Report

Training Workshop on Diagnostics of Plant Parasitic Nematodes

23 February - 7 March, 2020

Applied Research Institute of Agricultural Quarantine (ARIAQ)
Bekasi, Indonesia

This project on “Taxonomic capacity building to support market access for agricultural trade in the ASEAN region – Phase 2” is supported by the Government of Japan through the Japan-ASEAN Integration Fund (JAIF)

2020

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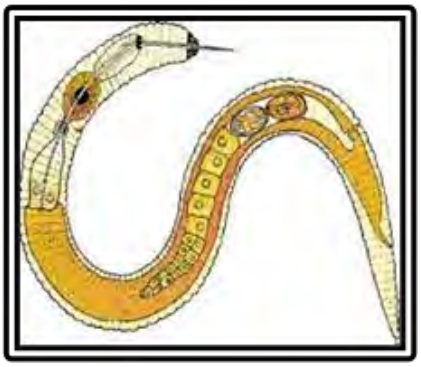


Training Workshop organized by:

2020

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Foreword

The Training Workshop on Diagnosis of Plant Parasitic Nematodes represents a key activity of the Project on Taxonomic capacity building to support market access for agricultural trade in the ASEAN region – Phase 2, supported by the Japan ASEAN Integration Fund. The topic of Diagnosis of Plant Parasitic Nematodes was one of the priorities selected by the Project Steering Committee in recognition of the paucity of skilled plant nematologists in the ASEAN region.

The Report of the Training Workshop on Diagnosis of Plant Parasitic Nematodes is prepared as one of the requirements of the JAIF Project that contains the summary of the training activities in the class, laboratory and field, organised for the participants, including the group presentations at the end of the training, as well as the evaluation (pre- and post-evaluation for technical subjects and evaluation for the non-technical, especially in the logistic preparation and implementation, quality of the training and also resource persons.

The training workshop was organized successfully at the Applied Research Institute of Agriculture Quarantine (ARIAQ) in Bekasi, Indonesia from 23rd February to 7th March 2020 and attended by 23 officers from the ASEAN Member States. On this occasion I would like to sincerely thank Dr. Ali Jamil, Director General, Indonesia Agency for Agriculture Quarantine (IAQA), Ministry of Agriculture Indonesia for permission to use ARIAQ for the venue and accommodation of participants and resource persons and to Dr. Wawan Sutian, Director of ARIAQ for allowing his officers to work with us in the organizing committee and for the use of the Agency's excellent facilities and guesthouse during the training.

Lastly, I hope that this report will be useful as a reference for the members of ASEAN in future organization of similar activities in their own country.

15th April 2020

Dr Lum Keng Yeang
Chairperson and Project Manager
ASEANET

EXECUTIVE SUMMARY

This Training Workshop on Diagnostics of Plant Parasitic Nematodes is one of the attachment programs of the APHCN - ASEANET Project Phase 2 on ***"Taxonomic capacity building to support market access for agricultural trade in the ASEAN region***". The training workshop is fully funded by the Japan - ASEAN Integration Fund (JAIF) and in collaboration with Applied Research Institute for Agriculture Quarantine (ARIAQ), Bekasi, West Java, Indonesia. This two-week training workshop was participated by 23 selected plant protection and plant quarantine officers whose main function is to secure the identity, assess, and tract down plant parasitic nematodes (PPNs) of export and import crops with of potential threat to the exchange of goods. Participants are competent representatives from 10 South-East Asian countries such as Brunei Darussalam, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand, and Vietnam.

The main objective of the training workshop is to enhance the technical capability of our South-East Asian phyto-nematologists in identifying PPNs at morphological and molecular levels, and draw basic management tactics to avoid further spread or introduction of the pathogen across South-East Asian regions. To fulfill the said objective, interactive lectures and laboratory practical including technique demo were conducted. Pre- and post-evaluation tests were also administered to gauge the technical capacity of participants on basic nematological works.

A total of 19 lectures distributed in 9 sessions were given by invited resource persons from Ryukoku University in Shiga, Japan, National Crop Protection Center of the University of the Philippines - Los Baños, Philippines, and ARIAQ, Bekasi, Indonesia. Discussions followed right after each lecture topic and proceeded to laboratory practical. Lectures encompassed from the general nematology concept, distribution of important plant parasitic nematodes, important diseases caused by PPNs, field sampling and nematode extraction techniques, morphological and molecular identification, and nematode management strategies. Fourteen (14) laboratory practical were performed by participants individually and in group (5 groups). Practical started with a field collection trip in Fruit Garden Mekarsari on the second day. During the whole day field trip, each group collected soil and plant samples after assessing and documenting typical symptoms. Working samples were used for the nematode extraction, fixation, and morphological identification. Most of the practical activities dealt with substrates processing, nematode extraction, and morphological identification, since these are pre-requisites to disease diagnosis and molecular identification of PPNs using Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) assays.

Results of all activities from day 1 to 10 were presented and discussed during the group report. Impressive presentations were successfully met wherein constructive comments and suggestions were incorporated as further references. In morphological identification, pictorial key to the genera of plant parasitic nematodes by Mai et al. (1968) and online reference (http://nematode.unl.edu/key/nemakey.htm) are of huge help on basic nematode identification and were used during the practical.

One of the notable results from this training workshop is the abundance of bulb nematode, *Ditylenchus dipsaci* on onion bulbs imported from China as commercially available in local markets in Bekasi, Indonesia. Although *D. dipsaci* has been detected and identified in Indonesia and other neighboring regions, the continues mass importation of bulbs may eventually further increase the nematode population in the locality, or it would be alarming in regions where *D. dipsaci* has not been reported yet. Thus, the simple practical technique taught during the training workshop is very important and necessary for nematode diagnosis. Aside from this, improvised mist chamber as demonstrated in the training is necessary particularly in quarantine facilities that are not fully equipped with extraction equipment but dealing with large number of samples.

Molecular assays such as Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) are reliable enough to detect and identify PPNs at the genus level. The use of

ISOHAIR Genomic DNA Extraction kit (Nippon Gene, Japan) is very efficient and easy-to- use in attaining high quality nematode DNA for PCR and RFLP analyses. This commercially available DNA extraction kit is fast enough for routine nematode identification suitable and economical for large number of samples.

The training workshop was concluded by closing remarks from the Director of ARIAQ, Dr. Wawan Sutian, Secretary of APHCN-ASEANET, Dr. Soetikno S. Sastroutomo, and Dr. Marita S. Pinili as representative of resource persons. Testimonies from two representatives from the Philippines and Brunei Darussalam were also given, wherein they expressed their utmost gratitude to the organizers and resource persons for imparting pertinent knowledge in the field of nematology, and also shared their experiences about the versatility of Indonesian food, conducive training center of ARIAQ, accommodating people particularly staff of ARIAQ, and camaraderie among participants. This was followed by giving certificates of accomplishment to all participants and certificate of appreciation to all resource persons. The event did not finish without the farewell party organized by ARIAQ and was attended by Heads of different offices and with some entertainment performances from the local organizers.

1. TECHNICAL DESCRIPTION

A. Project Title: Training Workshop on Diagnostics of Plant Parasitic Nematodes

B. Project Proponents:

Dr. Lum Keng Yeang, Chairperson, APHCN - ASEANET c/o CABI-SEA, Building A-19 MARDI Complex, 43400 Serdang, Selangor, Malaysia

Dr. Soetikno S. Sastroutomo, Secretary, APHCN - ASEANET c/o CABI-SEA, Building A-19 MARDI Complex, 43400 Serdang, Selangor, Malaysia

Dr. Marita S. Pinili, Regional Training Coordinator, National Crop Protection Center (NCPC), College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna 4031 Philippines

C. Implementing Agency:

Applied Research Institute of Agricultural Quarantine (ARIAQ), Bekasi, West Java, Indonesia

D. Project Duration: 2 weeks (February 23, 2020 to March 6, 2020)

E. Funding Agency: Japan - ASEAN Integration Fund (JAIF) through ASEAN Secretariat

2. OBJECTIVE/OUTCOME

Plant-parasitic nematodes (PPNs) cause serious problems in agricultural crops throughout the world from tropical to cold part of agricultural region. Especially, in tropical and subtropical regions, such as South-East Asia where agriculture occupies an important position in the economy. PPNs proliferate so quickly at high growth rate that crops sometimes suffer considerably high yield loss. Because the symptoms caused by PPNs resemble to that of plant disease or physiological disorders, diagnosis is very important to deal with the damage appropriately.

There are several species of PPNs, such as root-knot nematodes (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.) and cyst nematodes (*Heterodera* and *Grobodera* spp.). However, detection and identification of PPNs are difficult even for researchers because they are so tiny, have few morphological characters that microscopes and special skills in nematology are needed. Even in such situations, there are very few researchers of PPN in South-East Asia. For this reason, fostering of nematologists is urgently required in this area.

In this workshop, participants are going to study the biology and ecology of PPNs and the importance and difficulties in control through lectures. In the laboratory and field work, participants are going to practice to collect and extract PPNs from the field and learn morphological and DNA analysis techniques for identifying PPNs.

3. RESULTS/OUTPUTS

This workshop aims to provide participants with the knowledge of basic and practical understanding of general nematology and PPNs, how to identify important PPN species and several management methods for them, such as chemical, physical, biological, cultural methods, and so on. To achieve these aims, the training course covers the following topics: collection method and theory, basic taxonomy and ecology of major species, families and genera of PPNs, collecting and preparation of specimens, identification based on morphological characters and DNA analysis, impacts of PPNs on major agricultural crops, and available options of management. Thereby, upon completion of training, participants will have enough knowledge on nematology to design effective management plans for controlling PPNs.

4. MAIN ACTIVITIES

Lecture:

a) To become aware of the importance of PPNs on agriculture in the world, especially in tropical and sub-tropical area.
b) To acquire knowledge on the biology, ecology, and population distribution of PPNs.
c) To learn how to identify the symptoms (above and below grounds) of PPN-infected crops as one cause of poor growth due to continuous cropping.
d) To acquire knowledge on the taxonomy and classification of PPNs in relation to other nematode taxon; morphological characteristics and DNA barcoding of major genera and species.
e) To acquire knowledge on recent advances in identifying PPNs; focused on DNA extraction method, and analyses such as PCR, RFLP, real-time PCR and DNA sequencing method.
f) To learn how to protect crops from PPNs by chemical, physical, biological, cultural (including use of crop rotation, resistant varieties, nematode-suppressive or non-host plants) methods, and so on.

g) To become aware of the importance of international migration of PPNs as a serious problem on plant quarantine.

Laboratory:

a) To learn the symptoms (shoot wilting, root gall, root lesion, etc.) caused by different genera of PPNs.
b) To learn the basic techniques in soil and plant sampling, and proper handling of samples.
c) To learn how to extract PPN from soil and plant parts (Maceration - Baermann funnel method and sieving method)
d) To learn the morphological characters of major genera of PPNs for tentative identification and how to assess their population by nematode count them under a microscope.
e) To learn how to extract DNA from an individual nematode, perform PCR and RFLP assay for species identification.

5. TRAINING METHODOLOGY

The training workshop was conducted by a combination of interactive lectures, laboratory practices and field visits. Field observation and sampling were carried out in crop fields.

WORKSHOP PROGRAM

DAY 1 – 24 February 2020 (Monday) OPENING AND INTRODUCTION		
	Activity	**Resource Person(s)/Facilitator**
08.30 – 08.45	**Registration**	Dr. Ummu S. Rustiani (and her team), *ARIAQ, Bekasi*
	OPENING AND INTRODUCTION	
08.45 – 09.00	Workshop Introduction Purpose and scope of workshop	Dr. Soetikno Sastroutomo *Technical Secretary* *APHCN-ASEANET, Malaysia*
09.00 – 10.00	Opening Remarks and Official Opening	Dr. Ali Jamil, *Director General, IAQA, Jakarta* represented by Mr. Maman Suparman, Head of Seed Quarantine Division, Centre for Plant Quarantine & Biosafety

DAY 1 – 24 February 2020 (Monday) OPENING AND INTRODUCTION		
	Participants and Training Team Introductions – Participants will be invited to introduce themselves, identify the agencies they work for and state the nature of their work.	Dr. Marita S. Pinili *Regional Training Coordinator* *National Crop Protection Center, University of the Philippines Los Baños*
	Group photograph	
10.00 – 10.30	**Morning tea**	
10.30 – 11.00	Administration of pre-evaluation tests	Dr. Nurjanah
11.00 – 11.45	Introduction to the JAIF Funded Capacity Building Project.	Dr. Soetikno Sastroutomo
11.45 – 12.15	Lecture 1: General introduction to nematology	Dr. Hideaki Iwahori *Ryukoku University, Japan*
12.15 – 13.15	**Lunch**	
13.15 – 13.45	Lecture 2: Diseases caused by plant-parasitic nematodes: Signs and Symptoms	Dr. Marita S. Pinili
13.45 – 14.15	Lecture 3: Economically important plant-parasitic nematodes	Dr. Hideaki Iwahori
14.15 – 15.00	Lecture 4: Sampling: Principles and Methods	Dr. Marita S. Pinili
15.00 – 15.30	**Afternoon tea**	
15.30 – 16.00	Lecture 5: Nematode physiology and anatomy, and their implications for quarantine and survival	Dr. Hideaki Iwahori
16.00 – 17.00	Lecture 6: Introduction to nematode extraction: Passive and active techniques	Dr. Nurjanah
19:00 - 21:00	**Official Workshop/Welcome Dinner**	

DAY 2 – 25 February 2020 (Tuesday) FIELD TRIP & NEMATODE EXTRACTION		
08.30 – 09.30	Field trip to collect nematode specimens - Set up extractions facilities	Dr. Nurjanah
09.30 – 10.00	Preparation for field trip to several agricultural farms	All
10.00	Depart for nematode collection	
12.00 – 13.00	**Lunch** (in the field)	
13.00 – 17.00	On-site field sampling and collection of root and soil samples	All
17.00	Return to accommodation.	
DAY 3 – 26 February 2020 (Wednesday) SYMPTOM ASSESSMENT AND NEMATODE EXTRACTION		
08.30 – 10.30	Practical 1: Below ground and above ground symptom assessment	Dr. Marita S. Pinili
10.30 – 11.00	**Morning tea**	

11.00 – 12.00	Discussion	Dr. Nurjanah
12.30 – 13.30	**Lunch**	
13.30 – 15.30	Practical 2: Nematode extraction from soil	Dr. Nurjanah
15.30 – 16.00	**Afternoon tea**	
16.00 – 17.00	Practical 2: Nematode extraction from soil (continuation)	Dr. Nurjanah
DAY 4 – 27 February 2020 (Thursday) EXTRACTION TECHNIQUES		
08.30 – 09.00	Lecture 7: Extraction technique for endoparasitic nematodes	Dr. Marita S. Pinili
09.00 – 10.00	Practical 3: Extraction of plant-parasitic nematodes from roots, seeds, and bulbs	Dr. Marita S. Pinili
10.00 – 10.30	**Morning tea**	
10.30 – 12:30	Practical 3: Extraction of plant-parasitic nematodes from roots, seeds, and bulbs (continuation)	Dr. Nurjanah
12.30 – 13.30	**Lunch**	
13.30 – 14.00	Lecture 9 : Extraction using Sucrose-centrifuge method	Dr. Hideaki Iwahori
14.00 – 15.30	Practical 5: Sucrose-centrifuge method	Dr. Hideaki Iwahori
15.30 – 16.00	**Afternoon tea**	
16.00 – 17.00	Lecture 8 and Practical Demo: Extraction using Mist chamber technique	Dr. Nurjanah
DAY 5 – 28 February 2020 (Friday) NEMATODE FIXATION AND MOUNTING		
08.30 – 09.30	Lecture 10: Miscellaneous techniques: Handling, killing, fixing, staining, and mounting	Dr. Marita S. Pinili
09.30 – 10.00	Practical 6: Killing-fixation of nematodes	Dr. Marita S. Pinili
10.00 – 10.30	**Morning tea**	
10.30 – 11.00	Practical 6: Killing – fixation of nematodes (continuation)	Dr. Marita S. Pinili
11.00 – 12.00	Practical 7: Mounting of nematodes	Dr. Nurjanah
12.00 – 13.00	**Lunch**	
13.00 – 15.00	Practical 7: Mounting of nematodes (continuation)	Dr. Nurjanah
15.00 – 15.30	**Afternoon tea**	
15.30 – 17.00	Practical 7: Mounting of nematodes (continuation)	Dr. Nurjanah
13.00 – 15.00	Practical 7: Mounting of nematodes (continuation)	Dr. Nurjanah
DAY 6 – 29 February 2020 (Saturday) NEMATODE FIXATION AND MOUNTING		
08.30 – 10.00	Practical 7: Mounting of nematodes (continuation)	Dr. Nurjanah/Dr. Marita S. Pinili
10.00 – 10.30	**Morning tea**	
10.30 – 12.00	Practical 8: Tentative identification of nematodes based on morphological features	Dr. Nurjanah/Dr. Marita S. Pinili

