DNA Sequences from Formalin-Fixed Nematodes: Integrating Molecular and Morphological Approaches to Taxonomy[1]

W. Kelley Thomas,[2] J. T. Vida,[2] Linda M. Frisse,[2] Manuel Mundo,[3] and James G. Baldwin[3]

Abstract: To effectively integrate DNA sequence analysis and classical nematode taxonomy, we must be able to obtain DNA sequences from formalin-fixed specimens. Microdissected sections of nematodes were removed from specimens fixed in formalin, using standard protocols and without destroying morphological features. The fixed sections provided sufficient template for multiple polymerase chain reaction-based DNA sequence analyses.

Key words: DNA sequence, formalin, microdissection, molecular biology, nematode, polymerase chain reaction, taxonomy.

Surveys including nematodes, the most abundant metazoans, are a high priority (Freckman, 1994; Systematics Agenda 2000, 1994), but understanding species diversity in nematodes is often limited by the huge size of the phylum, and by the inadequate taxonomic understanding of many groups. Urgently needed advances in taxonomy may be encumbered by the relative lack of easily utilized, diagnostic morphological characters, and a paucity of specialists. Although estimates of the number of species vary by author (Andràssy, 1992; Pearce, 1995; Poinar, 1983; Systematics Agenda 2000, 1994), there is wide agreement that the majority of nematode species have not been taxonomically named and described. To address this problem, new tools for species identification must be integrated into nematode taxonomy. DNA sequences represent one of the most promising sources of new taxonomic characters.

While the sequencing of DNA can be a useful approach in taxonomy, a limitation has been the small size of most nematodes (0.3–2.0 mm in length). The isolation of DNA from such small individuals, while maintaining morphological voucher speci-

mens for linkage between DNA and classical characters, is not a trivial problem. Nevertheless, with new methods of molecular analysis, very small nematode tissue samples can provide sufficient DNA for polymerase chain reaction (PCR)-based analysis (Williams et al., 1992).

Standard protocols for the collection, preservation, and identification of nematodes typically include fixation in a formalin-based solution. Unfortunately, formalin and other fixatives are known to damage DNA and represent a potential problem for the integration of molecular characters as part of a standard survey of nematode species. Previous studies with paraffin-embedded tissues have shown that some methods of formalin fixation can yield tissues that are reliable sources of DNA for amplification by PCR, even when the tissues were fixed for more than 8 days (Greer et al., 1991). However, De Giorgi et al. (1994) have recently reported that nematodes fixed in formalin are not useful for PCR-based analysis.

Alternatives to the use of formalin-fixed tissue could include culturing individual species for molecular analysis or microdissecting nematodes before fixation to recover a small tissue sample for DNA amplification. However, culturing nematodes is not economically feasible for large-scale surveys and excludes numerous unculturable nematode species. Furthermore, the microdissection of unfixed nematodes, which are under hydrostatic pressure, is extremely destructive to the morphological integrity of the specimen. This is in contrast to fixed

specimens, where it is possible to remove a slice from a carefully selected region of the nematode with little or no impact on the diagnostic morphological characters.

For any valuable use of molecular information in nematode taxonomy, the DNA sequences must be linked to morphological voucher specimens. Our goal was to establish a method of molecular characterization using formalin-fixed nematodes that minimizes the destruction of the morphological specimen and does not compromise the quality of the molecular data to be obtained.

MATERIALS AND METHODS

Nematodes: Five genera from three families were used for these tests: *Caenorhabditis elegans* Maupas, 1900 (Dougherty, 1953) (Rhabditidae), PS1010 (Rhabditidae, unnamed species), *Zeldia punctata* (Thorne, 1925) Thorne, 1937 (Cephalobidae), *Aduncospiculum halicti* Giblin and Kaya, 1984 (Diplogasteridae), and JB100 (Diplogasteridae, unnamed species). The lengths of the intact nematodes used in this study ranged from 0.6–1.2 mm. All species were obtained from cultures on *Escherichia coli*-seeded agar petri dishes at the University of California, Riverside (UCR).

