Marine Benthic Nematode Molecular Protocol Handbook (Nematode Barcoding)

Technical Study: No. 7

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Marine Benthic Nematode Molecular Protocol Handbook (Nematode Barcoding)

ISA TECHNICAL STUDY: No. 7

International Seabed Authority

Kingston, Jamaica

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Foreword

The International Seabed Authority is responsible for protecting the deep-sea environment from damage and excessive exploitation. To succeed in this task, procedures and guidelines have to be implemented for the establishment of environmental baselines. Nematodes are the dominant animals of the potential mining regions; they can make up between 50 and 90 per cent of the measurable deep-sea fauna. Clearly, any monitoring of industrialization of the deep sea will involve nematode identification. However, marine nematode taxonomy is woefully underdeveloped. Few species outside of the north-western European continental shelf have been described and most descriptions are poor and rely on superficial characters.

Nematode identification using traditional morphological methods and light microscopes is difficult, time consuming and expensive. Recent molecular research has raised doubts about whether the traditional morphological characters used are adequate to distinguish sibling species or those demonstrating convergent evolution. There are insufficient traditional marine nematode taxonomists to undertake an extensive deep-sea monitoring campaign, and training new experts would be an extraordinarily slow and expensive business. New molecular technology offers the potential of a cheap, fast and, more importantly, objective way of identifying deep-sea nematodes. Furthermore, molecular identification, or 'barcoding' to use the jargon, does not require taxonomic expertise but uses more widely distributed technical skills.

This is a practical guide for undertaking a molecular survey of deep-sea nematodes including protocols for collecting deep-sea nematodes, preservation and shipping of samples, slide mounting, image capture and the production of individual molecular barcodes. This manual was prepared following a meeting hosted by the International Seabed Authority on 8-10 November 2005, and attended by the experts listed below. New technologies are discussed such as microarray-based identification systems and massively parallel sequencing (MPS). These future developments could conceivably be integrated into the system presently proposed for even faster and more accurate results.

The Authority wishes to express its appreciation to the following people for their participation in the workshop and the preparation of this manual: Professor Eualeme Abebe, University of New Hampshire (USA); Dr Paul Angel, University of Glamorgan (UK); Professor James Baldwin, University of California (USA); Mr Stephen Blenkin, University of Glamorgan (UK); Dr Adam Cook, formerly The Natural History Museum (UK); Dr Simon Creer, University of Bangor (UK); Mrs Nicola Debenham-Cook , formerly The Natural History Museum (UK); Dr Paul De Ley, University of California (USA); Professor John Lambshead, The Natural History Museum (UK); Dr Manuel Mundo-Ocampo, University of California (USA); Ms Margaret Packer, The Natural History Museum (UK); Professor Kelley Thomas, University of New Hampshire, (USA).

CHAPTER ONE

Introduction

Introduction

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The International Seabed Authority is responsible for protecting the deep-sea environment from damage and excessive exploitation. To succeed in this task, procedures and guidelines have to be implemented for the establishment of environmental baselines. Nematodes are the dominant animals of the potential mining regions; they can make up between 50 and 90 per cent of the measurable deep-sea fauna. Clearly, any monitoring of industrialization of the deep sea will involve nematode identification. However, marine nematode taxonomy is woefully underdeveloped. Few species outside of the north-western continental shelf have been described and most descriptions are poor and rely on superficial characters.

Nematode identification using traditional morphological methods and light microscopes is difficult, time consuming and expensive. Recent molecular research has raised doubts about whether the traditional morphological characters used are adequate to distinguish sibling species or those demonstrating convergent evolution. There are insufficient traditional marine nematode taxonomists to undertake an extensive deep-sea monitoring campaign, and training new experts would be an extraordinarily slow and expensive business. Another problem with traditional morphological methods is that they are subjective and, given the low state of the art of nematode taxonomy, it is highly likely that genuine disagreement will occur between equally competent authorities. Such disagreement is unfortunate when it involves legal and contractual obligations.

New molecular technology offers the potential of a cheap, fast and, more importantly, objective way of identifying deep-sea nematodes. Furthermore, molecular identification, or 'barcoding' to use the jargon, does not require taxonomic expertise but uses more widely distributed technical skills. Successful use of barcoding requires three phases. The first is for research organizations to develop the basic methods – to find the right gene, the right probes, the right way of sampling and preserving nematodes and so on. All this has now been achieved, and appropriate methods are described in this manual. 18S rDNA, in particular, has proved to be highly valuable for barcoding nematodes. It types animals across the taxonomic range from species to family, which means that it not only identifies a nematode species but also gives useful information about its taxonomy – from which biological inferences can be drawn.

The second phase is the population of a nematode library containing video-captures and barcodes of individual nematode species. Before a region can be monitored using nematode barcoding, a database of the majority of the species likely to be encountered needs to be assembled. The priority, for the development of a practical molecular monitoring technique for the mining zones, is to implement a programme to barcode on a mass scale. We need to record the majority of the resident species. This manual has been produced as a first component of such a programme.

The final phase is the monitoring itself. Here, speed and cost, as well as accuracy, are important. Monitoring is likely to employ mass screening methods. This aspect is still in the research phase of development but it is now clear that mass screening is now a practical proposition.

This peer-reviewed document is a practical guide for undertaking a molecular survey of deep-sea nematodes. We discuss protocols for collecting deep-sea nematodes, preservation and shipping of samples, slide mounting, image capture and the production of individual molecular barcodes. New technologies are discussed such as microarray-based identification systems and massively parallel sequencing (MPS). These future developments could conceivably be integrated into the system presently proposed for even faster and more accurate results. It provides the key first step in the development of: (a) a library of molecular barcodes; and (b) the development of practical mass molecular screening methods for fast, cost-effective and objective monitoring of the deep sea using nematode worms.

CHAPTER TWO

Collecting Techniques

Chapter 2 – Collecting Techniques

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Procedures for collecting deep-sea nematode mud core samples:

2.1 Sampling using a megacore device.
2.2 Retrieving the sample cores.
2.3 Processing the core.
2.4 Further processing of the samples.

2.1 Sampling using a megacore device

Figure 2.1. Megacorer

The megacorer is a device that is capable of taking deep-sea sediment core samples suitable for baseline surveys and impact monitoring. The megacorer contains 4 to 12 clear perspex tubes with a 10cm or 6cm internal diameter that will take the core samples (Figure 2.1). These tubes are fitted onto the body of the corer that, upon reaching the sediment surface, will be triggered, forcing the tubes into the sediment. The megacorer is mounted on a frame, which provides protection and ensures deployment in an upright position upon reaching the sea floor. Penetration of the sediment will vary depending on its density, and can be altered by the addition or removal of weight on the megacorer. Often, not all of the cores will be successful for a variety of reasons, including debris on the sediment surface (i.e. manganese nodules), and device failure. Deployment and recovery of the megacorer requires a winch, and the ship's crew are usually responsible for this. The average time for deployment and recovery of the megacorer is roughly one hour for every 1,000 metres depth. The megacorer can only be deployed in calm conditions. The rate of deployment is 50 metres per minute until the last 50 metres, when the speed is reduced to 10 metres per minute to reduce the risk of damage to the device on reaching the seabed and to prevent a 'bow-wave' from the corer disturbing the sediment surface.

2.2 Retrieving the sample cores

Once the megacorer has been recovered from the seabed it will require two people to retrieve the sample:

a) The first person should disengage the mechanism that holds the bottom plate on the core tube.

b) The second person must be ready to seal the bottom of the core tube with a suitably sized rubber bung/stopper, to prevent the sample spilling onto the deck (Figure 2.2).

Figure 2.2.
Core with bung in rack

c) The first person then removes the retaining ring located midway around the core tube, thus releasing the core tube from the megacorer housing.

d) The core sample must be kept in an upright position, be transferred to a rack (handling with care to avoid disturbance) and processed immediately in a temperature of no more than ~4°C, either on deck in a cold temperate region, or in a constant temperature room in a tropical region if possible.

Acceptable cores should be no less than 20cm deep and have an undisturbed surface with clear water above. It is advisable to take a photographic record of the cores and a description of condition, for example, mud colour, clear or murky surface water, disturbed or undisturbed mud surface, depth of core, stratification and any other noticeable features.

2.3 Processing the core

Figure 2.3.
Using the extruder

a) Place a bung/stopper on the top of the core to form a seal (and prevent the mud slipping from the core tube) and line up the bottom of the core tube with the core extruder (Figure 2.3).

b) Carefully remove the bottom bung/stopper whilst placing the core tube on the plunger. Once positioned on the extruder, remove the top bung/stopper and gently push the core tube onto the extruder. Remove the clear water from above the mud core until only 2cm remains, and discard. This task must be performed with great care to ensure that the surface of the core remains undisturbed. The remaining 2cm of water should be retained with the top section of the mud sample; a large syringe is suitable for this task.

Figure 2.4. Mud core slicing

c) Place the measuring ring on top of the core tube and gently ease the plunger into the core tube until the top 1cm of mud has been extracted from the top and slice with the metal slicing plate and remove to a labelled container (Figure 2.4).

d) The measuring ring and slicing plate should be rinsed into the sample container using filtered seawater (seawater that has been filtered through a 45µm sieve).

