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Specific Detection and Localization of Microsporidian Parasites in Invertebrate Hosts by Using *In Situ* Hybridization

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We designed fluorescence *in situ* hybridization probes for two distinct microsporidian clades and demonstrated their application in detecting, respectively, *Nosema*/*Vairimorpha* and *Dictyoceola* species. We used them to study the vertical transmission of two microsporidia infecting the amphipod *Gammarus duebeni*.

Microsporidia are important parasites of invertebrates that cause losses in beneficial insects such as pollinators (1, 2) and farmed Crustacea (3, 4), and they are also utilized for biological control of insect pests (5). While many microsporidian species are transmitted horizontally to new hosts, others are transmitted vertically (i.e., female-to-offspring transmission) or by a combination of the two modes (6, 7). Such variation in the mode of transmission affects the evolution of virulence, which is generally reduced in vertically transmitted microsporidia (6, 7). For example, parasites of the genus *Nosema* that infect crustacean hosts cause little pathogenesis; they form low-burden localized infections in the reproductive tissue, are vertically transmitted, and cause feminization of the host offspring, leading to distorted sex ratios (8, 9). In contrast, *Vairimorpha disparis* (also from the *Nosema* clade) causes high-density infection of the fat body, leading to death of the gypsy moth host and subsequent horizontal transmission (10).

Understanding and managing the impact of these parasites on their hosts requires an ability to both discriminate between strains and map the distribution and burden within host tissues. PCR-based detection of microsporidian parasites is well established and can be combined with either restriction fragment length polymorphism (11) or sequencing analysis (12) to identify parasite species. Although quantitative PCR techniques have been developed to monitor parasite burdens in hosts (13), they are a weak tool for the investigation of tissue distribution. Transmission electron microscopy (TEM) is an excellent tool for the visualization of parasites within tissues but is very time-consuming when looking at the distribution of microorganisms across a whole organism or whole tissues because only thin (<100-nm) sections of tissues can be visualized. Since its development as a method to detect microorganisms (14), fluorescence *in situ* hybridization (FISH) has been used to detect and localize many endosymbionts, notably in arthropod hosts (15, 16). It allows the precise localization and distribution of microorganisms within a particular tissue, as well as evaluation of their density. FISH probes target particular regions of the rRNA and can be designed to detect a broad range of microorganisms (if the targeted rRNA region is well conserved across these microorganisms) or to be very specific (if the targeted region is unique); they are thus considered to be phylogenetic stains (17). While this method is widely used to detect and localize bacteria, only a few studies have applied this method to microsporidia (18–20). This is partly due to the fact that microsporidia are eukaryotes with unusual rRNAs (21, 22) and many of the tools developed for bacteria to design FISH probes are therefore unsuitable (23–25).

In this article, we present two FISH probes designed to detect microsporidia of the genera *Nosema*/*Vairimorpha* and *Dictyocoela*. We tested the specificity of these probes by applying them to three economically important microsporidia of the genus *Nosema* and to two unrelated clades. We then used these probes to study the distribution of two vertically transmitted microsporidia, *Nosema granulosis* and *Dictyocoela duebenum* (26), within the ovaries of the crustacean host *Gammarus duebeni* in order to elucidate the route of transmission to developing oocytes.

To design these probes, we first used MAFFT (27) to align 34 microsporidian small rRNA sequences across the microsporidian phylogeny, including several *Dictyocoela* and *Nosema* species (accession numbers are shown in Fig. 1) in order to identify the regions that were conserved among the members of the genus of interest but distinct from others. rRNA regions are known to be more or less accessible to FISH probes (23). As an accessibility map does not exist yet for microsporidia, whose small rRNA is distinct in structure from that of other eukaryotes and from that of bacteria, we chose to target rRNA regions that are known to be accessible for both yeasts and bacteria. These regions were identified for our species by using the secondary structure of the microsporidian small subunit (SSU) found at the comparative RNA website (http://www.rna.ccbb.utexas.edu/) (28). Finally, we checked the *in silico* specificity of the two probes by using ProbeCheck (29) and BLAST. By using this method, we designed two probes, Ng02 (ATAGGTCA AGTTTCGCCC), with specificity for the *Nosema*/*Vairimorpha* clade, and Dd04 (GACCTTGGTCCTGGTAGC), with specificity to the genus *Dictyoceola*. The probes matching the targeted areas of each species are illustrated in Fig. 1.