Formalin fixation: Nematodes were killed and fixed at UCR by means of standard preparation techniques for light microscopy. Specimens were killed and straightened by placing them for approximately 1 minute in 5 ml of tap water heated to 50 °C. An equal volume of 10% formalin was added to the tap water for a final solution of 5% formalin. Samples were fixed for 48 hours at room temperature before processing for PCR. The region selected for DNA amplification, and considered to be of the least value for morphological diagnosis, was a portion of the intestine posterior to the esophagus but excluding the anterior end of the gonad. After photographing the specimen to record overall form and length, the nematode specimen, in a drop of fixative, was placed on the lid of a plastic petri dish and a 50-µm slice was removed with a small scalpel. The slice was transferred with a cap-

illary tube to a 0.5-ml plastic microfuge tube containing 10 µl of digestion buffer. As a control, a similar minute volume of fixative was transferred to buffer without a nematode slice. The nematode slices in digestion buffer were placed on dry ice and shipped to the senior author for PCR analysis. The remaining portions of each nematode were processed to glycerin with standard procedures (Golden, 1990), mounted on Cobb slides, and deposited as vouchers in the UCR Nematode Collection for future morphological investigations.

Template preparation: Nematode sections are prepared for amplification using "single worm" digestion buffer (Williams et al., 1992). Each section was placed in 10 µl of digestion buffer (10 mM Tris [pH 8.2]; 2.5 mM $MgCl_2$; 50 mM KCl; 0.45% TWEEN 20; 0.05% gelatin; 60 µg/ml Proteinase K) and frozen at −70 °C for 15 minutes to several days. The extracts were thawed, overlaid with a drop of mineral oil, and warmed to 60 °C for at least 60 minutes. Proteinase K was denatured by heating to 95 °C for 15 minutes. A 2.0-µl aliquot of the extract was used in subsequent PCR amplifications.

Amplification and sequencing: Amplifications were performed in 25 µl of a solution containing 67 mM Tris (pH. 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, each dNTP at 1 mM, each primer at 1 µM, 2–5 units of Ampli*Taq* (*Thermus aquaticus*) polymerase (Perkin-Elmer/Cetus, Foster City, CA), and 2 µl of DNA extract (Thomas and Wilson, 1991). For the ribosomal DNA (rDNA) expansion segment the primers D3A (5'-GACCCGTCTTGAAA-CACGGA-3') and D3B (5'-TCGGAAG-GAACCAGCTACTA-3') were used with an annealing temperature of 52 °C. PCR products were separated in 2.0% NuSieve agarose (FMC, Rockland, ME), and a small agarose plug was taken from each band and diluted in 100 µl of dH_2O. The plug was subsequently melted at 65 °C for one to several minutes. In subsequent asymmetric PCR amplifications, 1.5 µl of the diluted plug was used as template.

Sequencing templates were generated by asymmetric amplification using the same

primers with one diluted 1:100 relative to the other. Each 25-μl amplification was desalted on 30,000 NMWL filters (Ultrafree-MC, Millipore, Bedford, MA) and resuspended in 12 μl of dH₂O. A template of 3.5 μl was used in each subsequent sequencing reaction. Sequencing reactions were performed with Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH) following manufacturer's instructions, except that each reaction was performed in half the prescribed volume. Primers for sequencing were the same as those used in the original amplification and an internal sequencing primer ID3B (5'-TAGT/ATCRCCATCTTT-CGGGT-3').

RESULTS AND DISCUSSION

Microdissection of the morphological sample: Diagnostic morphological characters were preserved, even in small, relatively young specimens, with judicious selection of the region from which the tissue was excised for PCR (Fig. 1A,B). A central question concerns the exact section of the nematode that should be sacrificed for molecular analysis. The primary concern is to minimize the loss of taxonomically important morphological characters. For these selected taxa, the region dissected was a portion of the intestine posterior to the esophagus and excluding the anterior end of the gonad (Fig. 1A,B).