Repeat this procedure until the top 5cm has been recovered from the core in 1cm horizons. The rest of the core is divided into 5cm horizons comprising three further samples of 5-10cm, 10-15cm and 15-20cm.

Figure 2.5.
Sieving and storing

2.4 Further processing of the samples

The samples must then be rinsed through a 45µm sieve using filtered seawater to remove the fine silt. When the water runs freely through the sieve, tilt the sieve to 45° and move the sample to the edge using a gentle flow of water (Figure 2.5). To drive off any remaining water from the retained sample, add some DMSO/EDTA salt solution (DESS – see section 3.2) using a squeeze bottle and allow the solution to penetrate the sample. Transfer the sample to a labelled container using a gentle flow of DESS and a funnel if necessary. Top the sample container up with the DESS and gently agitate (do not shake) to ensure thorough mixing of the preservative in the sample. DESS will preserve at room temperature (~20C) but use refrigeration if available, especially in warm conditions.

CHAPTER THREE

Preservation and Shipping

Chapter 3 – Preservation and Shipping

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Procedures for preparing nematodes for preservation and transportation include:

3.1 Extracting specimens from sediment.
3.2 Preserving specimens in DESS for video capture followed by DNA extraction and sequencing.
3.3 Shipping in DESS from sample site to laboratory for further processing.

The goal of preservation in DESS followed by video capture is to provide a morphological record of fragile specimens when they have been freshly prepared. This record then provides a diagnostic morphological voucher of a given specimen linked to diagnostic DNA sequence data for that specimen.The digital video-capture morphological data (section 4.2) and any corresponding preserved slides (section 4.3) are then managed as vouchers through a recognized taxonomic museum for future verification of extant diagnostics. Linkage between specific morphologies with diagnostic DNA sequence data also provides a basis for the population of a reference database designed to simplify the time and cost of future sampling and monitoring. In addition to a video record, it is advisable to keep formalin-preserved voucher specimens on permanent slides (section 4.3) as representatives of an inventory in a recognized taxonomic museum for nematodes.

3.1 Extracting specimens from sediment

The goal is to separate specimens from sediment, taking special care to conserve morphology and DNA and to avoid losing small individuals:

a) Transfer each sediment sample (500 ml) to a large container (~20 litre clean plastic bucket) and fill it with seawater.

b) While continuously agitating the mixture, pour the contents through a 40-mesh sieve (425 m opening) into a clean, empty container (his section may be omitted if there is no large debris to remove from the sample).

c) Allow the suspension to settle undisturbed for about one minute.

d) Pour the contents (excluding heavier particles that settle) through a 325-400 mesh (45-38 m opening) sieve.

e) Recover fine sediment retained in the sieve, using a stream of filtered sea water from a wash bottle, and transfer the sediment/solution to a 50ml. vial for either of the following:

i) Fixing with DESS (see 3.2 below) where it can be held in solution for further processing including shipping (see 3.3 below) to a site where preparation of temporary slides, video capture, and DNA sequencing can be accomplished.

ii) Fixing in 5% formalin for temporary slides and video-recording or to be processed for permanent museum vouchers; material prepared with formalin, however, is not typically useful for DNA sequencing.

3.2 Preserving specimens in DESS for future video capture followed by DNA extraction and sequencing (Yoder et al., 2006)

a) Preparing DESS:

i) Mix 23.265 g of disodium ethylenediaminetera-acetic acid (EDTA) with formula weight (FW) 372.24 for a 250 ml solution (the formula may vary depending on the FW of the EDTA salt). Add 50 ml de-ionised water to the EDTA salt and stir. At this point the pH of the solution will be about 3-4.

ii) Using sodium hydroxide (NaOH), adjust the pH of the EDTA solution to 8.0. About 50 ml of 1M NaOH will be needed. As the pH increases the EDTA will dissolve slowly; the rate of dissolution can be increased by heating to 30°C.

iii) Using de-ionised water, increase the volume of the solution to 200 ml. Add DMSO to a final product to achieve 20% DMSO by volume, for example, 50 ml DMSO for a 250 ml solution.

iv) Add NaCl to the solution to saturation (until it no longer dissolves; the rate of dissolution can be increased by heating to 30°C). Decant the solution into a capped bottle, discarding any undissolved salt crystals).

b) Concentrate the nematodes from step 3.1 on one side of a 500 mesh sieve (25 m pore size) using filtered sea water; be careful not to allow the nematodes to dry out.

c) Using a wash bottle, rinse the concentration of nematodes into a small, labelled 50 ml vial or beaker using the DESS solution. The nematodes can remain in DESS for shipping (see 3.3 below) or until further processing for video microscopy (see 4.2 below) and DNA sequencing. Where possible, storage with refrigeration is advisable.

3.3 Shipping in DESS from sample site to laboratory for further processing

a) Small vials with nematodes in DESS can be individually packed in sealed (for example, Ziploc) plastic bags.

b) Wrap the bags in bubble wrap to avoid breakage and pack in a strong container.

CHAPTER FOUR

Morphology

Chapter 4 - Morphology

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Procedures for preparing nematodes for species identification with suitable vouchers include:

4.1 Preparation of temporary slides for video capture.
4.2 Video capture of morphology.
4.3 Permanent slides for deposit in a recognized voucher collection.

4.1 Preparation of temporary slides for video capture

A good temporary slide includes specimens that are live or preserved in DESS, 5% formalin or other fixative. The specimens must be in good condition, and oriented appropriately (typically laterally) to enable the observation and photography of key structures. An important application is to mount DESS-preserved specimens for video-capture and editing (VCE) prior to DNA extraction. In contrast to permanent mounts (see 4.3 below), temporary slides typically last for only a few hours. In some cases, the time can be extended by storing the temporary slides in a refrigerator.

a) Killing and fixing:

  i) DESS: specimens are killed simultaneously with fixation in DESS. These specimens are suitable for VCE and DNA sequencing.

  ii) 5% formalin: specimens are hand picked into a small container with about 5 ml of clean, fresh water. To this, an equal volume of 10% buffered formalin is added, resulting in killing and fixing in a final solution of 5%. These specimens are suitable for VCE but typically not suitable for DNA sequencing. Note: better fixation can be achieved by using hot formalin but this is hazardous and special protection, such as a fume hood, is required; cold formalin is normally adequate for fixing marine nematodes. Formalin mixed with seawater can give rise to carcinogenic vapour so this combination should be avoided.

b) To avoid crushing the nematode with the coverglass, it is crucial to have supports that approximate the thickness of the nematode (too thick a support results in floating specimens). For this purpose, commercially prepared microslides can be used or supports can be prepared using glass fibers or rings prepared from paraffin wax or resin materials such as fingernail polish (De Ley and Mundo-Ocampo, 2002).

c) Carefully transfer specimens to a small volume of mounting media (water, DESS, formalin or other fixative) using a fine needle or mounted eyelash.

Cover with a coverglass, lowering it at an angle to avoid bubbles (De Ley and Mundo-Ocampo, 2002; see 4.3c)i) and 4.3c)ii)).

d) Sealing the slide. An unsealed temporary slide in DESS or 5% formalin will last only a very short time due to evaporation. The life of the slide can be extended by sealing the periphery of the coverglass with melted wax or fingernail polish. To seal a slide properly, the interface between the coverglass must be dry and free of films that might interfere with adhesion of the sealant (i.e. start with a carefully cleaned slide).

4.2 Video capture of morphology

(Summarized primarily from De Ley et al., 2005)

(VCE consists of a multifocal series of images through the transparent specimen; these images are saved as a single video clip for conservation on the internet or on a CD (De Ley and Bert, 2002; De Ley et al., 2005).

a) Equipment: video capture equipment can range from modest laboratory microscopes with an analogue video camera and standard computer and software, to high-end research microscopes with Differential Interference Contrast (DIC), also known as Nomarski Interference Contrast (NIC), optics, digital cameras and commercial grade software for automated control of the focal plane of the microscope as well as VCE (see Tables 4.1 and 4.2). While there is considerable flexibility in developing such a system (Eyualem et al., 2004; De Ley et al., 2005), it is crucial that the resulting images are adequately resolved for routine taxonomic diagnostics.

b) Approach:

   i) General approach: the goal is to obtain a video clip that extends from the top to the bottom (generally from one lateral side to the other) of the nematode. This can be obtained manually by changing the focus at a continuous pace during video capture. In the case of an automated system, the software allows calibrating the upper and lower limits of capture and the number of frames between.

   ii) Low magnification map: a carefully mounted specimen is first captured at a magnification of 4X or 10X. The size of the objective used depends on the size of the nematode, the goal ideally being to capture the entire worm in a single field. This low magnification capture serves as a map to show and label regions subsequently captured at higher magnifications; ultimately these can be hot-linked to the various high magnification clips (Figure 4.1).

   iii) High magnification captures: repeat the capturing approach using sufficient magnification and resolution to capture all diagnostic structures. A magnification of 40X will generally be sufficient to capture the entire nematode, although key areas such as the head end and male copulatory structures are best captured at a magnification of 100X.

