Spore morphotypes of *Thelohania solenopsae* (microsporidia) described microscopically and confirmed by PCR of individual spores microdissected from smears by position ablative laser microbeam microscopy

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INTRODUCTION

*Thelohania solenopsae* Knell Allen and Hazard (1977) is a microsporidian pathogen of the red imported fire ant *Solenopsis invicta* Buren, which was introduced into the southern United States from South America in the early 1930s (Williams *et al*., 1999). *T. solenopsae* is promising for biological control of *S. invicta* (Oi & Williams, 2002; Cook, 2002), but a lack of knowledge of its life cycle is hindering attempts to mass-produce the microsporidium and infect ants in the field.

*T. solenopsae* development originally was thought to include formation of two types of spores: unicellular meiospores, maturing inside sporophorous vesicles in sets of eight (octospores); and *Nosema*-like binuclear free spores. Megaspores, discovered in 2001, develop primarily in alates and are morphologically distinct from the two previously known types of spores. The role of megaspores in the *T. solenopsae* life cycle, as well as their existence, has been questioned (Shapiro *et al*., 2003). The current research includes light and electron microscopic descriptions of the three major spore morphotypes characteristic of *T. solenopsae* development. In addition, individual octospores and megaspores were isolated into groups of 8–20 from methanol-fixed and Calcofluor-stained smears of the infected ants for subsequent PCR analysis by the laser pressure catapulting function of a position ablative laser microbeam microscope, a technique applied for the first time to research of microsporidia. The PCR-amplified SSU rDNA nucleotide sequences from octospores and megaspores were identical. This, along with the consistency with which megaspores are detected in infected ants, demonstrates that megaspores are integral to the life cycle of *T. solenopsae*.
specifically targeted procedure was developed in the late 1990s to capture individual cells from tissue cultures, as well as their nuclei and chromosomes, for genomic and proteomic analyses (Grant & Jerome, 2002). Numerous attempts were made to isolate megaspores from other spore types by conventional methods, including centrifugation in Percoll and Ficol gradients, but they always resulted in a mixture of spore types (Y. Y. Sokolova, unpublished data). PALM microscopy offered a possible solution to the problem of isolating T. solenopsae spore types for further study.

The goals of the current research were to: (1) demonstrate genetic identity of octospores and megaspores, isolated by the laser pressure catapult (LPC) function of a PALM microscope, by comparison of PCR-amplified partial SSU rDNA nucleotide sequences; (2) describe the morphology and fine structure of the three major T. solenopsae spore types; (3) determine tissue tropism of the different spore types; and (4) determine megasporae prevalence among castes and in fire ant colonies obtained from areas with native or introduced isolates of T. solenopsae.

METHODS

Source of microsporidia. Microsporidia-infected colonies of fire ants were removed from either naturally infected field populations near Rosepine, LA, USA, or the sites of experimental releases of the microsporidium near St Joseph and Clinton, LA, USA. Brood for both releases was obtained originally from infected colonies in Florida. Colonies were maintained in the laboratory with standard rearing methods (Banks et al., 1981).

Microdissection by LPC. A PALM microscope (Carl Zeiss) equipped with a UV laser microbeam for microdissection, LPC system and epifluorescence, was used to isolate individual T. solenopsae spores from methanol-fixed smears of infected S. invicta. Calcofluor-stained spores were viewed at a wavelength of 395–415 nm with a UV filter. Eight to twenty single megaspores, Calcofluor-stained spores were viewed at a wavelength of 395–415 nm with a UV filter. Eight to twenty single megaspores, spores from methanol-fixed smears of infected T. solenopsae were individually catapulted into a 0.5 ml microfuge tube (Zeiss) containing 2–5 µl sterile deionized water with the LPC function of the microscope. The water containing the spores was centrifuged to move the spores to the bottom of the tube. Tubes with megaspores were frozen for 1–24 h at −20 °C and then thawed at room temperature.

