4-1-2008

Use of phylogenetic stains to determine the feeding preferences of microbivorous soil nematodes

E. Harlan Michelle

Use of Phylogenetic Stains to Determine the Feeding Preferences of Microbivorous Soil Nematodes

by

E. Harlan Michelle

Honors Thesis

in

Department of Biology
University of Richmond
Richmond, VA

April 23, 2008

Advisor: Dr. Amy M. Treonis

INTRODUCTION

Due to their numerous and diverse roles in soil ecological processes – most notably decomposition and nutrient mineralization – nematodes have long been recognized as important biotic indicators of soil health (Ekschmitt *et al.*, 2001; Ritz and Trudgill, 1999). The predominate means of assessing the information that these organisms might contain has been through nematode community analyses, a methodology that seeks to convert the vast amount of data regarding features such as diversity, maturity and richness into a series of indices which can be used to evaluate soil health and make comparisons across samples. Performance of these analyses typically involves the sorting of nematodes morphologically into taxa or functionally by trophic level (Neher, 2001; Bongers and Ferris, 1999). The latter method has recently been recognized as potentially the more valuable as trophic level tends to correspond more directly to ecological role. Additionally, as a broader means of classification, sorting in this manner does not require the rigorous knowledge of nematode morphology necessary for sorting by taxa (Ritz and Trudgill, 1999).

Nonetheless, both of these methods of classification necessitate that an accurate knowledge of nematode biology be in place. More specifically, it is of crucial importance, especially in regards to trophic level sorting, to have a definite knowledge of the food source preference of nematode groups and/or species (Neher, 2001; Wood, 1973; Yeats, 1993). While such information is well-established for certain groups, for others – some of them surprisingly common – it is the subject of much ambiguity and debate. Resolving this issue for these groups will be important for future community analyses studies as it will help to prevent the generation of conclusions flawed by the misinterpretation of ecological roles or the omission of these nematodes from the analysis.

The *Tylenchidae* are one of the most widespread and abundant families of nematodes, being reported to comprise up to 30 percent of all nematodes in soils worldwide (Okada and Kadota, 2003). Given this prevalence, the *Tylenchidae* almost certainly play significant roles in soil ecological processes; however, an accurate assessment of these roles has been hindered by a lack of definitive data concerning the feeding preferences of the members of this family. At present, the *Tylenchidae* are variously designated as plant feeders, fungal feeders, or root and fungal feeders. The basis for these assignments comes predominately from morphological examinations and laboratory culture-based experiments. Yeats *et al.* (1993) classify the family as plant feeders based upon their possession of a slender stylet and their presence within the plant rhizosphere. Wood (1973) reports the growth and reproduction of several *Tylenchidae* species on both plant and fungal substrates, and Okada *et al.* (2002, 2003, 2005) describe the growth and reproduction of six species within the genus *Filenchus* on several fungal substrates. However, the observational and culture-dependent nature of these studies leaves the central question of preference unanswered. These data inform only about what the *Tylenchidae* are able to consume in a controlled and artificial laboratory setting rather than what they prefer to ingest in their natural environment.

To address this issue, we employed the molecular technique of phylogenetic staining [a fluorescent *in situ* hybridization (FISH) technique] to visualize food source genetic material within the nematode. This technique offered three distinct advantages

over the previous morphological and culture-based methodology. First, it allowed analysis of nematodes extracted directly from soil samples without necessitating a period of feeding or growth in the laboratory which meant that the natural feeding habits of the nematodes would remain undisturbed. In addition, the ability to visualize the material inside of the nematode provides a clear indication that the nematode actually ingested that material for consumption. Second, FISH has already been used extensively to characterize and identify species within a variety of microbial communities including activated sludge (Wong *et al.,* 2004; Snaidr *et al.,* 1997), anoxic basins (Lin *et al.,* 2006), freshwater, marine water (Glockner *et al.,* 1999), soil (Fierer *et al.,* 2005), blood cultures (Kempf *et al.,* 2000) and the murine intestine (Scupham *et al.,* 2006). Vandekerckhove *et al.* (2002) have even utilized the technique to visualize and identify endosymbiotic bacteria within the ovaries of *Xiphinema* nematodes. Through these efforts, numerous genetic sequences of varying degrees of phylogenetic specificity have been identified, and probes targeting them have been reported to be efficacious in discerning between diverse and sometimes closely related taxa using FISH techniques. Many of these sequences are located within the ribosomal ribonucleic acid (rRNA) making them especially suitable for the present study as stylet-bearing nematodes such as the *Tylenchidae* are unable to ingest the nucleus but almost certainly take in the much smaller ribosomes. In addition, many ribosomes exist within a single cell making the likelihood of ingestion, as well as fluorescence detection, much greater (Amann, 1995; DeLong *et al.,* 1989). Finally, the use of genetic sequences to distinguish food source material allows for a much more definite conclusion as to its identity than reliance on morphological or observational techniques could provide, especially as the degree of phylogenetic specificity may be altered by selection of different target sequences.

In summary, the aim of the present study is to investigate the potential of molecular techniques, specifically FISH, to resolve the controversy surrounding the feeding preferences of soil nematodes within the *Tylenchidae*.

