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Infection of *Gammarus duebeni* populations by two vertically transmitted microsporidia; parasite detection and discrimination by PCR–RFLP

J. C. HOGG[1], J. E. IRONSIDE[1], R. G. SHARPE[1], M. J. HATCHER[2], J. E. SMITH[1] *and* A. M. DUNN[1]*

[1] *School of Biology, University of Leeds, Leeds LS2 9JT, UK*
[2] *School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK*

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SUMMARY

We screened a population of the brackish water crustacean *Gammarus duebeni* from the Isle of Cumbrae for the presence of vertically transmitted microsporidia. We compared 2 screening techniques; light microscopy and PCR-based detection using generic 16S rDNA microsporidian primers. Fifty percent of females from this population tested positive for vertically transmitted microsporidia. The PCR screen was 100% efficient in comparison with existing LM based screening. In addition, the PCR screen produced bands of 2 sizes suggesting that more than 1 species of microsporidian was present. Sequencing revealed 2 distinct species of vertically transmitted microsporidia; 33% of females were infected with the feminizer *Nosema granulosis* and 17% were infected with a new species which we provisionally designate *Microsporidium* sp. On the basis of sequence information, we developed a discriminatory PCR–RFLP test based on *Msp*I and *Hae*III digests. This screen allows rapid detection and discrimination of vertically transmitted microsporidia in natural field populations. We applied the PCR–RFLP screen to a second *G. duebeni* population from the Isle of Man. This population also hosted these 2 parasite species. In total 45% of females harboured *N. granulosis* and 10% harboured *Microsporidium* sp. No dual-infected individuals were found in either population. The occurrence of 2 vertically transmitted parasites within a population has implications for our understanding of parasite–host relationships in the field and we discuss factors affecting the dynamics of parasite–parasite competition and coexistence.

Key words: *Nosema granulosis*, *Microsporidium* sp., microsporidia, PCR–RFLP, *Gammarus duebeni*, vertical transmission.

INTRODUCTION

Vertically transmitted parasites are of interest as they may affect host population size, stability and sex ratio and inform our understanding of the evolution of virulence (Dunn *et al*. 1995; Hatcher, Taneyhill & Dunn, 1999; Dunn & Smith, 2001). As most vertically transmitted parasites are uniparentally inherited, they are under strong selection pressure to manipulate host reproduction to favour the transmitting sex (Hatcher, 2000; Dunn, Terry & Smith, 2000). The most frequently studied vertically transmitted parasites are *Wolbachia* which induce a range of effects on host reproduction including feminization (Rigaud, 1997), male killing (Hurst, 1991), cytoplasmic incompatibility (Hoffman & Turelli, 1997) and induction of parthenogenesis (Stouthamer, 1997). In addition, parasites of the phylum Microspora are exceptional amongst the eukaryotes, as they are also capable of sex ratio distortion as part of a vertical transmission strategy (reviewed by Dunn *et al*. 2000; Dunn & Smith, 2001). Male killing microsporidia cause benign infections in female mosquito hosts which survive to transmit the parasite transovarially, whereas a virulent infection kills male hosts to release spores for horizontal transmission (Kellen *et al*. 1965; Hurst, 1991). Feminizing microsporidia of amphipod crustacea rely solely on vertical transmission (Dunn *et al*. 2000) and convert genetic male hosts into phenotypic females capable of transmitting the parasite to future generations of hosts (Bulnheim, 1978; Dunn, Adams & Smith, 1993; Terry, Smith & Dunn, 1998).

Vertically transmitted microsporidia are difficult to detect in the host population as parasite burden is low and these parasites cause no obvious pathology (Terry *et al*. 1998; Dunn, Terry & Taneyhill, 1998; Franzen & Muller, 1999). Previous studies of microsporidia in the crustacean *Gammarus duebeni* have identified 3 species of vertically transmitted microsporidia. *Octosporea effeminans* was described by light and electron microscopy (Bulnheim & Vavra, 1968) and *Thelohania hereditaria* was described via light microscopy (Bulnheim, 1971). More recently, we have described *Nosema granulosis* from a population at Millport, Isle of Cumbrae using molecular phylogenetic techniques and electron microscopy (Terry *et al*. 1999). All 3 species are

* Corresponding author: School of Biology, University of Leeds, Leeds LS2 9JT, UK. Tel: +44 (0) 113 3432856. Fax: +44 (0)113 3432835. E-mail: a.dunn@leeds.ac.uk

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feminizers and so females harbouring these parasites produce strongly female-biased broods (Bulnheim, 1978; Dunn *et al*. 1993; Terry *et al*. 1998).