We used the probes on tissues of *G. duebeni* infected with either

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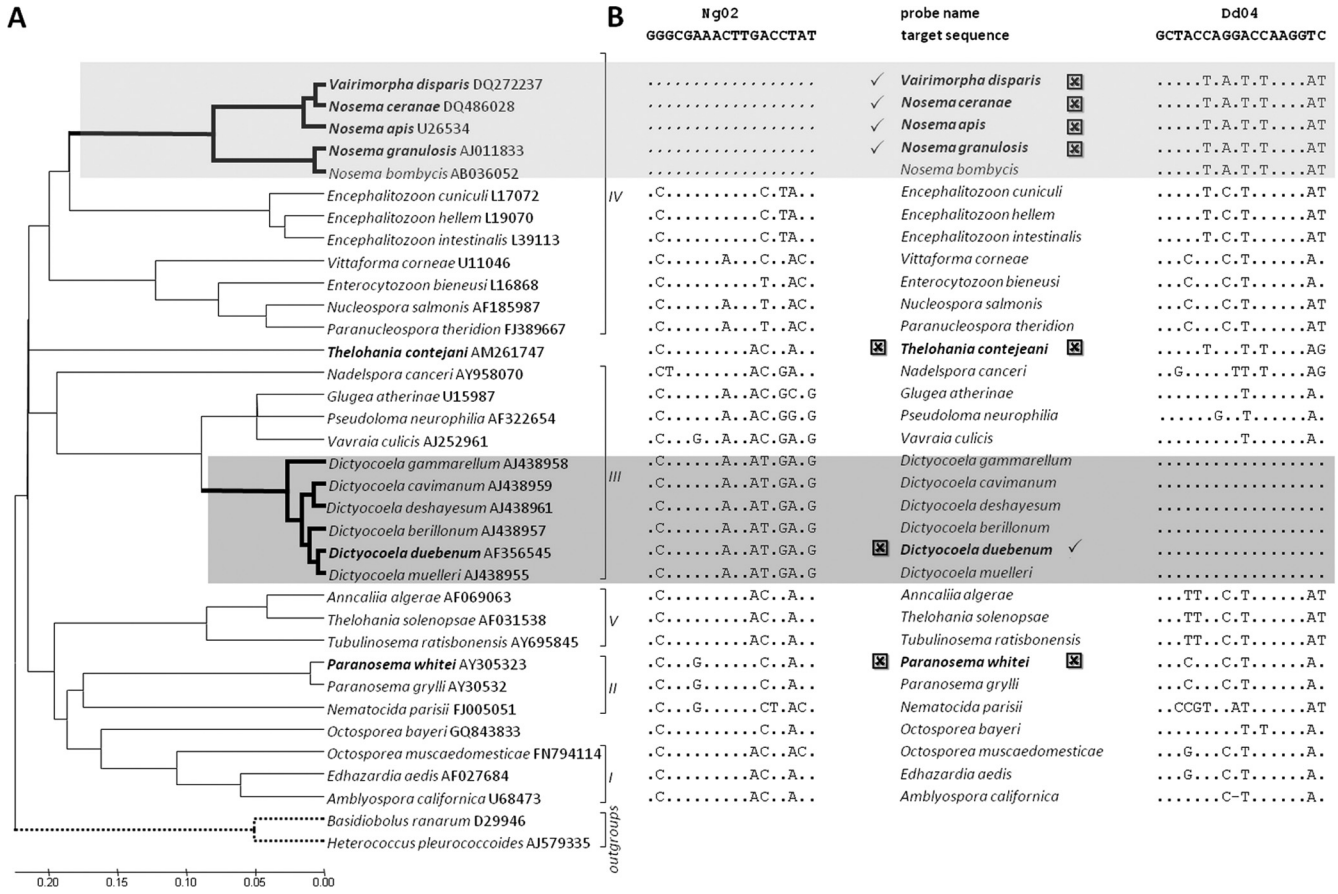

