**

DNA was extracted from individual Pseudacteon flies by the method of Valles et al. (2002). Individual flies were homogenized in 150 μL of digestion buffer (50 mM Tris-HCl, pH 8, 4% sodium dodecyl sulfate, and 5% 2-mercaptoethanol) in a 1.5-mL microcentrifuge tube with a disposable plastic pestle for 15 s and the mixture was incubated at 100°C for 15 min. The mixture was allowed to cool on ice for 1 min, followed by the addition of 200 μL of phenol:chloroform:isooamyl alcohol (Tris-HCl-saturated, pH 8). The tube was inverted several times to mix the contents and then centrifuged at room temperature for 5 min at 16,000g. The supernatant was removed and nucleic acids precipitated with iced-cold isopropanol (750 μL) and the pellets were washed twice with 500 μL of 70% ethanol. Pellets were dried for 5 min at 37°C, and suspended in 30 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Multiplex PCR was conducted with DNA preparations from Pseudacteon flies to detect the presence of a portion of the K. solenopsae 16S rRNA gene (Valles et al. 2002) and a portion of the Pseudacteon 18S rRNA gene to verify satisfactory DNA extraction and amplification (i.e., an internal positive control). Amplifying the 18S rRNA gene of Pseudacteon serves as a method to eliminate false negatives from failed PCR reactions.

Oligonucleotide primers for the Pseudacteon flies were designed to conserved regions of the Pseudacteon 18S rRNA gene. DNA was purified from flies of a known species (n = 3) which was used as template to amplify a 1,288 nucleotide region of the 18S rRNA gene. Amplification was accomplished with an oligonucleotide primer set designed to conserved regions from an alignment of Diptera sequences for this gene in GenBank (primer 51, 5’GACTCATCCGAGGCCCGTAATC and primer 54, 5’CGGGCGGTGTGTGCAGG) and the 18S rRNA gene of Pseudacteon serves as a method to eliminate false negatives from failed PCR reactions.

Oligonucleotide primers specific to the 16S rRNA gene (Moser et al. 1998; Moser et al. 2000; Valles et al. 2002; accession number: AF031538) of K. solenopsae (P1: 5’CGAAGCATGAAAGCCG-GAGG and P2: 5’CAGCATGTATATGCAC-TACTGGAGG) and the 18S rRNA gene of Pseudacteon flies as described above (P800: 5’TAGT- TACACCTATACATTGGGTTCTCATCAT- TACTCATA and P801: 5’ATAAGTTTCAACGCTATAATCCCTGAAAGCATC) were included in the same reaction. Multiplex PCR was conducted 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 40 s, followed by a final elongation step of 5 min at 68°C. The reaction was conducted in a 25 μL volume containing 2 mM MgCl₂, 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 50 to 200 ng of the genomic DNA preparation (depending on the experiment). PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. For all experiments, positive (DNA mixture purified from P. curvatus and K. solenopsae) and negative (non-template) controls were run alongside treatments.

Limits of detection of K. solenopsae were determined by combining DNA preparations of K. solenopsae-negative Pseudacteon DNA and DNA prepared from a known quantity of purified K. solenopsae spores (Valles et al. 2002). The fly DNA was held constant at 200 ng (generally 0.5 fly equivalents) and K. solenopsae DNA was diluted geometrically from 8 ng (15,060 spore equivalents) to 0.0078 ng (14 spore equivalents). Altogether, 10 dilutions of the stock solution (15,060 spore equivalents) were completed. Multiplex PCR was conducted as described above.