12.00 – 13.00	**Lunch**	

01 March 2020 (Sunday) - Holiday

DAY 7 – 02 March 2020 (Monday) NEMATODE IDENTIFICATION AND CLASSIFICATION		
08.30 – 09.00	Lecture 11: Digestive System of Nematodes: Key to Morphological Identification	Dr. Marita S. Pinili
09.00 – 10.00	Practical 9: Morphological Identification of nematode specimens collected during survey	All
10.00 – 10.30	**Morning tea**	
10.30 – 11.30	Lecture 12: Useful Taxonomic Characters used in Nematode Identification: Keys and other identification resources	Dr. Hideaki Iwahori
11.30 – 12.30	Practical 9: Morphological Identification of nematodes collected from S-E countries	All
12.30 – 13.30	**Lunch**	
13.30 – 17.00	Practical 9: Morphological Identification of nematodes collected from S-E countries	All

DAY 8 – 03 March 2020 (Tuesday) NEMATODE BIOLOGY		
08.30 – 09.00	Lecture 13: Plant-parasitic nematode life cycle	Dr. Marita S. Pinili
09.00 – 10.00	Practical 9: Morphological Identification of fixed nematodes	All
10.00 – 10.30	**Morning tea**	
10.30 – 11.30	Lecture 14: Plant-parasitic nematode bionomics	Dr. Hideaki Iwahori
11.30 – 12.30	Practical 9: Morphological Identification of nematodes	All
12.30 – 13.30	**Lunch**	
13.30 – 14.00	Lecture 15: Extraction and *in vitro* culture techniques: Case of *Radopholus similis*	Dr. Marita S. Pinili
14.00 – 15.00	Practical 10: Observing, counting and population assessment of nematodes	Dr. Marita S. Pinili
15.00 – 15.30	**Afternoon tea**	
15.30 – 16.30	Lecture 16: Molecular Techniques: Progress and Limitations	Dr. Hideaki Iwahori

DAY 9 – 04 March 2020 (Wednesday) EXTRACTION TECHNIQUES AND NEMATODE ASSAY		
08.30 – 10.30	Practical 11: Extraction of Nematode DNA	Dr. Hideaki Iwahori
10.30 – 11.00	**Morning tea**	
11.00 – 12.30	Practical 11: Extraction of Nematode DNA (continuation)	Dr. Hideaki Iwahori
12.30 – 13.30	**Lunch**	
13.30 – 15.00	Practical 12: Molecular Identification of Nematodes: PCR assay	Dr. Hideaki Iwahori
15.00 - 15.30	**Afternoon tea**	
15.30 – 17.00	Practical 12: Molecular Identification of Nematodes: RFLP assay	Dr. Hideaki Iwahori

DAY 10 - 05 March 2020 (Thursday) MOLECULAR IDENTIFICATION OF NEMATODES		
08.30 – 11.00	Practical 13: Gel Electrophoresis and Analysis	Dr. Hideaki Iwahori
11.00 - 12.00	Practical 14: Viewing and Interpretation of RFLP Results	Dr. Hideaki Iwahori
12.00 – 13.30	**Lunch**	
13.30 – 14.30	Lecture 17: Nematodes recorded worldwide and their global distribution	Dr. Hideaki Iwahori
14.30 - 15.30	Lecture 18: Management Strategies on Plant-Parasitic Nematodes I – Physical and Cultural Approaches	Dr. Nurjanah
15.30 – 16.00	**Afternoon tea**	
16.00 – 17.00	Lecture 19: Management Strategies on Plant-Parasitic Nematodes II – Biological and Plant Resistance Approaches	Dr. Marita S. Pinili

DAY 11 – 06 March 2020 (Friday) NEMATODE DAMAGE ASSESSMENT		
TIME	**EVENT**	
08.30 – 09.30	Discussion 2: Storage and cataloguing of images and specimen	Dr. Nurjanah
09.30 – 10.00	Group Report (Group 1)	Representative
10.00 - 10.30	Group Report (Group 2)	Representative
10.30 – 11.00	**Morning tea**	
11.00 – 11.30	Group Report (Group 3)	Representative
11.30 – 14.00	**Lunch Break and Friday Prayer**	
14.00 - 14.30	Group Report (Group 4)	Representative

14.30 - 15.00	Group Report (Group 5)	Representative
15.00 – 15.30	**Afternoon tea**	
POST-EVALUATION AND CLOSING SESSION		
15.30 – 16.00	Post-workshop evaluation test	Dr. Nurjanah
16.00 – 16.30	Group Discussion and Workshop conclusion (Testimony from participants)	Dr. Marita S. Pinili
16.30 – 17.30	Presentation of Certificates and Closing remarks	Dr. Wawan Sutian, Director of ARIAQ, Bekasi Dr. Soetikno Sastroutomo
17.30	**Workshop Close**	

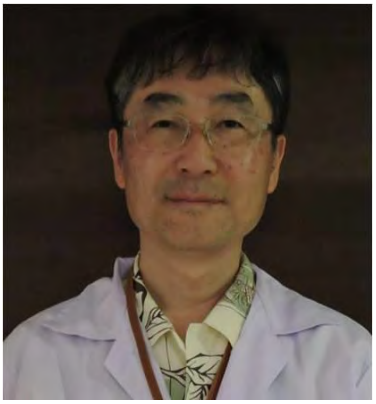
6. TRAINING TEAM & PARTICIPANTS

SOETIKNO S. SASTROUTOMO, DSc,
Senior Scientist
Technical Secretary
ASEAN Network on Taxonomy (ASEANET)
c/o CABI-SEA, Building A-19
MARDI Complex, 43400 Serdang,
Selangor, Malaysia
Phone: +60 3 89432921/3641; Mobile: +60 12 6342945
E-mail: ssoetikno@gmail.com

LUM KENG YEANG, PhD, Senior Scientist
Chairperson
ASEAN Network on Taxonomy (ASEANET)
c/o CABI-SEA, Building A-19
MARDI Complex, 43400 Serdang,
Selangor, Malaysia

MARITA S. PINILI, PhD, University Researcher III
Regional Training Coordinator
Nematology Laboratory
National Crop Protection Center
College of Agriculture and Food Science
University of the Philippines Los Banos
College, Laguna 4031 Philippines
Phone: +63-917-821-6856
E-mail: maripinili@gmail.com

RESOURCE PERSON (LECTURERS)

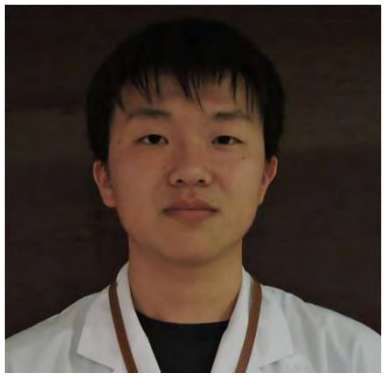


HIDEAKI IWAHORI, PhD, Professor
Department of Bioresource Sciences
Faculty of Agriculture, Ryukoku University
1-5 Yokotani, Seta Oe-cho, Otsu,
Shiga 520-2194, Japan
Phone: +81-77-599-5699 Fax: +81-77-599-5608
mail: iwahori@agr.ryukoku.ac.jp

MARITA S. PINILI, PhD, University Researcher III
Nematology Laboratory
National Crop Protection Center
College of Agriculture and Food Science
University of the Philippines Los Banos
College, Laguna 4031 Philippines
Phone: +63-917-821-6856
E-mail: maripinili@gmail.com

NURJANAH, PhD, Head
Sub Division of Seed Imports
Directorate of Plant Quarantine and Biosafety,
Indonesian Agricultural Quarantine Agency (IAQA),
Ministry of Agriculture
E Building, 5th Floor, Jl. Harsono RM. No. 3, Ragunan,
Pasar
Minngu, Jakarta Selatan 12550 Indonesia
Phone/Fax: +62 21 77816482
E-mail: Nurjanah7608@gmail.com or
Nurjanah0876@pertanian.go.id

RESOURCE PERSON (LABORATORY ASSISTANT)



KASUHO NARUO, MS Student
Department of Bioresource Sciences
Faculty of Agriculture
Ryukoku University
1-5 Yokotani, Seta Oe-cho, Otsu,
Shiga 520-2194, Japan
Mobile: 090 3464 9922
E-mail: kazuho.0922@gmail.com

LOGISTICS/LOCAL ORGANIZERS

UMMU SALAMAH RUSTIANI, PhD, Research Scientist
Plant Pathology
Applied Research Institute of Agricultural Quarantine (IAQA)
Jln Raya Kampung Utan-Setu, Mekar, Wangi, Cikarang Barat, Bekasi, West Java, 17520 Indonesia
Mobile: +62 8136220195
E-mail: ummu@buttmkp.org or ummurustiani@gmail.com

MS. LENY PANJAITAN, Research Scientist
Applied Research Institute of Agricultural Quarantine (IAQA)
Jln Raya Kampung Utan-Setu, Mekar, Wangi, Cikarang Barat,
Bekasi, West Java, 17520 Indonesia
E-mail: leny@buttmkp.org or Lenny6165@yahoo.com

MS. SALBIAH, Scientist
Applied Research Institute of Agricultural Quarantine (IAQA)
Jln Raya Kampung Utan-Setu, Mekar, Wangi, Cikarang Barat,
Bekasi, West Java, 17520 Indonesia
E-mail: salbiah@buttmkp.org or salbiahmadi85@gmail.com

MS. NURSUSILAWATI, Scientist
Applied Research Institute of Agricultural Quarantine (IAQA)
Jln Raya Kampung Utan-Setu, Mekar, Wangi, Cikarang Barat,
Bekasi, West Java, 17520 Indonesia
E-mail: usi@buttmkp.org or ussy.46@gmail.com

LIST OF PARTICIPANTS

BRUNEI DARUSSALAM

MS. HAJAH SAFWANAH BINTI ABDULLAH
Senior Agriculture Assistant
Plant Pathology Unit,
Crop Protection Division, Brunei Agricultural Research Centre (BARC)
Department of Agriculture and Agrifood Kilanas BF 2520, Brunei Darussalam
Tel: +673 8 8152662
E-mail: crop.protection@agriculture.gov.bn or safwanah.abdullahmp@gmail.com

MS. ADI LISEA BINTI MOHD ADDLY
Plant Pathologist, Crop Protection Division, Brunei Agricultural Research Centre (BARC)
Department of Agriculture and Agrifood Kilanas BF 2520, Brunei Darussalam
Tel: +673-8977073
E-mail: lisea.addly@agriculture.gov.bn

CAMBODIA

MR. YA PHAUK
Technical Officer, Department of Plant Protection Sanitary and Phytosanitary, General Directorate of Agriculture, Toeuk Laak 3, Phnom Penh, Cambodia
Tel: +855-92340431
E-mail: ya.phaukrua@gmail.com

MR. NIT TI
Technical Officer, Department of Plant Protection Sanitary and Phytosanitary, General Directorate of Agriculture, Toeuk Laak 3, Phnom Penh,Cambodia
Tel: +855-17459812
E-mail: nittifc1@gmail.com

INDONESIA

MS. ELMI MULIYA
Plant Quarantine Inspector
Quarantine Station of Tanjung Priok
Indonesian Agricultural Quarantine Agency (IAQA), Ministry of Agriculture Republic of Indonesia,J1. Enggano No. 17 Tanjung Priok, North Jakarta, 14310, Indonesia
Tel: +62 214 393 1012; +62 852 1698 1320
E-mail:muliyaelmi@gmail.com or elmimuliya@pertanian.go.id

MR. HAPPY CAHYA NUGRAHANA
Laboratory Analyst
Quarantine Station of Surabaya
Jln. Ir. H. Juanda, Semambung, Sidoarjo 61253, Indonesia
Tel: +62 318 67399
E-mail:happy_cahya@rocketmail.com or
happycahya@pertanian.go.id

LAOS

MS. PHETSAMONE SONGVILAY
Technical Officer, IPM Unit
Plant Protection Center, Department of Agriculture
Ministry of Agriculture and Forestry
Lane Xang Avenue, Patuxay Square, P.O. Box 811
Vientiane Capital
Tel: + 856 20 77312198; Fax: + 856 21 812164
E-mail: Phetsamone98@live.com

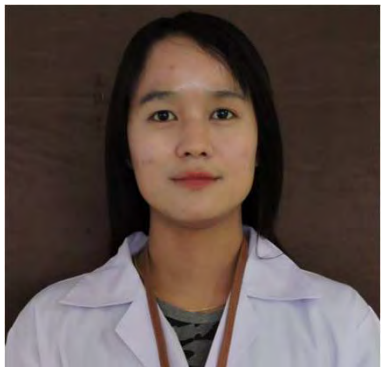
MS. SOUPHATSONE VORAVONG
Technical Officer, IPM Unit
Plant Protection Centre, Department of Agriculture,
Ministry of Agriculture and Forestry
Tel: + 856-20-22244998; Fax: + 856-21-812164
E-mail: Souphatsonevoravong@yahoo.com

MALAYSIA

MR. IKHWAN HARRIS BIN RAMLI
Assistant Director
Plant Biosecurity Division, Department of Agriculture,
Level 1-3, Wisma Tani, Jalan Gallagher, 50632 Kuala Lumpur, Malaysia
Tel: + 60-3-26977151; Fax: + 03 26977205
E-mail: ikhwanharris@gmail.com

MR. MOHD AZRUL BIN ALIAS
Agriculture Officer, Biosecurity Screening Unit, Plant Biosecurity Division
Department of Agriculture,
43400 Serdang, Selangor, Malaysia
Tel: + 60-13-4666964
E-mail: royz1020@gmail.com

MYANMAR



MS. KHINE THANDA MOE
Deputy Staff Officer
Plant Quarantine Section, Plant Protection Division,
Department of Agriculture
Baying Naung Rd, West Gyogon, Insein P.O. Box 11011,
Yangon Myanmar
Tel: + 95-9-451659020; Fax: + 95-1-644019
E-mail: khaingtdm@gmail.com

MS. THEINGI SOE
Deputy Staff Officer
Plant Quarantine Section, Plant Protection Division,
Department of Agriculture
Baying Naung Rd, West Gyogon, Insein P.O. Box 11011,
Yangon Myanmar
Tel: + 95-9-963281777; Fax: + 95-1-644019
E-mail: theingisoe1987@gmail.com

PHILIPPINES

MR. ALDWIN L. MENDOZA
Agriculturist
National Plant Quarantine Services Division
Bureau of Plant Industry, Los Banos
Laguna, Philippines
Tel: + 02 244 2950
E-mail: almendoza5@up.edu.ph

MR. MAR J. DE GUZMAN
Agriculturist II
Crop Pest Management Division
Bureau of Plant Industry
692 San Andres St., Malate,Manila, Philippines
Tel: + 02 85232426/85247211
E-mail: mardeguzman57@gmail.comor
croppestmanagementdivision@gmail.com

SINGAPORE

MR. AZMAN BIN AHMAD
Senior Inspector
National Park Board
6 Perahu Road Singapore 718827
Tel: +65 90918281
E-mail : Azman_Ahmad@nparks.gov.sg

