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Chapter 3

Helminth Identification and Diagnostics: Basic Molecular Techniques

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Introduction

Molecular systematics, that is, the use of DNA sequences to address a variety of questions on the identity, species boundaries, and relationships of organisms has now become a powerful and useful approach that complements traditional systematics based on morphology. A perusal of the literature on parasite systematics suggests that much but not all recent understanding and hypotheses of parasite identification and phylogenetic relationships have been obtained through the application of molecular methods (for example, Olson et al., 2003; Nadler et al., 2010). This review summarizes some key protocols in molecular systematics as are used for studying helminth parasites.

Collection of Specimens

The first step in doing molecular systematics is the proper recovery of helminths from the host. Although the specimens used for DNA extraction and subsequent processing need not be handled in the same gentle manner as specimens for morphological studies, they should be collected live, cleaned in 0.6% saline or PBS (phosphate buffered saline) by gentle pipetting or agitation in a petri dish to wash off adhering debris, and then preserved and stored for subsequent processing. Specimens that are to be used for DNA work should be stored directly in 95% or 100% ethanol, making sure that the ethanol does not contain denaturing agents such as ketones, aldehydes, methanol, or kerosene, which are harmful to DNA. A careful reading of the label on the ethanol bottle will indicate what denaturing agents were used. Often, commercially available 95% ethanol is preferred because it may not contain any denaturing agents. Isopropanol can be allowed as a denaturing agent. The sample should be stored in ethanol in a cryovial or in a similar suitable vial and should be kept chilled in a regular freezer (at –20 °C) if possible or in a regular refrigerator (approximately 4 to 8 °C) until use. As a cautionary note, formalin is very harmful for DNA work and the worms being used for DNA analysis should never be brought in contact with formalin. See Gardner and Jiménez-Ruiz (2009) for details on collection methods.

Note that each time a sample of worms is collected with the intention of doing molecular work, a small subsample of worms from the same batch should also be separately fixed for a corresponding voucher sample to confirm the identity of the worms being studied using morphological examination. These specimens should be fixed by the proper techniques that will allow good stained whole mounts to be produced and be suitable for histology or scanning electron microscopy (SEM). For certain helminths (cestodes, trematodes, nematodes), using hot (steaming) 5% or 10% neutral buffered formalin is an easy way of producing relaxed and well-fixed specimens for subsequent stained whole-mounts. If a fume hood or proper ventilation is not available, killing helminths with hot PBS (or saline) and then placing them in unheated fixatives (formalin alcohol acetic acid (FAA) and so on) will suffice for producing adequate stained whole mounts, but worms fixed in this way are not suitable for histology and not ideal for SEM work.

In certain cases, for example, in the case of cestodes, a piece of the worm may be collected in ethanol for DNA analysis and the rest of the worm fixed for morphology, which now allows the specimen to be treated as a **hologenophore** (meaning, a vouchered specimen for which there is corresponding DNA sequenced data) (Pleijel et al., 2008). Occasionally, acanthocephalans, nematodes, monogeneans, and larger trematodes can also be treated in this manner (Gardner and Jiménez-Ruiz, 2009).

Another technique that is now often used is killing the worms in hot water or hot PBS and immediately placing them in 95% ethanol. This saves time and desired portions of the worms can be later excised in the lab for DNA extraction. The disadvantage of this method is that ethanol is only a preservative and is not a fixative, and 95% ethanol can cause worms to shrink, become rubbery, and collapse.

While collecting and fixing specimens for morphological and molecular studies, it is important that vials, Petri dishes, and pipettes that have come in contact with formalin or other fixatives such as Bouin's or FAA (AFA) be kept separate from instruments and glassware used for handling worms being collected for DNA work.

Several specimens should be collected for molecular analysis but even 1 specimen is better than none. For worms that are less than 0.5 mm in length, 2–5 specimens are usually enough to guarantee sufficient DNA on extraction. For specimens 3–5 mm in length, 1 or 2 specimens is/are usually sufficient. DNA can be even extracted from single worms as small as 0.2 mm. Specimens can be stored in 100% molecular grade ethanol in a refrigerator or freezer for years but the quality of the DNA does decline with length of storage time unless the sample is stored at less than −85º C.

Another important aspect is the proper recording of data and the proper labeling of tubes. Tubes or vials that contain specimens for DNA work should be labeled on the outside with paint markers or in other ways that will not be erased by freezing and thawing. Paper labels are often used for labeling specimens inside the vial but should not be used for specimens being stored for DNA analysis because the labels may introduce contaminants.

DNA Extraction

DNA can be extracted from collected worms using standard techniques, such as phenol-cholorform extraction or a variety of commercially available kits. The phenol-chloroform extraction is a standard extraction technique, but phenol is a harmful chemical and the procedures have to be conducted with the proper precautions. As a result, scientists have switched to less toxic methods or easier and less toxic alternatives such as commercially available and fast extraction kits such as Qiagen's DNEasy DNA extraction kit. Other companies, such as Invitrogen, Promega, and others, also manufacture extraction kits. Such kits combine extraction with a subsequent cleaning step and each company provides a booklet with its kit that outlines the protocol. The extracted DNA can be stored in the freezer at −20 °C or at colder temperatures of −85 °C (or even lower).