DNA isolation and PCR. PCR was performed on each spore type in the original microfuge tubes. The initial PCR reaction mixture had a volume of 20 µl: 10 µl buffer H (FailSafe PCR kit), 1 µl of each 1 µM primer, 0.5 µl FailSafe enzyme, 5 µl water and 2.5 µl water containing the microdissected spores. Two pairs of primers were used for amplification and sequencing of SSU rDNA from T. solenopsae: (i) CGAAGCATGAAAGCGGAGC (1TSSf)–CAGCATGTATATGCACTACTGGAGC (2TSASr), amplicon size 318 bp (Valles et al., 2004); and (ii) AAGGACACACAAGGATGG (SSU41f)–CGCGAAGAAGTCCTACAACA (TS1059r), amplicon size 220 bp (Milks et al., 2004). The initial reaction mixture was placed into the cap of the microfuge tube. Once all components were added, the microfuge tube was vortexed and centrifuged. The PCR was run in an MJ Research thermocycler 100 with a heated lid, which held the cap of the microfuge tube in place. The initial PCR reaction included an initial denaturation at 98 °C for 6 min followed by 25 cycles (98 °C for 1 min, 55 °C for 30 s, 72 °C for 1.5 min) and an additional elongation at 72 °C for 5 min. The second PCR reaction mixture had a volume of 50 µl: 20 µl from the first reaction, 23 µl buffer H, 1 µl of each 20 µM primer, 4 µl water and 1 µl enzyme. The second PCR cycle parameters were the same as in the first reaction.

Agarose gel electrophoresis and DNA analysis. PCR products were loaded onto a 1% agarose gel, which was run at 100 kV and stained with ethidium bromide. Positive and negative controls in each experiment consisted of DNA isolated by phenol/chloroform extraction (Valles et al., 2002) from T. solenopsae-infected and uninfected ant colonies, respectively, and amplified as above.

Sequence analysis. Bands were extracted with a gel extraction kit (Zymo). The nucleotide sequences of the DNA samples were obtained with a Big Dye kit (Qiagen) and the corresponding forward primer. The reactant was vacuum-dried and sequenced at GeneLab (School of Veterinary Medicine, Louisiana State University). After direct sequencing of the amplicons, the resulting sequences were aligned with a T. solenopsae SSU rDNA sequence (accession no. AF134205) for comparison with each of the two spore types by the BLAST alignment analysis (www.ncbi.nlm.nih.gov).

Light microscopy. The ants were homogenized in sterile distilled water and smeared on glass slides. The smears were air-dried, fixed with absolute methanol for 5 min, and stained with Giemsa, modified trichrome stain, or Calcofluor white M2R (all stains from Sigma) as described previously (Undeen, 1997; Weber et al., 1992; Didier et al., 1995). Trichrome-stained spores were observed and measured electronically at ×1000 in bright field with a Nikon microscope equipped with a Meta-View imaging system (MetaView, 1998). The measurements were compared by one-way ANOVA with the Tukey HSD test among means (Statistica, 1996). The Giemsa stain was used to visualize both spores and prespore stages. Slides with Calcofluor-stained spores were used for spore isolation by the LPC technique. Phase-contrast microscopy was used for detection of infection in individual ants for subsequent electron microscopy.

Electron microscopy. For each ant, the gaster was separated from the head and thorax and placed in a fixative solution containing 2% glutaraldehyde and 1-25% paraformaldehyde in 0.1 M cacodylate buffer. The gaster was slightly squashed and observed under phase-contrast optics. If it was positive for T. solenopsae, the coverslip was carefully lifted and tissues were removed from that gaster and checked under a light microscope for microsporidia. Tissues removed included ovaries of the reproductive females, fat body and muscle adjacent to the ovaries, and fat body of major and minor workers. Large pieces of fat body and ovaries were processed for electron microscopy directly, while small (less than 0.5 mm) fragile pieces of tissue were first embedded in blocks of 2% agarose (Amresco) in buffer. Tissues or tissue-containing agar blocks were fixed for 2-24 h with the glutaraldehyde/paraformaldehyde solution, washed with cacodylate buffer, post-fixed with 1% osmium tetroxide in the same buffer, stained overnight with 2% uranyl acetate in water, dehydrated in an ethanol series, and embedded in Epon-Araldite resin. Thick (0.5–1 µm) and thin sections were cut with a Reichert Ultracut microtome and stained with methylene blue (1% methylene blue in 4% sodium borate in water). Thin sections were stained with lead citrate and viewed under a JEM 100CX microscope at 80–100 kV. Tissues of 11 infected insects (six reproductive females and five workers) from different localities (two reproductive females from St. Joseph, three reproductive females and two workers from Clinton, and one reproductive female and three workers from Rosepine) were studied ultrastructurally.