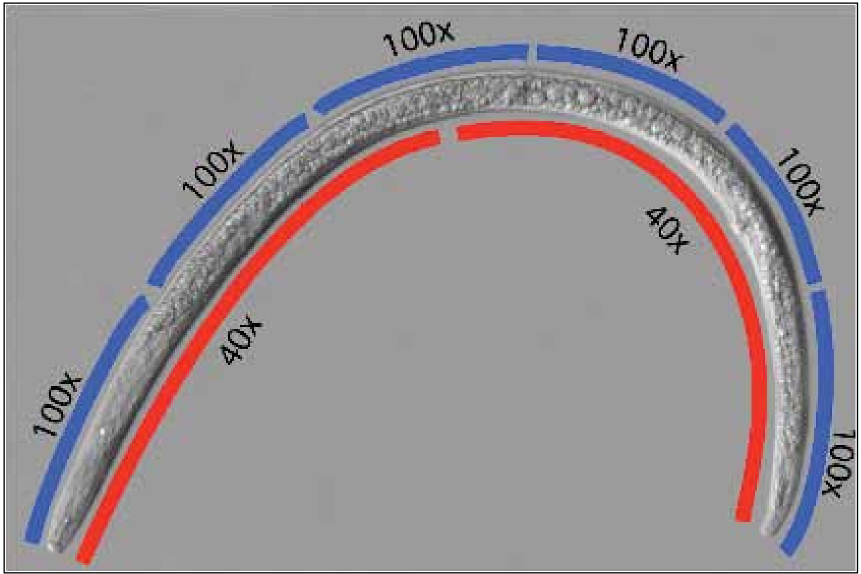
Table 4.1. Suggested options for a manual VCE system

Example components	PC configuration		Macintosh configuration	
	Low end	High end	Low end	High end
Microscope	Leitz Dialux 20 (bright field)	Olympus BX51 with DIC	Leitz Dialux 20 (bright field)	Olympus BX51 with DIC
Objectives	4X, 20X, 40X, 100X oil	4X, 10X, 20X, 40X oil, 60X oil, 100X oil	4X, 20X, 40X, 100X oil	4X, 10X, 20X, 40X oil, 60X oil, 100X oil
Camera	Hitachi KP-D20B (1/2" CCD analog)	Sony HDR-HC1 (HDV camcorder)	Hitachi KP-D20B (1/2" CCD analog)	Sony HDR-HC1 (HDV camcorder)
Capture device	Canopus ADVC110	Avid Pinnacle Studio Plus 700 (PCI or USB)	Canopus ADVC110	None required
Computer	Pentium 4, 1 GHz, 100 Gb, 512 Mb RAM	AMD Athlon 64 X2 3800+, 300 Gb, 2 Gb RAM	G4 PowerPC 1GHz, 100 Gb, 512 Mb RAM	G5 PowerPC, 300 Gb, 2 Gb RAM
Video software	QuickTime	Avid Pinnacle Studio Plus 10.5	iMovie, Final Cut Express, QuickTime	iMovie HD, Final Cut Pro HD, QuickTime Pro

Table 4.2.
Suggested options for a high-end automated VCE system (compatible with PC or Macintosh platform)

Example components	
Microscope	Nikon Eclipse E600 with DIC condenser
Objectives	4X, 10X, 20X, 40X plan apo oil, 100X plan apo oil
Automated focus control	Ludl Electronic (check current models)
Camera with microscope tube adaptor	Diagnostics Instruments: RT color Spot
Video capture and focus control software	Improvision (Jasco) OpenLAB V
Computer	Macintosh G5 4x2.5 500 Gb, Ghz, 2.5 RAM
Video editing software	Final Cut Pro, QuickTime

Figure 4.1. A low power photograph (taken at a magnification of 10X) of a nematode showing mapping of linked through-focus video clips at a magnification of 40X and 100X.



4.3 Permanent slides for deposit in a recognized voucher collection

(De Ley and Mundo-Ocampo, 2002).

Fixation and embedding:

a) Fix specimens in an aqueous solution of 5% formalin for at least 24 hours.

b) Transfer specimens from the fixative to a small, flat glass container with a mixture of: 20 parts, 96% ethanol; 1 part glycerin; and 79 parts distilled water.

   i) Place the container with nematodes in a desiccator or other large jar. The container with nematodes should be surrounded by an excess of 96% ethanol (to slow evaporation) and kept at 35-40°C for at least 12 hours.

   ii) Remove the container with the specimens from the desiccator and replace the liquid with a solution of: 5 parts glycerin in 95 parts of 96% ethanol.

   iii) Place the container in a partly closed Petri dish at 40°C until all the ethanol has evaporated. At this point the specimens are fully embedded in glycerin. Uncounted vouchers thus prepared can be permanently stored in a labelled vial.

c) Mounting on permanent slides

   i) Prepare a supporting ring (see 4.1b) for each clean glass slide (De Ley and Mundo, 2002). A round coverglass requires a smaller

Figure 4.2. Making permanent wax ring slides

and thinner wax ring (Figure 4.2) or support than a square one. When using a wax ring, the size of the ring determines the size of the drop of glycerin and the size of the coverglass.

ii) Add a very small (~2 mm diameter) drop of glycerin to the center of the slide. The drop should be as convex as possible, and large enough (when covered), where a ring is used, to fill the center of the support ring but small enough not to spread beyond the edge of the ring or coverglass. Transfer the nematodes to the drop using a fine needle or mounted eyelash. The nematodes should lie on the surface of the glass rather than float within the drop. If recovered specimens are abundant, a total of 6-8 nematodes (preferably of the same species if possible) per slide, is acceptable.

iii) Gently place a coverglass onto the glycerin drop with the specimens over the slide supports. It is helpful to lower the coverglass with only one side first touching the slide, to minimize bubbles. If using wax, heat the slide gently untill the wax ring melts.

iv) Ring the edge of the coverglass with a thin layer of sealant such as fingernail polish or another resin, allowing it to seal the space between the layer of glycerin, slide and coverglass.

v) Allow the seal to dry and apply a second layer of sealant.

vi) Slides should be labelled with important information, including: species name; number of individual females, males or juveniles mounted; date of mounting; and collection site and sampling parameters.

CHAPTER FIVE

Virtual Collections

Chapter 5 - Virtual Collections

Dr Paul Angel, School of Computing, University of Glamorgan, Pontypridd, Wales, UK.

Recommendations regarding image data storage:

Managing the storage, retrieval and backup of large image datasets is problematic given the range of standards and storage media that exists. In developing any system that will have long-term application, it is important to consider a number of factors, including:

a) There is no one definitive data/image format or compression technique. Technology and standards change. As such, any image format that is chosen must be an open standard. This will allow migration of the image datasets to future data formats/standards and avoid problems inherent in managing bespoke solutions.

b) The fidelity of the dataset must be accurate. As such, lossless compression of image data is most important.

To address these key issues, the following recommendations are proposed:

a) Adopt a lossless image compression format for storing image data. TIFF is a good example that can be adopted. This is an open standard and provides lossless compression. This addresses the problems of bespoke solutions, future migration and data fidelity.

b) A backup policy for the image dataset must be established.

c) A strategy should be in place to periodically review current hardware and software standards in order to facilitate future migration of the image datasets to new storage mediums/file formats.

CHAPTER SIX

Molecular Techniques

Chapter 6 - Molecular Techniques

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The molecular pipeline described below begins with the process of specimen fixation as described above. In the collection of specimens, a method of fixation that preserves morphology until the specimen is imaged is critical. The other critical aspect of fixation is that the specimen be amenable to subsequent molecular analysis. The steps described below are appropriate to a broad range of specimen conditions. The focus here is on individual nematodes, but in cases where populations of nematodes are used, the methodology can be adapted appropriately.

6.1 Extraction

6.1.1 Lysis buffer (to obtain DNA suitable for PCR)

There are several approaches to the preparation of nematode samples for amplification of target loci based on the polymerase chain reaction (PCR). The primary focus of this section is to describe the production of PCR competent material from an individual nematode specimen that has been fixed in DESS (Yoder et al., 2006), and whose morphology has been captured as described above (section 4.2). Below, we describe a commonly used approach that has been selected because it minimizes sample handling and the opportunities for cross contamination, while producing sufficient material for several PCR-based genotyping reactions from the vast majority of nematode samples that will be encountered. The process is a simple one that results in the release of DNA from the cells into a PCR-compatible solution that can be added directly to the amplification reaction. While the DNA is not actually extracted or purified, the process destroys most inhibitors and nucleases that would limit the use and lifespan of the DNA sample. The process described is amenable to both direct amplification of specific PCR products (section 6.3) and genomic amplification (section 6.2). In addition, we describe a second approach that can be applied to specimens that are recalcitrant to the primary methodology.

Single worm lysis:

In advance, prepare sufficient lysis buffer (Table 6.1) for all samples and controls. Control lysis reactions are critical to monitor potential sources of contamination. The controls are simply lysis reactions in which no worm is placed. All subsequent PCRs with this control template should be negative.

Single worms should be placed in at least 20 µl of a digestion buffer. The volume of the lysis will allow at least 10 subsequent amplifications with 2µl each. Keep in mind that nematodes vary greatly in size. Additional volume of lysis buffer can be used for larger worms.