MATERIALS & METHODS

Cultures. Three model systems consisting of a single nematode species maintained on a single food substrate were established for the purpose of assessing the efficacy of the FISH procedure and the reported phylogenetic specificity of the selected oligonucleotide probes. The bacterial model system consisted of *Caenorhabditis elegans* (Dr. Scott Knight, University of Richmond) maintained on colonies of *Escherichia coli* grown on NGM agar. These cultures were stored at 11°C and nematodes were transferred to fresh plates approximately every ten days to maintain culture viability. The fungal model system was composed of *Aphelencus avenae* maintained in wheat jars on a substrate of *Rhizoctonia solani*. Jars were inoculated with the nematodes approximately one week after their inoculation with the fungi and cultures were allowed to incubate at room temperature several weeks before harvesting. The plant model system consisted of *Meloidogyne hapla* cultivated on *Capsicum annum* plants grown from seed in the laboratory. Plants were inoculated with *M. hapla* approximately three weeks following germination and were allowed to grow 2-3 months before harvesting.

Probes. Oligonucleotide DNA probes were labeled at their 5’ end with fluorescein or AlexaFluor 546 (Invitrogen, Carlsbad, California). Probes were diluted in

TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4) to a concentration of 10 μM. The probe solution was then divided into 100 μM aliquots and stored at -80°C.

The following five probes were utilized: Euk516 (5'-ACCAGATTGCCCTCC-3'), a universal eukayotic probe complementary to eukaryotic 18S rRNA and used as a positive control (Amann *et al.*, 1992); Non338 (5'-ACTCCTACGGGAGGCAGC-3'), a nonsense probe used as a negative control to assess non-specific binding (Glockner *et al.*, 1999); Eub338 (5'-GCTGCCTCCCGTAGGAGT-3') a bacterial-specific 16S rRNA probe (Amann *et al.*, 1990); FR1 (5'-CTCTCAATCTGTCAATCCT-TATT-3') a fungal-specific probe complementary to a sequence on 18S rRNA (Zhou *et al.*, 2000; Hagn *et al.*, 2003), and 28KJ (5'-GGCGGTAAATTCCGTCC-3'), a plant-specific probe complementary to 28S rRNA (Cullings, 1992). Samples with no probe added were also run to assess potential autofluorescence.

Extraction. *C. elegans and A. avenae* were removed from their respective cultures by rinsing with de-ionized water. *M. hapla* and nematodes derived from local soil samples were extracted via the sugar centrifugation method using a sucrose solution of 454 g/L. Local soil samples were obtained from the University of Richmond, VA on the slope of a small wooded ravine between the Gottwald Science Center and Parking Lot C. All nematodes were transferred to centrifugation tubes fitted with filters in which all pre-hybridization washes were carried out. Between all washes, the tubes were centrifuged for several seconds at 6000 rev/min to force the supernatant through the filter.

Nematode FISH. FISH procedures were adapted from the work of Vandekerckhove *et al.* (2002). Nematodes were first washed in 0.1% benzalkonium chloride for one minute before being rinsed twice for two minutes each in sterile water (0.85% sodium chloride). Samples were then fixed for ten minutes in a 1:1 mixture of glacial acetic acid and ethanol after which they were rinsed twice for five minutes each in pure ethanol. Next, they were rinsed for ten minutes in a 1:1 mixture of methanol and phosphate-buffered Tween (PBT; 150 mM NaCl, 10 mM Na_3PO_4, 0.1% Tween 20, pH 7.4). Samples were then washed with 1.0% formaldehyde in PBT for 30 minutes, following which they were rinsed with PBT alone twice for two minutes each.

A sufficient amount of sheared herring sperm was added to the required amount of hybridization mixture (20 mM Tris HCl, 0.02% SDS, 0.9 M NaCl, 5 mM EDTA, 60% formamide, pH 7.4) to obtain a final concentration of 100 μg/mL. Each filter tube received 180 μL of hybridization mixture and was shaken gently to release the nematodes from the filter into the solution. The solution was then transferred to a non-filtered Eppendorf tube to prevent drying of the nematodes over the hybridization period. Twenty microliters of the desired probe were added to each tube to achieve a final concentration of 1 μM probe. The samples were then wrapped with foil to protect the fluorescent label from light exposure and placed in a darkened incubator at 46°C overnight to allow for hybridization. Following this incubation, samples were rinsed twice for 30 minutes each at 48°C in hybridization buffer (20 mM Tris HCl, 0.02% SDS, 0.008 M NaCl, 5 mM EDTA, pH 7.4).

Preparation of slides. Following the last hybridization buffer wash, the supernatant was removed and the nematodes were re-suspended in 30 μL DABCO (1,4-diazobicylco[2.2.2]octane) to preserve fluorescence. This solution was pipetted onto microscope slides that had been prepared by the placing of droplets of clear nail polish to hold the coverslip slightly above the sample to avoid flattening the nematodes. Cover

slips were sealed to the slides with a layer of clear nail polish. Slides were stored at 5°C until the samples could be examined.

Food substrate FISH. These FISH procedures were also adapted from the work of Vandekerckhove *et al.* (2002). Solutions, washes and times were employed as described above, however specimens were maintained on glass microscope slides during the procedure as opposed to centrifuge tubes. Both *E. coli* and *R. solani* samples were heat-fixed to the slide before beginning the FISH procedure. *C. annum* could not be used as the plant specimen for FISH due to an inability to obtain intact stem or leaf sections thin enough for subsequent microscopy. *Elodea canadensis* leaves were utilized instead due to their inherent thinness of approximately two cells. These samples were placed in centrifuge tubes for the FISH procedure and were placed on slides at its duration. All slides were ultimately prepared for microscopy as described above.