FIG 1 Use of probes Ng02 and Dd04 as phylogenetic markers. (A). Phylogenetic tree showing the relationships of microsporidian species belonging to various clades defined by Vossbrinck and Debrunner-Vossbrinck (39). The species with which probes Ng02 and Dd04 hybridized are highlighted in light and dark gray, respectively. The alignment of the small-subunit rDNA sequences of these 34 microsporidian species was performed with MAFFT (27). The tree, obtained by using the neighbor-joining method, was based on 578 positions. (B) Alignment of the microsporidian species in the tree in panel A focusing on the rRNA regions targeted by probes Ng02 and Dd04. The target region of each probe is shown. Periods indicate nucleotides in that region that match the corresponding probe, while letters indicate mismatches. A dash indicates a deletion in the SSU sequence. The species tested in this study are in bold. A check mark indicates that a hybridization signal was observed, and a boxed X indicates absence of hybridization.

D. duebenum or *N. granulosis* (infection confirmed by PCR-RLFP [11]). Tissues were fixed with acetone or Carnoy fixative and held at −20°C until use. The tissues were subsequently rehydrated in PBS-T (phosphate-buffered saline [pH 7.4] with 0.05% Triton X-100), incubated in a 1:1 solution of PBS-T and hybridization buffer (HB; 20 mM Tris-HCl [pH 8], 0.9 M NaCl, 0.01% SDS, 1× Denhardt's solution, 30% [for Dd04] or 35% [for Ng02] deionized formamide [P040.1; Roth]) for 10 min at 20°C, in HB for 20 min at 20°C, and then in HB for 30 min at 45°C before the addition of 5′-Cy5-labeled probes (Jena Bioscience) Ng02 and Dd04 at concentrations of 0.5 μM (Ng02) and 0.25 μM (Dd04). Following incubation at 45°C overnight, samples were washed in HB at 45°C 2 × 30 min and at 20°C 1 × 30 min and in HB–PBS-T at 20°C 1 × 30 min, stained for 30 min with PBS-T containing 300 nM 4′,6-diamidino-2-phenylindole (DAPI), and mounted in 40% glycerol. Images were taken with a Zeiss LSM510 confocal inverted microscope (Carl Zeiss Ltd., Herts, United Kingdom) and processed using Gimp. DAPI staining was visualized with a 405-nm laser and a 420- to 480-nm bandpass filter and Cy5-labeled probes using a 633-nm laser and a 650-nm low-pass filter. Autofluorescence of spores was transiently visible with a 488-nm laser and a 530- to 600-nm bandpass filter. Using our probes, we were able to detect all stages of these microsporidia (vegetative and spore stages; see Fig. S1 and S2 in the supplemental material). FISH is therefore a more powerful method to detect microsporidia than is immunofluorescence, which detects only spores (12, 30).

We assessed the specificity of these probes by applying them to *G. duebeni* whole-mount embryos infected with *N. granulosis* or *D. duebenum* and to spores of five other microsporidian species that had been stored in 50% glycerol (31). These included *N. ceranae* and *N. apis*, both of which cause significant disease in honeybees (1); *Vairimorpha disparis*, which also belongs to the *Nosema* clade (32) and is a parasite studied for biological control of the gypsy moth (5); and parasites from two additional unrelated clades, *Paranosema whitei*, which is a parasite of flour beetles (33), and *Thelohania contejeani*, which causes porcelain disease in crayfish (34). The protocol described above was used. The pattern of hybridization perfectly matched the phylogenetic associations (Fig. 1; see Fig. S3 and S4 in the supplemental material). Dd04 hybridized only with *D. duebenum*, and there was no cross-reactivity with other genera. Ng02 reacted with all three members of the *Nosema* clade; *N. apis*, *N. ceranae*, and *V. disparis*, and there