Detailed Protocol (modified from Williams, et al. 1992):

a) For a single worm:
   i) Add 20μl lysis buffer to a 0.5ml PCR tube or individual well of a 96-well plate.
   ii) Place at -70°C or otherwise freeze for 15 minutes or more. This can be stored for several days in its frozen state. In some cases, lysis efficiency can be improved by repeated freeze-thaw cycles.
   iii) Warm sample to room temperature and add one drop of mineral oil.
   iv) Incubate at 60°C for >1 hour to allow the digestion of membranes and proteins.
   v) Heat to 95°C for 15 minutes. This step is critical for the inactivation of protease and nucleases.
   vi) Cool to 4°C.
   vii) Pipette sample up and down to mix, and spin sample to separate cell debris into a pellet.
   viii) Use 2μl of supernatant as template for subsequent PCR amplification. Always include negative control lysate.

b) For many worms:
   i) Add 50-100 μl lysis buffer to tissue sample (10-100 worms) in a 0.5 ml PCR tube.
   ii) Place at -70°C for >15min. This can be stored for several days. Or place in liquid nitrogen followed by a 55°C water bath 10 times to help break down the nematode bodies.
   iii) Warm to room temperature and add 1 drop of mineral oil.
   iv) Incubate at 60°C for >1 hour. Vortex at least once during incubation to help break up tissue.
   v) Heat to 95°C for 15 minutes.
   vi) Cool to 4°C.
   vii) Vortex briefly (2-3 sec).
   viii) Spin at 6,000 rpm for 30 seconds.
   ix) Use 1-2μl supernatant as template for PCR amplification for 25μl reactions.

WLB (Worm lysis buffer):

The components of the lysis buffer are given with example vendors. In some cases, stock solutions belong to the general lab community. It has been our experience that making aliquots specifically for use in lysis buffer preparation has been beneficial. This reduces the possibility of introducing a contaminant. In all cases, fresh lysis buffer should be made daily.

Table 6.1. Lysis buffer components

Chemicals	F.W.	Stock	Final	1 ml	2 ml**	5 ml
KCl (Sigma, P-9541)	74.56g	1M	50 mM	50 µl	100 µl	250 µl
Gelatin* (Dicto Bacto, 0143-02 ¼ lb)		1%	0.05 %	50 µl	100 µl	250 µl
Tris pH 8.2 (BioRad, 161-0719)	121.14g	1M	10 mM	10 µl	20 µl	50 µl
Tween 20 (Fisher, FL-04-0796)	1227.54g	100%	0.45 %	4.5 µl	9 µl	22.5 µl
Proteinase K (Roche, 0 092 766, 1gm)		20mg/ml	60µg/ml	3.3 µl	6.6 µl	16.5 µl
$MgCl_2$ (From PCR reagents)	95.21g	1M	2.5 mM	2.5 µl	5 µl	12.5 µl
ddH_2O				880 µl	1760 µl	4400 µl

* made fresh, 100mg gelatin in 10ml water and heat in microwave;
** 2ml is sufficient for a 96-well plate

6.1.2 DNA extraction using Geneclean III

An alternative method of extracting PCR competent DNA from individual nematodes uses the commercially available 'glassmilk' protocol kit available from Q-Biogene. While this method is more time consuming and requires many additional steps, for the subset of nematodes where recalcitrant inhibitors exist, this approach may be successful. This method removes the DNA from the other components of the worm extraction and may allow analysis of worms that may otherwise be unsuccessful.

Turn on centrifuge to 4°C, set waterbath to 58°C, remove Proteinase K from freezer to thaw.

a) Lysis

   i) Transfer droplet containing nematodes stored in 1M NaCl to clean slide (cleaned with 70% ethanol and wiped with kimwipe). Slide must not have contaminating DNA.

   ii) Transfer 1 nematode by a needle picker to a 0.5ml microtube containing 18 µl Lysis Buffer. Can also be done in 96 well plates.

   iii) Crush nematode using pipette tip about 40 times.

   iv) Spin at 13,000 rpm for 1 minute to bring the solution down to the bottom of the tube.

   v) Freeze at -20°C for 20 minutes.

   vi) Soak for 5 minutes at 95°C in thermal cycler.

b) Proteinase K digestion

  i) Burst spin.

  ii) Add 2µl Proteinase K (20 mg/ml stock) and vortex.

  iii) Incubate at 58°C for 3 hours.

  iv) Adjust water bath to 51°C

c) Gene clean

  i) Burst spin.

  ii) Add 60µl NaI solution and vortex.

  iii) Add 0.8µl EZ-Glassmilk and vortex.

  iv) Incubate at room temperature for 5 minutes.

  v) Spin for 30 seconds at full speed and remove supernatant.

  vi) Add 60µl New Wash and vortex to re-suspend all EZ-Glassmilk.

  vii) Burst spin and remove supernatant.

  viii) Repeat New Wash two more times.

  ix) Spin for 30 seconds at full speed and remove supernatant.

  x) Leave the cap open for 10 minutes at room temperature or place the tube under vacuum for 2-5 minutes.

  xi) Add 10µl GCIII Elution and re-suspend EZ-Glassmilk.

  xii) Incubate at 51°C for 3 minutes.

  xiii) Spin for 30 seconds at full speed to make a solid pellet and collect supernatant into a new 0.5ml microtube.

  xiv) Add 5µl GCIII Elution and re-suspend EZ-Glassmilk.

  xv) Incubate at 51°C for 3 minutes.

  xvi) Spin for 30 seconds at full speed to make a solid pellet and collect supernatant into the previous microtube.

  xvii) Spin for 30 seconds at full speed again to get rid of the glass milk and collect supernatant into a new microtube.

  xviii) PCR immediately or freeze at -80°C for later use.

Potential problems:

a) Two aspects are critical in relation to the potential problems involved in the above process. First, it is imperative to avoid contamination of templates. Because the subsequent use of these templates will involve PCR amplifications, even very small amounts of contamination can make the process invalid. To best avoid contamination, all samples should be prepared in an environment free of contaminating DNA using dedicated pipettes. Special hoods are available that allow the area to be UV-irradiated on a regular basis. In addition, reagents should be made using

the same care and stored in small aliquots that can be discarded after each use.

b) As discussed above, inhibitors may make some samples difficult to amplify in subsequent steps. Aside from the alternative DNA extraction method suggested, other methods of extraction can be employed but it is not always practical to focus much attention on individual specimens that do not amplify. A commonly used approach is NaOH extraction: (http://zeldia.cap.ed.ac.uk/npg/naoh.html).

c) Typically, specimen IDs will be established at the imaging step. Tracking samples is a critical aspect of this process. It is very important that all information be kept with each specimen throughout processing. A labeling methodology and tracking system must be established first. As individual tubes are used, labeling devices are critical. If 96-well trays are used, standard labeling protocols can be applied.

6.2 Genomic amplification

Whole Genome Amplification is a means of increasing the amount of template DNA available for future molecular analysis. This process employs a highly processive polymerase and random primers to amplify long products randomly from a starting DNA template. Because it allows for the extreme dilution of the original stating material, the process may also help minimize problems that result from inhibitors as discussed in the section above. As with all amplification processes, the potential for contamination and amplification of unintended target templates requires special attention to keeping the process free of contamination. The amplification of total genomic DNA from cell lysates as produced in section 6.1 above is possible using the GenomiPhi DNA amplification Kit (Amersham Biosciences) The application of this step may ultimately be a routine aspect of processing samples but as yet has only been applied to nematodes in a limited way (Skantar and Carta, 2005). The specific protocol described below assumes the use of either nematode lysate or Gene-Cleaned nematode DNA.

Protocol: Modified from GenomiPhi DNA amplification Protocol (Amersham Biosciences).

a) Mix 1 µl of template (either nematode lysate of GeneClean DNA) to 9 µl of GenomiPhi sample Buffer (provided). In parallel add 1 ul of control template to 9 µl of sample buffer.

b) Denature samples at 95°C for 3 minutes, cool to 4°C and place on ice.

c) To each sample tube and control add 9 µl of GenomiPhi reaction Buffer and 1 µl of GenomiPhi enzyme mix.

d) Incubate at 30°C for 16-18 hours.

e) Incubate samples at 65°C for 10 minutes to inactivate enzyme (Phi29 polymerase) and prevent future degradation of DNA by exonuclease activity.

f) Samples can be diluted in 100 µl of TE, aliquoted into multiple tubes and stored frozen at -20°C.

Problems:

The main concerns about genomic amplification are: (1) the potential for amplification of contaminating material; (2) the generation of amplification artefacts such as mutations and chimeric molecules; and (3) the cost of an additional (in most cases unnecessary) step.

a) To minimize the potential for amplification of contaminating material negative controls should be amplified in all cases. However, it has been our experience that virtually all reactions result in the creation of products. Careful comparison of these products and those resulting from nematode extracts should be made.

b) If sufficient starting material was present in the original lysate, any chimeras or mutations should be in low frequency compared to the intact original genetic composition. As long as direct analysis by PCR is used in the genotyping without cloning the results should reflect the starting template genotype.

c) The addition of an extra step costs both time and money, and adds the potential for error. However if this step can be routinely applied, it has the potential to produce a valuable resource for re-analysis.

