# METHODS AND TECHNIQUES IN NEMATOLOGY

Ebrahim Shokoohi

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# Methods and Techniques in Nematology

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## Methods and Techniques in Nematology

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# FOREWORD I

Nematodes, also known as roundworms, are essential to soil ecology and play a crucial role in maintaining soil health. These organisms have various feeding groups, including bacterivores, fungivores, herbivores, predators, and omnivores.

Despite the herbivores or plant-parasitic nematodes, many of them, such as free-living nematodes, are beneficial for agriculture and crop production as they help in breaking down organic matter, such as dead plant material and animal waste, into nutrients that plants can absorb. In conclusion, nematodes are more than just tiny worms in the soil. They are critical to the health and productivity of agricultural ecosystems and play a significant role in ensuring global food security.

“Methods and Techniques in Nematology” is an excellent book for anyone interested in the nematology discipline. This book is specifically designed to help lecturers, researchers, farmers, and students deal with nematode problems. The book contains a variety of techniques with detailed explanations and high-quality photographs to make the learning process easier and more engaging. These photographs help bring the topic to life and make learning more enjoyable. You will be able to see the nematodes up close and appreciate their unique features. You will find all the essential information you need to understand nematodes and learn how to address any related problems. The book covers various techniques, from conventional to molecular, particularly for those wanting to start with nematology, all of which are explained in simple and easy-to-understand language.

In conclusion, “Methods and Techniques in Nematology” is an excellent resource for anyone interested in nematology. It is an easy-to-use practical guide that will help you understand nematodes and learn how to address any related problems.

Prof. Ebrahim Shokoohi's book on nematology is an invaluable academic resource that provides students with a comprehensive understanding of the subject. With years of experience teaching at various educational levels, ranging from BSc to Ph.D., Prof. Shokoohi's book is a reliable source that would appeal to a wide audience seeking a deeper understanding of the subject matter; I feel he accomplished it brilliantly.

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# FOREWORD II

Nematodes are remarkable organisms with amazing abilities that have made them the most common animals on the planet; however, they are rarely seen because they are microscopic. Yet people who observe them often share a common bond and instant friendship. My relationship with Dr. Ebrahim Shokoohi is just that, a brotherhood of camaraderie that is based on our mutual admiration of all things nematode. We have worked together describing the wonderful world of nematodes.

Because nematodes are so small, they are difficult to work with. For this reason, they are often neglected, but with proper techniques, they become extremely important in many different types of studies. Fortunately, Dr. Ebrahim Shokoohi has made an effort to assemble various methods of handling and investigating nematodes. They range from sampling and separating them from soil, making slides and examining them with a microscope, designing greenhouse experiments and analyzing the results, to extracting their DNA for taxonomic studies.

This book contains valuable information for anyone interested in working with nematodes because it clearly illustrates many techniques that are described in a logical, step-by-step manner, which makes it easy to follow. The nematology community will be very happy to have this valuable resource for their use.

**Jonathan D. Eisenback,**
Professor of Nematology
Former President of the Society of Nematologists, Virginia Tech
Blacksburg, VA 24061, U.S.A.

# PREFACE

The research on nematology is significant for researchers, students, and everyone interested in this science discipline. While I was teaching nematology for about ten years, I came up with the idea of documenting the methods and techniques that would be useful for everyone. In this book, I have presented the relevant methodology within a conceptual framework of different scopes within nematology that renders technical information that is needed for students and researchers. Methods ranging from sampling to advanced techniques, including molecular surveys, are discussed in this book. The methods are presented in a way that is adaptable for the students to use in formal courses, which can also be functional when used daily by academics and educational institutions. In almost part of the book, the author's experience and the available knowledge of the expert in nematology create an opportunity to easily run the experiments and surveys.

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# ACKNOWLEDGEMENTS

I am grateful to “Allah” for blessing me with a healthy mind to think about the creations and learn from nature. I am thankful to all my teachers and professors for all I have learned in nematology and plant pathology. I would like to thank Prof. Annette van Aardt for revising this book. I would also like to thank Mr. Panahi for providing some of the high-quality pictures for this book. I would especially like to thank my lovely wife for the excellent atmosphere and courage to finish and publish this book. Finally, I would like to thank my parents for the endless support, enthusiasm, and love they have given me. I dedicate my book to my beloved, my wife and son, Adrian.

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# CHAPTER 1

# Introduction

Nematology is an important branch of biological science that focuses on the study of a diverse group of roundworms known as nematodes. In addition, nematology plays a crucial role in agriculture. It is integral to the management of crop pests and the enhancement of agricultural productivity through the study of nematode interactions with crops. Additionally, in the medical field, nematology is vital for controlling diseases caused by parasitic nematodes. Nematodes also serve as important bio-indicators of environmental health in environmental studies, offering insights into soil quality and ecological balance. These organisms can be found in virtually all environments around the world. The term nematode has its roots in Greek, deriving from the words "nema" and "oides", which mean thread and resembling, respectively. Nematodes are an amazingly diverse group of organisms that can either be beneficial or parasitic to plants and animals alike. Generally, they have a slender body that is transparent and lacks segments, exhibiting bilateral symmetry. The study of nematology has made significant strides in the field of medicine. Notably, it has played a pivotal role in developing new antibiotics for the treatment of bacterial infections. Furthermore, nematology has been instrumental in identifying that tropical diseases like elephantiasis and ascariasis are caused by a type of nematode. Ascariasis is a parasitic infection caused by *Ascaris*. The disease occurs when individuals ingest food or water contaminated with the eggs of *Ascaris* species, typically found in soil, vegetables, fruits, and other foods. Research has revealed that *Ascaris* infections can potentially lead to impaired cognitive function in certain school-aged children. This underscores the importance of proper hygiene and food safety measures in preventing the spread of this disease. Several nematodes cause diseases in animals, such as fish, where they reduce the quality of the meat and pose a risk to food security. Nematodes are a type of parasite that can infect various types of fish in freshwater, marine, and brackish water environments. Some nematodes can have devastating effects on wild fish populations, leading to significant fish mortality. These parasites can infect fish in their adult stage, but their larval forms can also infect fish species after passing through birds, mammals, or reptiles that consume fish, or even through predatory fish. Certain nematodes, like *Anisakis*,

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are zoonotic, meaning they can be transmitted to humans. Consumption of raw/undercooked infected fish meat poses a risk of infection to humans. Nematodes also pose a threat to the economic value of fish due to consumer concerns about their presence in food products. Infected fillets are often rejected, leading to increased production costs. Therefore, research on nematology brings valuable insight into the fish/animal parasites aiming to secure food.

In agricultural research, nematology plays a crucial role in providing a wide range of ecosystem services that have a significant impact on the nitrogen cycle, the ability of soil to decompose waste, and the control of pests within soil systems. Although, certain female plant-parasitic nematodes can take on a spherical shape, such as cyst and root-knot females. All plant-parasitic nematodes possess a stylet in their anterior end that helps them to pass the food through the plant cells to their body. This feature enables plant-parasitic nematodes to inject the enzymes into plant cells, which digest the food and help nematodes to develop and cause damage to plant cells. Plant-parasitic nematodes pose a significant threat to various plant types, including vegetables, trees, turfgrass, and foliage plants. They can cause extensive damage and significantly reduce crop yield. Root-knot, cyst, root lesion, spiral, burrowing, bulb and stem reniform, dagger, bud and leaf, and pine wilt disease are among the most harmful nematodes. These pests are responsible for an average loss of 12.3% annually in 40 major crops worldwide, with developing countries bearing the brunt of the losses, estimated at 14.6%, compared to 8.8% in developed nations. Plant-parasitic nematodes result in global economic losses of nearly $125 billion per year, affecting all agricultural crops. The impact of harmful nematodes on agricultural production is regularly undervalued, as their symptoms are frequently mistaken for other issues such as water stress, nutritional disorders, virus infection, soil fertility problems, or complex diseases caused by interactions of fungal/bacterial with nematodes. The severity of their impact depends mostly on the population density in the soil and roots, the cultivar susceptibility, and the ecological circumstances. Root-knot and cyst nematodes are the main destructive plant-parasitic nematodes. The implications of these misinterpretations can be significant, as they can lead to the implementation of inappropriate corrective measures, resulting in further damage to crops and reduced yields. Therefore, an accurate diagnosis of nematode infestation is critical in ensuring optimal agricultural productivity and profitability. To achieve this, it is essential to utilize appropriate diagnostic techniques to differentiate nematode symptoms from those of other conditions. This approach can help farmers and agricultural experts make informed decisions regarding the management and treatment of nematode-infested crops, ultimately leading to better outcomes for all involved.