DNA Amplification

The next step in the process is the amplification of the desired genes of the specimens from which the DNA is extracted. In helminth systematics, the ribosomal RNA gene array (sometimes referred to as rRNA) and the cytochrome *c* oxidase subunit 1 gene (*CO1*) are commonly targeted for obtaining sequences. The most common regions of the rRNA gene array are usually parts of the small subunit (18S) and large subunit (28S) but also portions of the internal transcribed spacers (ITS-1 and ITS-2) as well as the 5.8S region. In the absence of full-length sequences, partial sequences of certain regions of these genomes are still useful. The method that is most widely used for amplifying portions of the target genes is the polymerase chain reaction (PCR). The **PCR reaction** requires several key ingredients:

1) A **polymerase enzyme** that will not denature at high temperatures. The successful isolation and commercial production of a polymerase from thermophilic prokaryotes allowed such enzymes to be used in the high temperature conditions encountered in the reaction. Several types of polymerase enzymes are available of which the polymerase isolated from the hot springs bacterium *Thermophilus aquaticus* (Taq polymerase) is the most common. This enzyme can be purchased from a variety of biotech companies.
2) **Primers**: These are small (usually 20–30 bp long; bp = base pairs) strands of DNA with sequences that are identical to portions of the genes that are being targeted for amplification. In a PCR reaction, primers are used in pairs (a forward primer and a reverse primer), and prescribed quantities of each primer are used. The forward primer binds upstream on the target gene and the reverse primer binds downstream and they work in opposite directions on each of the 2 complementary single strands of the double stranded DNA (ds DNA); the denaturing of DNA is part of the PCR reaction. Primers are usually made to order by supplying the biotechnology company that manufactures primers the letter sequences needed. There are several standard primer sequences that have been published in the literature.
3) **Magnesium buffer**: A special buffer that contains the required amount of magnesium for the enzyme to work adequately is supplied by the company that supplies the polymerase enzyme.
4) **DNA substrate**: This is the DNA that was extracted from the parasites using the protocol outlined before.

The reagents listed above are mixed in prescribed amounts in special PCR tubes and the reaction mixture is placed in a thermocycler. Numerous models of thermocyclers are commercially available from biotech companies, such as the ones made by Perkin-Elmer. Thermocyclers can be programmed and users have to specify the reaction conditions. Most published papers specify the PCR conditions. The PCR method, once standardized for a certain pair of primers, can be repeatedly used with success. Once amplification is completed, the PCR tube is removed from the thermocycler and the amplified DNA is first tested by running (electrophoresis) a small

aliquot (~ 5 µl) on a mini gel along with a DNA ladder appropriate for PCR products. PCR products can range anywhere between 300 to 2,000 bp, depending on the primers, the gene being targeted for amplification, etc. If the electrophoresis gives positive results and there is no evidence of mispriming (multiple amplified products on the gel), the remaining PCR product is purified by passing it through a membrane or column which binds the amplified DNA, which is then eluted out in a buffer or sterile deinonized water. There are standard kits for purification that are available commercially from biotech companies. This amplified and purified DNA sample can be stored in −20 °C or −80 °C (or lower) and a small amount of this is usually used for sequencing.

When the sample is ready for sequencing, it is thawed and a small aliquot of the purified PCR product is sent along with an aliquot of the primers but separately (unlike the PCR reaction, the sequencing reaction only uses one of the primers at a time). The sequencing reaction usually requires ~ 40 ng of purified amplified DNA and so the purified DNA has to be quantified first. Quantification can be done using DNA quantification ladders in a mini gel electrophoresis.

Sometimes the sequencing primers may be different from the PCR primers but most times the PCR primers are also used for the sequencing reaction. The sequencing can be done manually but this is time-consuming and no longer cost effective. Instead, most sequencing is now done on automated sequencers but due to the high cost of purchasing, maintaining, and operating automated sequencers (both material and personnel costs), many labs send their PCR products and primers in a standardized mixture to biotech labs that offer sequencing services. The turn-around time is usually fast. In the United States, many such sequencing facilities are able to send back the sequences within 2–3 days of receiving the samples.