Microscopy. Samples were viewed and recorded using a Leica SP2 laser scanning confocal microscope equipped with a krypton-argon laser (excitation filter wavelength 488 nm) for use with fluorescein, a helium-neon laser (excitation wavelength 546 nm) for use with AlexaFluor 546, and both 40X and 63X oil immersion objectives.

Experimental Design. Efficacy of the FISH technique was first assessed by application of EUK516 and NON338, positive and negative controls, respectively, to the organisms composing the three model systems. In subsequent trials, at least one sample for each of these probes was run to ascertain procedural success and non-specific binding levels for that particular trial. Probe specificity was determined by the performance of a series of cross-checking experiments in which each phylogenetically-specific probe was applied to samples of each type of food substrate and the resulting fluorescent signal was assessed. All three probes were tested on different samples of a particular substrate in the same trial – along with samples for EUK516 and NON338 – in order to minimize any variation due to potential unconscious procedural differences between trials.

Potential for the application of this FISH technique to nematodes was established by applying all three phylogenetically-specific probes to each of the nematode components of the three model systems. As above, all three probes were applied to different samples run during the same trial to minimize inter-trial variation in detected fluorescent signal. Performance of the FISH technique on directly extracted *Tylenchidae* nematodes followed this same scheme.

RESULTS

Application of the universal eukaryotic probe EUK516 yielded a very bright fluorescent signal with a generalized staining pattern for all eukaryotic components of the three model systems. In contrast, application of the nonsense probe NON338 on these same organisms yielded only a weak, general signal easily distinguishable from that obtained using EUK516 (Figure 1). The sole prokaryotic organism investigated in these systems, *E. coli*, exhibited a fluorescence staining pattern similar to that of NON338 when EUK516 was applied, but exhibited a strong signal localized to individual bacterial cells upon application of EUB338 which was considered to be the positive control for this particular case (Figure 2).

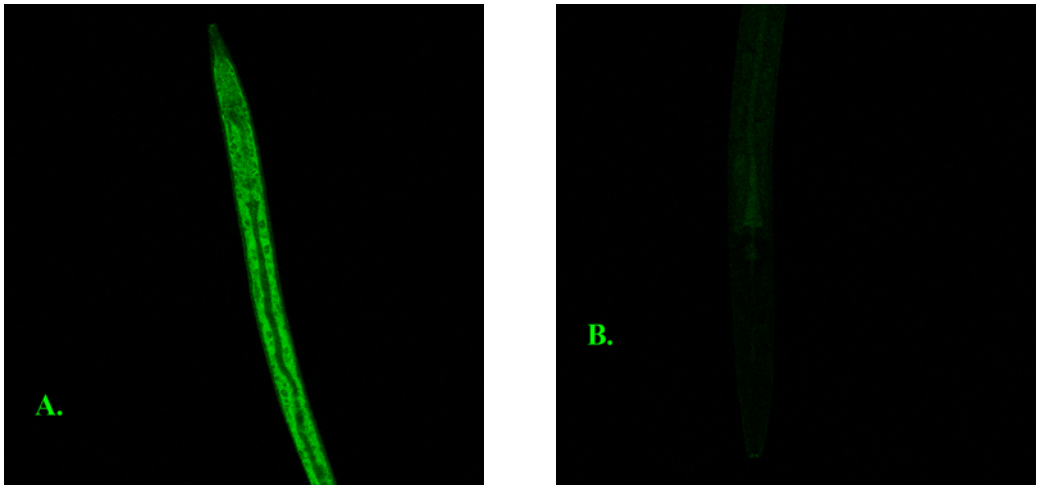

Figure 1: Positive and Negative Control Probes. (A). EUK516 on *C. elegans*. (B). NON338 on *C. elegans.* Both pictures were taken from slides processed during the same FISH trial. The difference in fluorescence is evident between positive control (A) and negative control (B). Only slight fluorescence is seen for the negative control indicating minimal levels of non-specific binding.

Cross-checking FISH experiments performed using EUB338, FR1 and 28KJ verified the specificity of these probes as reported in the literature (Table 1). Distinct and reproducible staining patterns were observed. Hybridization with EUB338 caused the entire *E. coli* bacterial cell to fluoresce strongly and individual cells could clearly be seen. When applied to *R. solani*, FR1 caused the entire fungal hyphae to fluoresce strongly. *E. canedensis* hybridized with 28KJ exhibited a less generalized staining pattern. Strong fluorescent signal was not seen to emanate from the entire cell, but rather from structures within it that appeared to correspond to nucleoli and/or potential amalgamations of ribosomes scattered throughout the cell (Figure 2). Fluorescent signal observed upon the application of any probe to a substrate not its target was no stronger than that seen upon application of NON338 which in turn exhibited only very weak signal, quite distinct from that obtained from the application of the probe targeting that substrate.