On the other hand, beneficial or free-living nematodes play a critical role in soil health due to their contribution to soil nutrition, nitrogen fixation, and microbial balance. The soils in a hectare of all agroecosystems typically contain billions of both plant-parasitic and beneficial nematodes, which can significantly affect crop yields. In conclusion, nematology discipline is an attractive and principal field of study that plays a vital role in understanding the complex relationships between nematodes, plants, humans, animals, and their environment.

# CHAPTER 2

# Sampling

**Abstract:** The process of sampling nematodes has become a crucial aspect of agricultural research. The accurate identification of these pests is essential, and the method of sampling is dependent on whether they are plant-parasitic or free-living. To ensure that errors are minimized and samples are reliable and representative, the sampling pattern is based on the area being studied. This can range from randomized to systematic sampling techniques. Furthermore, the final results are influenced by various factors such as the timing of sampling, depth of samples, and the total number of samples taken.

**Keywords:** Depth, Pattern, Sampling tool, Soil sampling.

## GENERAL CONSIDERATION

Useful nematode sampling depends on the time and the target group of the nematodes, which can differ. Several factors, including ecological consideration and distribution patterns, should be considered for plant-parasitic nematodes [1, 2].

As nematodes cannot scurry off within the soil, their damage to crops related to symptom expression on aerial plant parts appears as spots of general low growth (*e.g.*, stunted and/or wilted, yellowish plants), which fluctuates throughout the growing season. The best time for the sampling is when the soil is damp, not too wet or too dry. Nematodes are present in soil rhizosphere samples during the entire growing season, although their population densities fluctuate according to various abiotic and biotic factors. Although nematodes may exist in the soil in all seasons, the larval stages of plant-parasitic nematodes are more apparent in the winter samples. Free-living nematodes of the family Cephalobidae and many belonging to the family Rhabditidae appear more frequently in the samples during autumn.

On the other hand, plant-parasitic nematodes have high population densities during the growing season when climatic conditions and plant growth are optimal.

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Bacterivores nematodes belonging to the Rhabditida family mainly exist in substrates with high organic material contents such as manure or dung. Predator nematodes of the order Mononchida mostly live in the wetlands or the borders of aquatic areas (*e.g.*, rivers, lakes, lagoons, etc.). All nematode groups generally occur in the larval stages during winter. Concerning plant-parasitic nematodes, in particular, sampling should be done both around patches where plants appear to be growing optimally and where plants are stunted, wilted, or yellowish [3]. This will ensure a proper comparison of the nematode status of 'healthy' versus 'infected' plants.

## TOOLS FOR SAMPLING

Augers, Edelman, and Helical (Fig. **1**) are some of the tools that can be used to sample nematodes at a depth of 30 cm or more [4]. However, in dry soil, it is difficult to insert an auger properly for accurate sampling. Garden trowels, narrow-bladed shovels, big kitchen spoons, or spades are also useful tools to sample nematodes, mostly when sampling is done in areas with rocky soil (Fig. **1**). All equipment should be cleaned or appropriately sanitized, *e.g.*, washed with or soaked in a 1% NaOCl solution, after each sampling activity to avoid contaminating nematode populations that occur in different areas where sampling is done for separate projects.

A proper auger is essential for taking soil samples from various depths associated with multiple crops (Fig. **2**).

## SAMPLING FROM AQUATIC AND WETLAND

Nematodes are present in benthos belonging to various groups and families. However, they cannot be collected from enough individuals. Therefore, constant and hard work is required to assess the proper number of nematodes. The device for sampling from benthos is shown in Fig. (**3**).

## SIZE OF THE SAMPLING AREA

The size of the sampling area must be adapted to fit the purpose of the study. The sampling location should typically be divided into smaller pieces, for example, one hectare. Generally, a nematode sample (soil, roots/other below-ground plant parts and/or aerial plant parts) should consist of several sub-samples (at least 10) of each area being sampled. About 20 sub-samples can be combined to form one mixed sample of 1-2 kg for 5 acres [5]. For example, mixing soil can be done by hand to avoid mechanical damage to the nematodes. A sub-sample may consist of at least 100 g of soil and 5-50 g of plant material. Plant material should be chopped into small pieces, *e.g.*, 1cm, and appropriately mixed to obtain a

representative sub-sample. In or near aquatic areas, the sample that is in a solution form should be transferred to a plastic container while the soil is kept in a plastic bag (Fig. **4**).

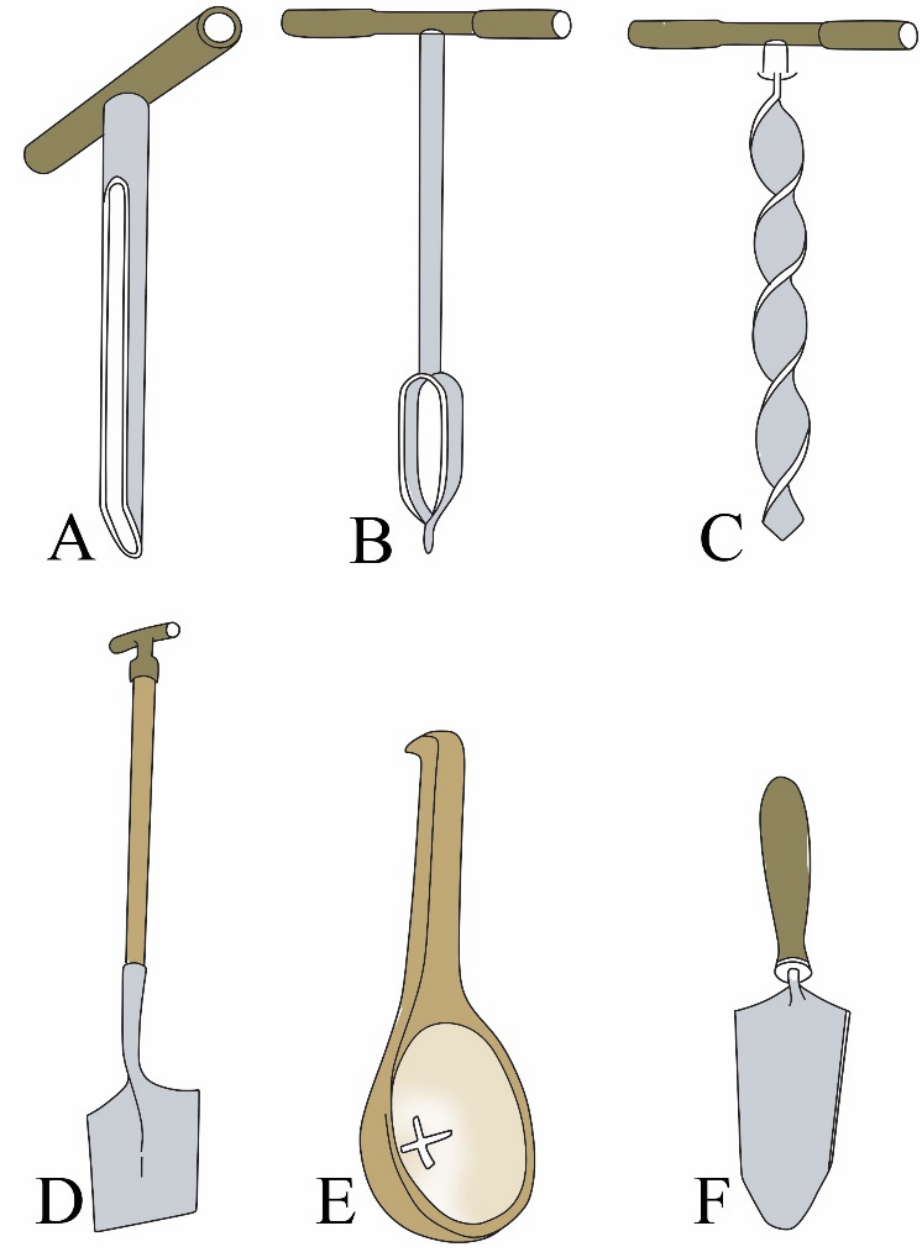


**Fig. (1).** Tools generally used for nematode sampling (adapted from Kleynhans [4]).

CHAPTER 3

# Nematode Extraction

**Abstract:** Obtaining an adequate number of nematode specimens is crucial for various studies related to biodiversity, taxonomy, and management. The extraction method chosen for the nematodes depends on the specific study being conducted. In regards to sedentary endoparasites, the roots are the primary source for nematode extraction, while for other purposes, the soil is the preferred option. It is essential to evaluate the efficiency of the extraction method used. Nematode extraction must be performed promptly after sample collection since the specimens tend to deteriorate with time. In this chapter, some of the commonly used techniques for nematode extraction are given.