To determine the K. solenopsae infection rate of P. curvatus, field collections of flies were conducted by disturbing fire ant mounds and collecting flies by aspiration that were attracted to the ants (Porter et al. 2004). A hand-held mechanical aspirator (Clarke Mosquito Control, Roselle, Illinois) was used by adding a fine mesh screen (52 × 52 mesh) to contain the flies. Flies were returned to the laboratory where they were segregated by species and sex. DNA was extracted from flies individually. Collections were made from 4 sites in Alachua County, Florida, with varying K. solenopsae infection rates among the S. invicta community: Site 1, Whitehurst pasture (southeast gate), Williston, Florida, K. solenopsae infection rate of S. invicta: 0/20 nests = 0%, flies collected 7 and 12 Nov 2008; Site 2, University of Florida, near the microbiology building, K. solenopsae infection rate of S. invicta: 5/17 nests = 29%, flies collected 2 Oct 2008; Site 3, field by former University of
Florida poultry unit, *K. solenopsae* infection rate of *S. invicta*: 18/28 = 64%, flies collected 25 Sep 2008 and 16 Oct 2008; Site 4, Hilton Hotel, NW 34th Avenue, Gainesville, FL, *K. solenopsae* infection rate of *S. invicta*: 12/19 nests = 63%, flies collected 6 Nov 2008.

In order to determine the rate of *K. solenopsae*-infection in *P. curvatus* that developed in *K. solenopsae*-infected *S. invicta* workers, we exposed *S. invicta* workers from 2 *K. solenopsae*-infected *S. invicta* colonies to *P. curvatus* in the field. Each colony contained approximately 10,000 adult worker caste ants, and the *K. solenopsae* infection rate was 90 and 60%, which was determined by examining wet mounts of 10 individual ants per colony for the presence of spores under phase-contrast microscopy. In each case, a 1,288-nucleotide amplicon was produced. These sequences have been deposited in the GenBank database under the following accession numbers: *P. curvatus* (EU851871), *P. litoralis* (EU851872), *P. obtusus* (EU851869), and *P. cultellatus* (EU851870). The *Pseudacteon* sequences exhibited significant identity (99.5%) with each other. An alignment of these sequences with the 18S rRNA sequence for *S. invicta* (AY334566) was used to develop oligonucleotide primers that would amplify the 18S gene of any of the 4 *Pseudacteon* fly species equally while not recognizing or amplifying the ant 18S rRNA gene. Thus, oligonucleotide primers P800 and P801 exclusively recognized and amplified DNA (small subunit 18S rRNA gene) from *Pseudacteon* flies. DNA prepared from *S. invicta* and *K. solenopsae* (3 independent preparations) failed to yield an amplicon with these primers (data not shown). However, the DNA from these samples did yield an amplicon with other (species-specific) oligonucleotide primers indicating that failure was not the result of insufficiencies in the PCR conditions or the DNA preparation (i.e., that the oligonucleotide primers were specific for the *Pseudacteon* 18S rRNA gene). An 800-nucleotide amplicon was consistently produced from DNA prepared from *Pseudacteon* flies or from *S. invicta* workers parasitized by *Pseudacteon* larvae.

Although *K. solenopsae* meiospores are monokaryotic, the 16S rRNA gene is typically present in multiple copies within the genomes of microsporidia, either as tandem repeats (Kawakami et al. 1994; Belkorchia et al. 2008) or found discontinuously on multiple chromosomes (Biderre et al. 1997). Indeed, the small subunit rRNA element was found on every chromosome of *Nosema bombycis* (Liu et al. 2008) and is thought to be present in multiple copies in all microsporidia (O’Mahony et al. 2007). Thus, the true limit

**RESULTS AND DISCUSSION**

Amplification with oligonucleotide primers P51 and P54 yielded a significant portion of the 18S rRNA gene from the 4 *Pseudacteon* species.
of detection of the assay is unknown because the number of copies of the 16S rRNA gene in *K. solenopsae* is not known currently. However, based on spore equivalents, this level of detection is commensurate with similar studies whose objective was to determine infection levels of microsporidia in their hosts (Leiro et al. 2002; Valles et al. 2002).