Cross-checking experiments were also performed for the nematode components of the model systems to further verify their specificity and also to test the applicability of the technique to nematodes (Table 1). Interestingly, two discrete, reproducible staining patterns were observed depending on the type of nematode being examined. When EUB338 was applied to samples of *C. elegans*, the fluorescent signal appeared as a large splotch localized within the pharynx of the nematode representing the recent consumption of a mass of bacterial cells. However, when FR1 was applied to *A. avenae* fluorescent signal was seen to emanate only from the stylet region. Signal was never observed beyond the posterior end of the stylet for these nematodes. Due to difficulties in isolating sufficient numbers of currently plant-feeding adult *M. hapla*, plant-feeding nematodes directly extracted from local soil samples were used for these experiments.

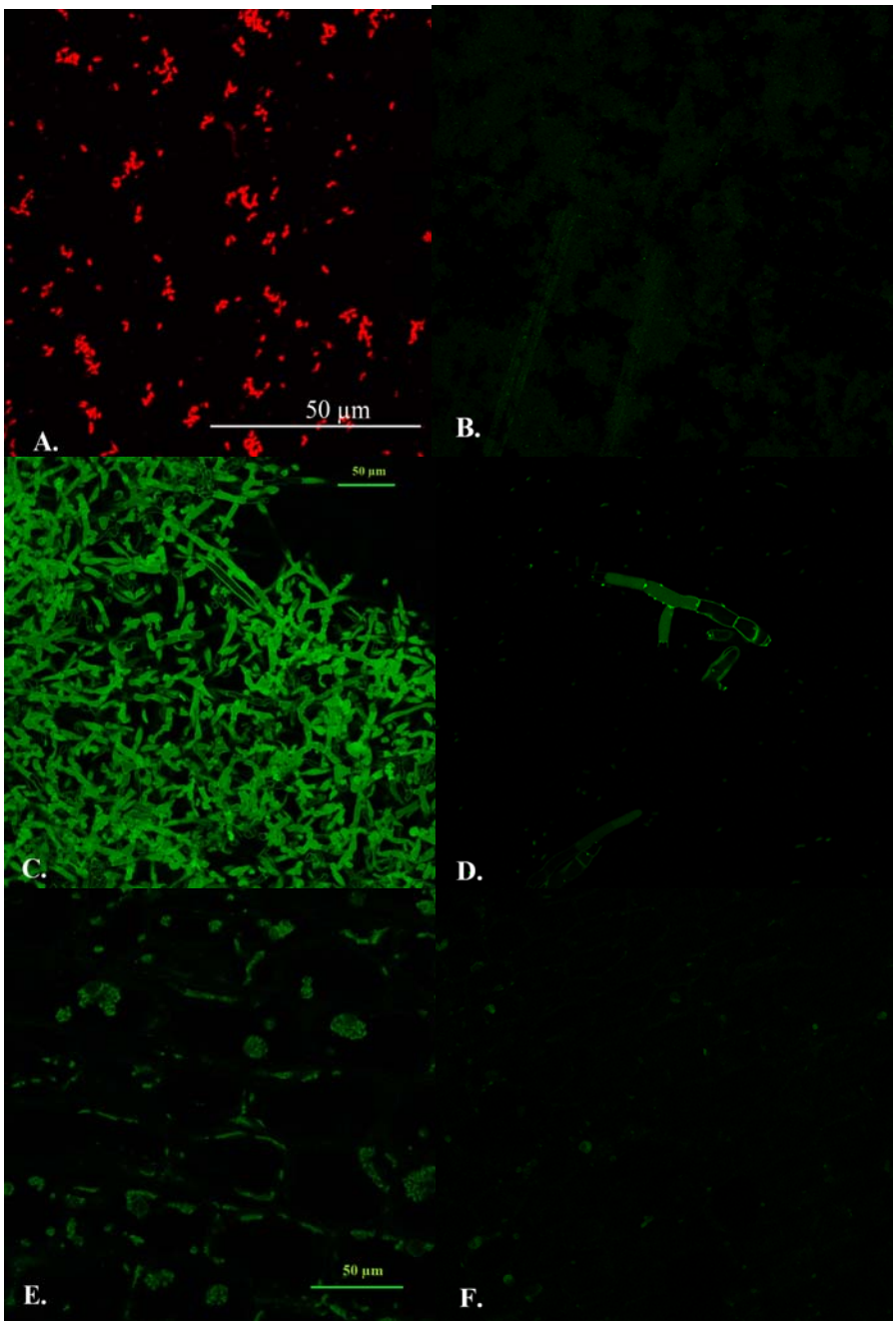

Figure 2: Phylogenetically-specific probes applied to target food substrates. Application of EUB338 to *E. coli* (A) resulted in strong localized staining of bacterial cells while application of NON338 (B) yielded only a weak generalized stain. FR1 applied to *R. solani* (C) caused the entirety of the fungal hyphae to fluoresce strongly while NON338 (D) resulted only in a weak, incomplete staining pattern. Application of 28KJ on *E. canadensis* resulted in the bright, localized staining of cellular structures while applying NON338 (F) yielded only weak, spotty staining that did not appear to correspond closely to cellular structures. Scaling in the NON338 images is not necessarily identical to that displayed in the corresponding substrate-specific probe images.