**Keywords:** Efficiency, Extraction, Root, Soil.

## EXTRACTION OF SOIL AND ROOT NEMATODES

### Tray Method

This is the simplest and cheapest method that is adapted from the Baermann Funnel technique. Even slow-moving nematodes, such as Criconematids, can be extracted using this technique. This method is relatively fast for yielding large numbers of alive and active nematodes and is suitable for ecological and taxonomical studies of free-living and plant-parasitic nematodes [10].

### Procedure

1. Put on gloves for safety (Fig. **1**).

2. Weigh 200 g of soil for biodiversity investigation and up to 500 g for a classical study.

3. Mix the soil.

4. Spread the soil on tissue or towel paper supported on a coarse meshed plastic screen kept in a plastic container.

5. Add tap water to cover the soil surface.

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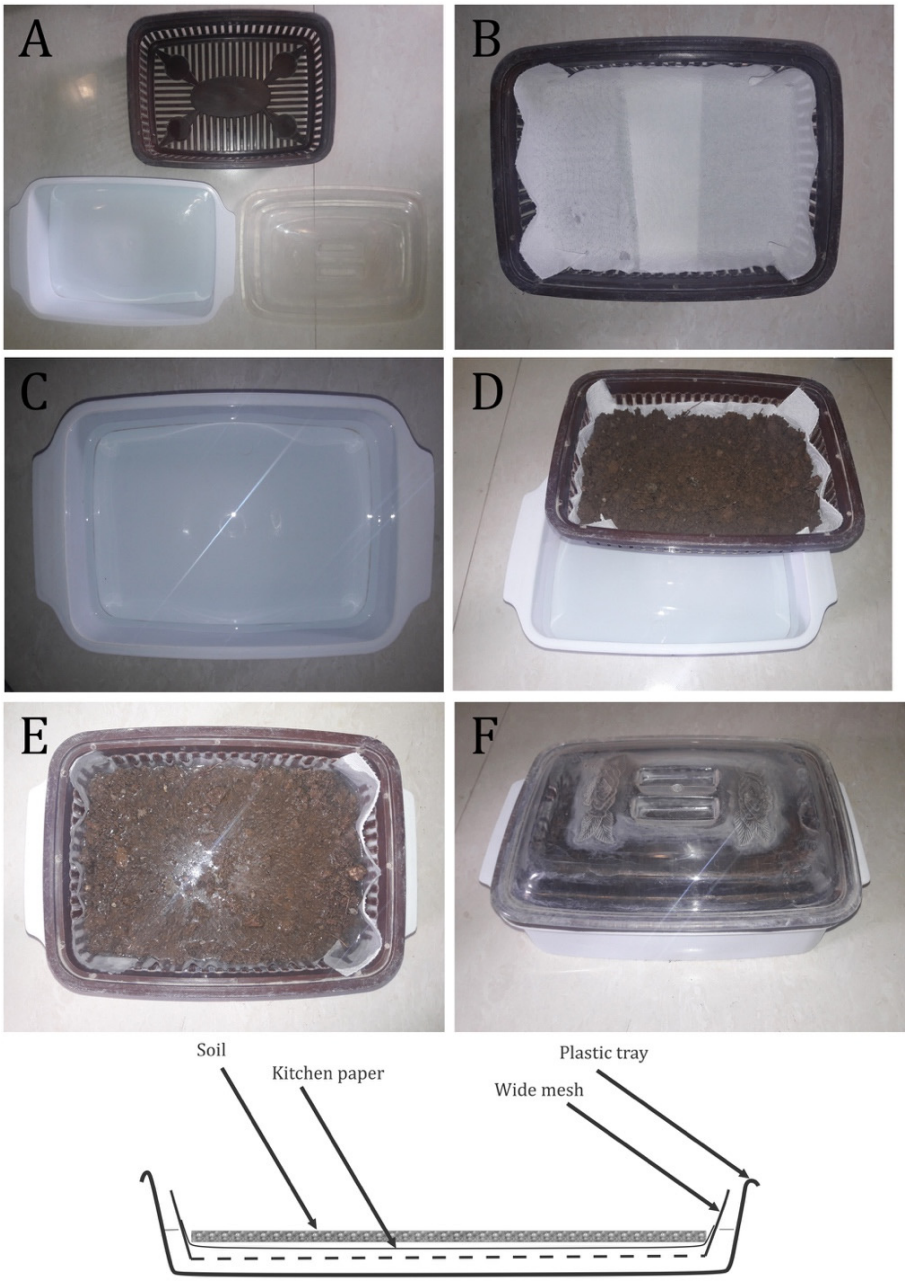


**Fig. (1).** The tray method that is used to extract nematodes from soil using a tray, including (**A**) three plastic parts; (**B**) a plastic mesh part covered with tissue- or towel paper; (**C**) the tray filled with an appropriate amount of water; (**D**) soil over the tissue paper; (**E**, **F**) soil over the tissue paper covered by a thin layer of water; (down illustration) schematic plan of entire tray method.

6. Leave the soil to soak in water in the container for 18-24 hours or at least overnight.

7. Cover the container to avoid evaporation of the water.

8. Collect the solution at the bottom of the container into Petri dishes.

**Sugar Flotation Method**

This method is fast and yields a high number of nematode individuals [2, 10]. However, more equipment and materials are needed than the tray method. By using this method, sluggish and active nematodes are extracted successfully. This method is very suitable for studies on plant-parasitic nematodes for ecological and classical purposes.

**Procedure**

1. Mix the soil with water and pass the solution through a 60-mesh size sieve to remove the debris material.

2. Next, pass the soil solution through the 100, 120, and 400 mesh size sieves, stacked from bottom to top.

3. Collect the residue on the 400-mesh size sieve and transfer it to a 500-1000 beaker or graduated cylinder and leave it for 20-30 minutes for dust, hummus, and debris to move up to the water surface.

4. Remove the debris from the water surface.

5. Shake the solution in the graduated cylinder and pour 300-500 ml of that into centrifuge tubes.

6. Centrifuge the solution for 5 min at 5000 rpm.

7. Take the tubes out and remove the water.

8. Prepare the sugar solution as follows: Put 700 ml of sugar into a 1000 ml graduated cylinder, then add water up to a volume of 1000 ml (hot water is preferable); shake the cylinder to dissolve the sugar in the water.

9. Add the sugar solution to the centrifuge tubes, then mix it with the soil that had settled at the bottom of the tubes.

10. Centrifuge for 1 min at 1000 rpm.

# CHAPTER 4

# Nematode Observations

**Abstract:** Preservation of nematodes is a crucial element of research endeavors, and it can be accomplished either temporarily or permanently. In order to conduct a rapid examination, researchers may place a minute droplet of water onto a glass slide for temporary observation. However, for the purpose of long-term preservation and collection, permanent slides are requisite. Prior to studying nematodes under a microscope for taxonomic purposes, it is necessary to first kill and fix them. This method is particularly useful for taxonomic studies involving light, electron (SEM), or transmission electron (TEM) microscopy.

**Keywords:** Fixative, Glass slide, Permanent, Preserving, Temporarily.

## FORMALIN-GLYCERINE METHOD

Formalin harms humans; however, most fixatives include formalin to kill the nematode [10]. It should be noted that dehydration is critical for nematode preservation [10].

### Materials

A volume of 100 ml can be prepared for each of the three fixative solutions:

Fixative I: 88 ml distilled water, 10 ml formaldehyde (40%), 1 ml acetic acid, 1 ml glycerine.

Fixative II: 95 ml ethanol (96%), 5 ml glycerine.

Fixative III: 50 ml ethanol (96%), 50 ml glycerine.

### Procedure

1. Remove the additional water from the petri dish (in which the nematodes are to be suspended (Fig. **1A-H**) using soft paper (Fig. **1B**), and soak it until only a thin layer of water is present in the petri dish (Fig. **1C**).