MS. UMASHANKARI CHANDRA SEGARAN
Scientist
National Park Board
6 Perahu Road Singapore 718827
Tel: +65 63165173/85719641
E-mail : uma_chandra_segaran@nparks.gov.sg

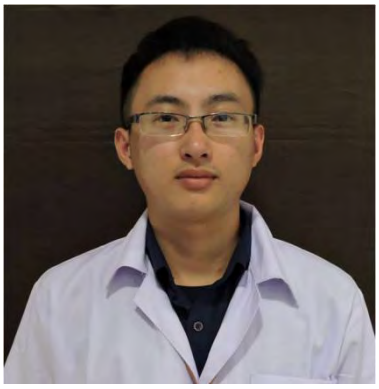
THAILAND

MS. UNGKANA POUNGNGENMAK
Plant Pathologist (Nematodes)
Plant Protection Research and Development Office,
Department of Agriculture,
50 Phaholyothin Rd. Chatuchak, Bangkok, Thailand
10900
Tel: + 66-895-584497
E-mail: its.me.booke@gmail.com

MR. SURASAK SAENKHOT
Agricultural Research Officer,
Practitioner Level
Plant Protection Research and Development Office,
Department of Agriculture, 50 Phaholyothin Rd.
Chatuchak , Bangkok, Thailand 10900
Tel: +66 844 247 843; Fax: +66 0 2561 2146
E-mail: Surasak028ss@gmail.com

VIETNAM

DR. TRINH THI THU THUY
Senior Nematologist
Plant Quarantine Diagnostic Centre
Plant Protection Department, MARD
7A Le Van Hien-Duc Thang, Bac Tu Liem,
Ha Noi Capital, Vietnam
Tel: + 84-4-38571064; Fax: + 84-4 38513746
E-mail: thuytt47@yahoo.com



MR. LE NGUYEN TUAN
Technical Officer
Post Entry of Plant Quarantine Center No. 1
Plant Protection Department, MARD
Vien Street – Bac Tu Liem District-
Ha Noi, Vietnam
Tel: +84-365268211
E-mail: Tl7742@gmail.com

OBSERVERS (INDONESIA)

MR. ABDUL MUBARAQ IRFAN
Plant Quarantine Officer
Applied Research Institute for Agriculture Quarantine (ARIAQ),
Indonesian Agricultural Quarantine Agency (IAQA),
Ministry of Agriculture Republic of Indonesia
Jl. Raya Kampung Utan-Setu, Desa Mekar Wangi,
Barat, Kab. Bekasi, West Java, Indonesia
Tel: +62 21 82618923; Mobile: +62 8114804812
E-mail: irfan@buttmkp.org or irfanmubaraq@gmail.com

MS. NELLY PARETANGA TANGARAN
Plant Quarantine Officer
Laboratory Analyst
Agricultural Quarantine Major Centre of Makassar,
Ministry of Agriculture
The Agricultural Quarantine Major Centre of Makassar
Jl. Perintis kemerdekaan KM.12 Makassar, South
Sulawesi, Indonesia
Phone: (0411) 581323; Mobile: +62 81355181054
E-mail: nellytangaran30@gmail.com

MS. MASAYUN EKA MAYLANDARI
Plant Quarantine Officer
Standard Testing Laboratory Institute, Indonesian
Agricultural
Quarantine Agency (IAQA), Ministry of Agriculture
Jl. Pemuda No. 64 Jakarta, Indonesia

GROUPINGS

Group 1	***Nationality***
Dr. Trinh Thi Thu Thuy	Vietnam
Ms. Masayun Eka Maylandari	Indonesia
Ms. Theingi Soe	Myanmar
Mr. Ikhwan Harris Ramli	Malaysia
Hajah Safwanah Abdullah	Brunei

Group 2

Mr. Aldwin L. Mendoza	Philippines
Ms. Nelly Paretanga Tangaran	Indonesia
Ms. Adi Lisea Mohd Addly	Brunei
Ms. Phetsamone Songvilay	Laos

Group 3

Ms. Ungkana Poungngenmak	Thailand
Mr. Ya Phauk	Cambodia
Mr. Azman Bin Ahmad	Singapore
Mr. Mohd Azrul Bin Alias	Malaysia
Mr. Abdul Mubaraq Irfan	Indonesia

Group 4

Mr. Nit Ti	Cambodia
Ms. Souphatsone Voravong	Laos
Ms. Khine Thanda Moe	Myanmar
Ms. Elmi Muliya	Indonesia

Group 5

Mr. Happy Cahya Nugrahana	Indonesia
Mr. Mar J. de Guzman	Philippines
Mr. Le Nguyen Tuan	Vietnam
Mr. Surasak Saenkhot	Thailand
Ms. Umashankari Chandra Segaran	Singapore

7. ACTIVITY REPORT

The two-week training workshop was divided into 9 major sessions. Each session comprised of series of activities including lectures and practicals, and with technique demonstration, and pre- and post-evaluation tests.

SESSION I - Opening and Introduction

Registration and issuance of training kits were done during the first day, followed by the opening ceremony (*Photo on the left*). Opening remarks were given by Dr. Ali Jamil, Director General of IAQA, Jakarta and Dr. Wawan Sutian, Director of ARIAQ, Bekasi. Introduction of the training team headed by Dr. Marita S. Pinili, Regional Training Coordinator from the NCPC, UPLB, gave brief remarks including acknowledgement of the logistic team of ARIAQ. Each participant from 10 South-East Asian regions including 3 observers from Indonesia gave also their brief background. Resource persons headed by Dr. Hideaki Iwahori from Ryukoku University, Shiga, Japan was also introduced with his assistant student Mr. Kazuho Naruo, and Dr. Nurjanah from Indonesian Agricultural Quarantine Agency (IAQA), Ministry of Agriculture, Indonesia.

Pre-evaluation test was administered by the resource persons to gauge the technical capacity of each participants on nematological works. The 30-minute pre-evaluation test reflects basic questions on symptomatology, disease identification, nematode general body parts and characteristics, and management strategies.

Dr. Soetikno S. Sastroutomo, Technical Secretary of APHCN-ASEANET introduced the JAIF-Funded Capacity Building Project and reported past accomplishments of the said project during Phase1. After the brief report on project accomplishments and plans, the lecture proper of Dr. Iwahori on the General Introduction in Nematology followed.

Photos 1. Left photo. Welcome remark from the Director of ARIAQ, Dr. Wawan Sutian during the Opening Ceremony. Right photo. On-going pre-evaluation test for the participants right after the opening program).

Photo 2. Group photo taken right after the Opening Program. Inclusive in the photo are the organizing team headed by Dr. Soetikno, invited guests Director General of IAQA (represented by Mr. Maman Suparman) and Director of ARIAQ (Dr. Wawan), resource persons, Dr. Hideaki Iwahori, Dr. Marita S. Pinili, and Dr. Nurjanah, and Mr. Kazuho Naruo, logistic team headed by Dr. Ummu Rustiani, and 23 participants from 10 South-East Asian countries.

Series of lectures were delivered by the 3 resource persons including Lectures 2 and 4: Diseases caused by plant-parasitic nematodes: Signs and Symptoms and Sampling: Principles and Methods (Dr. MS Pinili), Lectures 3 and 5: Economically important plant-parasitic nematodes and Nematode Physiology and anatomy, and their implications on quarantine and survival (Prof. Iwahori), and Lecture 6: Introduction on nematode extraction: Passive and Active Methods (Dr. Nurjanah). These lecture series gave the participants an overview on the importance of plant parasitic nematodes not only in agricultural crops but also in plant quarantine and biosecurity. Advanced discussions on proper soil and plant sampling and extraction methods were given prior to the actual field sampling activities on the following day. All sampling tools and materials were also prepared ahead.

SESSION 2: Field Trip, Symptom Assessment and Nematode Extraction

Despite of heavy rainfall in Bekasi, the field trip to the Taman Buah Mekarsari (Botanical Garden) *(Left photo)* was proceeded due to unpredictable weather condition. Participants were divided into 5 groups. Each group brought their complete set of field sampling materials and provided with raincoat each. Proper coordination was done by the logistic team with resource persons in the botanical garden to maximize the whole-day field sampling and collection activities. The field tour started with a welcome lecture from a well-known plant taxonomist where he introduced all plant species collections in the botanical garden. Most of the plant collections are various species and varieties of fruit trees maintained and utilized as food. Some collections were derived from other South-East Asian regions. Field tour in the snake fruit orchard with free tastes was experienced by all participants. Right after the orchard field tour, each group was deployed on designated sampling areas to collect soil and plant samples. Plant symptoms were assessed first by photo documentation prior to destructive sampling. Areas visited and sampled include banana, papaya, citrus, guava, oil palm, and organic vegetable garden (tomato, eggplant, ginger and upland kangkong).

Photos 3. Activities during the field visit and sample collection in Fruit Garden Mekarsari. General information given by the lead Botanist of the Mekarsari, Mr. Greg Hambali (upper left) where all participants are welcomed and given instructions prior to field sampling (upper right). Actual sample collection from organic vegetable crops (lower left) and citrus orchard (lower right).

Collection trip ended with a group lunch held in traditional Indonesia restaurant.

All collected samples were washed and assessed in the laboratory prior to processing. Symptoms such as presence of galls, root lesions, rotting, stunted growth of the plant and others were noted and properly documented. Additional specimen were presented in the class during practical. These include onion bulbs commercially available in Bekasi, potato tubers, carrots, and bottle gourd roots brought from Myanmar. Below and above ground symptom assessments were done during this session followed by nematode extraction from soil. Soil extraction method for active nematodes was performed using the combination of Sieving-Baermann Funnel technique. The technique critically

emphasized the required chronological pore sizes of sieves and the importance of proper setting-up of the Baermann funnel.

Since the space in the laboratory for soil processing is not enough for the 23 participants, the class were divided into two activities. The first 2 groups performed the nematode extraction from soil, while the others were introduced on how to extract nematodes from rice seeds and onion bulbs. Each group were provided with rice seeds to be processed for possible rice seed nematode, *Aphelenchiodes besseyi* and onion bulbs for extracting *Ditylenchus dipsaci.*

All processed samples (soil, onion bulbs, and rice seeds) were incubated for overnight prior to collecting nematode suspension.

Photos 4. Left photo. Despite of heavy rain, quick sampling soil and roots from oil palm was conducted. Possible nematode infestation was also assessed on guava orchard by collecting soil and root samples (Right photo).

Photo 5. Group photo after the sample collection activities in Fruit Garden Mekarsari.

SESSION 3: Extraction Techniques

Each group have worked on 5 crops collected during the field trip. Roots and other plant samples were processed using the combination of maceration-sieving-Baermann Funnel method. Samples of approximately 5 g were processed based on the given protocol for extracting nematodes from root samples. However, samples with less than 5 g were cut finely and directly placed onto Baermann funnel set-up. Incubation period of samples was done overnight prior to nematode suspension collection.

Photo 6. Lecture on nematode extraction techniques conducted by Dr. Marita S. Pinili. Here, principles, advantages and disadvantages of each extraction method were discussed prior to laboratory practicals. Post laboratory discussion was also held a day after the practical activity.

Aside from the maceration-sieving-Baermann funnel method, sucrose-centrifugation technique was taught in the class during the practical. Soil samples from potato field in Indonesia were used as materials for extracting cyst nematodes, *Globodera* sp. This technique is not dependent on nematode mobility (passive method) instead on the density of the nematode relative to the substrate which is sucrose. Along with the practicals on extraction from roots and other plant parts and sucrose-centrifugation technique, lectures (Lectures 7 to 9) regarding these topics were discussed including the actual demonstration of the mist chamber technique. The improvised mist chamber was designed by ARIAQ to compensate processing of large number of soil and plant samples for nematode extraction.

Post laboratory discussion was also conducted to assess the advantages and disadvantages of extraction techniques used and how to deal with unexpected results.

Photo 7. Pre-laboratory discussion delivered by Dr. Marita S. Pinili on nematode extraction from soil using the sieving method.

Photos 8. Symptom assessment by inspecting root samples for lesions, galls, etc. prior to nematode extraction (upper and lower left photos). Participants also assessed symptoms from other crops from Bekasi, Indonesia and samples brought by participants (upper right). Baermann funnel set-up ready for overnight incubation (lower right).

Photos 9. Some of the test materials used for symptom assessment and nematode extraction. Garlic commercially available in the local market in Bekasi showing lesions in bulblets (upper left), galled roots of bottle gourd from Myanmar (upper right), carrots showing small root knots (lower left), and pototo tubers and roots with few galls (lower right).

Photos 10. Hands on activity during nematode extraction from soil using sieving method (left), and preparation of rice seeds for possible extraction of seed nematode, Aphelenchoides besseyi (right).

Photo 11. Prof. Iwahori demonstrating the use and principle of sucrose centrifugation method for extracting potato cyst nematodes from soil.

Photos 12. Participants performing the sucrose-centrifugation method and preparation of sucrose solution for extracting potato cyst nematodes from infested soil (above). Improvised mist chamber set-up for extracting nematodes from soil and root substrates. This method was demonstrated during the laboratory practicals (below).

Photo 13. Group photo after the mist chamber demonstration held in ARIAQ.

SESSION 4: Nematode Fixation and Mounting

Lectures on miscellaneous technique: killing, fixing, staining, mounting, and culturing nematodes (Lecture 10) were introduced to the class followed by practicals on how to fix nematodes in preparation for slide mounting and tentative morphological identification.

Nematode suspensions collected after the overnight incubation period were killed in 80C water batch for 2-3 minutes by quick plunging in water *(Left photo)*. After quick killing all nematode suspensions were fixed in an equal volume of freshly prepared Formalin-Acetic acid (FA) fixative. These fixed nematode suspensions served as the working specimen all throughout the training activities.

Preparation of live slide mounts was done first for the participants to experience the live movement of nematodes, including the protrusion of stylet. After the live slide mounts, each participant started preparing their individual semi-permanent mounts for tentative morphological nematode identification. Shapes of nematodes at rest were noted as their reference for the initial taxonomic identification.

Photo 14. During the laboratory practical, each group is performing the heat-killing of nematodes collected from the Baermann funnel set-up.

Photo 15. Microscopic examination of nematodes for live mount preparation.

Photo 16. Picking of fixed nematodes for semi-permanent slide mounts preparation.

SESSION 5: Nematode Morphological Identification and Classification

Photo 17. Left photo. Practical activities related to nematode morphological identification were supplemented with lectures (Lectures 11 and 12) and reference materials either printed handouts or website. Lecture 11 which discussed the Digestive System of Nematodes: Key to Morphological Identification was primarily used for basic taxonomic identification. Lecture 12 i.e. Useful Taxonomic Characters used in Nematode Identification: Keys and other identification resources,

gave additional characteristic features in identifying PPNs. Most of the practicals dealt with morphological characterization since this is a pre-requisite to correct identification and molecular assay.

During this session, samples brought by each participants from their respective countries were verified for correct identification with the aid of taxonomic keys. Participants were also introduced to the use of morphometrics computer program for them to measure important anatomical features of PPNs.

Photo 17. Left photo. Morphological identification of plant parasitic nematodes lectured by Prof. Iwahori. Important anatomical parts of nematodes were discussed in the class for tentative diagnostic identification.

Photo 18. Multi-channel compound microscope with 5 -paired eyepiece and attached to a computer for morphometrical analysis and photo documentation of nematodes (above). Participants while performing the actual measurements of nematode body parts for morphological identification (below).

SESSION 6: Nematode Biology

PPN life cycle as well as its bionomics (Lectures 13 and 14) were discussed during this session. The importance of the developmental stages of nematodes in relation to their parasitic habits was given emphasis on these topics. Infective stages of PPNs based on their feeding habit or behaviour i.e. ectoparasitic, endoparasitic, and semi-endoparasitic nematodes must take into consideration before conducting field surveys and sampling and deploying management tactics.

Additional lecture on Extraction and In vitro culture technique: Case of *Radopholus similis* (Lecture 15) was given. This introduced the participants to a technique in which in vitro culture is

possible for selected nematode group such as migratory endoparasites, *R. similis* and *Pratylenchus* sp. This aseptic carrot disc culture technique is being used for maintaining pure culture necessary for genetic studies, population dynamics, and screening for nematode resistance.