Prevalence of megaspores in field populations of fire ants. Ants were examined from 48 colonies from St. Joseph (sampled April and November 2002, and February and April 2003), 99
colonies from Clinton (same dates and May 2003), and 141 colonies from Rosepine (March, April, May and June 2002). Ants were sampled in 20 ml glass vials that were inserted into each mound with the mouth of the vial level with the soil surface. The vials were coated inside at the opening with liquid fluoropolymer resin PTFE 30 (Teflon) to prevent the ants from escaping. The vials were transported on ice to the laboratory and then stored at $-20^\circ$ C. Fifty to one hundred ants from each vial were homogenized in 200 $\mu$l sterile distilled water in a 1.5 ml microtube with a disposable pestle (Koates Glass). Twenty microlitres of homogenate was spread on a glass slide over 2.5 cm$^2$ (area of a standard cover slip) to obtain a thin transparent layer without solid particles, and the smears were then methanol-fixed and trichrome-stained. For each sample representing one colony, the presence or absence of infection as well as the spore types were recorded. The percentage of colonies with megaspores was compared among the three sites by a one-way ANOVA with the Tukey HSD test among means (Statistica, 1996).

Prevalence of spore types among ant castes. A polygynous colony of S. invicta was maintained in the laboratory for 11 months after experimental infection with the Florida isolate of T. solenopsae by introduction of infected brood (Williams et al., 1999). Individual insects were smeared, methanol-fixed, stained with Giemsa and observed under the microscope for various spore types as well as for pre-spore stages.

RESULTS

PCR-based analysis of spores isolated from smears by LPC microdissection

Electrophoretic patterns of fragments amplified by each of the two primer sets were similar regardless of whether the DNA was obtained from individual octospores, octets of octospores, megaspores or from ant colonies infected with both spore types (Fig. 1a). The number of spores, 8–20, did not affect the success of amplification. The clear bands resulting from PCR of spores isolated by PALM and LPC (Fig. 1a, b) demonstrated that isolation of DNA by this method was as effective as the method of isolating DNA by phenol/chloroform extraction from homogenates of ants infected with T. solenopsae. DNA from octets of octospores and individual octospores was isolated and amplified consistently if slides with tissue smears had been stored at 4–10 $^\circ$ C (10 successful amplifications in 11 experiments). We occasionally failed to amplify DNA when methanol-fixed and Calcofluor-stained smears had been stored at room temperature. Our observations also suggest that UV light might destroy DNA; successful amplifications of DNA isolated from catapulted spores decreased after a slide remained for more than 3 h under a fluorescence microscope with a UV filter.

Amplification of DNA from megaspores was successful only 50 % of the time (five successes in 10 experiments), and four of the successful cases were achieved after addition of the freeze–thaw procedure. Sequences derived from octospores were of slightly better quality than the ones from megaspores; the lengths of the regions suitable for alignments were longer (Table 1), and numbers of mismatches were fewer for octospores. SSU rDNA sequences of spores from Rosepine (Louisiana isolate) did not differ from those from Clinton (Florida isolate). In all cases, the sequences of regions amplified by the 1TSSf–2TSASr and TS841f–TS1059r primer pairs were highly homologous between octospores and megaspores, because BLAST alignment analysis (Table 1) indicated that both spore types closely matched the T. solenopsae SSU rDNA sequence (Moser et al., 1998, 2000), accession numbers AF134205, AF031537 and AF031538.

Light microscopy

Octospores, Nosema-like spores and megaspores were consistently present in smears prepared from ant samples from all of the localities and laboratory colonies. Uninuclear octospores developing inside a sporophorous vesicle, eight spores in each, and Nosema-like free diplokaryotic spores (Fig. 2a, b, c, f, g, h) were similar to those described previously (Knell et al., 1977; Moser, 1995; Moser et al., 2000). Nosema-like spores were the only spore type that easily discharged their polar filament on fresh smears. Like previous authors (Knell et al., 1977; Williams et al., 1998) we occasionally observed elongated macrospores (teratospores) (Knell et al., 1977), which were approximately 70 % longer than octospores (Table 2). These macrospores occurred outside and inside sporophorous vesicles with octospores.