In summary, here are the steps in PCR-based identification and systematics:

1) DNA extraction
2) PCR-based amplification
3) Purification of PCR product
4) Sequencing
5) Retrieval and evaluation of DNA sequences
6) Alignment of sequences
7) Comparisons and phylogenetic analyses

Working with the Sequences

Sequence data are usually received in 2 formats: As chromatograms and as actual nucleotide (letter) sequences. Each sequence is first manually checked for accuracy by checking the chromatogram, using a viewing or editing software package such as FinchTV (Geospiza, Inc.) or ABI EditView, or any number of other packages for manipulation of molecular sequences. These programs can generally be downloaded from the web. Undetermined nucleotides in the sequences to be examined are either left as "N" or are replaced by the correct nucleotide if this is apparent from the chromatogram. Careful examination and proper judgment are necessary to determine how much of the sequence is usable. The usable portion is extracted and copied and pasted into a sequence manipulation program. Such a program allows the assembly of a database of sequences for further comparison and analysis.

Often, one of the first steps in using any DNA sequence that is generated is finding what that sequence is most similar to among the vast number available in GenBank. GenBank is a repository of sequences deposited by researchers from published and unpublished studies (https://www.ncbi.nlm.nih.gov/genbank/). The search is done using the Basic Local Alignment Search Tool (BLAST) through the NCBI BLAST portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This search, which provides results usually in a few seconds to minutes, allows one to see a list of taxa with sequences that match the sequence that has been generated. The BLAST search also shows pairwise comparisons between the sequence submitted and the sequences that match it as well as other details of the comparisons.

One popular program that allows working with the sequences is MEGA (Molecular Evolutionary Genetic Analysis). It is also updated in a timely fashion by the authors, the latest version being MEGA 11.0. This program can be downloaded without cost from https://www.megasoftware.net. In MEGA, sequences from GenBank can be downloaded into an alignment file for additional comparisons. Once an alignment file with the sequences of various species of interest has been compiled, the next step is to align these sequences, that is, to have the nucleotide bases lined up in a homologous corresponding manner (since we do not know the exact position of the sequences in the genome); different sequences may start and end at different base positions in a gene or genome. There are several stand-alone programs that can also be used to align sequences, such as 'ClustalX' (Thompson et al., 1997). In MEGA, the sequence alignment programs 'ClustalW,' and 'Muscle' are embedded within the MEGA software. Alignments are performed on the assembled sequences from the various species using parameters that are set by the program or by manipulating certain parameters depending on the nature of the sequences (Hall, 2001). A copy of the unaligned raw sequences should always be saved and not overwritten by the aligned file because if a new sequence is added to the database, it must be added to the unaligned (meaning, raw) sequence database and the alignment performed again.

Systematic Applications

Once the sequences have been aligned, the unaligned extra overhanging portions on either side are pruned or trimmed and this new dataset can now be used for a variety of purposes, including:

1) The sequences of species can be compared to determine the similarity. This may provide clues as to whether or not 2 samples belong to the same species or can be used to study variation between populations. For example, if consistent molecular differences among isolates from the same geographical area correlate with morphological differences and/or different levels of host range, then a case can be made for different species.
2) The aligned sequences can be used for identification purposes or to determine the evolutionary relationships among the species being studied. There are several programs that can be used for such analyses and several are available in MEGA. There are various settings that can be chosen while doing a phylogenetic analysis, and there are various methods to evaluate how robust the resulting tree of relationships is; the bootstrap analysis is perhaps the most common.

Examples of Explanations about How to Identify Particular Species

Correct application of species names to specimens by biologists is critically important, because species are named according to the agreed-upon rules of scientific naming using the system of **binomial nomenclature** developed by Linnaeus (1758) with the publication of the 10th edition of Systema Naturae. Each species with a unique binomial (**bi** = 2; **nom** = name, from Greek; in this case, **genus** and **species**) provides an instant means to know what species are being referred to anywhere in the world (ICZN, 2024). Following are descriptions of a few sources of methods for species identification.

A useful example of the application of molecular techniques to address questions of helminth systematics is a paper by Hernández-Mena et al. (2019) that examines the relationships of species in the family Allocreadiidae. Pertinent references as well as details of the methods used can be found there.

Methods for collecting and processing mammals for museum collections can be found in Wilson et al. (1996). Specific techniques for collecting parasites from vertebrates can be found in Gardner and Jiménez-Ruiz (2009), which is focused on obtaining and processing parasites from bats; however, the methods can be applied to collections of helminths, ectoparasites, protozoans, and blood parasites from any of the vertebrate classes. Additional methods are found in a book chapter specifically written for reptiles by Gardner et al. (2012), and for mammals in general by Gardner (1996) and Galbreath et al. (2019).

Examples of descriptions of species of *Eimeria* (phylum Apicomplexa: family Eimeriidae) include Jensen et al. (2015) and Tinnin et al. (2012). Some examples of descriptions of nematodes (phylum Nemata) can be found in Drabik and Gardner (2019) and Rodrigues et al. (2020). For descriptions of some of the phylum Platyhelminthes including cestodes, see Caira et al. (2017), and for those in the family Arostrilepididae, see Dursahinhan et al. (2022). For descriptions of trematodes of the family Dicrocoeliidae, see Gardner and Pérez-Ponce de León (2002). This is just a small sampling of available valid descriptive literature.

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