SESSON 7: Molecular Identification

Molecular assays in the form of Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) were used to identify *Meloidogyne* spp. and *Radopholus similis*. This session started with a lecture on Molecular techniques: Progress and Limitation (Lecture 16), wherein the usefulness and restrictions of molecular assays were emphasized.

Photo 19. Prof. Iwahori during the lecture class on understanding the DNA gene concept of nematodes for molecular identification.

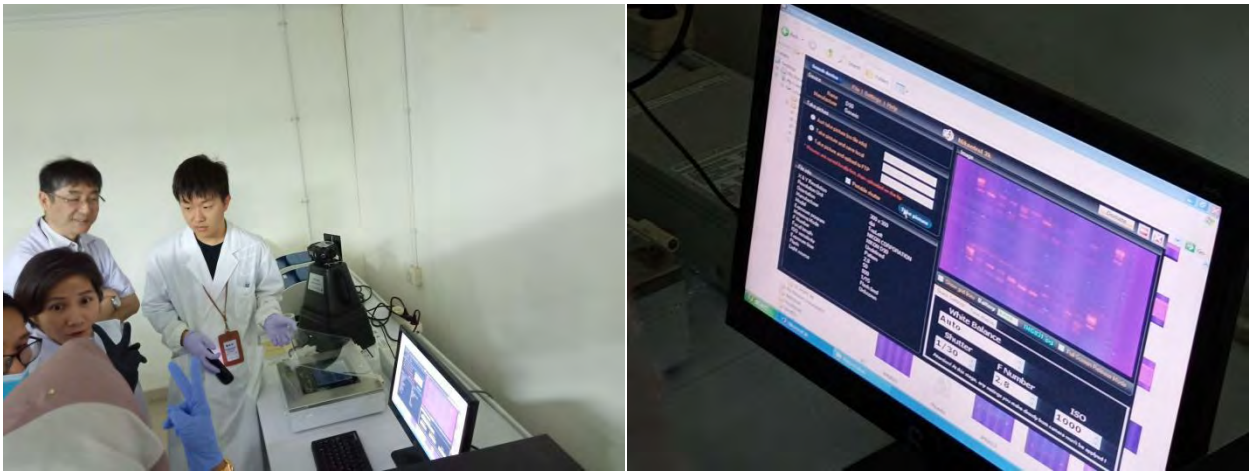
Genomic DNA of nematodes were discussed as well as target sites used for the sequence analysis. DNA extraction using genomic DNA extraction kit ISOHAIR (Nippon Gene, Japan) was taught in the class during practicals. This was followed by PCR assay using universal primers in Thermal

Cycler set at optimized conditions. Gel electrophoresis was done and viewed the target bands under UV transilluminator.

To determine banding patterns among nematode species, RFLP assay was conducted using specific restriction enzymes. Gel electrophoretic analysis was also done under UV transilluminator.

Photos 20. Pre-laboratory discussion on PCR assay conducted by Prof. Iwahori (above). Demonstration on nematode DNA extraction using ISOHAIR DNA genome extraction kit (lower left). Actual preparation of PCR cocktail for PCR assay (lower right).

Photos 21. Loading of PCR products into prepared agarose gel for gel electrophoresis.



Photos 22. Gel electrophoretic analysis using UV transilluminator (left). Expected band sizes from PCR products were analyzed and documented.

SESSION 8: Nematode Damage Assessment

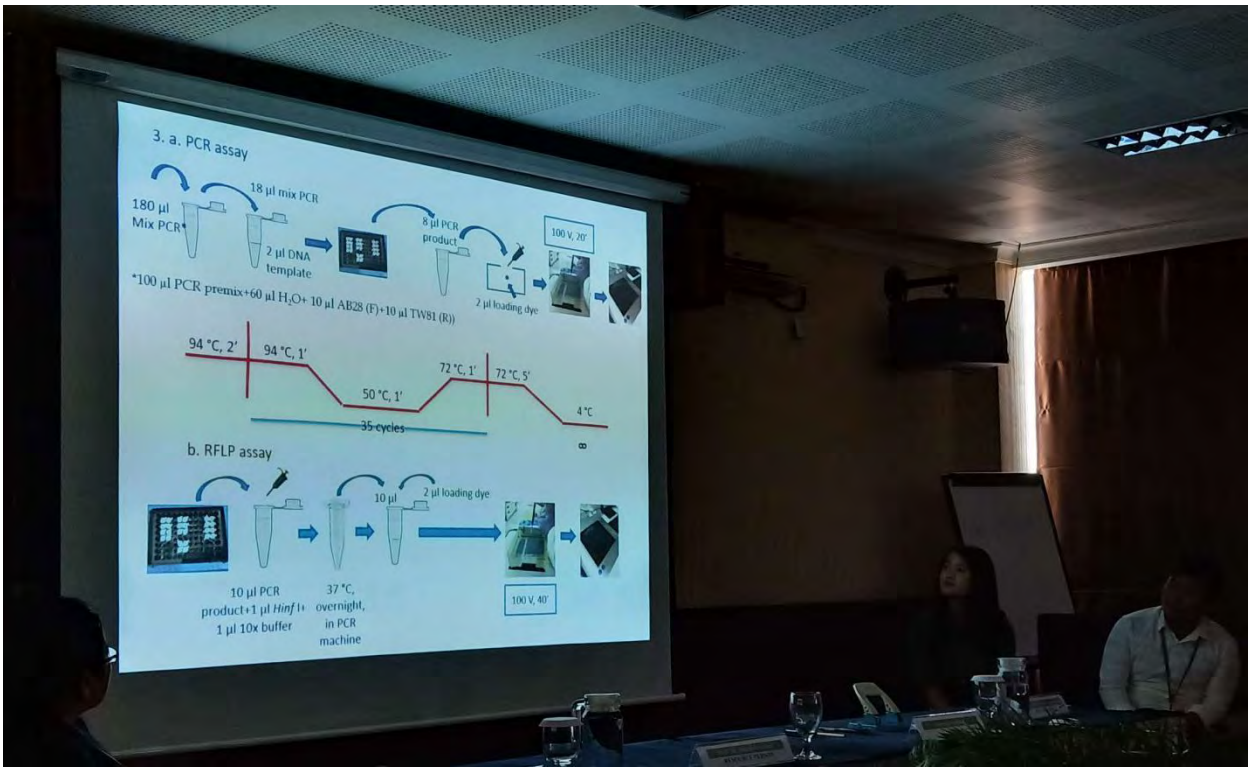
To realize the impact of nematode damage, nematodes recorded worldwide and their global distribution were discussed (Lecture 17). This was supplemented by some lectures on management strategies including physical, cultural, chemical, biological control agents, and resistant varieties (Lectures 18 and 19). These approaches were given to design appropriate strategies in lowering nematode population in a particular crop and cropping system.

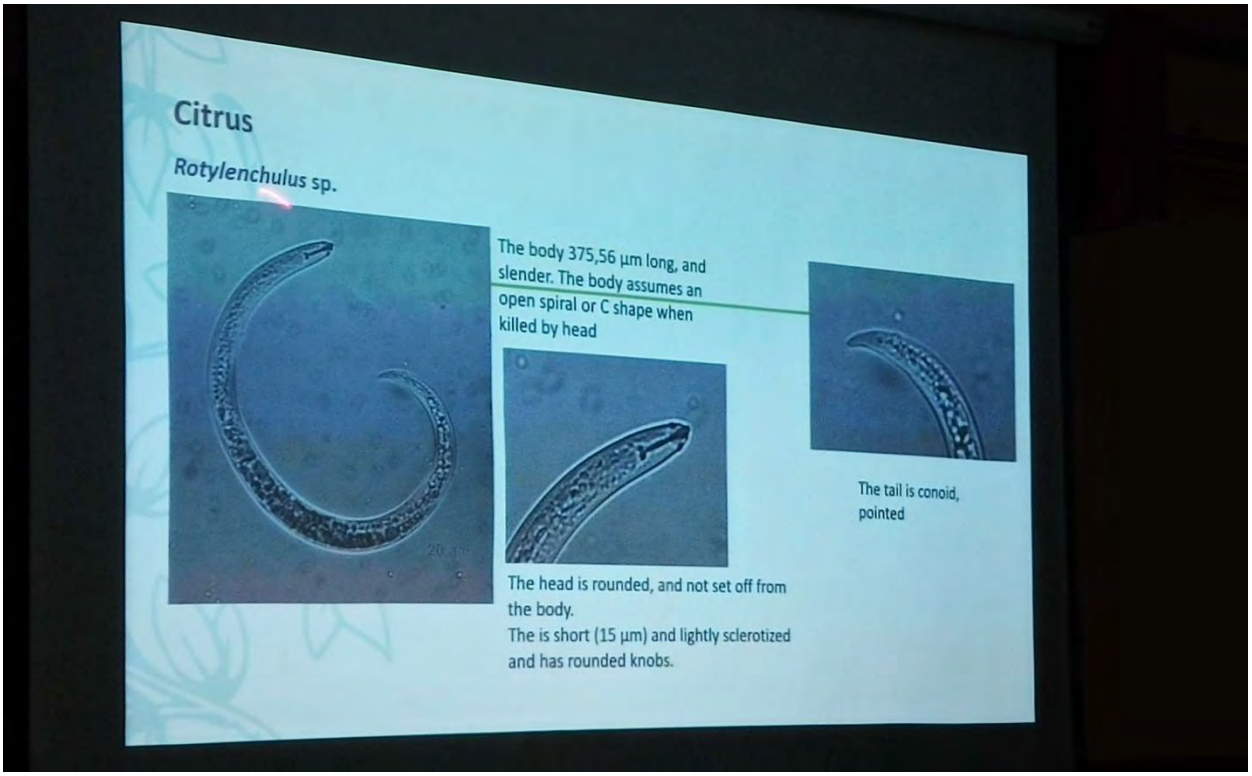
Photo 23. Lecture delivered by Dr. Nurjanah on nematode management strategies using physical, cultural, and chemical methods.

Results of this two-week activities were presented during the group report. Each group were given 30 minutes to present their accomplishments from day 2 - Field sampling collection, substrate processing and nematode extraction, morphological identification and molecular assays. Conclusions and recommendations were also presented and questions and comments from the resource persons were also asked.

General comments addressed during the presentations are as follows: incomplete label of extraction method used, minor misidentification of nematode species, and incomplete details of PCR methods. However, each group had their own strength in presenting their reports. Most of them had a unique style of presentation, simplified and easy to convey information, and some of them were able to do correct and substantial morphological and morphometrical identification of PPNs (All presentations of the Group 1 to 5 were given in the Attachments 1-5).

Photos 24. Group report during the last day of the training workshop where each group presented their output from the activities since day 1.





Photos 25. Group presenting the step by step procedures in molecular identification of plant parasitic nematodes (above). Notable results on the morphological identification of plant parasitic nematodes showing the basic morphometrical data and body description (below).

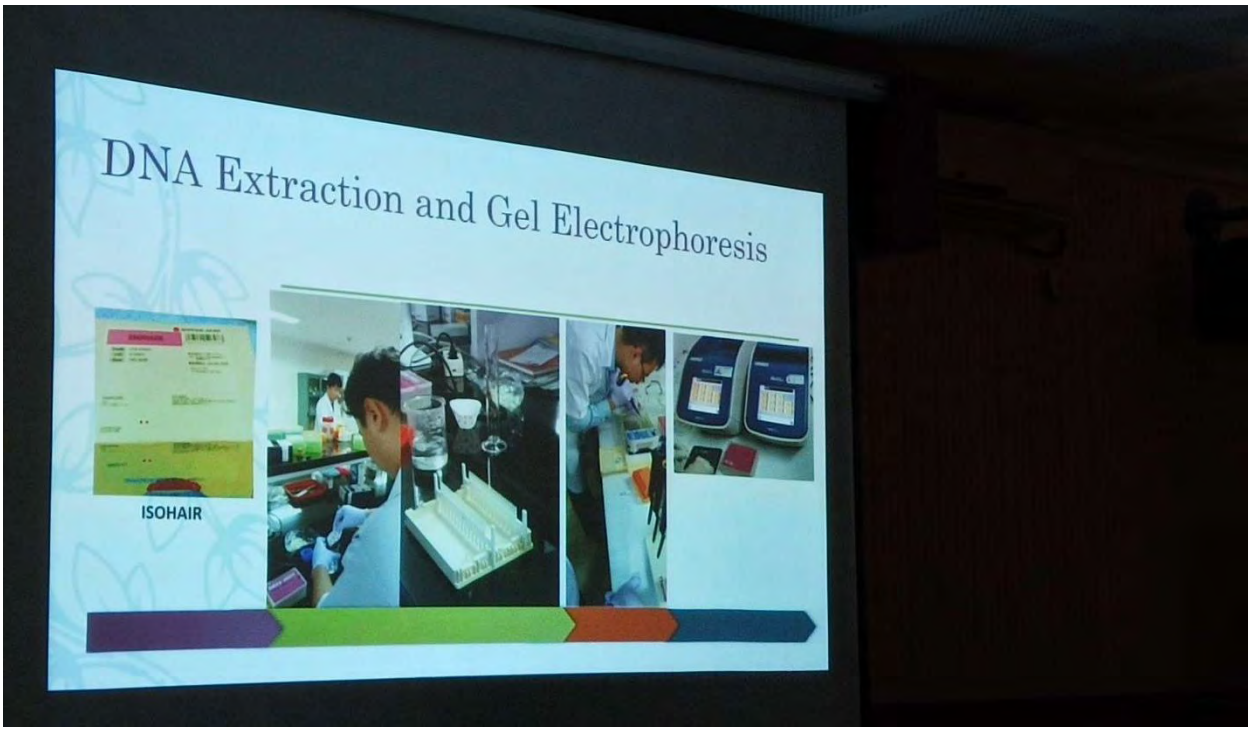


Photo 26. Presentation of molecular works of the group from DNA extraction, PCR and RFLP assays, and gel electrophoresis.

SESSION 9: Workshop Conclusion

Prior to the formal closing of the training workshop, a post-evaluation test was administered. A 45-minute written test was given to determine how far the participants learned from the lectures and practical activities. The post-evaluation test includes tricky questions given during the pre-evaluation test and with some basic computation related to molecular assays, and personal assessment on how the participants will deal with nematode problem as a plant quarantine officer.

Photo 27. Administering the post-evaluation test to the participants during the last day of the training workshop.

After the post-evaluation test, general impression or assessment coming from the resource person was delivered as well as remarks from the organizing team. The closing message was given by the Director of ARIAQ as the host agency.

Photo 28. Final remarks and assessment from the organizing team headed by Dr. Soetikno Sastroutomo of APHCN-ASEANET

Photo 29. Participants during the closing ceremony listen attentively from the remarks given by the organizing team, resource persons, and guests.

Testimonies coming from Ms. Adi Lisea Mohd Addly of Brunei Darussalam and Mr. Aldwin Mendoza from the Philippines were read by themselves. Hearth-warming messages came out from the two representatives by acknowledging the effort, hardwork and preparedness of the training team. The training facility of ARIAQ was also commended for its very conducive venue in nematology training including the accommodating staff, tasty and variety of Indonesia food, and the participants accommodation.

Photos 30. Heart-warming testimonies given by Ms. Adi Lisea Mohd Addly from Brunei and Mr. Aldwin Mendoza from the Philippines.

Right after the testimonies, certificates of completion/participation were given to each participant as well as certificates of appreciation for the resource persons.

The two-week training workshop ended with an entertaining farewell party held at ARIAQ hall during the last night of the event.

Photos 31. Awarding of certificates of completion and appreciation to each participants and training team during the closing ceremony (above). Final words of gratitude and appreciation from the lead resource person Prof. Hideaki Iwahori (below).

Photo 32. Training team from Japan headed by Prof. Iwahori and his student Mr. Kazuho, and Dr. Marita S. Pinili from the Philippines during the concluded training workshop held in ARIAQ.