6.3 PCR amplifications

The amplification of target loci is the first step in genotyping specimens. The use of near universal primers for the amplification of specific loci (DNA "barcodes") is key to the process. Nevertheless, as yet, not all nematodes have been amenable to any single set of primers. For each target locus a set of alternative primers are suggested. This problem stems largely from the great diversity in the phylum. One solution has been to design primers for the specific nematode clades. This process will be greatly aided by preliminary morphological analysis, which will allow the selection of primers specific to that group of nematodes, significantly increasing the rate of positive genotyping (barcoding). We recommend three distinct loci: small subunit ribosomal DNA (SSU rDNA); large subunit ribosomal DNA (LSU rDNA); and mitochondrial DNA (for example, MtCO1) as a starting point. Each locus has an increasing capacity for resolution of more closely related taxa. SSU rDNA is a standard locus for general placement of nematodes into a phylum wide context and also capable of species-level resolution for some taxa (Floyd et al. 2002; Blaxter, et al. 1998, 2005). LSU (D2-D3) locus is more highly resolving at near the species-level (Inserra et al., 2001; Kanzaki and Futai, 2002; Nadler, 1992; Nadler and Hudspeth, 1998), and the mitochondrial locus is often highly variable within species (Blouin et al. 1998; Courtwright et al., 2000).

The PCR amplification can be conducted in essentially the same protocol for all nematodes, with the only variables being the selection of specific primer pairs based on taxonomic group and the selection of the optimal annealing conditions for each unique primer pair.

In all cases, negative control amplifications are a critical component of quality control. All reagents should be used in small aliquots. PCR should be set up in

a special environment and physically separated from the PCR product analysis. The pipettes, reagents and work space used should be kept free of contaminating DNA.

6.3.1 Amplification of the SSU rDNA

The amplification of the SSU locus can involve either the entire locus (approximately 1.4kb) or using either the 5' fragment or the 3' fragment. We recommend that the amplifications at the beginning of the data collection focus on full-length SSU genes and that subsequent highly efficient approaches focus on the most informative sub-fragment(s). For the SSU rDNA amplification, we often use a cocktail of primers for amplification, and have routinely had better success amplifying the fragment in three parts. The 5' end is amplified with a cocktail of forward primers comprised of 18S-G18S4. 82F, EukF(10), and SSU_F_03 and the 530R primer. The 3' end is amplified using the 550F primer and a cocktail of reverse primers (Table 6.2) comprised of 18S-18P and EukR(10). These fragments are then sequenced using the 530R and 550F primers. From the results of the sequences, a pair of primers that match perfectly, typically 385F and 1108R, are used to amplify the middle portion of SSU. Other primers we have commonly used are included in Table 6.6.

Table 6.2. SSU rDNA PCR using Dynazyme (Finnzyme)

Reagents	Stock	Final 25µl	Per reaction 25µl	Per reaction 50µl	16 reaction 25µl
RNase/DNase free water			18.5µl	37µl	296µl
10X PCR reaction buffer (+Mg Cl_2)	10X	1X	2.5µl	5µl	40µl
Forward primer	10µM	0.4µM	1.0µl	2.0µl	16µl
Reverse primer	10µM	0.4µM	1.0µl	2.0µl	16µl
dNTP mix	10mM	0.2mM	0.5µl	1µl	8µl
Dynazyme	1U/µl	0.5 unit	0.5µl	1µl	8µl
DNA			1µl	2µl	1µl
Total			**25µl**	**50µl**	**25µl**

a) Thaw all the reagents completely, centrifuge before using. Keep samples on ice.

b) Dispense 24µl mix except for DNA to each PCR tube (0.2ml).

c) Add 1µl of DNA sample to the tubes.

d) Place in BioRad I-Cycler thermal cycler.

PCR programmes ~3 hours 10 minutes in total.

Step 1: 95°C for 4 minutes — Cycle number: 1

Step 2: 95°C for 30 seconds

50°C (for Forward/530R) or 58°C (for 550F/Reverse) for 30 seconds

Cycle number: 35

72°C for 3 minutes

Step 3: 72°C for 10 minutes — Cycle number: 1

Step 4: Hold temperature: 4°C

PCR products can be visualized on agarose gels. For each specimen a single, predominant band at the appropriate molecular weight should appear. Size markers allow the PCR product to be sized. Negative controls should not have any PCR product other than the primer-dimer artefact.

6.3.2 Amplification of the LSU rDNA D3 loci

PCR Amplifications by Locus: D3 expansion segment of the LSU rDNA gene. The D3a and D3b primers were designed by Gary Nunn (1992) and are among the most universal in their ability to amplify an informative segment of nematode rDNA (see Table 6.3).

Table 6.3. PCR of D3 LSUrDNA (AmpliTaq)

Reagents	Stock	Final 25µl	Per reaction 25µl	Per reaction 50µl
RNase/DNase free water			14.7µl	29.4µl
10X PCR reaction buffer	10X	1X	2.5µl	5µl
$MgCl_2$	25mM	2.5mM	2.5µl	5µl
Forward primer D3a	10µM	0.4µM	1µl	2µl
Reverse primer D3b	10µM	0.4µM	1µl	2µl
dNTPs	10mM	0.2mM	4x0.5µl	4x1µl
AmpliTaq (Applied Biosystems)	5 unit/µl	1.5 unit	0.3µl	0.6µl
DNA			1µl	2µl
Total			**25µl**	**50µl**

a) Thaw all the reagents completely and centrifuge before using. Work should be carried out on ice.

b) Dispense 24µl mix except for DNA to each PCR tube (0.5ml).

c) Add 1µl of DNA sample to the tubes.

d) Place in BioRad I Cycler thermal cycler.

PCR program (D3 57 PCR): ~3 hours 10 minutes in total.

Step 1: 95°C for 5 minutes — Cycle number: 1

Step 2: 95°C for 25 seconds
57°C for 30 seconds — Cycle number: 35
72°C for 2.5 minutes

Step 3: 72°C for 10 minutes — Cycle number: 1

Step 4: Hold temperature: 4°C

6.3.3 Amplification of the LSU rDNA D2/D3 loci

D2/D3 rDNA PCR (AmpliTaq). The inclusion of the D2 expansions segment by replacing the D3a primer with the D2a primer is commonly used to expand the sequence of this informative region (see Table 6.4 below).

Table 6.4. PCR of D2/D3 rDNA

Reagents	Stock	Final 25µl	Per reaction 25µl	Per reaction 50µl
RNase/DNase free water			13.7µl	27.4µl
10X PCR reaction buffer	10X	1X	2.5µl	5µl
$MgCl_2$	25mM	2.5mM	2.5µl	5µl
Forward primer D2a	10µM	0.4µM	1µl	2µl
Reverse primer D3b	10µM	0.4µM	1µl	2µl
dNTPs	10mM	0.2mM	4x0.5µl	4x1µl
AmpliTaq	5 unit/µl	1.5 unit	0.3µl	0.6µl
DNA			1µl	2µl
Total			25µl	50µl

a) Thaw all the reagents completely, burst spin and centrifuge before using. Work should be carried out on ice.

b) Dispense 24µl mix except for DNA to each PCR tube (0.5ml).

c) Add 1µl of DNA sample to the tubes.

d) Place in BioRad I Cycler thermal cycler.

PCR program (D3 55 PCR): ~3 hours 10 minutes in total.

Step 1: 95°C for 5 minutes — Cycle number: 1

Step 2: 95°C for 30 seconds
55°C for 40 seconds — Cycle number: 35
72°C for 2 minutes

Step 3: 72°C for 10 minutes Cycle number: 1

Step 4: Hold temperature: 4°C

6.3.4 Amplification of mitochondrial CO I- mtDNA

CO I- mtDNA PCR by AmpliTaq polymerase. Amplification of mitochondrial genes using a single set of universal primers has as yet been unsuccessful. Among the most universal and highly informative sets of mitochondrial primers has been the 12s (region). As with other barcoding projects, the design of mitochondrial primers to standard protein coding genes such as the COI will be a group-by-group process. We expect the application of this methodology will be on a limited basis considering the diversity of the phylum (see Table 6.5 below).

Table 6.5. PCR of mt COI

Reagents	Stock	Final 25µl	Per reaction 25µl	Per reaction 50µl
RNase/DNase free water			14.7µl	29.4µl
10X PCR reaction buffer	10X	1X	2.5µl	5µl
$MgCl_2$	25mM	2.5mM	2.5µl	5µl
Forward primer	10µM	0.4µM	1µl (10pmol)	2µl
Reverse primer	10µM	0.4µM	1µl (10pmol)	2µl
dNTPs	10mM	0.2mM	4x0.5µl	4x1µl
AmpliTaq (Applied Biosystems)	5 unit/µl	1.5 unit	0.3µl	0.6µl
DNA			1µl	2µl
Total			25µl	50µl

a) Thaw all the reagents completely, burst spin and centrifuge before using. Work should be carried out on ice.

b) Dispense 24µl mix except for DNA to each PCR tube (0.5ml).

c) Add 1µl of DNA sample to the tubes.

d) Place in BioRad I Cycler thermal cycler.

PCR program (51 CO): ~3 hours 10 minutes in total.