Currently, rapid detection of vertically transmitted microsporidia is carried out by DNA fluorescent staining of host embryos which reveals parasites within the host cytoplasm (Terry *et al*. 1998) but which cannot discriminate between species of microsporidia. Here we develop a sensitive PCR–RFLP-based protocol which enables us to detect and discriminate between vertically transmitted microsporidia in natural host populations. We screen 2 *G. duebeni* populations for vertically transmitted microsporidia and present evidence for multiple infection within the same population.

MATERIALS AND METHODS

Adult *G. duebeni* were collected from a field population in Fintray Bay, Isle of Cumbrae in December 1999, transported to the laboratory in aerated brackish water and maintained at 12 °C. Approximately 50 pairs of animals were placed in individual containers and checked daily until the eggs were laid into the female's brood pouch. A total of 30 females were screened for vertically transmitted parasites using both fluorescent staining of their embryos and PCR screening of the ovary. To detect parasites by fluorescent staining, eggs were flushed from the brood pouch of each female, permeated with 5 M HCl, rinsed in distilled water and fixed in acetone at −20 °C. The eggs were then transferred to a microscope slide and stained with DAPI (4,6-diamidino-2-phenyl-indole diluted 1:500 in 0·2 M NaH_2PO_4), which is fluorescent for DNA. The eggs were screened for parasites using a Zeiss Axioplan fluorescence microscope. In infected eggs, the diplokaryotic nuclei of the microsporidian parasite could be seen in the cytoplasm around the host nuclei (Dunn *et al*. 1995; Terry, Dunn & Smith, 1997).

For PCR detection of parasites, ovarian tissue was dissected from the females and genomic DNA extracted (Hogg & Lehane, 1999). Microsporidian 16S rDNA was amplified using microsporidian 16S primers V1 (forward) 5′ CACCAGGTTGATTCTGCCTGAC 3′ (Vossbrinck & Woese, 1986) and 530R (reverse) 5′ CCGCGGCTGCTGGCAC 3′ (Baker *et al*. 1995). PCR was performed in a 50 μl reaction using 1 μl (10 ng) of the DNA extract as template and 10 pmols of each primer. The reaction mixture was then adjusted to a final concentration of 1·5 mM $MgCl_2$, 0·04 mM dNTP's and 0·625 U of Gibco *Taq* polymerase and buffer according to the manufacturer's instructions. After an initial denaturing of 5 min at 95 °C, there were 35 cycles of denaturation at 95 °C (1 min), annealing at 50 °C (1 min) and extension at 72 °C (1 min 30 sec) with a final extension of 10 min at 72 °C, using a Perkin Elmer thermal cycler.

PCR bands of 2 distinct sizes (410 or 440 bp, Fig. 1, lanes 2 and 5) were found suggesting the presence of more than 1 type of microsporidia. Therefore, for each band size, we sequenced PCR products obtained from 10 individual hosts. For sequencing, each sample was PCR amplified using the microsporidian primers 18F (5′CACCAGGTTGATTCTGCC3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) (Baker *et al*. 1998). Each product was then submitted to direct sequence analysis in one direction by Dye Termination reaction, according to the manufacturer's instructions (Abi Prism™, Perkin Elmer).

To design a discriminatory RFLP of microsporidian 16S rDNA, the 16S sequence of each parasite was edited to give the region amplified by the 18F:1492R PCR and entered into the internet program Webcutter 2.0 (Heinman, 1997). From the suggested restriction enzymes that would cut these sequences, 2 four cutters giving different restriction patterns were selected, *Msp*I and *Hae*III. Then 10 μl of each PCR product from samples with either a 410 or 440 bp product were digested with the restriction enzymes *Msp*I and *Hae*III according to manufacturer's instructions (Gibco BRL). Digestion products were ethanol precipitated and re-suspended in 5 μl of 15 % Ficoll 0·2 % bromophenol blue and run at 150 V for 45 min on a 12 % acrylamide TAE minigel (3·9 % acrylamide TAE stacking gel) in TAE buffer using a Bio-Rad minigel system. Digestion products were visualized by UV illumination after staining with ethidium bromide. An RFLP profile for each isolate was compiled for each enzyme. Samples were then grouped according to RFLP profile and compared to the Webcutter 2.0 predicted RFLP profiles.

In April 2000, we collected *G. duebeni* from a second, undescribed *G. duebeni* field population from Gansey Point, Isle of Man and applied our new PCR–RFLP screen to 29 females from this population.