Other Activities

Aside from the collection trip in Botanical Garden in Mekarsari, leisure trip but educational in form was held on March 1, 2020, Sunday. With the assistance of ARIAQ staff headed by Dr. Ummu Rustiani, all participants were able to visit the Bogor Botanical Garden (above photo), some souvenir shops, shopping mall and the famous hand-made batik in Bogor, Indonesia (bottom photos).

1: PRESENTATION OF GROUP 1

TRAINING WORKSHOP ON DIAGNOSTICS OF PLANT PARASITIC NEMATODES

GROUP PRESENTATION : GROUP 1

GROUP 1

MEMBERS:

1. Dr. Trinh Thi Thu Thuy (Vietnam)
2. Ms. Masayun Eka Maylandari (Indonesia)
3. Ms. Theingi Soe (Myanmar)
4. Mr. Ikhwan Harris Ramli (Malaysia)
5. Hajah Safwanah Abdullah (Brunei)

SAMPLE COLLECTION

Collection site : Taman Buah Mekarsari

Date of sampling : 25th February 2020

Crops : Banana, Papaya, Citrus, Guava, Oil Palm And Ginger

CROP 1 : BANANA (Vegetative stage)

- **SYMPTOMS:**
  - Wilting
  - Yellowing
  - Toppling
  - Root Lesion

CROP 2 : PAPAYA (Vegetative stage)

- SYMPTOMS:
  - No symptom

CROP 3 : CITRUS (Fruiting stage)

- SYMPTOMS:
  - Yellowing

CROP 4 : GUAVA (Fruiting stage)

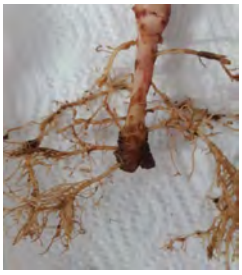
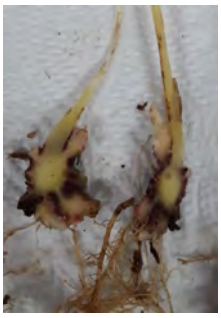
- **SYMPTOMS:**
  - Root lesion
  - Root crack

CROP 5 : PALM OIL (Fruiting stage)

- SYMPTOMS:
  - Less fruit

CROP 6: GINGER (Vegetative stage)

- SYMPTOMS:
  - Stunted growth
  - Yellowing
  - Root knot
  - Darken rhizome

SIEVING AND BAERMANN FUNNEL TECHNIQUE FOR NEMATODE EXTRACTION


Stir 300g of soil + water

Sieve the soil/plant tissue using 250µm, 106µm and 45 µm sieve

Mount the funnel on the support, set the basket on the top of the funnel and pour water until it reach bottom of the basket

Place tissue paper on top of the screen and submerged it


Wash root or plant tissue to remove the soil then cut it into small pieces

Spread sieved soil or plant tissue on the tissue paper and gently pour more water until it covered the lower part . Let it stand for 24-48 hours

Open clamp to withdraw 5-10ml water containing nematodes and transfer it to viewing dish

CENTRIFUGAL FLOATATION ASSAY FOR NEMATODE EXTRACTION

Fill 50 ml centrifuge tube with 10g of soil +20ml water + 1 tea spoon kaolin and stirred thoroughly

Tube was centrifuged at 1800G for 4 minutes

After pour off the supernatant, add 20 ml sucrose solution with specific gravity of 1.18 into the tube

Centrifuged again for 4 minutes

Sucrose solution was decanted through 20µm sieve and rinse with tap water

Water containing nematodes from the sieve was stored at 4°C until ready to be counted

Centrifuge machine used

Centrifuge tubes need to be in equal weight

IDENTIFICATION TECHNIQUE

MORPHOLOGICAL CHARACTERISTICS	MOLECULAR
1. Body length and shape	1. PCR assay
2. Lip region and head	2. RFLP assay
3. Tail shape	
4. Male / Female / Juvenile	
5.	

DNA EXTRACTION

55 C (30')

95 C
10 '

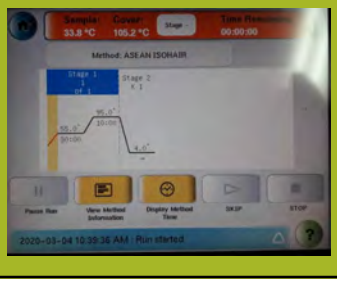
4 C

40µl H2O

Mix well

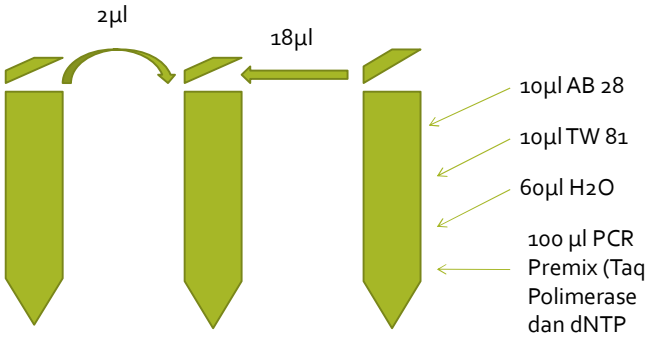
DNA
Templete

10 µl
ISOHAIR
SOLUTION



50µl

PCR ASSAY



1.2 Agarose Gel

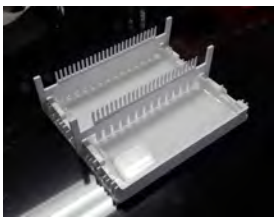



100µl TBE 1x

1.2gr Agaarose S

Shake well

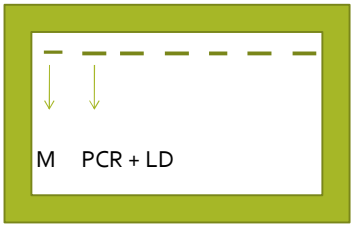
Microwave 1' in 3 time

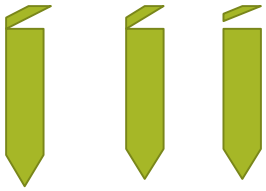



20- 30 '

Add Gel Red 10µl

Elektrophoresis

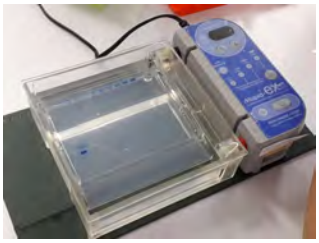




Marker 500 bp 5µl

PCR product 8µl

Loading dye2µl



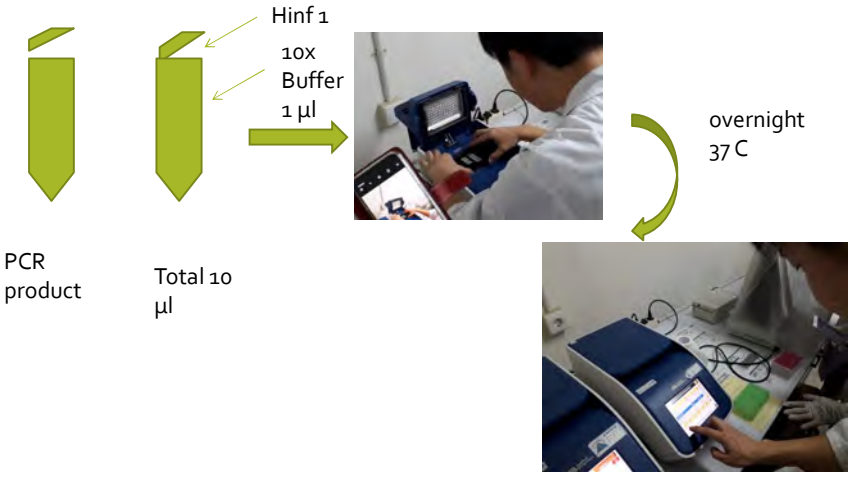

30'

RFLP ASSAY

- **RESTRICTION FRAGMENT LENGTH POLYMORPHISM**

is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination.

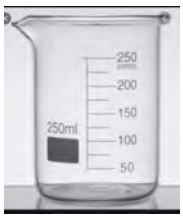
RETRICTION ENZIME



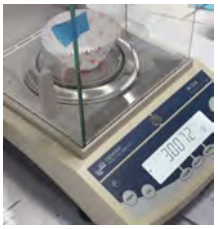
Prime Gel

TBA
1x

100 µl



Beaker glass



Agarose
3 gr

Pour to elektrophorensis tank incubate 20 – 30'

Red Gel

Microwave 1' three time

RESULTS

MORPHOLOGICAL IDENTIFICATION
TABLE -1. SAMPLE COLLECTED

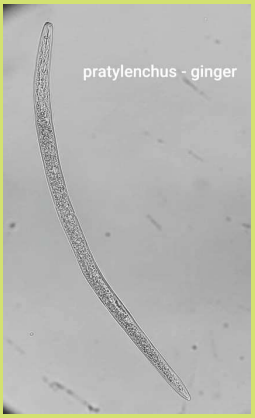
CROP	CROP STAGE	SYMPTOMS	
		ABOVE GROUND	BELOW GROUND
Banana	Growing stage	Yellowing Wilting Toppling	Lesion root
Payaya	Growing stage	No symptom	No symptom
Citrus	Fruiting	Yellowing	-
Guava	Fruiting	No symptom	Dark brown and crack roots
Oil Palm	Growing stage	Less fruit	Dark brown roots
Ginger	Growing stage	Stunted, Yellow leaf	Brown rhizomes and roots with galls

MORPHOLOGICAL IDENTIFICATION
TABLE -2: NEMATODES ASSESSMENT

CROP	EXTRACTION TECHNIQUE	NEMATODES IDENTIFIED		
		SOIL	ROOT / TUBER	SEED
Banana	Sieving and Baermann funnel	*Helicotylenchus* sp. *Aphelenchus* sp.	*Aphelenchoides* sp.	
Payaya	Sieving and Baermann funnel	*Helicotylenchus* sp.	-	
Citrus	Sieving and Baermann funnel	Tylenchidae	-	
Guava	Sieving and Baermann funnel	*Rotylenchulus* sp. Tylenchidae	-	
Oil Palm	Sieving and Baermann funnel	*Aphelenchus sp.* Tylenchidae *Helicotylenchus* sp.	*Helicotylenchus* sp.	
Ginger	Sieving and Baermann funnel		*Meloidogyne* sp. *Rotylenchulus* sp. *Pratylenchus* sp.	
Potato	Centrifuge method	*Globodera rostochiensis*	-	
Garlic	Sieving and Direct Examination		-	
Rice	Modefied Baermann funnel			*Aphelenchoides* sp.

2. Nematode Identification

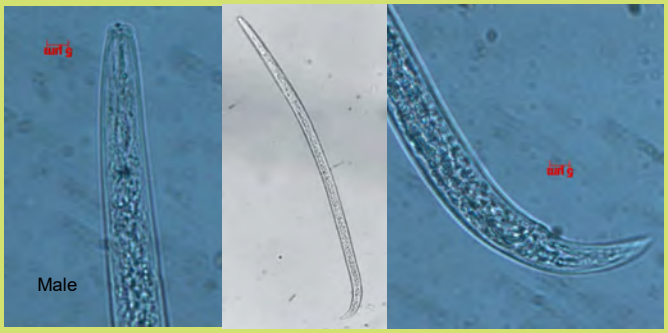
Banana, Oil Palm



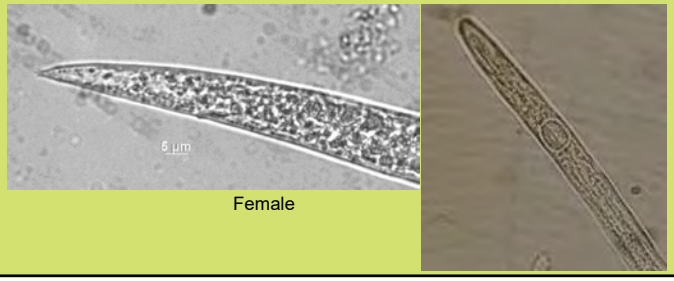
Ginger

Helicotylenchus sp.

2.Nematode Identification



Aphelenchoides besseyi - Rice

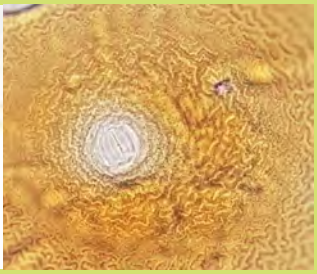


Female

2.Nematode Identification

Tylenchidae
Citrus, Oil palm, Guava

2.Nematode Identification



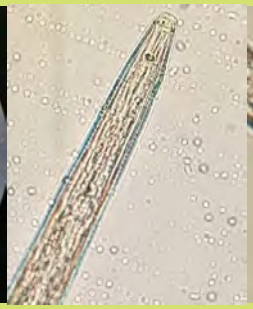
Wet Soil
1 Cyst

Globodera rostochiensis

Dry Soil
3 Cysts

2.Nematode Identification

Meloidogyne sp.
Ginger



2.Nematode Identification

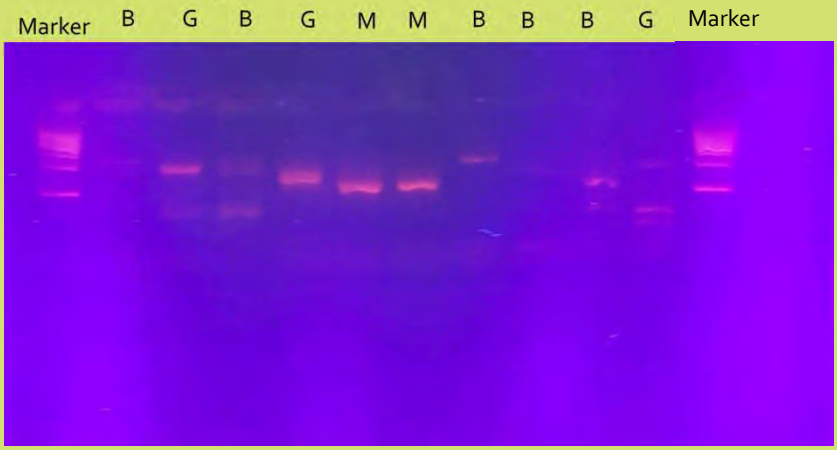
Rotylenchulus sp.
Ginger, Guava

2.Nematode Identification

Aphelenchus sp.

3. MOLECULAR IDENTIFICATION

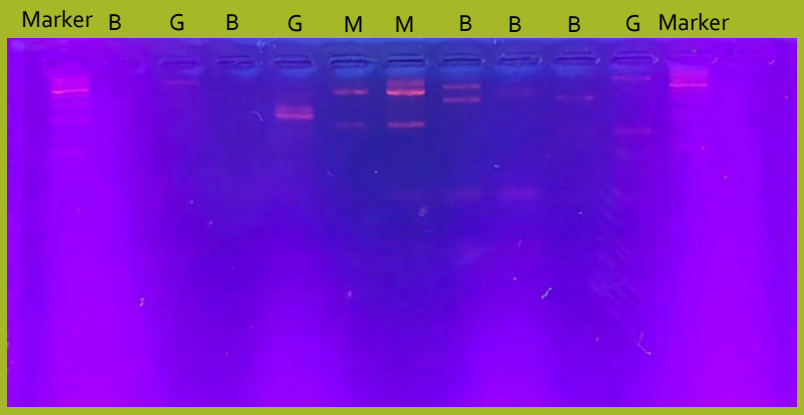
3.1 PCR assay



B : Banana
G : Garlic
M : Meloidogyne

3. MOLECULAR IDENTIFICATION

3.2 RFLP assay



B : Banana
G : Garlic
M : Meloidogyne

4. CONCLUSION

- Six soil and 5 root samples collected from 6 crops (Banana, Payaya, Guava, Citrus, Oil Palm and Ginger) in Mekarsari Fruit Garden
- Three methods used to extracted nematode from soil and root samples
- Morphology and Molecular Biology Technique used for identification of nematode
- Eight genus of nematodes found on 6 crops, garlic bulb and rice

THANK YOU
FOR
YOUR ATTENTION!