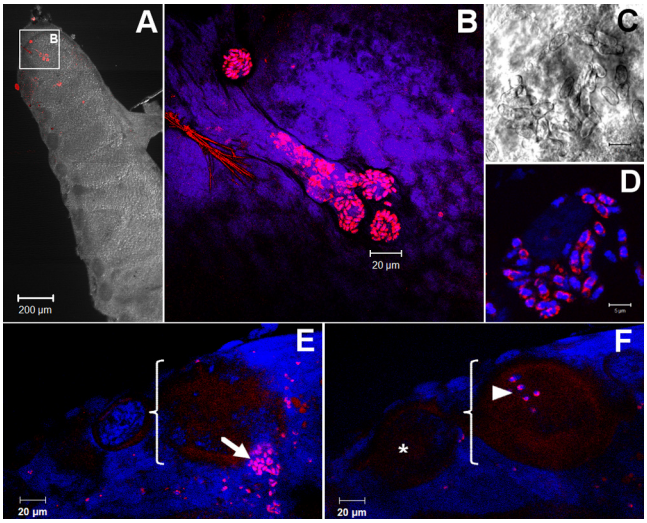

FIG 2 Transovarial transmission of *D. duebenum* in its *G. duebeni* host. (A) *G. duebeni* ovary. The red signal (FISH) indicates the presence of *D. duebenum*. (B) High magnification of the *G. duebeni* ovary shown in panel A. Clusters of microsporidia are visible. (C, D) Group of microsporidia. Spore walls are visible in panel C. (E) A follicle cell containing *D. duebenum* spores (arrow) is visible in the vicinity of a maturing oocyte (bracket). Oocyte yolk is lightly autofluorescent. (F) Same tissue as in panel E observed in a deeper *z* plane. An immature oocyte is visible (*), while the maturing oocyte contains *D. duebeni* meronts (arrowhead). Panel C, differential interference contrast image; B, D, E, and F, fluorescence microscopy images; A, overlay of differential interference contrast and fluorescence images; red, FISH signal; blue, DAPI staining.

was no cross-reactivity with other genera. These results not only show that these probes can be used as phylogenetic tools to detect *Dictyocoela* or *Nosema* species but highlight their potential use to detect microsporidian species of economic relevance. These probes could also be used to detect these species in environmental samples such as honey, soil, or water in order to follow their spread in the environment.

Finally, we applied these probes to whole-mount ovarian tissues of *G. duebeni* to investigate the mechanism of transovarial transmission by *N. granulosis* and *D. duebenum*. We observed a high density of *N. granulosis* spores in follicle cells (see Fig. S5 in the supplemental material), which are adjacent to developing oocytes, in accord with previous TEM studies that suggested that spores invade secondary oocytes during their maturation (35). Furthermore, we observed a similar proliferation of *D. duebenum* spores in follicle cells, as well as the presence of meronts in maturing oocytes (Fig. 2). These data led us to conclude that these two phylogenetically distant microsporidia have evolved convergent vertical transmission strategies.

Our study shows that FISH can be applied successfully to detect and precisely localize microsporidian species within host tissues. So far, most of the few studies that have applied FISH to microsporidia have focused on the detection of spores of microsporidia infecting vertebrates, especially humans (18, 19, 36). Our study shows that not only the spores but all of the stages of the microsporidian life cycle can be detected by FISH (see Fig. S1 and S2 in the supplemental material). Moreover, previous studies applied this method to stool samples, intestinal biopsy samples, or environmental samples (18, 36). Our study shows that this method is also suitable for use with whole-mount tissues, allowing the study of the dynamics of cell invasion of microsporidia within tissues. Applications of FISH to microsporidia are broad. For example, coinfections with different microsporidian species have been reported in many hosts (13, 37, 38). With the use of specific probes, one could easily determine the tissue specificity of multiple microsporidian species within a host to understand their respective impact on the host's biology. Moreover, as vertical transmission is widespread among microsporidia (9), FISH could help to decipher the various mechanisms used to achieve such vertical transmission. Owing to the diversity of rRNA sequences in microsporidia (39), FISH probes could also be designed for other important clades or species. We believe that the FISH method applied to microsporidia is only at its beginning and that a variety of studies will benefit from its application.

Probe sequence accession numbers. The sequences of probes Ng02 and Dd04 have been deposited in ProbeBase (29) under accession numbers pB-03882 and pB-03883.

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