Step 1: 95°C for 5 minutes Cycle number: 1

Step 2: 95°C for 30 seconds
51°C for 45 seconds Cycle number: 35
72°C for 2 minutes

Step 3: 72°C for 10 minutes Cycle number: 1

Step 4: Hold temperature: 4°C

Table 6.6. PCR and sequencing primers used in the Thomas Lab

Primer	Amplified gene	Sequence (5'-3')	Reference
D2a	28S	ACAAGTACCGTGAGGGAAAGT	Nunn, 1992
D3b	28S	TGCGAAGGAACCAGCTACTA	Nunn, 1992
Ferg-ID2B	28S	AGTAACCTCTTGCACCAAAC	Weimin Ye
D2B	28S	GACCCGTCTTGAAACACGGA	Nunn, 1992
D3aR	28S	TCCGTGTTTCAAGACGGGTC	Nunn, 1992
D3bR	28S	TAGTAGCTGGTTCCTTCGCA	Nunn, 1992
D2F1-Ferg	28S	AGTACCGTGAGGGAAAGTTGAA	Weimin Ye
D2R-Ferg	28S	GATAGTTCGATTAGTCTTTCGCCC	Weimin Ye
D2F2-Ferg	28S	GGAAAGTTGAAAAGCACTTTG	Weimin Ye
COI-F1	Cytochrome oxidase subunit I	CCTACTATGATTGGTGGTTTTGGTAATTG	Kanzaki & Futai, 2002
COI-R2	Cytochrome oxidase subunit I	GTAGCAGCAGTAAAATAAGCACG	Kanzaki & Futai, 2002
COI-R1	Cytochrome oxidase subunit I	GTTGAGGGAAAAATGTTAAATTAACTCC	Kanzaki & Futai, 2002
COI-F2	Cytochrome oxidase subunit I	CCTGTCTTGGCTGGTGCTATTAC	Kanzaki & Futai, 2002
COI-F3	Cytochrome oxidase subunit I	TGAGCTCACCATATGTATACAGTAGG	Kanzaki & Futai, 2002
COI-R3	Cytochrome oxidase subunit I	CAATGTAGAGCAAAAATAACTAAATC	Kanzaki & Futai, 2002
CO1FD	Cytochrome oxidase subunit I	ATRGTNATRCCNACNATRATYGGNGG	Weimin Ye
CO1RD	Cytochrome oxidase subunit I	CCRGCRAARTGNARNGGRAARAANGT	Weimin Ye
CO1InF	Cytochrome oxidase subunit I	ATGATTGGTGGTTTTGGTAA	Weimin Ye

CO1InR	Cytochrome oxidase subunit I	ACAACCAATTAAACCAATTC	Weimin Ye
18S-G18S4	18S	GCTTGTCTCAAAGATTAAGCC	De Ley et al., 2002
18S-18P	18S	TGATCCWKCYGCAGGTTCAC	De Ley et al., 2002
18S AR (420)	18S	CATCTAAGGGCATCACAGACCTGTT	Thomas Lab
SSU_F_03		GCTTGTCTCAAAGATTAAGCCATGC	Blaxter Lab
18S-530R	18S	GCG GCT GCT GGC ACC ACA CTT	Blaxter Lab (rev. comp of SSU F11)
18S-550F	18S	GGC AAG TCT GGT GCC AGC AGC C	Thomas Lab
18S-82F	18S	CTC AAR GAY TAA GCC ATG CA	Lopez-Garcia, et al. 2003
eukR (10)	18S	TGA TCC TTC TGC AGG TTC ACC TAC	Medlin, et al. 1988
eukF(10)	18S	AAC CTG GTT GAT CCT GCC AGT	Medlin et al. 1988
18S-F-385	18S	CGG TGG TTA CGG GTA GAG	Thomas Lab
18S-R-1108	18S	CCA CTC CTG GTG GTG CCC TTC C	Thomas Lab
18S-F-377	18S	TGC GCC TAC CAT GGT TGT AAC GGG T	Thomas Lab
18S-F-447	18S	GGC TAC CAC ATC CAA GGA AGG CAG C	Thomas Lab
18S-R-1650	18S	CAT CTA AGG GCA TCA CAG ACC TGT T	Thomas Lab
18S BR (440)	18S	CATCTAAGGGCATCACGGACCTGTT	Thomas Lab
18S_SSU F_11*	18S	AAGTCTGGTGCCAGCAGCCGCGGT	Thomas Lab Modified from Blaxter SSU_F_11
18SF-Burs	18S	ATGCATGTCTAAGTGGAGTATTATA	Weimin Ye
18SF-342	18S	GTC TGC CTT ATC AAC TTT CGA TGG	Thomas Lab

18SR-1837	18S	CTT ACT GGG AAT TCC TCG TTC AAG	Thomas Lab
18SR-1410	18S	AAC YAA GAA CGG CCA TGC AYC AC	Thomas Lab
18SR-Burs	18S	CTACGGCTACCTTGTTACGACTTTT	Weimin Ye

6.4 Sequencing

Sequencing of PCR products from target loci is a two-step process. First the PCR products must be purified to remove the unincorporated primers, nucleotides and inappropriate buffer components. This cleaned product is then ready to be sequenced using standard approaches with either of the PCR primers or an internal sequencing primer.

There are several methods to prepare PCR products as templates for sequencing. In cases where the PCR products are clean (one dominant size fragment), Solid Phase Reversible Immobilization (SPRI) Purification of PCR Products is routine and produces high-quality sequencing template (Hawkins et al., 1994, Elkin et al., 2001). In this method, DNA will reversibly bind to the surface of carboxyl coated magnetic beads under conditions of high salt and polyethylene glycol concentration. The basic procedure binds DNA to the magnetic beads and then the beads are removed from the solution by use of a magnet. The DNA is then washed off the beads with low salt solution. This procedure is also highly automatable and simple routines can be developed for robotic purification.

A solution of magnetic beads can be made and stored for months. Preparation of magnetic beads:

a) Dilute beads (Eastapor SuperParMagnetic Microspheres [ME03N, Bangs Laboratories, INC, 317-5707020, Fax 317-5707034]) from bottle at 1:5 in 0.5M EDTA (pH8.0): 2 tubes, 10ml each using 2ml beads and 8ml EDTA.

b) Wash beads three times with 0.5M EDTA, using magnetic separation plate.

c) Thoroughly re-suspend beads prior to use.

6.4.1 Protocol for the high throughput (96-well) preparation of sequencing template

PCR products can be purified by SPRI using a 96-well plate compatible magnet, e.g. Qiagen:

a) Transfer PCR samples to a 96-well plate (Falcon 353911) compatible with the magnetic separation system.

b) Add 25µl hybridization buffer (2.5M NaCl, 20% PEG8000) and 5µl washed beads to each 25µl PCR reaction, re-suspend mixture thoroughly.

c) Incubate for 10 minutes at room temperature. Re-suspend mixture thoroughly again.

d) Place tubes on magnetic separation plate, allow to separate for 3 minutes or until beads have completely separated. Beads (rust coloured material) will gather at the magnet side of the tube.

e) Remove supernatant from tubes using an aspirator or pipette tip and discard.

f) Wash bead pellet with 150µl 70% Ethanol while still on the magnetic plate and repeat wash once more. Be careful to remove all Ethanol from the tube.

g) Allow beads to air dry for 1 hour. The elimination of all traces of Ethanol is critical to the subsequent sequencing reactions.

h) Add 25µl elution buffer (10mM Tris) to each well and thoroughly re-suspend bead pellet. Save pipette tips.

i) Incubate for 3 minutes at room temperature. Re-suspend mixture thoroughly, then place tubes on the magnetic separator.

j) Allow beads to separate for 5 minutes and remove the supernatant (contains PCR product) to a new plate. Store at –20 °C.

k) Prepare 4µl for each sample and transfer to a plate for gel electrophoresis.

Potential problems:

The most common error in this preparation is the incomplete elimination of Ethanol. Careful removal of Ethanol and sufficient drying time is critical.

A second problem centres on the size selective nature of this procedure. As described the process has the advantage of selectively isolating only DNA fragments >300bp. This is useful when significant amounts of primer-dimer and secondary amplification products are present. However, it is important to apply the approach only to products >300bp in length.

Alternative approaches:

In some cases where the PCR product is too small or alternative products interfere with successful sequencing, the isolation of the Target PCR product by gel purification is necessary. In these cases, simple gel purification approaches such as the Qiagen method are appropriate.

6.4.2 QIAquick gel extraction kit protocol

Cat. No. 28704 (US$100 for 50 samples at time of press), Qiagen, made in Germany.