2: PRESENTATION OF GROUP 2

GROUP 2 REPORT

TRAINING WORKSHOP OF PLANT PARASITIC NEMATODES

ARIAQ, BEKASI, INDONESIA

24TH FEBRUARY – 6TH MARCH 2020

MEMBERS

- Aldwin L. Mendoza, Plant Quarantine Officer (Philippines)
- Nelly Paretanga Tangaran, Plant Quarantine Officer (Indonesia)
- Adi Lisea Mohd Addly, Plant Pathologist (Brunei)
- Phetsamone Songvilay, Technical Officer (Laos)

Sample Collection

Table 2. Samples collection summary and other necessary information

CROP	SITE	CROP STAGE	SOIL	ROOTS
*BANANA 1+3	1	PRE-HARVEST	/	
*BANANA 2	1	PRE-HARVEST	/	/
*CITRUS	2	VEGETATIVE	/	
*GUAVA	3	FRUITING		/
*OIL PALM	4	VEGETATIVE	/	/
*PAPAYA	1	FRUITING		/
*TOMATO	5	VEGETATIVE	/	/

*Collected site: Mekarsari Fruit Garden, Cileungsi, Bogor, West Java, Indonesia
Date of sampling: 25th February 2020
Legend:
/ - samples processed

SAMPLING PICTURES



METHODOLOGY - SUMMARY

Table 1. Nematode extraction methods conducted

METHOD	SAMPLES PROCESSED			
	SOIL	ROOTS	SEED	BULB
Sieving – Baermann Funnel	/	/		
Water soaking method			/	/
Sucrose Centrifugation Method	/			
Mist – Chamber Technique		/		

NEMATODE EXTRACTION METHOD
1.a. SIEVING – BAERMANN METHOD(SOIL)

Sample Collection

Table 2. Samples collection summary and other necessary information

CROP	SITE	CROP STAGE	SOIL	ROOTS
*BANANA 1+3	1	PRE-HARVEST	/	
*BANANA 2	1	PRE-HARVEST	/	/
*CITRUS	2	VEGETATIVE	/	
*GUAVA	3	FRUITING		/
*OIL PALM	4	VEGETATIVE	/	/
*PAPAYA	1	FRUITING		/
*TOMATO	5	VEGETATIVE	/	/

*Collected site: Mekarsari Fruit Garden, Cileungsi, Bogor, West Java, Indonesia
Date of sampling: 25th February 2020
Legend:
/ - samples processed

NEMATODE EXTRACTION METHOD(Active)
1.a. SIEVING – BAERMANN METHOD(SOIL)

Unclumped 300g of soil in basin filled with water to clean off root debris →

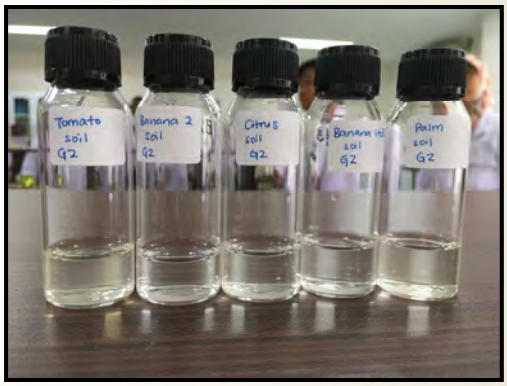
Sieved ~200uM mesh, carefully collecting eluded; suspension 2 →

Sieved ~100uM mesh, carefully collecting eluded suspension

NEMATODE EXTRACTION METHOD
1.a. SIEVING – BAERMANN METHOD(SOIL)

Sieved 45uM mesh, sieve removing excess water and small soil particles. Wash sieve surface with wash bottle or pressured water (tap) carefully to assist sieving → Washed off sieve surface with wash bottle and collected remaining suspension into beaker → Baermann Funnel technique incubated overnight at room temperature

NEMATODE EXTRACTION METHOD
1.b. SIEVING – BAERMANN FUNNEL (SOIL)



Collected 10ml of suspension by carefully turning the funnel tap → NEMATODE SUSPENSION

NEMATODE EXTRACTION METHOD
1.b. SIEVING – BAERMANN METHOD(ROOTS)

L-R: Diced roots of Guava, Papaya, Palm, Banana, and Tomato.

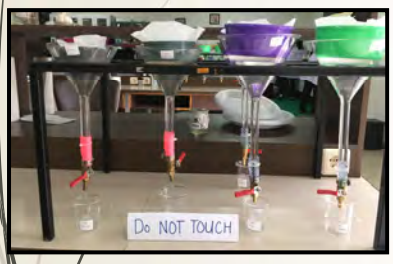
NEMATODE EXTRACTION METHOD
1.b. SIEVING – BAERMANN METHOD(ROOTS)

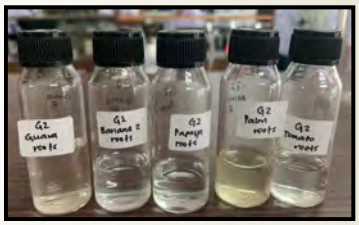
Roots were cleaned and cut into smaller pieces

Roots were macerated using blender at low speed for 10 seconds and repeated 3x.

Macerated roots were sieved

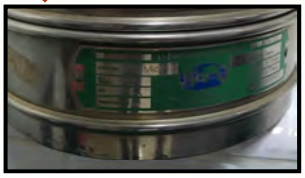
NEMATODE EXTRACTION METHOD
1.b. SIEVING – BAERMANN METHOD(ROOTS)



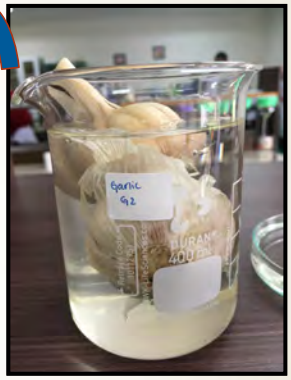


Baermann Funnel technique was conducted and Incubated overnight at room temperature → 10ml of suspension was collected by carefully turning on the funnel tap and were put into a snap cap bottle. → Live nematode suspension

NEMATODE EXTRACTION METHOD
2.a SIEVING OF OVERNIGHT SOAKED BULB(GARLIC)



40uM sieve



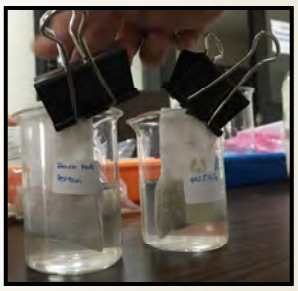
Garlic is immersed in cold water in petri dish and left overnight

Garlic bulbs can also be soaked fully in cold water and left overnight

NEMATODE EXTRACTION METHOD
2.b. SIEVING OF OVERNIGHT WATER SOAKED SEEDS (RICE)

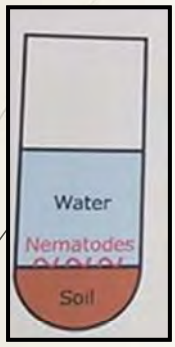
250 seeds were sampled in accordance to ISTA

Every seed were cut into 3 pieces using laboratory scissors



Seeds were soaked in water overnight at room temperature and were sieved and were collected

NEMATODE EXTRACTION METHOD
3. SUCROSE CENTRIFUGATION METHOD



50mL centrifuge tubes were filled with 10grams of wet and dry soil, separately and were added with 20mL water

A table spoon of kaolin was added for the separation of nematode to the sediment in the suspension.

The mixture were vortexed and were centrifuged at 1,800xg for 4 minutes

NEMATODE EXTRACTION METHOD
3. SUCROSE CENTRIFUGATION METHOD

The **supernatant** were gently poured off and discarded

Pellets were **resuspended with sucrose/sugar solution** with a density of 1.15-1.18

The mixture were vortexed and were centrifuged again at 1,800xg for 4 minutes

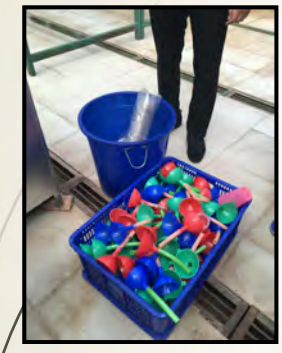
NEMATODE EXTRACTION METHOD
3. SUCROSE CENTRIFUGATION METHOD

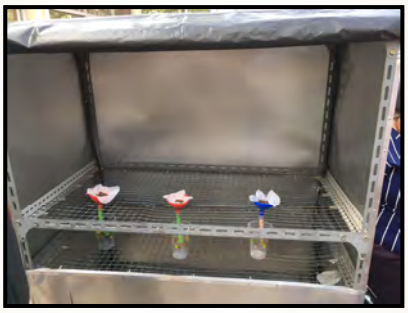
The **supernatant** were gently poured off over 20-25µM sieve

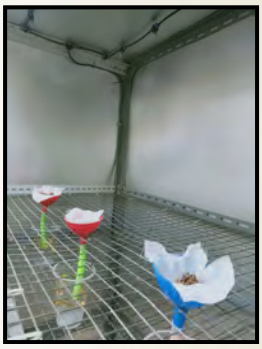
Sieve was rinsed immediately with water to remove the sugar solution

The mixture were vortexed and were centrifuged at 1,800xg for 4 minutes

NEMATODE EXTRACTION METHOD
4. MIST-CHAMBER TECHNIQUE (ROOTS)







Roots were cut, covered with net and were set-up with funnel and an open hose with a collection glass → Sprinkles were opened low. Chamber was covered and incubated for 5 days. → Samples in the form of suspension were collected from the set-up

FIXING AND KILLING OF NEMATODE

Water bath was pre-set to **80°C** → Live nematode suspension bottles were placed in the heated water bath from 2 – 4 minutes → Equal part of fixative (FA) was added to each **hot** nematode suspension

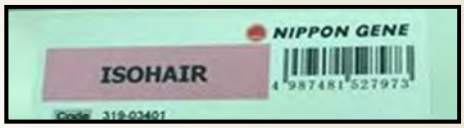
MORPHOLOGICAL IDENTIFICATION OF NEMATODES

Fixed nematodes were viewed on stereomicroscope and were mounted with FA in a microscope slide

→

Nematodes were viewed on compound microscope

DNA Extraction Technique



Ten microlitres (10uL) of **ISOHAIR** DNA extraction mixture was placed into a 200uL PCR tube

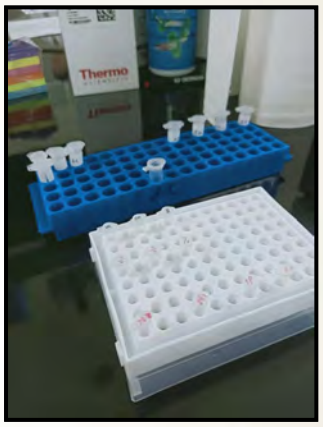
→

One (1) nematode from **garlic, banana and *Meloidogyne incognita*** suspensions was collected and put into the DNA extraction mixture **individually**.

→

The samples were subjected to undergo heating at **55°C for 30 minutes** and **95°C for 10 minutes** and incubated for **4°C.**

DNA Extraction Technique



After heating, **40µL of pure water** was added with a micropipette and mixed well.

Samples can now **be stored at -20°C** to prevent DNA degradation.

Polymerase Chain Reaction

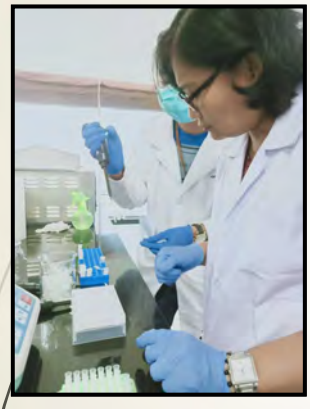


Table 3. PCR reagents and amount per reaction tube

Reagent	*Amount
PCR premix	100µL
sdH_2O	60µL
**Primer TW81	10µL
**Primer AB28	10µL

*Amount good for 10 preps
**rDNA ITS region primer concentration not indicated

Two microlitres (**2uL) of DNA template** was put into a PCR tube

To a 1.5mL microcentrifuge tube, the PCR reagents were added **(See Table 3).**

Set the PCR conditions in the thermal cycler equipment **(See table 4)**

Polymerase Chain Reaction

Table 4. PCR conditions for **primer TW81/AB28 ITS region**

Stage	Temperature	Time	
Initial denaturation	94°C	2 minutes	
Denaturation	94°C	1 minute	35X
Annealing	50°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	5 minutes	
Incubation	4°C	∞	

Restriction Fragment Length Polymorphism

In a 200µL tube, PCR product (8µL) was mixed with the digestion enzyme (1µL Hinf1) and 10x buffer (1µL)

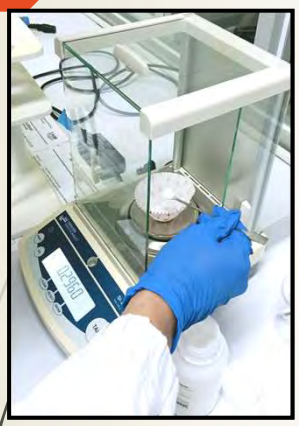
→

Restriction enzyme treatment was done overnight at 37°C using thermal cycler

→

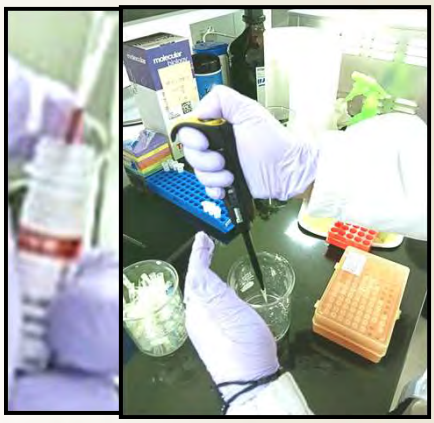
End product ready for gel electrophoresis and UV viewing

Gel Electrophoresis



Agarose was weighted and prepared using TBE for **1.2%/3%** (50mL TBE + 0.6Grams/1.2Grams Agarose)

To **dissolve the agarose** crystals, the mixture was **heated** using microwave oven



Hot mixture was added with **GelRed** as a **staining dye** of the amplified fragment

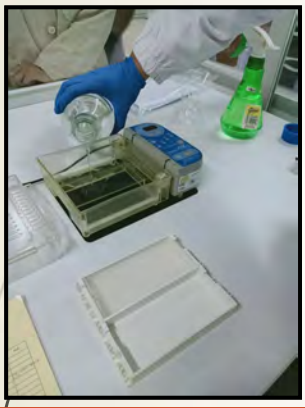
Gel Electrophoresis

The heated gel with dye was placed in a **gel tray with comb** on a horizontal surface. Bubbles were removed

The gel was covered with plastic foil to **avoid dust**

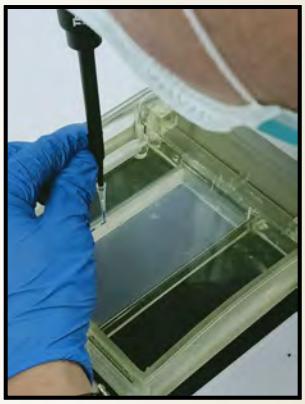
After 20-30minutes, the 13-well comb was removed

Gel Electrophoresis



Electrophoresis apparatus was **filled** initially with **TBE buffer**

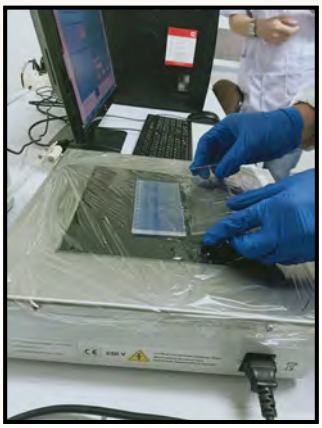
Gel was placed and **submerged in the tank** placing the wells on the negative side. Blue juice/dyes were prepared



After PCR/RFLP products were loaded together with the dye (2µL BJ+8µL template)