RESULTS

Of the *G. duebeni* screened from the Isle of Cumbrae population, 50 % of females were positive for vertically transmitted microsporidia by PCR screening and by microscopy. There was 100 % agreement between the DAPI and PCR results showing that PCR is as sensitive as microscopy in detecting microsporidia. PCR products fell into 2 distinct sizes (Fig. 1, lanes 2 and 5). Of the 15 positive PCR products, 10 (33·3 % of the total population) were approximately 410 bp in size (Fig. 1, lane 2) and 5 (16·7 % of the total population) were approximately 440 bp (Fig. 1, lane 5). Sequencing of the products revealed the existence of 2 different parasites. The sequence generated from the first product (410 bp

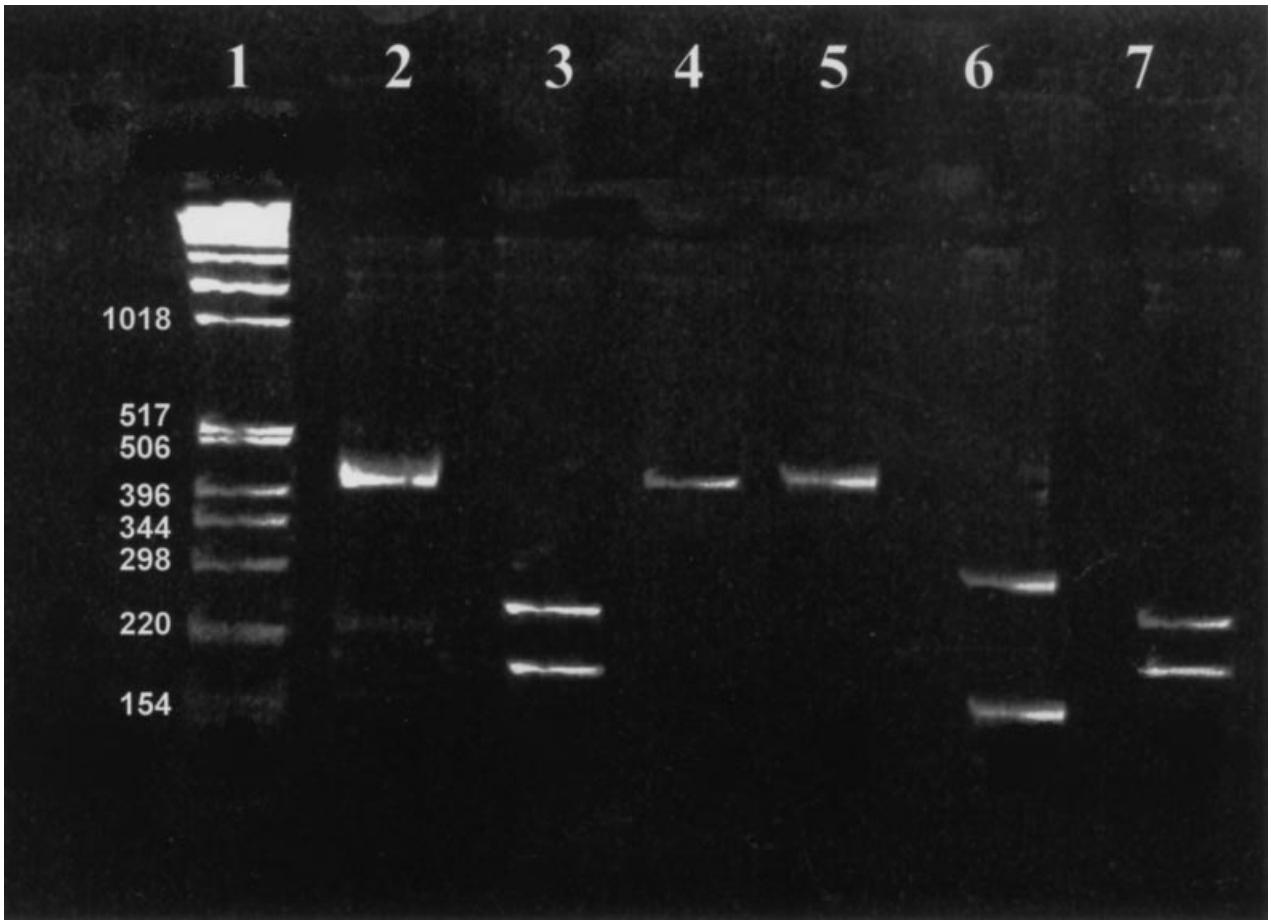

Fig. 1. PCR amplified regions of microsporidian 16S rDNA (primer combination V1: 530R) and their respective DNA fragments following digestion with the restriction enzymes *Msp*I and *Hae*III. Lane 1, 1 kb DNA ladder. Lane 2, *Nosema granulosis* 410 bp PCR product. Lane 3, *Msp*I digestion of the *N. granulosis* PCR product. Lane 4, *N. granulosis* PCR product digest with *Hae*III (no *Hae*III site). Lane 5, 440 bp PCR product of *Microsporidium* sp. and the DNA fragments produced by its digestion with *Msp*I (lane 6) and *Hae*III (lane 7). Samples are representative of those obtained from the ovaries of *Gammarus duebeni* from Fintray Bay, Isle of Cumbrae.