2. Heat Fixative I to approximately 80 ˚C (Fig. **1E**).

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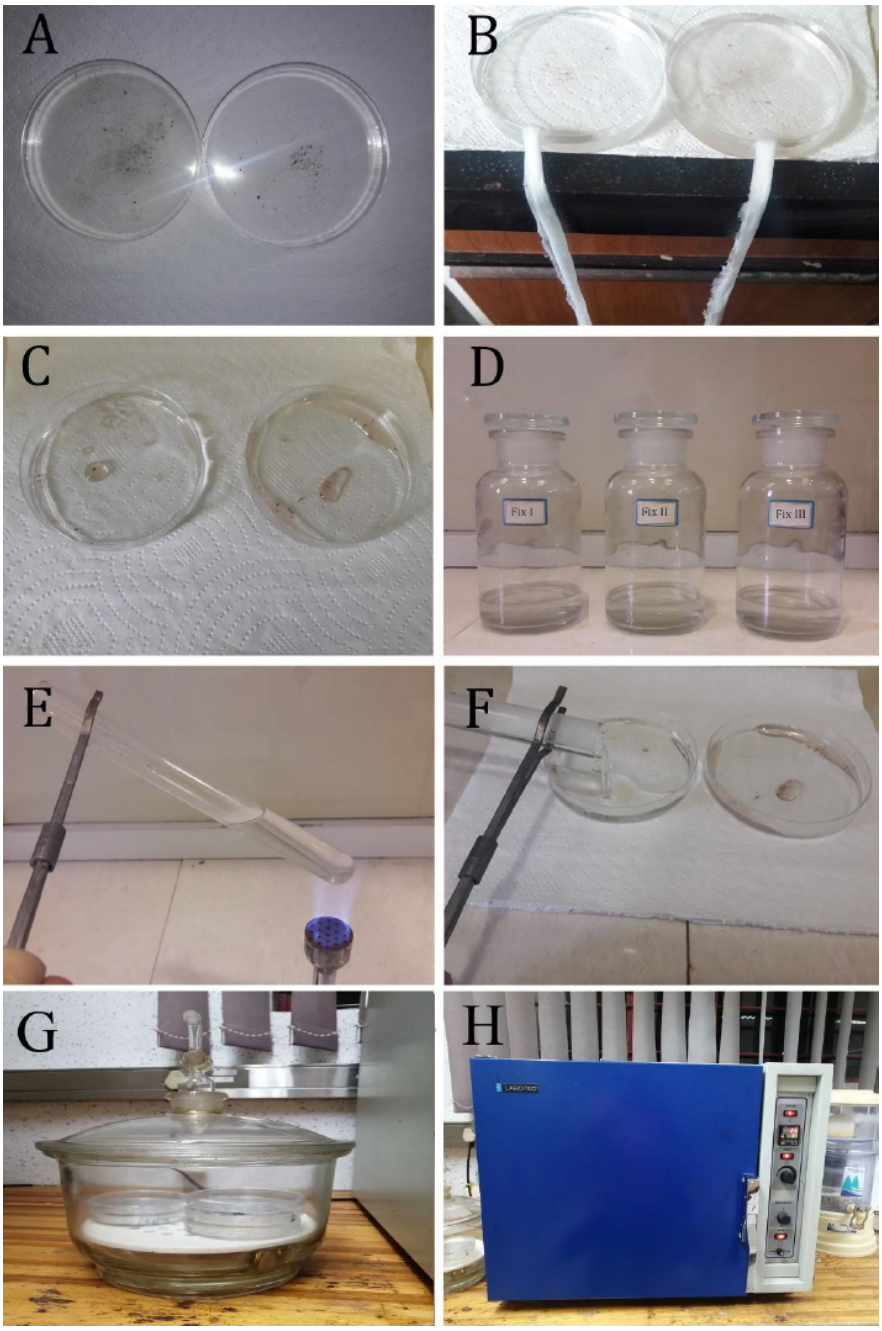


**Fig. (1).** Fixation of the nematodes. **A**: Nematodes in the water solution in the petri dish. **B**: Removing the water from the petri dish using soft paper. **C**: Petri dish with a thin layer of water after removing redundant water. **D**: Fixative I-III. **E**: Heating fixative I. **F**: Pouring the fixative into the petri dish. **G**: Desiccator with nematodes covered by glycerine after fixation processing. **H**: Incubator for fixation processing.

3. Add warm Fixative I to the petri dish in which the nematodes are suspended (Fig. **1F**).

4. Put the petri dish that contains the nematodes suspended in Fixative I into a desiccator, the bottom of which is filled with 96% ethanol (Fig. **1G**).

5. Put the desiccator into an incubator at 37 ˚C for 18-24 hr (Fig. **1H**).

**After 18-24 Hours**

6-Take out the petri dish from the desiccator.

7-Remove Fixative I by using soft paper to soak it up.

8-Add Fixative II to the petri dish.

9-Put the petri dish back into the desiccator.

10-Put the desiccator into the incubator for only 4 hrs (this step is critical).

**After 4 Hours**

11-Remove the desiccator from the incubator, take the petri dish with nematodes suspended in Fixative II out of the desiccator.

12-Add Fixative III to the petri dish or remove Fixative II, then add Fixative III.

13-Put the petri dish into the incubator (not into the desiccator) for at least 4 hrs until all the ethanol has evaporated.

Finally, all nematodes are suspended in glycerine to prepare permanent slides.

## TAF (Triethanolamine Formalin) Method

This method is frequently used by many researchers and nematologists to preserve nematodes [2]. However, long-term storage of specimens in TAF leads to cuticle degradation [2, 34]. The procedure is as follows [2]:

1) Prepare TAF fixative (8% formalin and 2% triethanolamine in distilled water).

2) Transfer live nematodes to a small glass vial or Petri dishes and allow them to settle to the bottom. Draw off additional water until they are left in about 2 ml water.

3) Kill the nematodes by stirring the vial for 20-30 secs in a 70-90 °C water bath until they are dead and stretched.

**CHAPTER 5**

# Nematode Morphological Observations

**Abstract:** In the field of nematology, a variety of microscopes are utilized for distinct purposes, including standard light microscopes and stereomicroscopes. Additionally, line illustrations are essential for the taxonomical examination of nematodes. As such, it is crucial to properly calibrate and maintain microscopes to ensure accurate outcomes. This section delves into the essential duties associated with microscope usage in nematological research. Differential interference contrast microscopy (DIC) is a useful technique to create a high-quality photograph of the nematodes. In regard to taxonomy and diagnosis of nematodes, accurate measurements are of utmost importance. Each nematode family, whether they are free-living or plant-parasitic, necessitates specific measurements of particular attributes. The identification of nematodes relies on the de Man indices. This section offers visual aids for different families to showcase the significant features that must be measured. The art of capturing images of nematodes holds immense significance in the fields of taxonomy and histological research. Nematology employs numerous methods to obtain top-notch visuals for analysis, among which is Scanning Electron Microscopy (SEM). Furthermore, phase contrast is widely used in nematology for a variety of purposes. This chapter endeavors to provide a comprehensive understanding of the microscope, measurements, DIC, and SEM techniques.

**Keywords:** Calibration, DIC, Microscope, SEM.

## MICROSCOPE JUSTIFICATION

### Microscope Types

After slide preparation, the nematologist always uses a microscope to study nematode specimens for identification purposes since these organisms are microscopic. Since all nematologists use a microscope, the quality of the microscope is essential. Two types of microscopes are used for research work: a dissecting/stereomicroscope and a light microscope. The “stereomicroscope” (Fig. **1**) has a limited number of lower magnification rates (10-80x), offering a wide-angled vision. This microscope is mainly used for analyzing nematode samples while counting at only the genus/family level and/or for dissection work.

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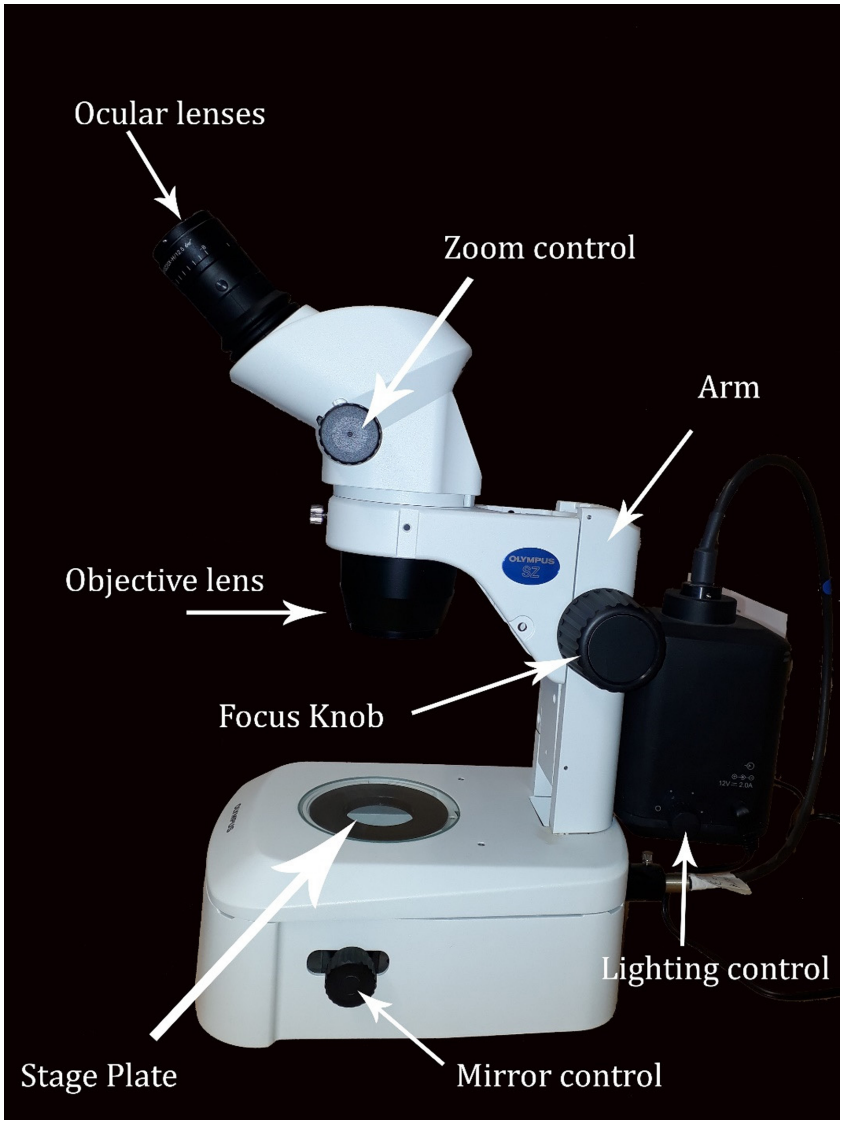