While this region may conserve the most useful morphological characters for these taxa, other sections may be chosen for other nematode groups. Given the distribution of nuclei within a typical nematode, any section should provide adequate template quantity and quality.

Based on the number and distribution of nuclei in *C. elegans,* we estimate that a typical 50-μm section will include approximately 20 nuclei. With this number of nuclei and an estimated 55 copies of the target rDNA sequence per haploid genome (Files and Hirsh, 1981), we expect that a typical *C. elegans* section would include a few thousand "potential" template molecules for rDNA loci. However, both the absolute number of target repeats per genome and the number of nuclei in a section are expected to vary from species to species and locus to locus. In addition, the degradation of DNA by the fixation process may vary depending upon factors such as cuticle permeability. For this reason, we included a taxonomically diverse set of nematodes in our analysis.

A potential problem with the microdissection sample is the existence of other organisms (and potential PCR templates) in the intestine. For the vast majority of nematodes, these are ingested bacteria and fungi, which can be excluded by use of PCR primers (such as the D3 primers, see below) that do not amplify homologous targets in these
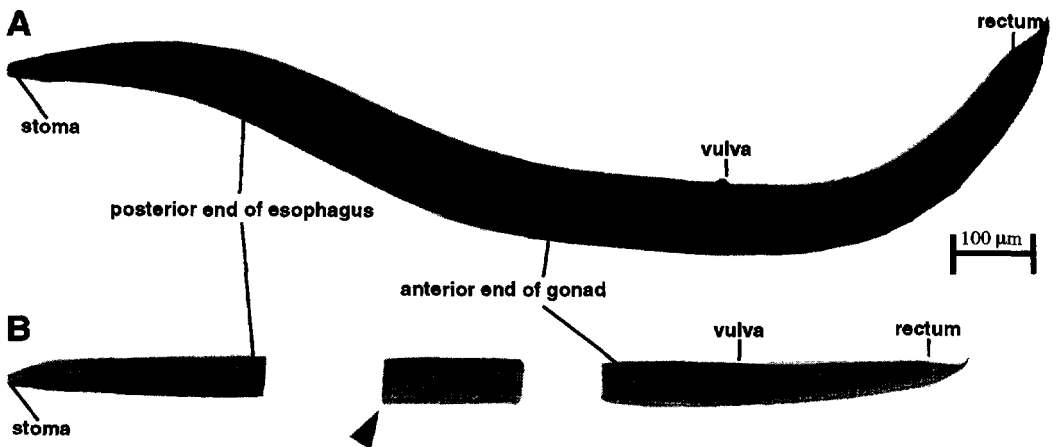

FIG. 1. Dissection of *Zeldia punctata.* A) A *Z. punctata* mature egg-laying female. B) A young *Z. punctata* female just at the point of reproductive maturity. Arrowhead indicates the region between the esophagus and gonad excised with a scalpel for PCR.

organisms. Nevertheless, in some groups of nematodes, particularly predators of other nematodes, such as Mononchida, this may represent a greater problem and require excised tissue from a more posterior region. In most such cases, it should be possible to eliminate the intestinal contents by starvation or during microdissection.

Amplifications: PCR amplifications were performed on each nematode extract using the primers D3A and D3B (Nunn, 1992). These primers are known to amplify a region of the genome that includes the D3 expansion segment of the large subunit of the nuclear rDNA repeat from a wide range of metazoans and all nematodes tested thus far (Litvaitis et al., 1994; Nunn, 1992). The expected size of the PCR product is 362 bp in *C. elegans* and does not vary in length significantly in other nematode taxa (Ellis et al., 1986; W. K. Thomas, unpubl.). In 10 attempts to amplify template prepared from a nematode fixed in formalin, all amplified after 35 cycles. In all cases, extracts from a nematode section gave products of the expected size (Fig. 2A, lanes 1–4).