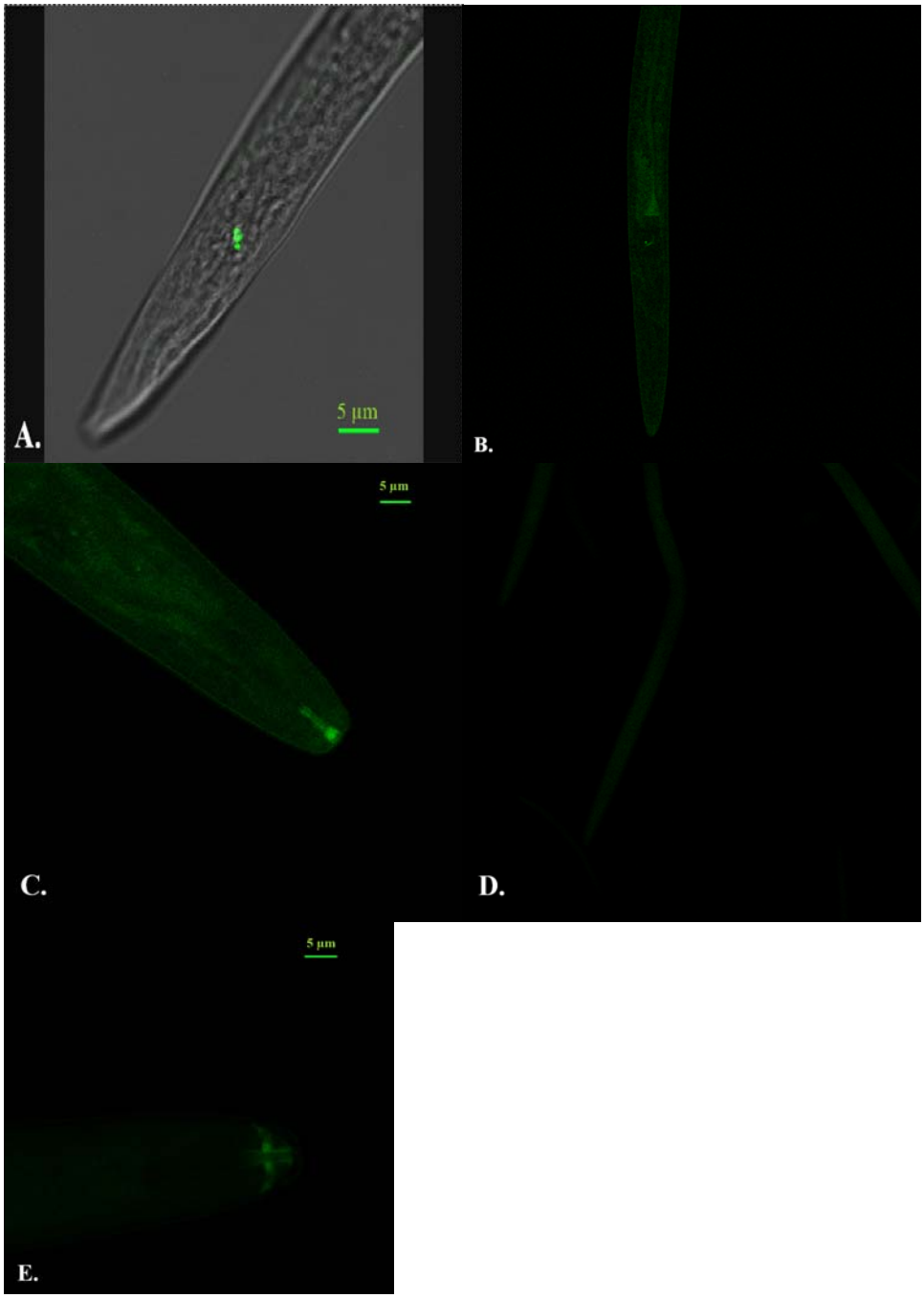

Figure 3: Phylogenetically-specific probes applied to nematode components of the model systems. Application of EUB338 to *C. elegans* (A) revealed a strong localized fluorescent signal within the pharynx of the nematode while FR1 on *A. avenae* (C) exhibited strong localized staining in the stylet region. For both species, application of NON338 yielded only a generalized, weak signal, (B) and (D), respectively. Application of 28KJ to directly extracted plant-feeding nematodes (E) also revealed localized staining in the stylet region. Scaling in NON338 images is not necessarily identical to that displayed in the corresponding substrate-specific probe images.

When 28KJ was applied to these nematodes staining was, as with *A. avenae*, localized to the stylet region (Figure 3). As with the substrate cross-checking experiments, application of a probe not specific to a particular substrate did not yield a fluorescent signal any stronger than that obtained after application of NON338 which itself was a general, weak signal readily distinguished from the strong localized signals obtained from application of the probe specifically targeting that substrate.

Table 1: Summary of Cross-Checking Experiments on Substrate and Nematode Components of Three Model Systems

	E. coli	*C. elegans*	*R. solani*	*A. avenae*	*E. canedensis*	Plant-feeder
EUK516	X	✓	✓	✓	✓	✓
NON338	X	X	X	X	X	X
EUB338	✓	✓	X	X	X	NA
FR1	X	X	✓	✓	X	NA
28KJ	X	X	X	X	✓	✓

Application of these probes to *Tylenchidae* nematodes directly extracted from local soil samples was next performed to initiate determination of the natural feeding preferences of this family. Hybridization of *Tylenchidae* nematodes with EUB338 and 28KJ did no yield any fluorescent signal stronger that that achieved upon application of NON338 which again was a very weak, general signal. However, upon application of FR1 to *Tylenchidae* specimens, faint but distinct staining was observed localized to the stylet region in a pattern highly similar to that seen for *A. avenae* (Figure 4). While such a staining pattern was not observed for every *Tylenchidae* nematode examined, the pattern was observed for multiple worms in two separate trials representing a degree of reproducibility comparable to that achieved with the model system nematodes.