![Fig. 1. Agarose gel electrophoresis of PCR products. (a) Fragments of SSU rDNA obtained from different numbers of spores and amplified with the TS841f–TS1059r primer pair. Lanes: 1, 100-base DNA mass ladder; 2, 20 megaspores; 3, 10 megaspores; 4, 20 individual octospores; 5, 10 individual octospores; 6, octet of octospores; 7, eight megaspores; 8, DNA isolated from uninfected ants (negative control); 9, DNA isolated from infected ants (positive control). (b) Comparison of SSU rDNA fragments obtained from eight individual octospores (lanes 2–4) or eight megaspores (lanes 5–7) amplified with three sets of primers. 1 and 9, DNA mass ladder; 2 and 5, V1F–530R (universal microsporidian primer pair); 3 and 6, TS841f–TS1059r; 4 and 7, 1TSSf–2TSASr; 8, DNA isolated from uninfected ants (negative control).](http://mic.sgmjournals.org)
Table 1. BLAST analysis of sequences obtained from primer pairs TS841f–TS1059r and 1TSSf–2TSASr

<table>
<thead>
<tr>
<th>Spore type analysed</th>
<th>Identity (%) to T. solenopsae sequence*</th>
<th>Alignment length (bp)</th>
<th>Mismatches</th>
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<tr>
<td></td>
<td>841–1059</td>
<td>1TS–2TS</td>
<td></td>
</tr>
<tr>
<td>Octospore octet</td>
<td>100·0</td>
<td>99·7</td>
<td>0</td>
</tr>
<tr>
<td>Eight octospores</td>
<td>99·5</td>
<td>99·3</td>
<td>0</td>
</tr>
<tr>
<td>Eight octospores</td>
<td>99·5</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Eight megaspores</td>
<td>98·0</td>
<td>96·0</td>
<td>2</td>
</tr>
<tr>
<td>Eight megaspores</td>
<td>94·4</td>
<td>96·7</td>
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<td>0</td>
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<tr>
<td>Control 3</td>
<td>97·4</td>
<td>97·7</td>
<td>5</td>
</tr>
</tbody>
</table>

*Accession no. AF134205.

(Fig. 2g). Some smear contained free macrospores mixed with Nosema-like spores, but the two spore types could be distinguished by their significantly different lengths and width/length ratios (Table 2). Both macrospores and Nosema-like spore types had two nuclei, which were clearly revealed by DNA-specific DAPI staining (not shown). Megaspores were easily recognized on fresh smears by their size, shape, characteristic trichrome-staining pattern and opaque refractive spore wall (Fig. 2a, f, g, h). Megaspores often occurred together with octospores on smears prepared from imagos; they were detected also in pupae and callow adults, in which octospores were never observed (Table 3). Megaspores were significantly larger than the other three types of spores (Table 2). Generally, octospores were the most abundant spore type in imagos; Nosema-like spores and megaspores were found consistently in smears, but in low prevalence (approximately 2%). However, both of these spore types can occur in high prevalence (e.g. Table 3), and occasionally individual ants were infected nearly exclusively with Nosema-like spores (Fig. 2b) or megaspores.

Spore ultrastructure

Octospores (Fig. 3a–c, i) were found in all of the examined tissues; their appearance and internal structure were similar to those described previously (Knell et al., 1977; Moser et al., 2000). They preserved their integrity after fixation and embedding much better than other spore types. In ultrathin sections, octospores measured $2·6 \pm 0·49 \times 1·5 \pm 0·19 \mu m$ (mean $\pm SD$, $n = 31$). They form inside sporophorous vesicles, and have a single nucleus surrounded by two or three layers of endoplasmic reticulum and an isofilar polar filament arranged in one row of 9–12 coils. Their envelopes were $0·15 \pm 0·062 \mu m$ thick, consisting of a wide endospore ($0·11 \pm 0·06 \mu m$) narrowing to approximately half thickness in the spore apex, and a thin ($0·04 \pm 0·011 \mu m$) undulating exospore, comprised of at least three layers (Fig. 3b). The polar sac was elongated (Fig. 3a, b, i) and often appeared as an elongated broad sacculus embracing the anterior part of the polaroplast (Fig. 3i, arrow). The polaroplast consisted of flattened membranes packed slightly more compactly in the anterior part. The posterior vacuole is not well expressed. The undulating, multilayered exospore (Fig. 3a–c, i, l) is the most characteristic feature distinguishing this spore type from others on thin sections. Sporophorous vesicles with sporoblasts and undeveloped spores contain filamentous material that eventually is replaced by a homogeneous matrix of average electron density (Fig. 3i, l).