Our results differ from those observed by De Giorgi et al. (1994) with respect to amplification success. In their study, only 20% (9 of 44) of fixed nematodes amplified even though entire nematodes or pools of approximately 100 nematodes were used. By contrast, all (10 of 10) of our amplifications were successful. Two distinct differences exist between methodology used in our study and that of De Giorgi et al. (1994) that may account for the difference in amplification success. First, De Giorgi et al. (1994) attempted to amplify a target twice the size of the one in this study. Previously, Greer et al. (1991) clearly demonstrated that the size of a PCR product that could be amplified is dramatically reduced with tissue fixation time. A second difference is that the DNA isolation from De Giorgi et al. (1994) involved extraction and precipitation steps that could result in substantial loss of template. By contrast, we have chosen a simple PCR-compatible tissue digestion that does not involve any steps that would remove potential template from the reactions.

A

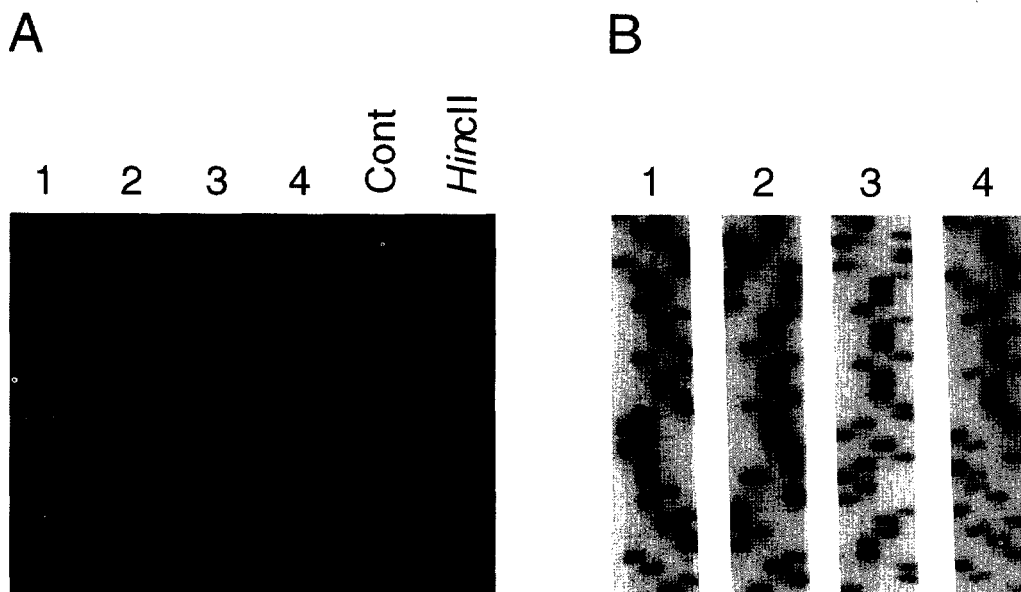1   2   3   4   Cont   *Hinc*II

B

1   2   3   4

FIG. 2. PCR amplification and sequencing results. A) A 2% agarose gel containing amplification products of the D3 expansion segment from four nematode species fixed in formalin for 2 days (lanes 1–4). 1: *Zeldia punctata*; 2: PS1010; 3: *Aduncospiculum halicti*; 4: *Diplogaster sp.* Lane 5 is a lysis extract-PCR negative control. Lane 6 is a *Hinc*II digest of φX174 as a size standard. B) A representative region of a DNA sequencing gel demonstrating the quality of results obtainable from formalin-fixed nematodes. Labeled lanes correspond to the same species as in Fig. 2A, lanes 1–4. Nucleotides are loaded in the order G, A, T, C.

Authenticity of sequences from formalin-fixed nematodes: It is critical that sequences faithfully represent those in the genome of the original specimen. Theoretically, although the PCR templates are highly modified and errors will be generated during the amplification process, it is not possible to generate an incorrect sequence when the sequences are determined for the entire population of amplified molecules (Thomas and Kocher, 1993). Nevertheless, problems such as contamination can lead to incorrect sequences when the proper controls are not used.