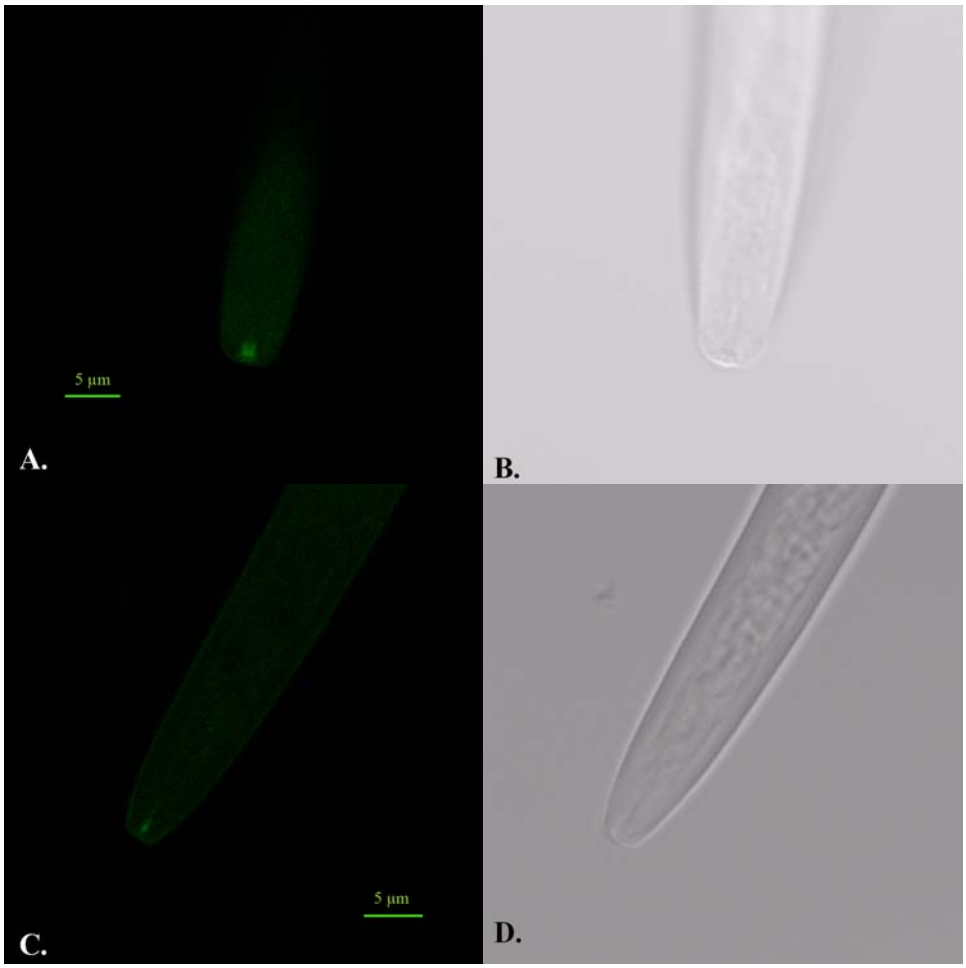

Figure 4: FR1 on *Tylenchidae* sp. Fluorescent (A, C) and transmitted light (B, D) images of two *Tylenchidae* nematodes showing localized fluorescent signal in the stylet region upon application of FR1. Nematodes were from separate FISH trials. Scaling in the transmitted light images is the same as that in the fluorescent images.

DISCUSSION

In order to utilize nematode communities as bioindicators of soil ecological processes, there must be in place strong foundation knowledge of nematode biology in order to perform the types of classification required by various analyses (Neher, 2001; Bongers and Ferris, 1999). In particular, it is important to have a definitive knowledge of the natural feeding preferences of species and/or groups of nematodes since trophic level tends to relate more directly to ecological role than does taxonal classification (Ritz and Trudgill, 1999; Wood, 1973; Yeats, 1993). Morphological (Wood, 1973; Yeats, 1993) and laboratory-based culture (Okada *et al.*, 2002, 2003, 2005) studies are not in themselves sufficient to resolve this question. These types of experiments tend either to draw conclusions based on inferences or to introduce artificial factors into the

investigation, respectively, meaning that they determine what the nematodes can eat rather than what they prefer to consume. Resolving this latter question calls for the development of molecular techniques that can eliminate the necessity for periods of growth and feeding in the lab and can provide definitive identification of the food organism without necessitating simplification of the system. To this end, the present research focused on the development and application of a FISH technique designed to resolve the controversy surrounding nematode feeding preferences with particular attention to the widespread and abundant *Tylenchidae* family.

Taken together, the strong fluorescent signal achieved upon application of EUK516 to all eukaryotic components of the model systems and the absence of such upon application of NON338 indicated that the developed FISH technique was efficacious (Figure 1). In particular, these results showed that permeabilization measures were sufficient for probe entry while still allowing for maintenance of morphological integrity of the specimen, an important consideration given that the aim of this technique was to visualize food source genetic material inside the nematode. Additionally, results from the application of these two control probes allowed the determination that the hybridization conditions employed were adequate for probe-target sequence binding and the verification that that binding was indeed specific. This latter point was further supported by the NON338-like binding pattern observed upon application of EUK516 to prokaryotic *E. coli*.