Gel Electrophoresis

Loaded PCR/RFLP products were **run for 20minutes and 40minutes**, respectively



The gel was placed in an ultraviolet apparatus

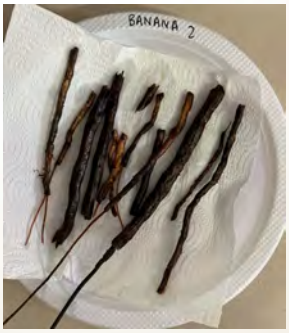
The gel was viewed under UV transilluminator, carefully

BANANA 1-3 - DETAILS

- Mekarsari Site:
- Crop: Banana
- Crop Stage: Pre-harvest
- Symptoms (AG): Yellowing of leaves
- Symptoms (BG): Blackening, few areas affect vascular part

BANANA 2 - DETAILS

- Mekarsari Site:
- Crop: Banana
- Crop Stage: Pre-harvest
- Symptoms (AG): toppled tree (dead)
- Symptoms (BG): Severe blackening, affect all parts

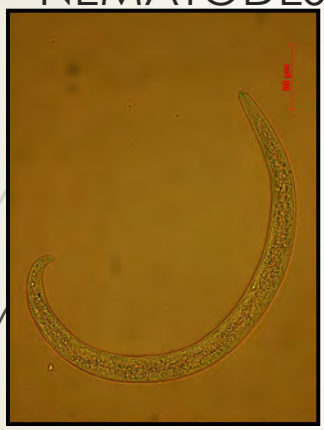


MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Banana roots

Free-Living

10x

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Banana soil



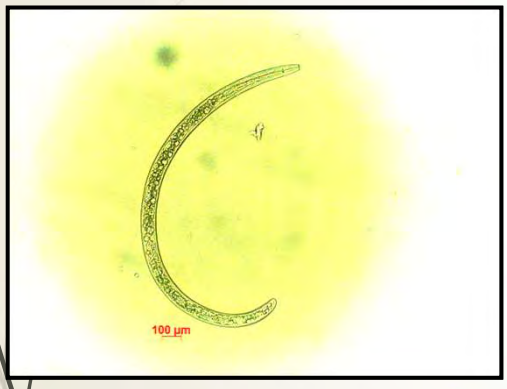
Juvenile Rotylenchulus

Female *Rotylenchulus sp.*

20x

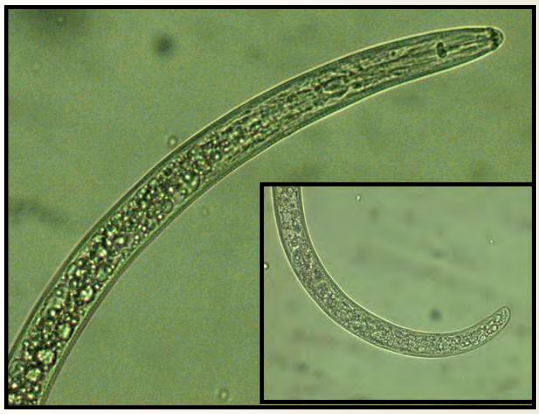
20x

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Banana roots



Helicotylenchus sp.

20x



***Helicotylenchus sp.* Tail and head**

40x

CITRUS - DETAILS

- Mekarsari Site:
- Crop: Citrus
- Crop Stage: Pre-harvest
- Symptoms (AG): Yellowing of leaves, not flourishing
- Symptoms (BG): Little browning of vascular bundle, otherwise healthy

GUAVA - DETAILS

- Mekarsari Site:
- Crop: Banana
- Crop Stage: Pre-harvest
- Symptoms (AG): Stunted
- Symptoms (BG): Root galling, blackening

OIL PALM - DETAILS

- Mekarsari Site:
- Crop: Oil Palm
- Crop Stage: Fruiting
- Symptoms (AG): Browning of leaves
- Symptoms (BG): Blackening at some parts, browning of vascular area

PAPAYA - DETAILS

- Mekarsari Site:
- Crop: Papaya
- Crop Stage: Pre-harvest
- Symptoms (AG): Yellowing of leaves
- Symptoms (BG): Root galling

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Papaya roots

Rotylenchulus sp.

10x → 40x

TOMATO - DETAILS

- Mekarsari Site:
- Crop: Tomato
- Crop Stage: Vegetative
- Symptoms (AG): Yellowing and curling of leaves
- Symptoms (BG): Healthy roots

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Tomato soil

Free-living nematode

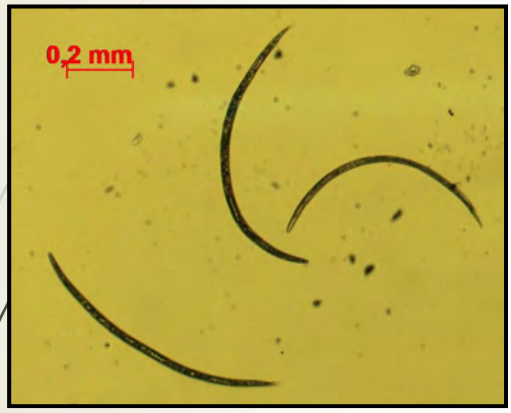
Rotylenchulus sp.

Scanner → 20x

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Rice - *Aplenchoides besseyi*

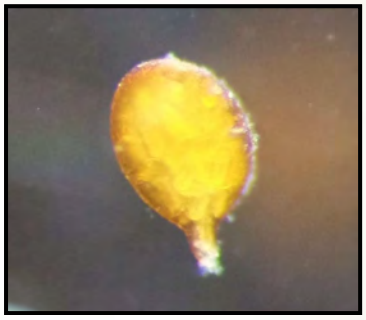
Scanner → High power objective → Oil Immersion

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Garlic – *Ditylenchus sp.*



Scanner → High power objective Male

MORPHOLOGICAL IDENTIFICATION OF NEMATODES: Wet soil VS Dry soil



Wet soil:
Globodera sp.

CYST

PERENNIAL PATTERN

40x → 10X STEREO → 40X

Table 7. Nematode collection and tentative identification summary

CROP /Sample	CROP STAGE	PART	
		SOIL	ROOTS/ OTHERS
BANANA 1-3	PRE-HARVEST	FL, *Rotylenchulus sp.*	*Helicotylenchu sp.*
CITRUS	VEGETATIVE	-	-
GUAVA	FRUITING	-	-
OIL PALM	VEGETATIVE	-	-
PAPAYA	FRUITING	-	-
TOMATO	VEGETATIVE	FL, *Rotylenchulus sp.*	-
GARLIC	-	n/a	*Ditylenchu sp.,*
RICE	-	n/a	*Aphelenchoides besseyi*
Wet soil	-	*Globodera sp.*	n/a
Dry soil	-	-	n/a

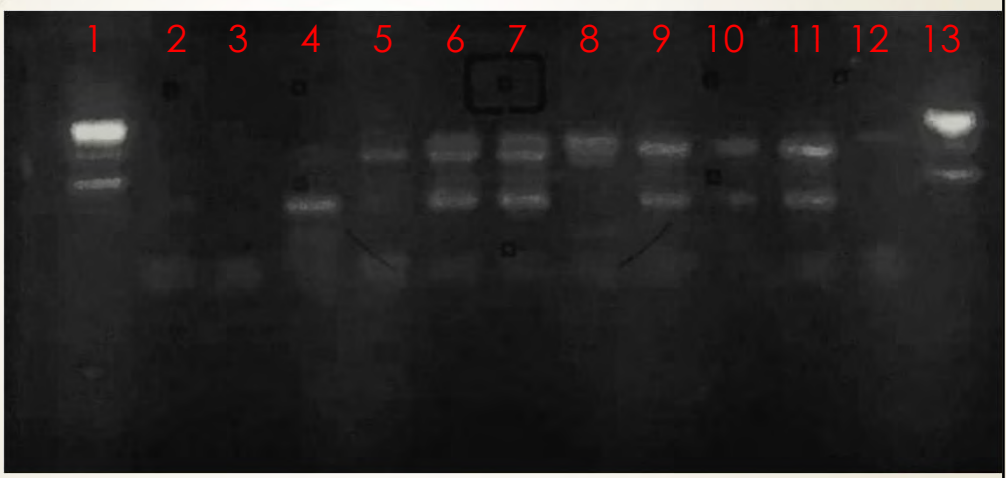
SAMPLES COLLECTED - SUMMARY

CROP	CROP STAGE	SYMPTOMS	
		AG	ROOTS
BANANA 1+3	PRE-HARVEST	/	
BANANA 2	PRE-HARVEST	/	/
CITRUS	VEGETATIVE	/	
GUAVA	FRUITING		/
OIL PALM	VEGETATIVE	/	/
PAPAYA	FRUITING		/
TOMATO	VEGETATIVE	/	/

Legend:
/ - samples processed

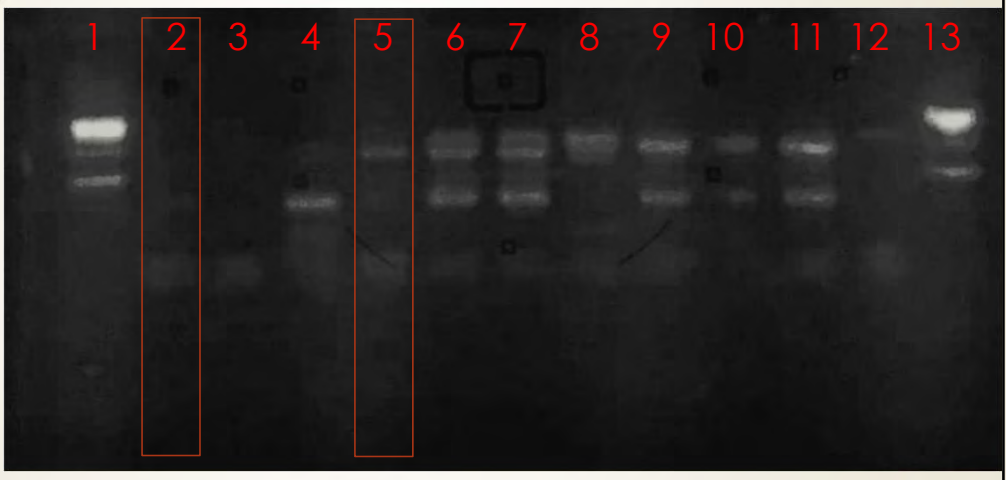
Polymerase Chain Reaction

Well	Description
1	LADDER
2	*Meloidogyne incognita*
3	Banana
4	Garlic
5	Meloidogyne incognita
6	Banana
7	Garlic
8	Banana
9	Garlic
10	Garlic
11	Garlic
12	Banana
13	LADDER



Polymerase Chain Reaction

Well	Description
1	LADDER
2	***Meloidogyne incognita***
3	Banana
4	Garlic
5	**Meloidogyne incognita**
6	Banana
7	Garlic
8	Banana
9	Garlic
10	Garlic
11	Garlic
12	Banana
13	LADDER

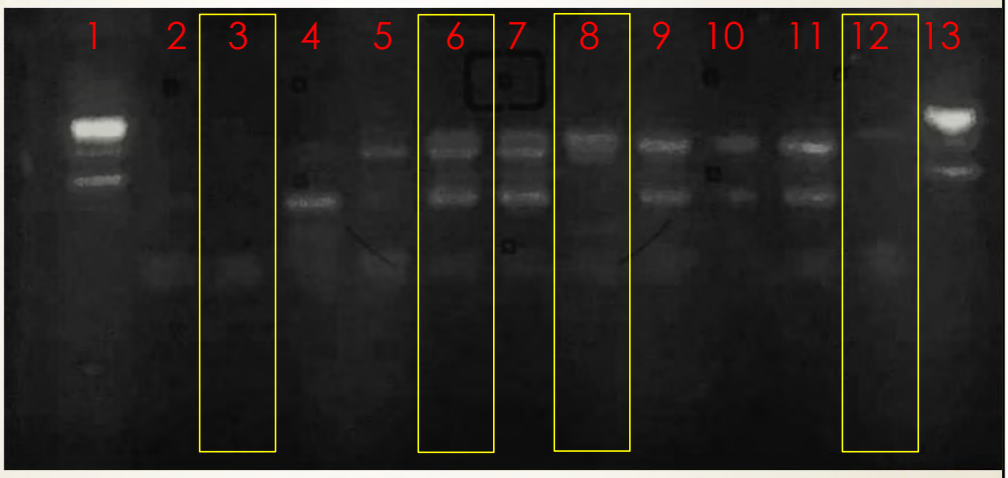


*1.2% Agarose

M. INCOGNITA

Polymerase Chain Reaction

Well	Description
1	LADDER
2	*Meloidogyne incognita*
3	**Banana**
4	Garlic
5	Meloidogyne incognita
6	**Banana**
7	Garlic
8	**Banana**
9	Garlic
10	Garlic
11	Garlic
12	**Banana**
13	LADDER

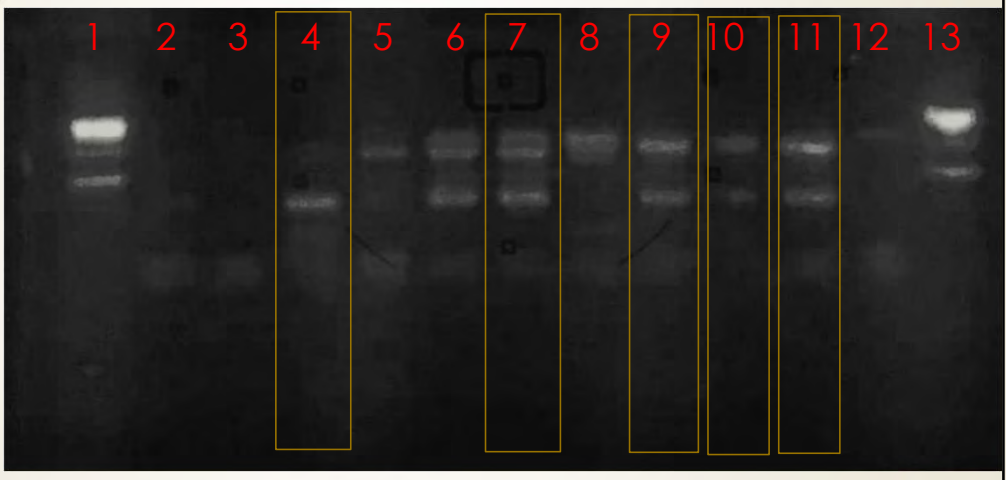


*1.2% Agarose

BANANA

Polymerase Chain Reaction

Well	Description
1	LADDER 500
2	*Meloidogyne incognita*
3	Banana
4	**Garlic**
5	Meloidogyne incognita
6	Banana
7	**Garlic**
8	Banana
9	**Garlic**
10	**Garlic**
11	**Garlic**
12	Banana
13	LADDER 500

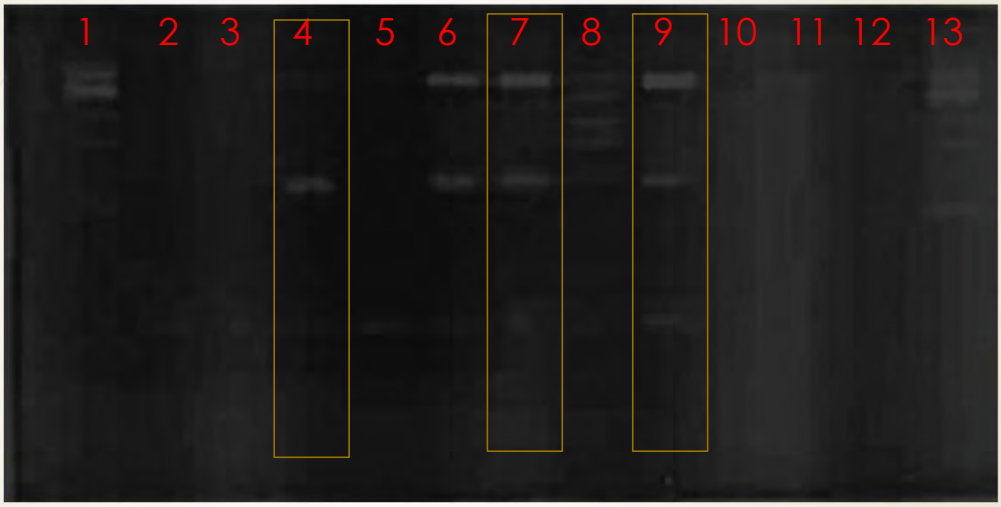


*1.2% Agarose

GARLIC

Restriction Fragment Length Polymorphism

Well	Description
1	LADDER 100
2	*Meloidogyne incognita*
3	Banana
4	**Garlic**
5	Meloidogyne incognita
6	Banana
7	**Garlic**
8	Banana
9	**Garlic**
10	No template
11	No template
12	No template
13	LADDER 500