product in our PCR screen with primers V1:530R) matched the existing DNA sequence of the feminizer *N. granulosis* using BLAST (Altschul *et al*. 1990). The sequence generated from the second (which produced a 440 bp product) was microsporidian in origin (but only 51% similar to *N. granulosis*) and gave an 82% match with the partial 16S sequence of *Pleistophora sp*. 3 (Genbank AF044390.1) using BLAST. This novel sequence was submitted to Genbank and we provisionally designate this species *Microsporidium* sp. (Genbank AF397404).

*Msp*I digestion of the *N. granulosis* 410 bp PCR product produced 2 bands of approximately 240 bp and 170 bp (Fig. 1, lane 3) whereas *Hae*III failed to cut the PCR product leaving a band of size 410 bp (Fig. 1, lane 4). Digestion of the *Microsporidium* sp. 440 bp PCR product with *Msp*1 gave 2 bands of 330 bp and 140 bp, (Fig. 1, lane 6). Digestion of the *Microsporidium* sp. product with *Hae*III gave 2 bands 210 bp and 230 bp (Fig. 1, lane 7).

Application of the PCR–RFLP screen to *G. duebeni* from the Isle of Man showed that 55% of females harboured vertically transmitted microsporidia. The 2 parasite species were also present in this second population; 45% of females harboured *N. granulosis* and 10% of females were infected by *Microsporidium* sp. There was no evidence of dual infection within an individual in either population.

DISCUSSION

The PCR method is a sensitive technique to detect infections by vertically transmitted microsporidia. The PCR screen detected 100% of infections (in comparison with LM screening) in our field sample and did not produce any false positives. We can, therefore, use the technique with confidence. We generated novel sequence data from a previously undescribed vertically transmitted microsporidian and demonstrated the presence of 2 distinct species of vertically transmitted microsporidia in both of our field populations, revealing that the relationship between vertically transmitted parasites and their

hosts may be more complex than previously thought. Our PCR–RFLP screen using the restriction enzymes *Msp*I and *Hae*III effectively discriminated between the 2 species. This technique provides a rapid means of discriminating between microsporidia in samples from the field and will facilitate our understanding of these host–parasite interactions.

The occurrence of 2 species of vertically transmitted microsporidia infecting a single host population has implications for our understanding of host–parasite relationships in the field. *N. granulosis* is unusual in that it relies solely on vertical transmission (Terry *et al*. 1998; Dunn & Smith 2001). The new parasite is also vertically transmitted between generations of *G. duebeni* hosts, although the possible additional use of horizontal transmission by this parasite remains to be tested. If both transmission routes are utilized, then we would predict a higher parasite burden and virulence for *Microsporidium* sp. than for *N. granulosis* (Dunn & Smith, 2001). However, if *Microsporidium* sp. is solely vertically transmitted, it must utilize some form of reproductive manipulation to persist in the host population (Howard, 1942; Bull, 1983; Werren & O'Neill, 1997).

Both male killing and feminization have been reported for vertically transmitted microsporidia (Dunn & Smith, 2001). Theoretical analysis predicts that 2 different feminizing parasites cannot coexist within a host population, because the most efficient feminizer will outcompete its rival (Bull, 1983). Two feminizers with identical transmission and feminization efficiencies could, in theory, coexist; but this seems unlikely because both parasites would have to arrive simultaneously in a population if both were to spread through it. In addition, any mutation enhancing the efficiency of one would lead to its spread at the expense of the other (Bull, 1983).

Mathematical models of other pairs of reproductive parasites suggest that outcomes may be complex with one parasite enhancing or impeding the spread of another (Werren & Beukeboom, 1993; Freeland & McCabe, 1997; Smith, 1998; Hatcher, 2000). The only other known form of reproductive manipulation by vertically transmitted microsporidia, male killing, has previously only been reported in mosquito hosts. The co-dynamics of male killers and feminizers in the same population have not been examined theoretically, but we suggest that the dynamics of such pairs will be highly dependent on the mechanism and timing of parasite-induced reproductive manipulation. If the male-killer were to act prior to feminization, the former would impede spread of the latter. If the feminizer were to act first, it would reduce the base for virulent horizontal transmission of the male killer, but the male killer would gain additional vertical transmission via feminized males. It is therefore clearly important to elucidate the transmission mechanisms and reproductive impact of this newly reported microsporidian and to examine the consequences of multiple infections for natural populations. The PCR–RFLP screen described here provides the tools with which to undertake these studies.

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