The results of cross-checking FISH experiments revealed that the chosen phylogenetically-specific oligonucleotide probes were highly specific as reported in the literature (Amann *et al.*, 1990; Cullings, 1992; Glockner *et al.*, 1999; Hagn *et al.*, 2003; Zhou *et al.*, 2000). The probes bound only to their target substrate and did not bind to any significant degree to other organisms (Figure 2). This was the case even for the fungal and plant probes for which much difficulty has been reported in attaining specificity between the two phyla while still attempting to achieve broad detection within each one (Camacho *et al.*, 1997; Cullings, 1992).

Applying these probes to the nematode components of the three model systems utilized in this experiment revealed that the FISH technique was able to detect food source genetic material within nematodes. Staining patterns observed appeared to reflect the method of feeding employed by the particular nematode species. *C. elegans*, a bacterial-feeder, is thought to consume large clumps of bacterial cells at a time (Yeats, 1993). FISH images of EUB338 application to *C. elegans* specimens revealed a large fluorescent splotch in the pharynx of the nematode which likely represents this large clump of ingested bacteria (Figure 3A). In contrast, stylet-bearing nematodes like the fungal-feeding *A. avenae* and the plant-feeding *M. hapla*, feed by puncturing fungal or plant cells with the stylet and using it as a straw to extract the cell's cytoplasm along with any organelles small enough to pass through the slender stylet opening (Yeats, 1993). The staining pattern observed upon application of FR1 or 28KJ to fungal-feeding or plant-feeding nematodes, respectively, was localized to the stylet region, presumably reflecting the presence of rRNA in the cytoplasm drawn into the stylet as the nucleus of fungal and plant cells is too large be ingested (Figure 3C, E). It is interesting to note that staining was never observed in the pharynx of these nematodes as it was with *C. elegans*, and in fact, staining was never observed to occur past the posterior end of the stylet. It is

possible that the rRNA is degraded by the time the ingested cytoplasm moves past the stylet region.

Given the above observation, it was not surprising that the fluorescent signal observed in the *Tylenchidae* specimens was localized to the stylet region. It has long been noted that the *Tylenchidae* possess – and presumably utilize – stylets in a manner similar to that of other stylet-bearing species (Wood, 1973; Yeats, 1993) which would seem to indicate similar feeding preferences. However, it has not been resolved as to whether those feeding preferences are aligned more closely with fungal-feeding or plant-feeding stylet-bearing nematodes. The results obtained through this FISH study suggest that *Tylenchidae* feeding preferences are fungal in nature (Figure 4). The veracity of this finding is supported by the fact that the particular *Tylenchidae* nematodes that exhibited fluorescent signal have tentatively been identified as belonging to the genus *Filenchus*, several species of which Okada *et al.* (2002, 2003, 2005) described as being able to grow and reproduce on several fungal substrates. However, these data are the results of only two independent FISH trials and further work must be performed both to ensure reproducibility and to definitely identify the nematodes investigated before more fixed conclusions can be drawn.

CONCLUSIONS

In summary, a FISH technique has been developed that has been shown to be suitable for the visualization of food source genetic material within nematodes. Moreover, this technique is applicable to both nematodes grown in culture and thus surrounded by an excess food supply, and to nematodes directly extracted from their natural soil environment where food sources are likely to be more disperse and thus the concentration of ingested food lower at any point in time. As such, this technique has the potential to resolve the controversy surrounding the feeding preferences of not only the *Tylenchidae* but also of other groups such as the *Dorylaimidae.* Answering these questions will be of significant value to the performance of nematode community analyses and thus the use of nematodes as bioindicators of soil ecological processes.

Application of this technique to *Tylenchidae* nematodes has yielded data suggesting that at least some of the members of this family may display fungal-feeding preferences in their natural environment. However, more FISH trials and more specific identification of nematode specimens is needed for definitive conclusions to be drawn. In addition, it will be helpful to develop a Multi-FISH technique in which multiple probes targeting different food substrates can be applied to a single sample. This method would be more time and resource efficient than the current method and would allow for the more reliable detection of potential omnivorous tendencies in the *Tylenchidae.* It will also be of interest to investigate the potential of more phylogenetically-specific substrate-targeted probes to provide better identification of the food source organism.

ACKNOWLEDGEMENTS

I would like to thank Dr. Amy Treonis for her mentorship on this project, Dr. April Hill for sharing her expertise on *in situ* techniques, Carolyn Marks for her assistance with confocal microscopy, Erin Austin for her aid on the cross-checking

experiments while I was abroad and Dr. Scott Knight for his provision of several plates of *C. elegans* cultures.

This research was supported by grants from the American Society for Microbiology, the Thomas F. and Kate Miller Jeffress Memorial Trust, and the University of Richmond School of Arts and Sciences.

REFERENCES

Amann, R. I., Binder, B., Chisholm, S. W., Olsen, R., Deveruex, R. & Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied Environmental Microbiology,* 56, 1919-1925.

Amann, R. I. (1995). Fluorescently labeled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Molecular Ecology,* 4, 543-554.