Turn the water bath to 50°C and make a 1% agarose gel.

a) Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

b) Weigh the gel slice in a colourless tube. Our 1.5 ml tube is 1.1 g/each although this may vary according to manufacturer. Add 3 volumes of Buffer QG to 1 volume of gel (Usually 500ul).

c) Incubate at 50°C for 10 minutes. To help dissolve the gel, mix by vortexing the tube every 2-3 minutes during the incubation.

d) Add 1 gel volume of isopropanol to the sample and mix (If DNA<500bp or >4kb).

e) Place a QIAquick spin column in a 2 ml collection tube (provided).

f) To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute.

g) Discard flow-through and place QIAquick column back in the same collection tube.

h) Add 0.5ml of Buffer QG to QIAquick column and centrifuge for 1 minute.

i) To wash, add 0.75 ml of Buffer PE to QIAquick column, let the column stand for 3 minutes and centrifuge for 1 minute.

j) Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at 13,000 rpm.

k) Place QIAquick column into a clean 1.5 ml microfuge tube.

l) To elute DNA, add 20µl water to the centre of the QIAquick membrane, let the column stand for 3 minutes and centrifuge the column for 1 minute at 13,000 rpm.

m) Store the sample in a -20°C freezer.

6.4.3 DNA sequencing

Sequencing generally follows a standard protocol. As described above, the purified PCR products should produce template sufficient for 2-6 sequencing reactions. In most cases, this allows for re-sequencing whenever necessary. Routine DNA sequencing is typically conducted by contractors, where templates are provided and data is returned within days. The process of sequencing produces a set of standard data files. These include: the trace file (or chromatogram); a text file containing the sequence of nucleotides called by the sequencing software; and a file of quality scores. The quality scores represent a numerical evaluation of the quality of the information upon which each specific base was determined. Typically, quality scores are non-randomly distributed along a sequence. Low-quality scores are clustered at both the beginning and end of sequencing runs.

In the application of high-throughput sequence analysis of nematode samples, the data for specific regions will focus on large numbers of specimens rather than depth of sequence coverage for any one individual. As such, the information regarding sequence quality is a critical aspect of the data. Sequences from each locus can exist as single reads or combinations of reads that must be combined into a single consensus sequence. A reliable analysis pipeline such as phred/phrap/consed (Gordon et al., 1998) can be established to rapidly produce consensus sequences that take into account quality scores and allow for editing and evaluation. The final product is a DNA sequence for each specimen from

the desired locus and some estimate of the quality of each nucleotide in that sequence. The implementation of a sequence analysis pipeline can take place as either a component of the sequencing process or as a subcomponent of the bioinformatics at NemAToL described below.

6.5 Curation of DNA material

To allow for the subsequent re-analysis, quality control or further refinement of approaches it is important to voucher all DNA extracts. Typically, PCR products and sequencing templates are not stored long-term. As described above, the potential use of Genome-wide amplification for the generation of large quantities of DNA will significantly impact this process and potentially affect the approach to long-term storage. The goal of a DNA voucher is to allow subsequent use of the DNA from a specific nematode specimen. As with long-term vouchers, the curation may best be accomplished in the context of other museum collections. Several specific methods of curation are available. The storage of individual worm lysates as developed above will potentially generate large numbers of samples. Storage in ultra-cold freezers may be cost-prohibitive. An alternative strategy for such samples is the use of FTA paper (Whatman Inc, Clifton, New Jersey). This paper is specifically treated to bind and stabilize nucleic acids in a dry state at room temperature. The product is available from numerous vendors for the high density and long-term storage of nucleic acid extracts. This methodology is common practice in the safe storage of environmental samples (Hide et al., 2003).

CHAPTER SEVEN

Bioinformatics

Chapter 7 - Bioinformatics

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In order to efficiently assay nematode biodiversity, we need to be able to identify each nematode specimen based on a combination of morphological and molecular (DNA sequence) data. The approaches to evaluation of nematode biodiversity described above require a sophisticated database structure that can efficiently organize and link the molecular, morphological and ecological information for each specimen. NemAToL (http://nematol.unh.edu) is a web-accessible relational database, developed with open source software, to support the nematode branch of the Tree of Life and nematode biodiversity studies. This database is intended to organize and archive morphological, molecular and ecological data in the context of nematode phylogeny. The site includes a set of databases linked together using unique specimen identifications (NemAToL ID).

7.1 Why a phylogenetic approach?

The taxonomy of nematodes is in a state of flux. There are no formal species descriptions for the majority of nematodes encountered in a survey of marine sediments. As such, it is critical that a set of practical operations be established for the definition of species. For nematode survey, a phylogenetic species concept, as described by De Ley et al. (2005) and Adams (1998), is particularly pragmatic and biologically meaningful. The biodiversity of the phylum is so rich that a traditional species description for each new species encountered is virtually impossible, and meaningful assessment of biodiversity will need to proceed in parallel with the establishment of taxonomic infrastructure.

Currently, the phylogenetic trees available on NemAToL are generated using the SSU gene of the rDNA repeat. SSU rDNA is a common metric for nematode phylogeny and biodiversity. However, plans to generate trees using other loci, such as LSU and mtCOI (mitochontrial cytochrome c oxidase subunit 1), among others, are in progress and will be critical for the establishment of a more finely resolved understanding of genetic relationships and more refined definitions of species and population-level variability.

The informatics pipeline for nematode species identification is shown in Figure 7.1. In this schematic, molecular data is the primary dataset for the analysis. Morphological data in this context serves two specific roles: the preliminary morphological analysis can aid the capture of molecular information by focusing the amplification steps on the use of taxon-specific primers; and the morphological data is an important aspect of quality control. The results of molecular analysis can be easily compared to the preliminary taxonomic assessment based on morphology and by expert inspection of the morphological information and the proposed molecular relationship.

The DNA sequence data is used initially to query the database for matching sequences by BLAST (sequence-similarity analyses algorithm). This is best accomplished in a sequential search beginning with SSU sequence. The search

is accomplished via the dedicated BLAST search engine in NemAToL (Altschul et al. 1990). The output of BLAST in NemAToL shows the best matches, links to the morphology of the best matches, and links to the subtrees that include the best matches. If the BLAST produces an exact match, subsequent analysis with other loci such as LSU can be used to further refine the identification. If no perfect match is found, the DNA sequences can be compared to other closely related sequences in a phylogenetic analysis. NemAToL supports this approach by providing already aligned DNA sequences for the set of closest relatives and tools such as CUTTER, which automate analysis of the sequence alignments. Finally, the output is produced in a format that is directly amenable to phylogenetic inference programmes. As new sequences are added to the database, they will be recognized in future searches as exact matches. As the database expands, the pipeline becomes streamlined with more and more sequences matching. However, the process remains capable of detecting new genotypes.

The database structure

Phylogenetic information:

Phylogenetic analysis of data is essential to developing meaningful inventories in groups of organisms where a large fraction of the species are unknown. Included as part of this information are the DNA sequence alignments (both raw and refined) and tree and image files associated with the phylogenies within NemAToL. The tree pages contain links to all of the information contained in NemAToL (such as sequence, images/videos and metadata) for the taxa in all trees.

Images:

Morphological information in the database is primarily in the form of high-definition, digitized QuickTime® videos. However, the database is capable of storing and organizing all types of morphological information. Every specimen whose image is in the website is given a unique identification number (nematode ID) and all the information on that particular specimen in the site is interconnected through this unique number.

Sequence information:

NemAToL contains all published sequence information for SSU, LSU, mitochondrial DNA and other loci that are common to studies of nematode biodiversity and phylogeny. A dedicated BLAST server enables rapid searches of an input sequence against databases of sequences. The BLAST results are integrated with the NemAToL to provide links to the NemAToL records and the phylogenetic trees. LinkOuts are also developed to link the information within NemAToL to the associated National Centre for Biotechnology information (NCBI).

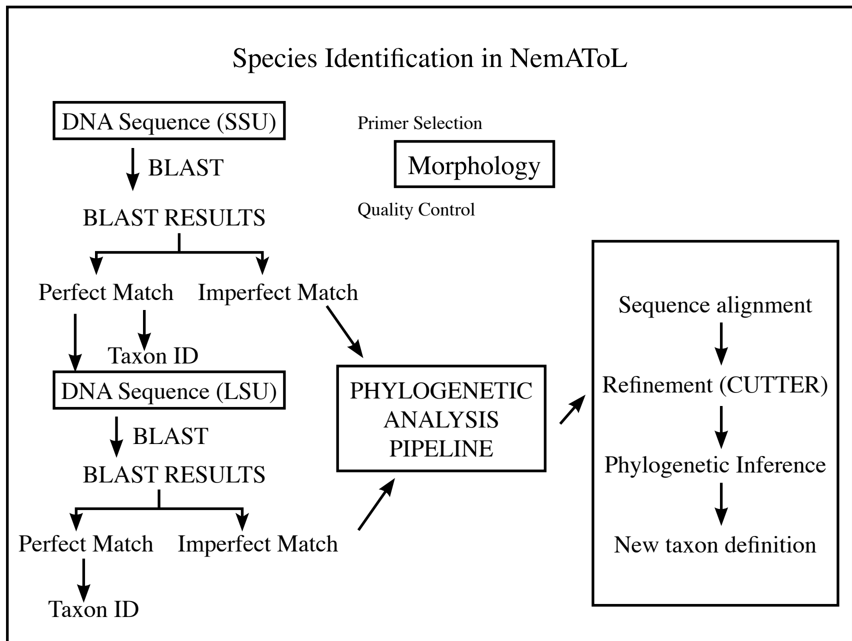
Metadata:

The database also provides information on many aspects of the nematodes whose images and sequences are deposited. It includes taxonomic information, GPS (Global Positioning System) coordinates and ecological information. Two future updates of NemAToL are planned. The first is to provide a true GIS integration of all data. This will be particularly important for the future support of biodiversity assessment. A second aspect that is being implemented in the next version is the ability to use methods of data integration (Remsen et al. 2006; Patterson et al., 2006) to bring together all information associated with each named nematode taxon. The ability to organize the known information associated with each taxon will be critical to the practical implementation of these approaches to survey and maximize the information context. The ultimate plan is to provide users with a comprehensive and integrated set of information on nematodes and their biological and physico-chemical environment, within the framework of an interactive GIS platform.