**Fig. (1).** Stereomicroscope and its different parts.

The light microscope (Fig. **2**) offers magnifications ranging from 10 to 1000x. Working with it at a magnification of 500 or 1000x requires an oil immersion lens. This type of microscope is generally used for the identification of nematodes. Usually, the nematodes studied with a light microscope are mounted using a dissecting microscope.

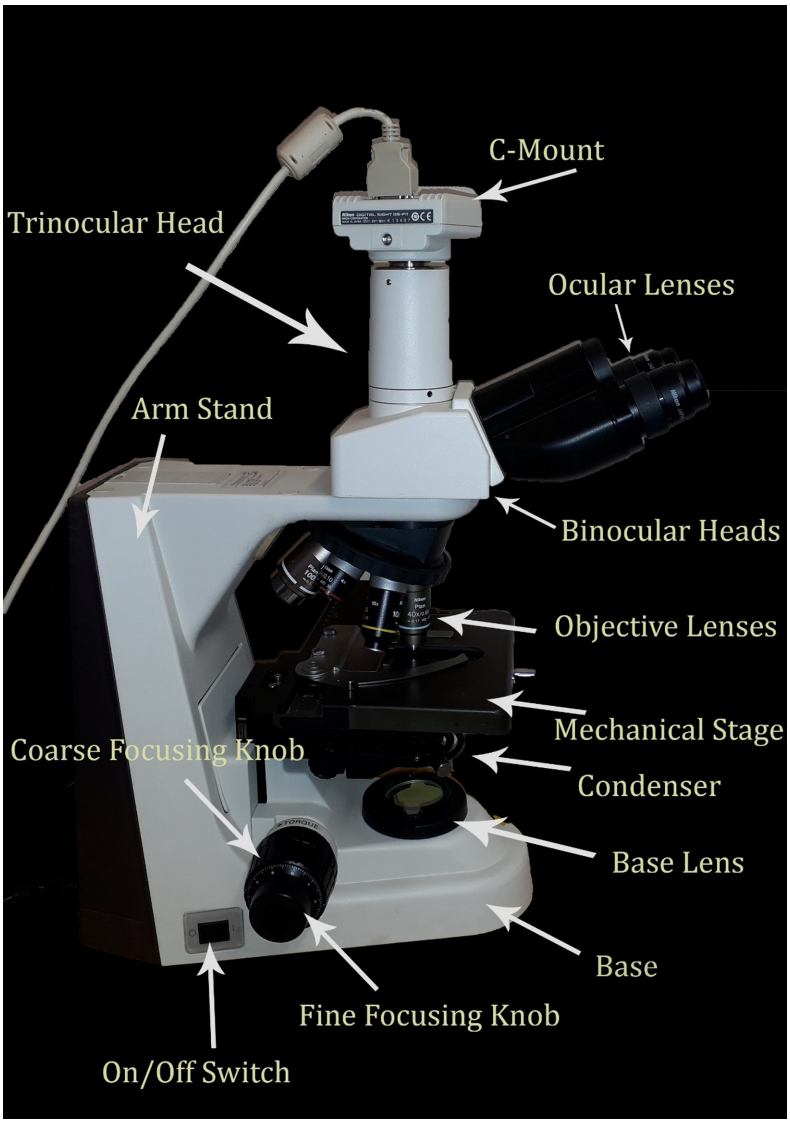


**Fig. (2).** The microscope and its different parts.

## Calibrating the Eyepiece Graticule

To ensure accurate measurements of nematode specimens, the eyepiece graticule (Fig. **3**) on a microscope must be calibrated prior to use. The following steps should be taken in order to properly calibrate the eyepiece graticule:

# CHAPTER 6

# Molecular Diagnosis

**Abstract:** Molecular techniques are crucial for research on nematology, and the processing of samples is essential for their long-term storage prior to analysis. This chapter focuses on DNA extraction, various DNA markers, and polymerase change reaction (PCR). To ensure the preservation of nematodes for extended periods, DESS can be utilized. Moreover, the quality of extracted DNA is vital for proper PCR processing. This chapter offers an overview of nematode isolation and the different techniques for DNA extraction. The utilization of molecular markers presents numerous benefits when studying the genetic makeup of nematode populations. Among these markers, sequence characterized amplified region (SCAR) stands out for its exceptional value in identifying nematodes belonging to the *Meloidogyne* species. Amplified fragment length polymorphism (AFLP), on the other hand, is a technique that can be employed to analyze the genetic variability of nematodes. It is important to note that these markers are primarily used for plant-parasitic nematodes, which have a significant impact on the economy. This chapter focuses on the most commonly utilized marker for nematodes. After extracting DNA, the next step involves amplifying the target genes through PCR. Various primers, including rDNA and mtDNA, are available for nematode genes and serve useful taxonomic purposes. However, precise primer design is critical for achieving accurate nematode diagnosis. Both DNA and primers must be of high quality to ensure successful PCR. Eco-friendly standard dyes like SafeView can be employed to visualize PCR products. This chapter offers a comprehensive guide to PCR processing and DNA visualization techniques.

**Keywords:** Chelex, DNA extraction, DNA marker, Genetic diversity, Primer, Sequencing, Thermocycler, Visualization.

## DNA EXTRACTION

### Isolation of Nematodes

Nematodes that are extracted from soil or plant tissues can be used for DNA isolation immediately, or they can be preserved in a solution until they can be processed. For the latter, the nematodes can be stored in a 70% ethanol solution for a short period of several months or in a DESS solution for a more extended period. In addition, the nematodes stored in the DESS solution can be used for morphological and molecular studies [41].

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## DESS Solution

1. Mix 23.265 g of disodium ethylenediaminetera-acetic acid (EDTA = FW: 372.24) for a 250 ml solution (the formula may vary depending on the FW of the EDTA salt). Add 50 ml of deionized water to the EDTA salt and stir (pH = 3-4).

2. Adjust the pH of the EDTA to 8.0 using sodium hydroxide (NaOH). At this stage, about 50 ml of 1M NaOH will be required. By increasing the pH, the EDTA will dissolve gradually, which will be increased by temperature up to 30°C.

3. Using de-ionized water, increase the volume of the solution to 200 ml. Add DMSO to a final volume of 20% DMSO; —for example, 50 ml DMSO for a 250 ml solution.

4. Add NaCl to the solution to be saturated (till it no longer dissolves; the rate of dissolution can be improved by warming to 30°C).

5. Transfer the solution into a container with a lid, and remove any salt crystals.

## How to Transfer Nematodes to Ethanol

1. Extract the nematodes from soil or plant tissue

2. Clean a plastic or glass petri dish using a 96 or 70% ethanol solution

3. Add three separated drops of distilled water to the inside of the petri dish

4. Transfer the nematodes to the drops of distilled water one after the other

5. If possible, before the third drop of water, soaking the nematodes in a drop of 70% ethanol would be helpful.

6. After the third drop of water, put the nematodes into a 1.5 ml tube with 20-30 µl of distilled water

7. Keep the tubes that contain the nematodes at 4 $^{o}$C if the processing can be done in a few days, or at -20$^{o}$C if the processing will be done only after a week

Note that all steps should be conducted in a sterilized place. The nematodes are ready for DNA extraction.