Nosema-like spores (Fig. 3d, e, f, l) in thin sections measured $2·8 \pm 0·69 \mu m$ ($n = 21$) $1·6 \pm 0·42 \mu m$ ($n = 26$). Their ultrastructure also was consistent with earlier descriptions (Knell et al., 1977; Moser et al., 2000). They are diplokaryotic and develop in direct contact with host cell cytoplasm, their polar filament arranged in one row of 12–13 isofilar coils. The posterior vacuole is prominent but appears in sections as a poorly fixed zone in the posterior part of the spore. Spore envelopes average $0·13 \pm 0·045 \mu m$ ($n = 25$); exospores are similar in size to those in octospores $0·04 \pm 0·014 \mu m$ ($n = 25$), but they are smooth (not undulating) and occasionally covered with a thin filamentous electron-dense layer (Fig. 3d, e). Intact Nosema-like spores were rare in the sections. However, empty envelopes presumably left after sporoplasm discharge often were found near the plasma membrane of adipocytes and in the periphery of immature cysts (Fig. 3f, l).

Megaspores (Fig. 3g, h, i, m) were easily differentiated from other spore types in ultrathin sections. They are larger, measuring $5·5 \pm 1·02 \times 2·8 \pm 0·73 \mu m$ ($n = 24$), and have thick envelopes ($0·40 \pm 0·10 \mu m$) with endospores $0·26 \pm 0·059 \mu m$ wide and exospores $0·14 \pm 0·053 \mu m$ wide. Exospores are smooth and one-layered, and never with a
filamentous external layer. The thick walls of megaspores presumably prevent penetration of fixatives and embedding media, which causes poor preservation of internal structures. Nevertheless, a diplokaryotic arrangement of nuclei (Fig. 3h) and an anisofilar polar filament arranged in 19–23 coils and two to four rows (Fig. 3g, h, i) could be distinguished.

Aberrant spores with two nuclei and 18–20 polar filament coils were seen repeatedly inside sporophorous vesicles. Their other features, including a polar sac embracing a polaroplast, envelope thickness and an undulating exospore, were similar to those of octospores (Fig. 3j). Additionally, free binuclear spores with undeveloped, poorly resolved envelopes and 18–20 polar filament coils arranged in one or two layers were observed rarely in sections (Fig. 3k).

**Tissue tropism**

**Fat body.** All three spore types were seen occasionally inside adipocytes of the fat body, which was the only previously reported site of *T. solenopsae* infection (Knell et al., 1977; Moser, 1995; Shapiro et al., 2003). In heavily infected minor and major workers, the fat body was replaced by masses of mature octospores. In certain major workers, males and reproductive females, ribbons of adipocytes filled the space between abdominal organs. These adipocytes were sporadically infected, mostly with

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**Fig. 2.** Light microscopy of *T. solenopsae*: (a–e) phase contrast; (f) Giemsa staining; (g) trichrome staining; (h, i) Calcofluor white M2R staining. (a) Megaspores on a smear from an alate female ovary. (b) Smear from a major worker, containing predominantly *Nosema*-like spores (elongated arrowheads) and sporophorous vesicles (arrows) with octospores. (c) Smear from an immature cyst. *Nosema*-like spores are indicated by elongated arrowheads, octets of octospores by short arrows, and megaspores by a long thin arrow. The asterisk shows the mass of developing parasites. (d) Adipocyte with *Nosema*-like spores. (e) Conglomerate of megaspores adjacent to ovaries of an alate female. (f) Left, sporophorous vesicle (SV) with eight octospores; middle, megaspore; right, immature *Nosema*-like spore with diplokaryon. (g) Upper row: left, octospores in SV; middle, *Nosema*-like spores; right, megaspore. Lower row: left, octospores after liberation from SV; middle, SV containing three undivided octospores (macrospores); right, an undivided octospore liberated from a SV. (h) Calcofluor-stained spores of three types are indicated with arrows as on (c). (i) Muscle fibre containing numerous megaspores. Bars, 5 μm (a, b, f, g) and 10 μm (c, d, e, h, i).
developmental diplonokaryotic stages and by Nosema-like spores and their empty envelopes (Fig. 2d). Uninfected haemocytes often attached to the infected adipocytes. In reproductive females, ribbons of fat body containing all spore types were attached to ovarioles, remaining so during dissections.