To evaluate the authenticity of the sequences, we included several control experiments. Controls incorporated in this amplification scheme included a negative PCR control, a mock extraction control, and the inclusion of four different species with distinct rDNA sequences. In addition, sequences from fresh, unfixed specimens were determined and compared to those of fixed tissue. Both the PCR control and the extract control resulted in no PCR product (Fig. 2A, Cont).

To test that products amplified from each extract were those of the original strain, we sequenced each of the amplified products directly after the asymmetric amplifications. Sequences from several nematode taxa clearly demonstrated that unambiguous discrete sequence differences can be identified from formalin-fixed tissue (Fig. 2B, lanes 1–4). In all cases, the sequences were identical to those previously determined from those strains (Ellis et al., 1986).

Integrating molecular analysis and morphological analysis: The goal of this study was to develop a technique for the effective use of nematodes collected with methods typical of standard nematode surveys. We have demonstrated that nematodes fixed for as long as 2 days before extraction can yield sufficient amounts of template DNA to allow accurate amplification of a typical target sequence. This length of fixing time is sufficient for preliminary processing of samples collected in the field and for preliminary morphological sorting of specimens. The size of the sequence amplified in this study (ca. 360 bp) is typical of many PCR-based analyses. We also have demonstrated that sufficient template is recovered from formalin-fixed tissue samples to allow for several amplifications, making it possible to amplify complete genes and (or) sequences from multiple different loci. Future investigations might explore both the maximum size of the sequence that can be amplified under various fixation protocols and the maximum time of fixation that can still yield amplifiable template.

LITERATURE CITED

Andrássy, I. 1992. A short census of free-living nematodes. Fundamental and Applied Nematology 15:187–188.

De Giorgi, C. M. F., M. F. Sialer, and F. Lamberti. 1994. Formalin-induced infidelity in PCR-amplified DNA fragments. Molecular and Cellular Probes 8:459–462.

Ellis, R. E., J. E. Sulston, and A. R. Coulson. 1986. The rDNA of *C. elegans*: Sequence and structure. Nucleic Acids Research 14:2345–2364.

Files, J. G., and D. Hirsh. 1981. Ribosomal DNA of *Caenorhabditis elegans.* Journal of Molecular Biology 149: 223–240.

Freckman, D. W., ed. 1994. Life in the soil. Soil biodiversity: Its importance to ecosystem processes. Fort Collins, CO: Colorado State University.

Golden, A. M. 1990. Preparation and mounting nematodes for microscopic observation. Pp. 197–205 *in* B. M. Zuckerman, W. F. Mai, and L. R. Krusberg, eds. Plant nematology laboratory manual. Amherst, MA: University of Massachusetts Agricultural Experiment Station.

Greer, C. E., J. K. Lund, and M. M. Manos. 1991. PCR amplification from paraffin-embedded tissues: Recommendations on fixatives for long-term storage and prospective studies. PCR Methods and Applications 1:46–50.

Litvaitis, M. K., G. Nunn, W. K. Thomas, and T. D. Kocher. 1994. A molecular approach for the identification of meiofauna. Marine Biology 120:437–442.

Nunn, G. B. 1992. Nematode molecular evolution. Ph.D. thesis. University of Nottingham, UK.

Pearce, F. 1995. Rockall mud richer than rainforest. New Scientist 147:8.

Poinar, G. O., Jr. 1983. The natural history of nematodes. Englewood Cliffs, NJ: Prentice-Hall.

Systematics Agenda 2000. 1994. Systematics agenda 2000: Charting the biosphere. New York: American Museum of Natural History.

Thomas, W. K., and A. C. Wilson. 1991. Mode and tempo of molecular evolution in the nematode *Caenorhabditis.* Genetics 128:269–279.

Thomas, W. K., and T. D. Kocher. 1993. Sequencing of polymerase chain reaction-amplified DNAs. Methods in Enzymology 224:391–399.

Williams, B. D., B. Schrank, C. Huynh, R. Shownkeen, and R. H. Waterston. 1992. A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics 131:609–624.