## Genomic DNA Extraction

Isolation of genomic DNA from any type of cell includes three steps [42]:

1. Lysis of cells and extraction of the DNA

2. Dissociation of DNA-protein complexes

3. Extraction of DNA from other macromolecules (carbohydrates, protein, and lipids)

DNA of the nematodes can be isolated manually or by using a DNA extraction kit. Generally, a kit for DNA extraction is more expensive than when the solutions are prepared manually. Moreover, more reactions can be done using manually prepared DNA extraction solutions, while reactions to be done using a DNA extraction kit are limited. Several DNA extraction kits are available, *e.g.*, ClearDetections DNA extraction kit (http://www.cleardetections.com/nematode-dna-extraction) and kits provided by Sigma and Promega companies.

## DNA Extraction Using the Chelex Method

Materials: Chelex resin (Sigma sells this as "chelex 100 or chelex 50"), Proteinase K, Sterile water. The schematic view of the process is given in Fig. (**1**).

1. Pprepare a 5% Chelex solution (*e.g.*, for a 10 ml solution)

2. Place a stirrer bar in a 50 ml conical tube held upright in a beaker

3. Place 0.5g Chelex resin into the conical tube

4. Add sterile water to obtain a volume of 10 ml

This Chelex solution can be kept in the fridge for up to 1 month. It is recommended to prepare a fresh work solution for each DNA extraction process [43].

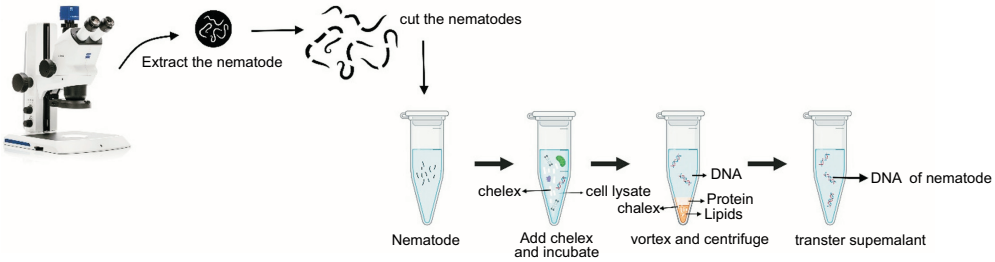


**Fig. (1).** Overview of DNA extraction of nematodes using Chelex.

CHAPTER 7

# Basic Bioinformatics in Nematology

**Abstract:** Bioinformatics is the interdisciplinary study of nucleic acid and protein sequences, which has proven especially useful for genomics, gene expression, and nematode diagnosis. Quality control of these sequences is essential, and bioinformatics plays a crucial role in their processing for phylogenetic analysis. Skilled analysis is particularly vital for molecular ecology and environmental DNA analysis, especially when working with next-generation sequences. Furthermore, the utilization of an online database and specialized software designed for the identification of nematode species serves as valuable resources for a proper diagnosis of nematode-related issues. In this chapter, we will explore the bioinformatics of Sanger sequencing, with an emphasis on the phylogenetic study of nematodes and online species identification of nematode species.

**Keywords:** Bioinformatics, DNA, Nematodes, Phylogeny, Sanger sequencing.

## GENERAL INFORMATION

A massive amount of data mainly related to molecular analysis indicates the challenges in computing in biological sciences. Bioinformatics is a field of study that leverages computational methods to analyze large datasets associated with biomolecules. It is widely recognized as a subfield of molecular biology and encompasses a range of subjects, including structural biology, genomics, gene expression, DNA barcoding, and phylogenetics.

The main scientific products of Nematology nowadays focus on DNA barcoding to identify the species and phylogenetic study. For this reason, the PCR products can be sequenced by different technologies. The DNA sequencing method, commonly referred to as Sanger sequencing or the chain termination approach, was introduced by Sanger and colleagues in 1977. This technique entails incorporating chain-terminating dideoxynucleotides (ddNTPs) selectively into DNA polymerase during in vitro DNA replication.

Nowadays, a large set of nucleotide data can be obtained by New Generation Sequencing or NGS [106]. A study of the organisms by using NGS can be done on different platforms [106]. DNA divides into some parts in the 454 Roche pyrosequencing method, and then sequencing of 400-600 megabases of DNA

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during the 10-hour running will be obtained. In Illumina sequencing, DNA is first cut, and then two adapters ligate to the end of each. This method yields millions of reads from the original DNA. Illumina is the most usable technique in the field of next-generation sequencing. In Nematological study, the nematode community is mainly studied by the pyrosequencing method of the Roche 454 platform [107, 108, 109, 110, 111]. NGS sequencing is beneficial for understanding the diversity of the nematodes in the related community [106]. An NGS study of nematodes by combining two genes, including SSU and LSU, leads to precisely identifying 97% of the species [107].

## CHECKING THE SEQUENCES

Sequencing technologies are imperfect, and quality control is necessary to confirm that the data used for the downstream study is not compromised of low-quality sequences, sequence artifacts, or sequence contamination that might lead to erroneous conclusions. The simplest way of quality control is by looking at summary statistics of the data. Different programs can yield those statistics.

The first step in checking the quality of the sequences is to open and observe the raw data and analyzed files. For this purpose, open the "*.ab1" file (the raw chromatogram trace file) using the "BioEdit" or "Chromatogram" program.

In the chromatogram presented for *Butlerius* (Superfamily: Diplogasteroidea) (Fig. **1**), some features, including well-formed and distinct single-colored peaks, separated peaks, and lacking background signals, are visible. This sequence quality is considered successful. The successful result could be due to the appropriate concentration of DNA template and primer, excellent purity of DNA, and optimum primer design.

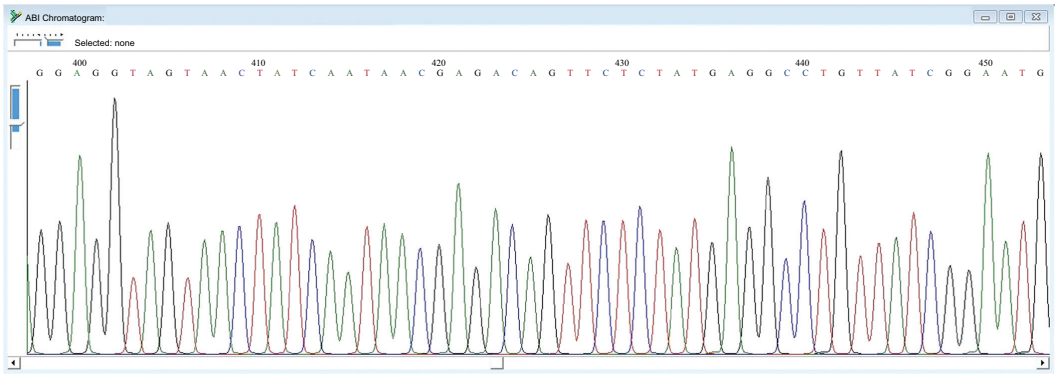


**Fig. (1).** Chromatogram of a normal rDNA sequencing read of *Butelrius butleri* [112].

In the chromatogram presented for *Helicotylenchus* (family: Hoplolaimidae) (Fig. **2**), some features, including the absence of clearly defined peaks in raw and analyzed data, the presence of excess dye peaks, and a high ratio of noise, are visible. This sequence quality is considered a failed one. The failed result could be due to an inadequate amount or poor quality of DNA template or the primers not binding well.

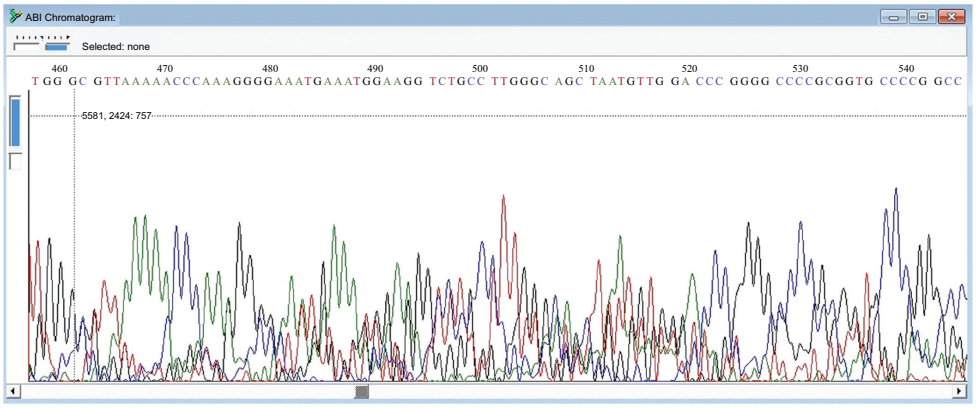


**Fig. (2).** Chromatogram of a failed rDNA sequencing read of *Helicotylenchus* sp. [21].

In the chromatogram presented for *Helicotylenchus* (family: Hoplolaimidae) (Fig. **3**), some features, including the unclean pick of the sequencing, multiple peaks with the same height or differing heights, overlapping one another, and artifacts beneath the peaks are visible. This sequence quality is considered a failed one. The failed result could be due to a contaminated DNA template or primer, poor DNA template and primer quality, or multiple priming.