7.2 Phylogenetic trees

A set of phylogenetic trees for all SSU sequences are maintained within NemAToL to promote the analysis of new sequences. To ease viewing and interactivity, all nodes with a 95 or greater bootstrap value were condensed and displayed as a triangle. Each terminal taxon or triangle is a link to the represented subtree. For a terminal taxon, the link goes to the NemAToL record. For each of these subgroups, the untrimmed sequences were aligned and refined (via CUTTER) and a 'subtree' for that node was generated. This process was repeated for each node with a bootstrap value greater than or equal to 95, until only terminal nodes remained. All raw data used to generate trees are provided as downloadable datasets.

Figure 7.1. The informatics pipeline for nematode species identification



CHAPTER EIGHT

New Technologies

Chapter 8 - New Technologies

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In the future, one of the outcomes of molecular barcoding projects could be the provision of new technologies that will enable the simultaneous molecular identification of mixtures of species, such as those found in nematode environmental core samples. Recent work has investigated the use of denaturing gradient gel electrophoresis (DGGE), but the method appears only to detect dominant taxa (i.e. less than two thirds of the species present). Therefore, the technique may be useful for detecting changes in communities that influence the abundance of the most common taxa. However, the method is not recommended for estimating species richness in marine communities that are often typified by the presence of many rare species (Cook et al. 2005). Presently, there appear to be two alternative research directions that may facilitate accurate biodiversity assessment.

8.1 Micro array-based identification systems

In its natural form, DNA exists as a double-stranded molecule in which the complimentary bases guanine (G), adenine (A), thymine (T) and cytosine (C) are complimentary. That is, Gs bind to Cs and As bind to Ts. Thus, a single DNA strand will only bind, or hybridize, effectively, to another exactly matching complimentary reverse strand.

Given this knowledge, it is now possible to generate species-specific stretches of DNA (for example, for the 18S small subunit and 28S large subunit rRNA genomic regions) and use them as probes that can be hybridized to DNA that has been extracted from the organisms present in a sample. A microarray (or DNA chip) usually consists of a solid substrate (commonly a glass slide) onto which species-specific DNA probes have been attached. The probes are spotted onto the glass in an ordered pattern so that each spot represents a unique stretch of DNA, and many thousands of probes can be attached to a single slide. In order to use the microarray, whole DNA can be extracted from all the organisms in the environmental sample and the targeted gene region amplified and labelled with a fluorescent dye. The labelled DNA regions can then be hybridized with the probes on the microarray and the results of the hybridisation experiment visualised using a scanner. The presence of a particular nematode species is represented by a fluorescent spot, as the corresponding probe spot and fluorescently labelled DNA target will have hybridized to each other. Microarrays are now commonly used in the identification of micro-organisms from environmental samples (Call et al., 2003) and the technique is becoming popular for the identification of other meio- and macrofauna (Pfunder et al., 2004; Welch and Welch, 2005), suggesting that nematode microarrays or 'phylochips' would be appropriate tools for the qualitative environmental assessment of nematode communities.

8.2 Massively parallel sequencing

Since 1977, the scientific community has sequenced DNA by visualising labelled DNA fragments that differ by single base pairs in size in order to read the sequence of individual bases present in the genome. The latter method, also known as 'chain-termination sequencing', can either be achieved by cloning DNA regions of interest (for example, using bacteria), or by directly sequencing an amplified fragment from a known region of the genome. However, new technologies (Margulies et al., 2005; Rogers and Venter, 2005) are emerging that may change the way in which scientists sequence DNA. It is now possible to simultaneously read the sequences of up to 200,000 different short DNA fragments in a four-hour period, which has not been previously possible. The DNA fragments can be from many different sources. An example may be a whole organismal genome that has been cut into many shorter pieces, or short specific gene regions that have been amplified from thousands of different individuals or species. The latter approach is of particular interest to the molecular barcoding community focusing on meiofaunal identification (see Rogers and Lambshead, 2004). Using this technology, it will be possible to generate and read molecular barcodes for thousands of nematodes from an environmental core sample, potentially supplying information on quantitative and qualitative estimates of nematode species assemblages.

Although the above technological innovations show extreme promise for the assessment of environmental nematode communities, their future use will only be possible if baseline data are gathered first. Morphological and genetic information will be required from throughout ranges of ecologically important taxa in order to confidently be able to assign a molecular barcode to a particular species. Once the information has been gathered and validated, microarray or massively parallel sequencing (MPS, or 'pyrosequencing') identification systems will serve as rapid, high-throughput solutions to the identification of complex mixtures of meiofaunal communities.

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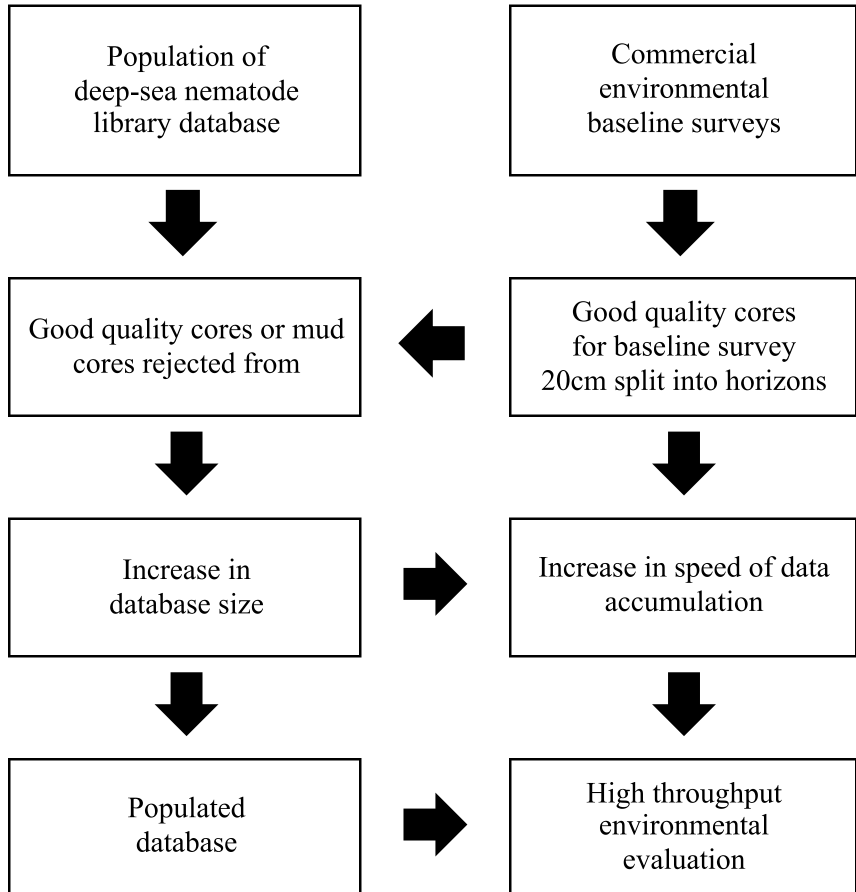
Annex

Annex

Parallel development of a nematode library database alongside baseline environmental surveys to ultimately provide a quick, informative assessment of Pacific deep-sea biodiversity.



The International Seabed Authority is responsible for protecting the deep-sea environment from damage and excessive exploitation. To succeed in this task, procedures and guidelines have to be implemented for the establishment of environmental baselines. Nematodes are the dominant animals of the potential mining regions; they can make up between 50 and 90 per cent of the measurable deep-sea fauna. Clearly, any monitoring of industrialisation of the deep sea will involve nematode identification. However, marine nematode taxonomy is woefully underdeveloped. Few species outside of the North West European continental shelf have been described and most descriptions are poor and rely on superficial characters.

Nematode identification using traditional morphological methods and light microscopes is difficult, time consuming and expensive. Recent molecular research has raised doubts about whether the traditional morphological characters used are adequate to distinguish sibling species or those demonstrating convergent evolution. There are insufficient traditional marine nematode taxonomists to undertake an extensive deep-sea monitoring campaign, and training new experts would be an extraordinarily slow and expensive business. New molecular technology offers the potential of a cheap, fast and, more importantly, objective way of identifying deep-sea nematodes. Furthermore, molecular identification, or 'barcoding' to use the jargon, does not require taxonomic expertise but uses more widely distributed technical skills.

This document is a practical guide for undertaking a molecular survey of deep-sea nematodes including protocols for collecting deep-sea nematodes, preservation and shipping of samples, slide mounting, image capture and the production of individual molecular barcodes. New technologies are discussed such as microarray-based identification systems and massively parallel sequencing (MPS). These future developments could conceivably be integrated into the system presently proposed for even faster and more accurate results.

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