Muscles. Megaspores were abundant in the subcuticular layer of abdominal musculature in workers and reproductive females (Fig. 3m), in which the fat body was infected with octospores and Nosema-like spores. Some muscle fibres were heavily loaded with megaspores (Fig. 2i). Electron microscopy revealed groups of mature and poorly preserved megaspores located inside partially destroyed muscle tissue (Fig. 3m).

Ovaries. Ovaries removed from 34 alate and 4 dealate infected females were examined for spores. Abundant spores of all types were found in adipose tissue attached to ovaries. Massive assemblages of megaspores (40–60 μm diameter) were present occasionally in close proximity to ovarioles (Fig. 2e). Infections of oocytes themselves were detected only in two cases. Both of these dealate females were heavily infected with numerous mature octospores in the fat body as well as megaspores in muscles. The single infected oocyte from the first female contained mature octospores exclusively. In the second female, two adjacent oocytes were heavily infected, one with octospores and the other with megaspores. Spores or vegetative stages of the microsporidium could not be detected in other oocytes, nurse cells or follicular epithelium cells in these two females.

Cysts. Approximately 5% of the examined infected imagos of all ant castes contained one to five large (200–350 μm diameter) ‘cysts’ (Knell et al., 1977), which were liberated from the abdomens during dissections. Light microscopic observation with phase-contrast or dark-field optics revealed that mature cysts were assemblages of tightly packed octets of meiospores, sometimes mixed with zones of megaspores. Electron microscopy showed that Nosema-like spores and their empty envelopes also occurred in the periphery of some immature cysts (Fig. 3l).

Prevalence of spore types
Megaspores were detected in 10–67% of T. solenopsae-infected S. invicta colonies at the following mean (±SD) prevalence rates: 40·2 ± 16·0% at Clinton; 26·9 ± 20·7% at St. Joseph and 31·3 ± 21·7% at Rosepine. There were no differences in megaspor prevalence among the three sites (F=0·55; df=2; P=0·59).

All three spore types were consistently present in all castes, although prevalence rates differed (Table 3). Megaspores

| Table 2. Measurements (μm) of T. solenopsae spore types on methanol-fixed smears stained with trichrome |

Means in each column followed by the same letter did not differ at P=0·05 (Tukey, HSD).

<table>
<thead>
<tr>
<th>Spore type</th>
<th>Length ± SD</th>
<th>Width ± SD</th>
<th>Width/length ± SD</th>
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<tr>
<td>Octospores</td>
<td>3·1 ± 0·38</td>
<td>2·1 ± 0·23</td>
<td>0·7 ± 0·08</td>
<td>148</td>
</tr>
<tr>
<td>Nosema-like spores</td>
<td>4·6 ± 0·29</td>
<td>2·3 ± 0·20</td>
<td>0·5 ± 0·05</td>
<td>30</td>
</tr>
<tr>
<td>Megaspores</td>
<td>6·2 ± 0·41</td>
<td>3·6 ± 0·29</td>
<td>0·6 ± 0·05</td>
<td>26</td>
</tr>
<tr>
<td>Macrospores</td>
<td>5·3 ± 0·44</td>
<td>2·2 ± 0·23</td>
<td>0·4 ± 0·06</td>
<td>22</td>
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ANOVA parameters  
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<tr>
<th>F</th>
<th>df</th>
<th>P</th>
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<tr>
<td>668·41</td>
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<td>345·99</td>
<td>3</td>
<td>&lt;0·001</td>
</tr>
<tr>
<td>102·43</td>
<td>3</td>
<td>&lt;0·001</td>
</tr>
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| SPV*  | 9·0 ± 0·46  |

*Sporogenous vesicles (SPVs); only the largest diameter was measured.

| Table 3. Prevalence of various spore types among castes in one experimentally infected colony |