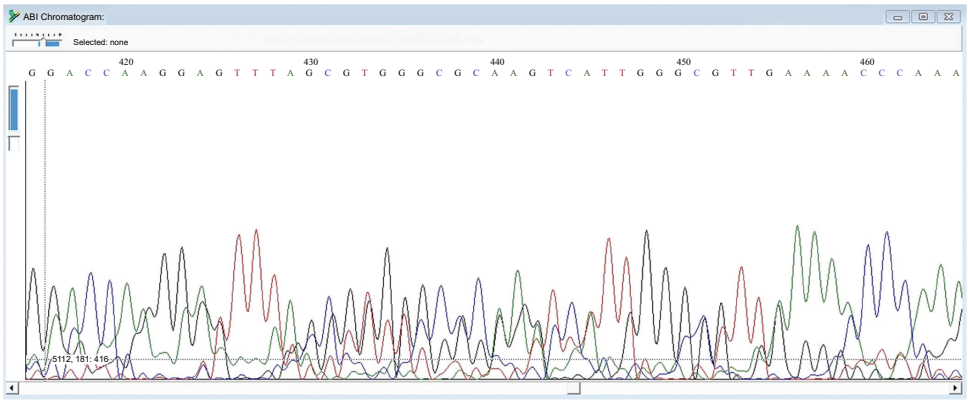


**Fig. (3).** Chromatogram of a multiple sequence signal of rDNA sequencing read of *Helicotylenchus* sp. [21].

# CHAPTER 8

# Biodiversity Analysis

**Abstract:** This chapter delves into the assessment of nematode biodiversity, which involves investigating the community of these organisms present in a given region. The biodiversity of nematodes can be studied through molecular or conventional approaches. In a conventional method, proper sampling is essential to accurately evaluate biodiversity, and this task is aided by the use of various indices, including Shannon, richness, evenness, and community indices. In the field of molecular biodiversity, researchers can investigate the diversity of nematodes by conducting metagenomic analysis of ribosomal DNA (*e.g.,* 18S rDNA) or cytochrome c oxidase subunit I (cox1) gene of mitochondrial DNA. These approaches provide significant insights into the identification and classification of nematode taxa present in soil ecosystems. Analysis of these genetic markers allows scientists to better understand the rich diversity of nematodes and their ecological roles within soil communities. In this chapter, relevant information on nematode biodiversity assessment is presented.

**Keywords:** Biodiversity, Free-living, Indices, Nematodes, Plant-parasitic.

## CONVENTIONAL BIODIVERSITY

### Sampling

The sampling pattern depends on the type of soil (*e.g.,* forest, agricultural, and natural fields). For deep sea, the samples should be taken from different depths [120]. Soil sediments from the deep sea can be carried by using a spade-corer at a specific time. The spade-corer with a 6-cm diameter collects the deposits after 7 hours [120]. For meiofaunal analyses, additional sediment samples can be collected by employing collectors connected to six large cages placed on the seafloor and left for 24 h before recovery [121].

Soil sampling should be done systematically across the area of the study. For instance, collect ten samples from each 40 × 5 m split at approximately 4m intervals. Two parallel zig-zag lines are marked along the field to take the samples, with ten equally spaced sampling points on each line. For each sample, two cores are taken at 30 cm depth (to cover the most free-living and plant-parasitic nematodes) at opposite points on the zig-zag line, using a carbon steel tube or an auger. Then, combine the soil to make up a quantity of about 500 g of

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soil. Soil samples should be bagged and sealed on a bright plastic carrier to avoid desiccation but keep them out of direct sunlight. Samples can be temporarily stored in a protected box for transportation to the laboratory and then at 4° C until extraction, which should be performed as soon as possible. In our nematology laboratory at the University of Limpopo, about 100-300 gr of soil for counting nematodes and about 200 gr for analytical purposes should be prepared. For seasonal fluctuation, soil samples should be taken from different seasons in the exact location for each season over several years. This is very important when you want to study the seasonal fluctuation of a specific plant-parasitic nematode in a particular locality. Geographical coordinates using the global position system or GPS should be noted for each sampling site.

Nematodes can be extracted from a specific quantity of soil taken from each composite sample using the Baermann funnel technique [122]. After 72 h of incubation at room temperature (25 °C), nematodes can be collected and counted using a stereo microscope/light microscope, and the average populations will be determined. Subsequently, nematodes should be fixed with a hot 4% formaldehyde solution and transferred to anhydrous glycerine [13, 123] method for species identification.

## Evaluation of Soil Samples

Rhizosphere soil samples from different fields should be analyzed for pH and electrical conductivity (EC), soil texture, C:N ratio, and various mineral analyses, which can be determined by the standard method [124].

## Counting of Nematodes

A counting dish or counting slide Fig. (**1**) is used for nematode counting. When the nematodes have been extracted from the soil and collected in a petri dish, adjust all containers to a specific volume of water, *e.g.,* 10 ml, 20 ml, *etc.* Then, stir the container or tubes using a magnetic stirrer to proportionate the nematodes in the water. Take 1 ml of the solution (or more, depending on the counting slide or counting dish) and count the nematodes. The number of nematodes should be multiplied by the total volume of the solution. For instance, if we have 20 ml of the solution, then the number of individuals in 1 ml should be multiplied by 20. The counting process should be repeated at least five times to obtain an average and make the analysis statistically acceptable. The results can be transferred to an Excel file for further study when the average for all sampling sites is acquired. The schematic view of nematode biodiversity analysis is given in Fig. (**1**).

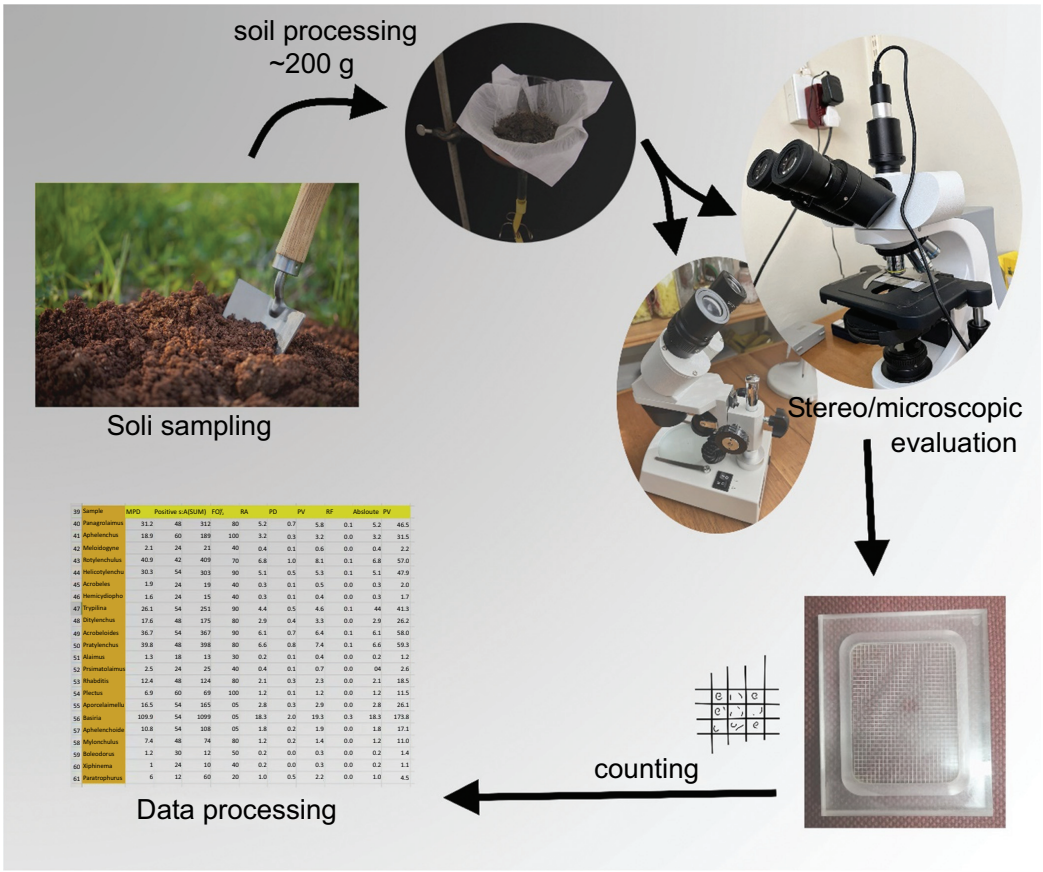


**Fig. (1).** Schematic view of nematode biodiversity analysis

## Biodiversity indices and data analyses

The prominence value (PV) per unit area is commonly used to calculate the correlation between the population density of nematodes (MPD) and the frequency of their occurrence (FO%) based on their genus/species [125]. PVs serve as an index for classifying the nematode genera/species found during surveys [125]. Thus, high PVs for nematode genera indicate the most abundant ones in the rhizosphere soil samples collected from each site. The following equation [126, 127] is used to calculate PV:

PV = Population density × √frequency of occurrence /10

Nematode biodiversity indices represent the Evenness Index (E) [128], Richness Index (SR) [128], Shannon Index (H) [129], and Simpson Index (S) [129].