Bongers, T. & Ferris, H. (1999). Nematode community structure as a bioindicator on environmental monitoring. *Trends in Ecology and Evolution,* 14, 224-228.

Camacho, F. J., Gernandt, G. S., Liston, A., Stone, J. K. & Klein, A. S. (1997). Endophytic fungal DNA, the source of contamination in spruce needle DNA. *Molecular Ecology,* 6, 983-987.

Cullings, K. W. (1992). Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molecular Ecology,* 1, 233-240.

DeLong, E. F., Wickham, G. S. & Pace, N. R. (1989). Phylogenetic Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells. *Science,* 243, 1360-1363.

Ekschmitt, K., Bakonyi, G., Bongers, M., Bongers, T., Bostrom, S., Dogan, H., Harrison, A., Nagy, P., O'Donnell, A. G., Papatheodorou, E. M., Sohlenius, B., Stamou, G. P. & Wolters, V. (2001). Nematode community structure as indicator of soil functioning in European grassland soils. *European Journal of Soil Biology*, 37, 263-268.

Fierer, N., Jackson, J. A., Vilgalys, R. & Jackson, R. B. (2005). Assessment of Soil Microbial Community Structure by use of Taxon-Specific Quantitative PCR Assays. *Applied and Environmental Microbiology,* 71, 4117-4120.

Glockner, F. O., Fuchs, B. M. & Amann, R. (1999). Bacterioplankton Compositions of Lakes and Oceans: a First Comparison Based on Fluorescence In Situ Hybridization. *Applied and Environmental Microbiology,* 65, 3721-3726.

Hagn, A., Pritsch, K., Ludwig, K. & Schloter, M. (2003). Theoretical and Practical Approaches to Evaluate Suitable Primer Sets for the Analysis of Soil Fungal Communities. *Acta Biotechnology,* 23, 373-381.

Kempf, V. A. J., Trebesius, K. & Autenrieth, I. B. (2000). Fluorescent In Situ Hybridization Allows Rapid Identification of Microorganisms in Blood Cultures. *Journal of Clinical Microbiology*, 38, 830-838.

Lin, X., Wakeham, S. G., Putnam, I. F., Astor, Y. M., Scranton, M. L., Chistoserdov, A. Y. & Taylor, G. T. (2006). Comparison of Vertical Distributions of Prokaryotic Assemblages in the Anoxic Cariaco Basin and Black Sea by Use of Fluorescence In Situ Hybridization. *Applied and Environmental Microbiology,* 72, 2679-2690.

Neher, D. (2001). Role of Nematodes in Soil Health and Their use as Indicators. *Journal of Nematology*, 33, 161-168.

Okada, H., Tsukiboshi, T. & Kadota, I. (2002). Mycetophagy in *Filenchus misellus* (Andrassy, 1958) Lownsbery & Lownsbery, 985 (Nematodo: Tylenchidae), with notes on its morphology. *Nematology*, 4, 795-801.

Okada, H. & Kadota, I. (2003). Host status of 10 fungal isolates for two nematode species, *Filenchus misellus* and *Aphelenchus avenae*. *Soil Biology and Biochemistry*, 35, 1601-1607.

Okada, H., Harada, H. & Kadota, I. (2005). Fungal-feeding habits of six nematode isolates in the genus *Filenchus*. *Soil Biology and Biochemistry*, 37, 1113-1120.

Ritz, K. & Trudgill, D. L. (1999). Utility of nematode community analysis as an integrated measure of the functional state of soils: perspectives and challenges. *Plant and Soil*, 212, 1-11.

Scupham, A. J., Presley, L. L., Wei, B., Bent, E., Griffith, N., McPherson, M., Zhu, F., Oluwadara, O., Rao, N., Braun, J. & Borneman, J. (2006). Abundant and Diverse Microbiota in the Murine Intestine. *Applied and Environmental Microbiology,* 72, 793-801.

Snaidr, J., Amann, R. I., Huber, I., Ludwig, W. & Schleifer, K. (1997). Phylogenetic Analysis and In Situ Identification of Bacteria in Activated Sludge. *Applied and Environmental Microbiology,* 63, 2884-2896.

Vandekerckove, T. T. M., Coomans, A., Cornelis, K., Baert, P. and Gillis, M. (2002). Use of the *Verrucomicrobia*-Specific Probe EUB338-III and Fluorescent In Situ Hybridization for Detection of "*Candidatus* Xiphinematobacter" Cells in Nematode Hosts. *Applied and Environmental Microbiology,* 68, 3121-3125.

Wong, M., Tan, F. M., Ng, W. J., & Liu, W. (2004). Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. *Microbiology,* 150, 3741-3748.

Wood, F. H. (1973). Nematode feeding relationships: Feeding relationships of soil-dwelling nematodes. *Soil Biology and Biochemistry,* 5, 593-601.

Yeats, G. W., Bongers, T., De Goede, R. G. M., Freckman, D. W. & Georgieva, S. S. (1993). Feeding Habits in Soil Nematode Families and Genera – An Outline for Soil Ecologists. *Journal of Nematology,* 25, 315-331.

Zhou, G., Whong, W., Ong, T. & Chen, B. (2000). Development of a fungus-specific PCR assay for detecting low-level fungi in an indoor environment. *Molecular and Cellular Probes,* 14, 339-348.