<table>
<thead>
<tr>
<th>Spore type</th>
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<td></td>
<td>Reproductive females (n=62)</td>
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<tr>
<td>Octospores</td>
<td>37·1</td>
</tr>
<tr>
<td>Megaspores</td>
<td>22·6</td>
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<tr>
<td>Nosema-like spores</td>
<td>12·9</td>
</tr>
<tr>
<td>One or more of above</td>
<td>37·1</td>
</tr>
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</table>
Fig. 3. Electron microscopy of *T. solenopsae* spores. (a) Octospore with the characteristic undulating profile of the exospore (short arrow). The polar sac is indicated by long arrow. (b) Anterior part of the octospore at greater magnification. The exospore (long arrow) displays multilayered structure; the endospore (short arrow) is narrowing anteriorly. (c) Transverse section through the octospore. The arrow indicates the undulating exospore. (d) *Nosema*-like spore with a diplokaryon and smooth exospore ornamented with electron-dense material (long arrows). The short arrow points to the endospore. (e) Transverse section through a *Nosema*-like spore, recognizable by its smooth exospore (arrow). (f) ‘Empty’ *Nosema*-like spore after firing; the arrow indicates the opening in the exospore through which the polar filament was extruded. (g) Oblique section through a megaspore, revealing numerous polar filament coils and smooth thick exospore. (h) Longitudinal section through the central part of the megaspore. Multiple polar filament coils are arranged in several rows, and two nuclei are visible in spite of poor fixation of internal spore structures. (i) Section through a central part of an immature cyst. A free megaspore and a sporophorous vesicle with octospores are visible. The arrow points to the characteristic structure of the polar sac in octospores. (j) Macrospheres (teratospores): octospores that failed to divide, containing two nuclei and 18–20 polar filament coils. (k) Teratospores with similar internal structure as in (j), but with poorly visible envelope, located outside the SV. (l) Section through the periphery of an adipocyte with empty (fired) *Nosema*-like free spores and a sporophorous vesicle containing mature octospores. (m) Muscle fibre infected with megaspores. AD, anterior disk; CW, cyst wall; En, endospore; ENS, empty *Nosema*-like spore; Ex, exospore; MPF, manubrial part of the polar filament; MS, megaspore; N, nucleus; OS, octospores; PF, polar filament coils; PP, polaroplast; PS, polar sac; PV, posterior vacuole; SV, sporophorous vesicle; SVE, sporophorous vesicle envelope; SW, spore wall. Bars, 0.5 μm (b), 10 μm (m) and 1 μm (other parts).
and Nosema-like spores were detected at least once in every caste examined. Octospores were the most common spore type in reproductive female and workers but were not detected in brood, which contained either Nosema-like spores or megaspores (Table 3).

DISCUSSION

Megaspores

The current study provides strong evidence that megaspores are integral to the life cycle of T. solenopseae. Sequence analyses of SSU rDNA from octospores and megaspores isolated by the LPC technique showed that megaspores belong to this species and are not a result of infection by another microsporidium. Sequence analysis also confirmed that the Florida and Louisiana isolates of T. solenopseae are identical. Furthermore, megaspores, as well as octospores and Nosema-like spores, were consistently present at high prevalence rates in certain tissues in experimentally infected laboratory colonies and in artificially and naturally infected populations of fire ants in the field in the current and previous (Sokolova & Fuxa, 2001) studies. It is almost inconceivable that an aberrant spore could be found at such high rates of infection. The fact that megaspores were not described before 2001, in spite of their prevalence and obvious differences from octospores and Nosema-like spores, might indicate that they were regarded as accidental teratospores, as suggested by Shapiro et al. (2003), or as belonging to another microsporidian species. Dipllokaryotic spores with large numbers of polar tube coils, similar in size to megaspores, are produced by Burenella dimorpha in the tropical fire ant Solenopsis geminate (Jouvenaz & Hazard, 1978) and by Vairimorpha invictae in S. invicta (Jouvenaz & Ellis, 1986).

The differences in success of SSU rDNA amplifications from octospores versus megaspores might be due to different envelope structures in the two spore types. As electron microscopy showed, the megaspores had thicker exospores, which might impede the release of DNA or prevent methanol penetration inside the spore, resulting in partial destruction of the template by endonucleases.