# CHAPTER 9

# Nematode Rearing and Greenhouse Studies

**Abstract:** The maintenance of live nematodes is a crucial task for various studies related to their biology, taxonomy, and genetics. It is essential to have a living source of nematodes for future research purposes. Researchers typically use media or plants to culture and multiply nematodes. While agar serves as a base ingredient for several nematodes, liquid or solid culture media is used for laboratory rearing. It is important to note that culturing plant-parasitic nematodes is more challenging in the lab than free-living nematodes. However, entomopathogenic nematodes can be cultured in large quantities for pest control purposes. This chapter provides a thorough discussion of the culturing methods of several nematodes. Greenhouses are an essential tool for nematologists to study nematodes in the field of integrated pest management. By using various types of greenhouses, researchers can achieve their goals in nematode biology. The Phytotron provides the most precise and controlled environment for biological studies, while certain nematodes, such as *Meloidogyne* and cyst nematodes, can be mass-reared in greenhouses for further molecular or biological surveys. This chapter delves into the important tasks associated with greenhouses for economically significant plant-parasitic nematodes.

**Keywords:** Bacteria, Biology, Culture, Greenhouse, Laboratory, Multiplication, Phytotron, Water agar.

## CULTURING OF NEMATODES

### General consideration

Some factors must be considered when determining the type of research, as well as molecular or biological approaches. The most integral is the question concerning the number of nematodes required. In vivo and in vitro cultures help maintain colonies of different nematodes. For large-scale rearing of nematodes, in vitro methods are the most practical. For this purpose, nematode species (free-living or plant-parasitic) and maintenance duration are essential. Concerning the free-living nematodes, for instance, *Panagrolaimus*, they could be reared simply in a WA2% (Water Agar medium) even in the absence of bacteria added to the medium. We tested this medium in our laboratory, and after one week, several individuals for the molecular study were collected (Shokoohi, unpublished data). Some methods for rearing specific nematodes are explained. The nematodes that

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have been carefully raised are utilized to conduct in-depth research in the areas related to biology, molecular taxonomy, and a wide range of physiological studies. The procedure for several nematodes is as follows.

### *Globodera* spp.

To rear *Globodera*, start by disinfecting potato tubers using a 5% NaOCl solution for 4 minutes and then rinse them with water. Next, dry the potato tubers and leave them at room temperature to allow the eyes to develop and shoots to form. Afterward, soak the cysts in root exudate for one to several days. Prepare a closed container with about 200g of dried, clean sand and 30ml of tap water. Place the germinated potatoes in the container. Crush the cysts to extract the eggs and juveniles and add approximately 1000 eggs and juveniles of *Globodera* spp. to the container with sand. Close the lid and store the containers in the dark at 20°C for about 14 weeks. The cysts can be collected after the roots have died [142].

### *Heterodera* spp.

To prepare for the cyst nematode of *Heterodera*, start by obtaining white mustard seeds (Sinapis alba). Then, sterilize the seed surface by using a 20% dilution of 3.6% sodium hypochlorite for 20 minutes and wash the seeds six times with sterile double-distilled water. Next, keep the seeds at 4°C overnight to enhance and synchronize germination. Prepare the Knop medium by pouring 1 liter of distilled water into a beaker, then adding 3 g of calcium nitrate and 1 g of each of the following chemicals: magnesium sulfate, potassium nitrate, and potassium phosphate, and dissolve them in the water. After that, autoclave the solution. Add 100 g of sucrose to 1 L of Knop's solution, and then soak the seeds in a petri dish containing the Knop medium. For female counting, dye the agar using various food coloring (e.g., Limino, DYL-ghm-20210126–324). For cyst counting, leave the agar undyed. Plants should be grown in a growth chamber with a 16-hour day at 21°C and an 8-hour night at 20°C cycle. Soak the cyst in 3 mM Zinc Chloride to promote egg hatching. Then, inoculate 300 second-stage juvenile individuals to the plants after 21-28 days of plant growth. The cysts will develop in 10–12 weeks at 20–25°C in darkness [143].

### *Meloidogyne* spp.

Root-knot nematodes aiming for molecular diagnosis are cultured in the greenhouse or in an in vivo environment. Therefore, a susceptible cultivar of tomatoes is the best option to rear this nematode. As the molecular diagnosis of root-knot nematodes can be made using eggs, juvenile, and adult stages, therefore, tomatoes should be transplanted into the proper pots and then inoculated with eggs and second-stage juveniles. For obtaining a pure culture of *Meloidogyne*, the

egg mass should be isolated from a single female, and then tomatoes must be inoculated. After 56 days, enough nematodes are extracted for various molecular studies [105].

### *Radophulus* spp.

To propagate burrowing nematodes, begin by extracting them from banana roots. Chop the roots into small pieces and collect the nematodes using extraction methods in a beaker. Then, peel the carrots using 70% ethanol and cut them into small 2 cm diameter pieces. Ensure all steps are conducted under laminar flow conditions and use glass Petri dishes to avoid contamination. Collect 60-100 female nematodes in a 10ml measuring cylinder. Add 6 mg of streptomycin sulfate to 10 ml of distilled water to make a sterilization solution for nematodes. Then, in the measuring cylinder, allow the nematodes to settle, and then remove 5 ml of the water. Add 5 ml of the streptomycin solution to the nematode cylinder and let it sit for 1 hour. Repeat this step two more times, leaving the nematodes for 1 hour and then 30 minutes. Reduce the water in the nematode cylinder to 2-3 ml. In a laminar flow hood, add *Radophulus* to the carrot discs. Place 15-50 nematodes per disc using a maximum of three drops of nematode suspension. Seal the Petri dishes with parafilm and incubate for 3-4 weeks at 25-28°C. The presence of callose indicates a successful culture of *Radophulus*. After 3-4 weeks, check the discs under a stereomicroscope without removing the parafilm. If the nematodes are visible, they can be collected [144].

### *Pratylenchus* spp.

Roo-lesion nematodes are very hard to culture on media or in vitro. However, under controlled conditions, these nematodes can be reared in the laboratory. For this purpose, after the isolation of *Pratylenchus* from soil or infected roots, carrot discs are a suitable option for the multiplication of *Pratylenchus* species. It should be noted that all equipment should be sterilized in autoclave. Additionally, carrot discs should also be treated with sterilization materials. Also, for sterilizing nematode, streptomycin sulfate can be used. Next, the nematode should be transferred to the carrot discs, and the Petri dishes should be sealed with parafilm. After 3-4 weeks, *Pratylenchus* can be extracted from carrot discs [144].

### *Aphelenchoides* and *Bursaphelenchus* spp.

To rear foliar nematodes, you should first extract them from the soil, seeds, or leaves. Next, disinfect the *Aphelenchoides* surface with streptomycin sulfate (0.1%) for 10 minutes, followed by rinsing with sterilized water three times. Then, prepare a potato dextrose agar (PDA) and culture some fungi, such as *Alternaria* sp., *Fusarium* sp., and *Botrytis cinerea*. After that, add about 25

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# SUBJECT INDEX



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## Ebrahim Shokoohi

Dr. Ebrahim Shokoohi, hailing from the city of Ahel in Fars Province, Iran, holds a BSc in Plant Protection (Agricultural Engineering) from Shiraz University, an MSc in Nematology-Plant Pathology from Tehran University, and a Ph.D. in Nematology from the same institution. With over a decade of dedicated work on nematodes, he has authored more than 100 papers in esteemed Nematology journals and related fields. Besides, he published two books and over four chapter books. Dr. Shokoohi has supervised over 14 MSc and three Ph.D. students in Iran and South Africa. He has made significant contributions by describing new species and records from South Africa, Iran, and other countries. His research focuses on the microbiome, biodiversity, morphology, and molecular identification of plant-parasitic and free-living nematodes in the agricultural and natural areas of Iran and South Africa. Notably, he is a recipient of the VC Research Award from the University of Limpopo and serves as an Editorial Board member for prestigious and highly accredited journals offering expert insights on submitted research papers. Dr. Shokoohi has held the position of Professor of Nematology at Kerman University in southeastern Iran and currently serves as an academic member at the University of Limpopo, South Africa.