Because *K. solenopsae* was detected in several *Pseudacteon* species present in the USA (Oi et al. 2008), our original intention was to examine the infection rate of *K. solenopsae* in all *Pseudacteon* species collected from the field in north-central Florida. Unfortunately, the combination of a low infection rate of *K. solenopsae* and the displacement of other *Pseudacteon* species previously present in this area of Florida by *P. curvatus* (SDP, unpublished) forced us to change our objective to determine the *K. solenopsae* infection rate in *P. curvatus* only. The mean *K. solenopsae* infection rate (Table 1) of *P. curvatus* from the 4 sites in Gainesville and Williston, Florida, was 12.3 ± 5.0%, a value close to the infection rate we estimated previously (9 to 13%) with pooled groups of flies reared in the laboratory (Oi et al. 2008). The infection rate for *P. curvatus* was independent of the infection rate among *S. invicta* colonies in the immediate area of the fly collections. For example, the *K. solenopsae* infection rate among field-collected flies was 15.6% in areas where *K. solenopsae* was not detected among *S. invicta* colonies and 14.5% in areas with high *K. solenopsae* infections among *S. invicta* mounds (>60%). It is possible that the *K. solenopsae* infection rate was higher, but could not be detected because of limitations with the assay. Also, the *K. solenopsae* infection rate in *P. curvatus* would be expected to be lower because not all ants serving as host for flies would be infected with *K. solenopsae*. In other words, the *K. solenopsae* intra- or inter-colony infection rate is not 100%. Indeed, *K. solenopsae* infection rates of adult *S. invicta* workers among field colonies can vary widely, from less than 1 to 88% (Briano et al. 1996; Cook 2002; Oi et al. in press). Furthermore, as shown in Table 2, not all *P. curvatus* flies that develop in *K. solenopsae*-infected fire ants are themselves positive for *K. solenopsae* upon eclosion. However, the *K. solenopsae* infection rate was higher among flies completing development within *K. solenopsae*-infected fire ant workers (24% males + females) compared with those flies sampled directly from the field (12.3% females only). An additional consideration is the possibility that many flies reared in infected ants do not develop successfully into adults; however, this does not appear to be a primary explanation because fly production rates

### Table 1. *Kneallhazia solenopsae* Infection Among Individual Field-Collected *Pseudacteon curvatus* Flies in North-Central Florida.

| Collection Date | Sex | n  | Number of flies infected | Infection rate (%) | Site       |
|-----------------|-----|----|--------------------------|-------------------|------------|
| 7/12 Nov 2008   | ♀   | 32 | 5                        | 15.6              | Whitehurst |
| 25 Sep/16 Oct 2008 | ♀   | 49 | 7                        | 14.3              | Poultry    |
| 2 Oct 2008      | ♀   | 63 | 3                        | 4.8               | Microbiology |
| 6 Nov 2008      | ♀   | 48 | 7                        | 14.6              | Hilton Hotel |
from groups of infected workers is about the same as those from uninfected colonies (Oi et al. in press). In this study, 79% (n = 63) and 72% (n = 66) of the ant heads from *K. solenopsae*-infected bodies from each colony had evidence of adult eclosion, while uninfected parasitized ants had an eclosion rate of 77% (n = 22; sum of both colonies).

Parasitoid infection by host microsporidia has been reported and in several instances these infections reduced the vigor and effectiveness of the parasitoid (Brooks et al. 1993; Hoch et al. 2000). Whether *K. solenopsae* is actually infecting cells of *Pseudacteon* flies and, if so, its possible effect on the parasitoid remain unanswered, although as mentioned above (Oi et al. in press) flies from infected ant colonies appear healthy and abundant. There is no doubt, however, that *Pseudacteon* flies possess *K. solenopsae* in some form. The more relevant question from our perspective is whether *Pseudacteon* flies are part of the normal life cycle of *K. solenopsae* and if the flies somehow vector the parasite to *S. invicta*. We have studies currently under way to address these questions.

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