Other spore types

The fine morphology of octospores and Nosema-like spores was identical between the current study and those of T. solenopseae isolates from Brazil (Knell et al., 1977; Moser, 1995; Moser et al., 2000), Argentina (Moser, 1995; Moser et al., 2000) and Florida (infecting fire ants in Florida or Louisiana) (Moser, 1995; Moser et al., 2000; Sokolova & Fuxa, 2001). The current ultrastructural analysis confirmed that macrospores, observed also by Knell et al. (1977), are teratospores, resulting from abnormal development of octospores. The formation of electron-dense spores with two nuclei, 17–20 polar filament coils and an undeveloped spore envelope (Sokolova & Fuxa, 2001) is rare and inconsistent; thus they are probably also teratospores.

Possible functions of spores discovered in S. invicta

The functional role of the numerous spore types discovered in T. solenopseae has not yet been resolved, in contrast to other microsporidian genera producing three or more spore types, such as Amblyospora, Culicosporella, Edhazardia, Hazardia, Parathelohania and Vairimorpha (Becnel & Andreadis, 1999).

Several observations suggest that Nosema-like spores may function in autoinfection: ready discharge of polar filaments of Nosema-like spores in distilled water; their occurrence with their empty envelopes and diplokaryotic stages in adipocytes; and their presence in peripheral regions of immature cysts and absence in mature ones. If so, sporoplasts discharged by Nosema-like spores in host tissues presumably would initiate a sequence of development resulting in mass production of octospores in adipocytes. These cells would either transform into cysts or be destroyed and replaced by the spore mass.

The functions of octospores are not understood. Neither they nor other known T. solenopseae spore types are able to initiate per os infection (Shapiro et al., 2003). Introduction of infected brood is the only known way to infect new colonies with T. solenopseae (Williams et al., 1999). Because octospores were never observed in brood (Oi et al., 2001; Shapiro et al., 2003; current study, Table 2), there is no evidence of their immediate role in horizontal transmission of the parasite. It is possible that an intermediate host is susceptible to infection with octospores (Oi et al., 2001), similar to Amblyospora species developing in mosquitoes and in copepods as alternate hosts (Becnel & Andreadis, 1999), but no such alternate host of T. solenopseae has been discovered. Our observations of octospores within oocytes of two different females suggest that they may function in transovarial transmission. On the other hand, ovarian infection was observed in only two reproductive females of 38 examined, both of which were heavily infected, raising the possibility that a generalized infection was carried into ovaries during the final stages of microsporidiosis.

Megaspores cannot be ruled out as functioning in horizontal (peroral) or vertical (transovarial) transmissions. They were found inside cysts in imago gasters, in ribbons of fat body surrounding ovaries, in an oocyte and, unlike other spore types, inside muscle fibres. They were more prevalent in reproductive females than in other castes. Unlike octospores, megaspores were observed in larvae, pupae and callow adults.

It is possible that megaspores are produced alternatively to octospores inside specific cell types with different intracellular environments. Both spore types originate from diplokaryotic meronts (Y. Y. Sokolova & J. R. Fuxa, unpublished data), which either undergo meiosis and...
produce sporonts developing into octospores inside sporophorous vesicles, or immediately switch to sporogony resulting in megasporogenesis.

The ultrastructural description of an additional type of diplokaryotic spore in pre-imaginal stages of ants (Shapiro et al., 2003) is another indication of the complexity of the *T. solenopsae* life cycle. Morphologically, these spores are much like the 'early' spores of several microsporidian species described from the gut epithelium of lepidopteran (Maddox et al., 1999) or dipteran (Becnel et al., 1989) hosts. This structural similarity may suggest a similar function, namely, fast redistribution of infection throughout the host.

**LPC techniques**

The current research represents the first application of LPC microdissection to microsporidian research. This is a relatively new technique that has been established in various fields of cell biology and biomedical science, including cancer research (Lehmann et al., 2000; Eltoum et al., 2002; Cor et al., 2002), haemopathology (Fend et al., 2000), diagnosis of bacterial and viral infections (Ryan et al., 2002), cytogenetics (Cao et al., 2001; Stark et al., 2003) and other fields (Grant & Jerome, 2002). Coupled with such methods as immunostaining, PCR, nucleotide sequencing, creation of cDNA libraries, proteomic analyses of dissected material and electron microscopy, this method has proved to be indispensable when specific cellular or subcellular targets must be isolated (Obiakor et al., 2002).

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