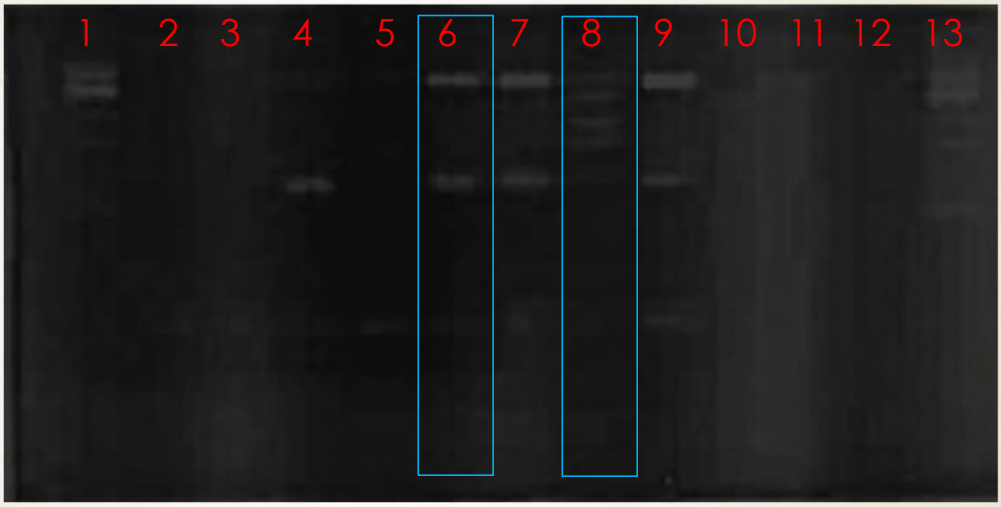


*3% Agarose

GARLIC

Restriction Fragment Length Polymorphism

Well	Description
1	LADDER 100
2	*Meloidogyne incognita*
3	Banana
4	Garlic
5	*Meloidogyne incognita*
6	**Banana**
7	Garlic
8	**Banana**
9	Garlic
10	No template
11	No template
12	No template
13	LADDER 500



*3% Agarose

BANANA

PCR & RFLP Results

1.2% Agarose

PCR Result

3% Agarose

RFLP Result

Conclusion

- There were some plants like guava, citrus and palm that shown no presence of nematodes
- Various methods can be chosen based from the mode of extraction of nematodes namely: Sucrose centrifugation (Cyst), Baermann (Active), or Sieving (passive-cloudy)
- ITS serves as a barcode primer for nematodes
- Various nematodes are present in one suspension that caused variation of banding pattern during enzyme digestion (RFLP)

Recommendation

- Sampling should have done not in a rainy weather
- Identification until family level should have been taught
- Perenial pattern ID for *Meloidigyne spp.* should have been tried

- LABORATORY
- Aseptic environment (touching of PCR tubes)
- Human error
- Cross contamination of samples
- More hands-on training on first hand DNA sequences and molecular techniques

3: PRESENTATION OF GROUP 3

Training Workshop on Diagnostic of Plant Parasitic Nematode

GROUP 3:

Ms. Ungkana Poungngenmak	Thailand
Mr. Ya Phauk	Cambodia
Mr. Azman Bin Ahmad	Singapore
Mr. Mohd Azrul Bin Alias	Malaysia
Mr. Abdul Mubaraq Irfan	Indonesia

ARIAQ, BEKASI, INDONESIA, 06/MAR/2020

METHODOLOGY

SAMPLE COLLECTION : SITE 1

- BANANA
- VEGETATIVE

Above Ground:
- Toppling
- Yellowing

Under Ground:
- Poor Root System
- Lesion on the cuticle and the middle of the root
- Dead roots

SAMPLE COLLECTION : SITE 1

- RAMBUTAN
- HARVESTED

Above Ground:
- Poor Growth
- Defoliation

SAMPLE COLLECTION : SITE 2

- CITRUS
- MATURING

Above Ground:
- Yellowing
- Defoliation
- Stunting

Under Ground:
- No Symptom

SAMPLE COLLECTION : SITE 2

- PAPAYA
- FRUITING

Above Ground:
- Yellowing
- Shortening of internodes causing bushy appearance.
- Necrotic spot leaves.

Under Ground:
- Galling
- Excessive secondary roots.

SAMPLE COLLECTION : SITE 4

- OIL PALM
- MATURING

Above Ground:
- Yellowing
- Stunting

Under Ground:
- No symptom

SAMPLE COLLECTION : SITE 4

- AUBERGINE / EGG PLANT
- FRUITING

Above Ground:
- Stunting

Under Ground:
- Severe galling
- Shallow rooting

SAMPLE COLLECTION : SOIL SAMPLING

Hand trowel, ~25-cm deep

Transfer to clean plastic bag + labelling

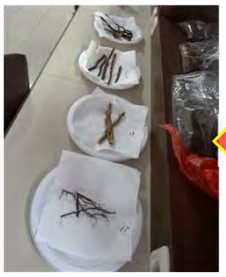
Arrange and prepare for processing

SAMPLE COLLECTION : ROOTS SAMPLING

Hand trowel, dig under the canopy (drip line for tree)

Transfer to clean plastic bag + labelling

Water cleaned



Drying

EXTRACTION : Sieving & Baermann Funnel

- Soil

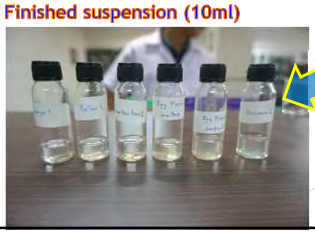
Add water & unclump soil

Collect supernatant

Serial sieving

Baermann Funnel 24 hrs

Finished suspension (10ml)



EXTRACTION : Maceration & Sieving & Baermann Funnel

- ROOTS

Cut roots

Roots + water in & macerate

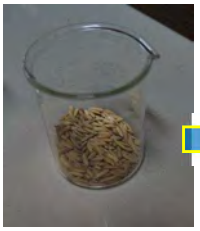
Sieve & collect sample suspension

Baermann Funnel

Collect nematode suspension

EXTRACTION : Water soaking

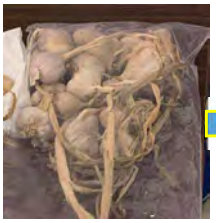
- Garlic & Rice



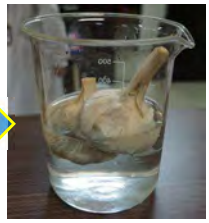
Cut the 250 seeds (each into 3 pieces)

Put in linen bag + add 40ml cold distilled water and incubate 24 hrs in darkness)

Collect ~10 ml nematode suspension, if cloudy suspension proceed sieving (25um)



Choose the sample



Submerge in cooled water

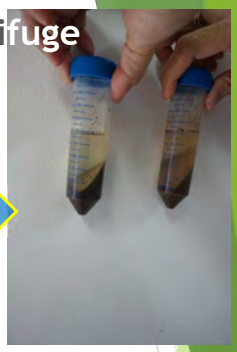
@

Put on cold water

EXTRACTION : Sucrose centrifuge

10g soil + 1tsp Kaolin + 20ml water and mix well

Centrifuge at 3,000 rpm 4min



Discard supernatant

Add 20ml sucrose suspension

Centrifuge at 3,000 rpm 4min

Sieve (25µm) supernatant (sucrose + nematode)

Collect nematode suspension

MOLECULAR ASSAY : DNA EXTRACTION

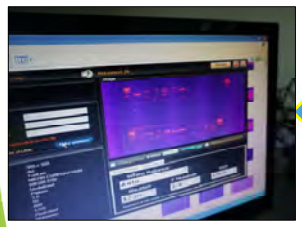
Pick nematode

Add master mix

Put in PCR machine

Placing agarose gel

Loading sample + run the gel doc



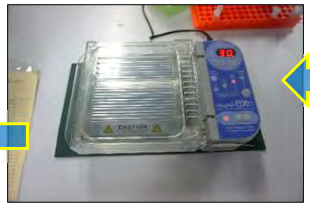
Decipher the result

MOLECULAR ASSAY : RFLP ANALYSIS

Add restriction enzyme

Incubate 37C overnight

Agarose gel prep



Run gel electrophoresis

Gel above UV light

RESULTS : Morphological Identification

Table 1 Symptom assessment of collected plants

CROP	CROP STAGE	ABOVE GROUND SYMPTOM	BELOW GROUND SYMPTOM
Banana	Vegetative	- Toppling - Yellowing	- Poor root system - Lesion on the cuticle and the middle of the root - Dead roots
Rambutan	Harvesting	- Poor Growth - Defoliation	- No symptom
Citrus	Maturing	- Yellowing - Defoliation - Stunting	- No symptom
Papaya	Fruiting	- Yellowing - Shortening of internodes causing bushy appearance - Necrotic spot leaves	- Galling - Excessive secondary roots
Oil Palm	Maturing	- Yellowing - Stunting	- No symptom
Aubergine/ Egg Plant	Fruiting	- Stunting	- Severe galling - Shallow rooting

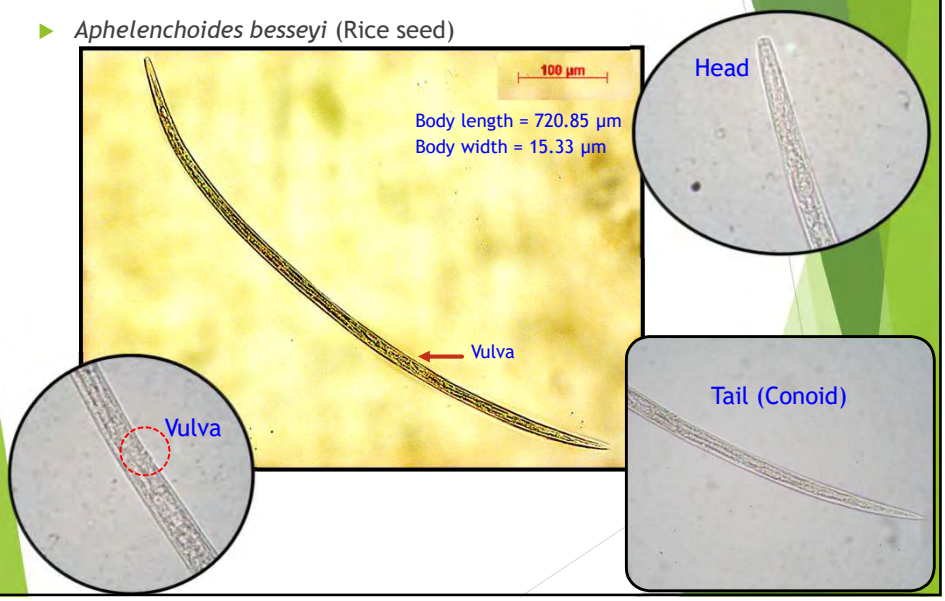
RESULTS : Morphological Identification

Table 2 Nematodes associated with collected samples

CROP	EXTRACTION TECHNIQUE USED	NEMATODE SPECIES
SOIL		
Banana	Sieving + Baermann Funnel	None
Rambutan		None
Citrus		None
Papaya		None
Oil Palm		None
Aubergine / Egg Plant		Non PPN / Free-living
ROOT/BULB/SEED		
Banana	Maceration + Sieving + Baermann Funnel	J2 RKN
Rambutan		None
Citrus		None
Papaya		None
Oil Palm		None
Aubergine / Egg Plant		J2 RKN
Rice seeds	Water Soaking	*Aphenlenchoides besseyi*
Garlic		*Ditylenchus dipsaci*

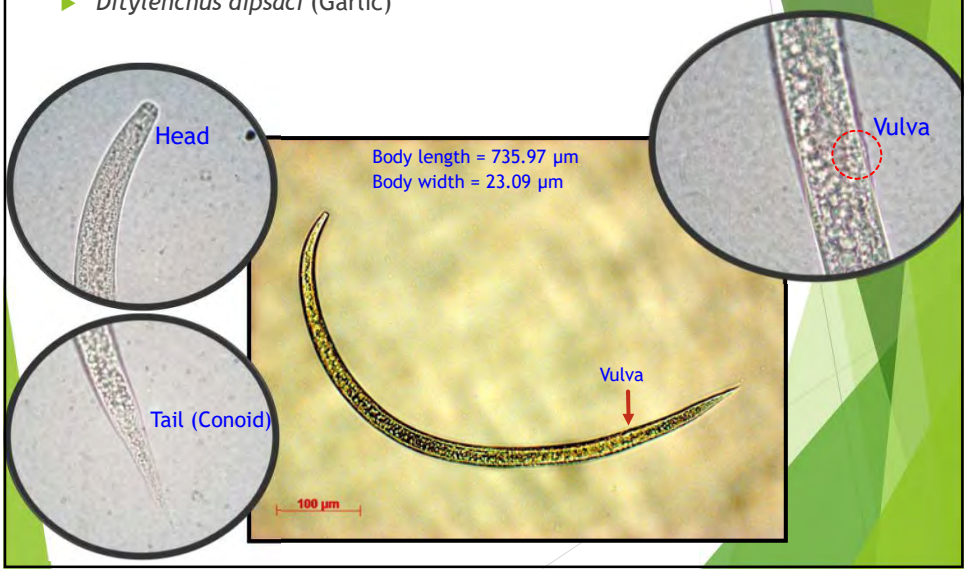
RESULTS : Morphological Identification

- *Aphelenchoides besseyi* (Rice seed)



RESULTS : Morphological Identification

- *Ditylenchus dipsaci* (Garlic)



RESULTS : Morphological Identification

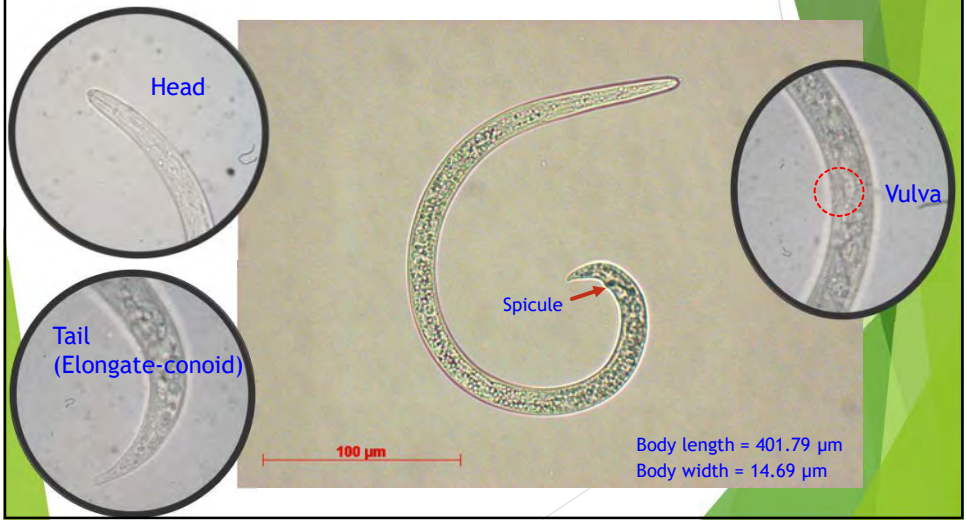
- J2 *Meloidogyne* sp.
  (Egg Plant roots & Banana roots)

RESULTS : Morphological Identification

- *Rhabditis* sp.
  (Egg Plant Soil)

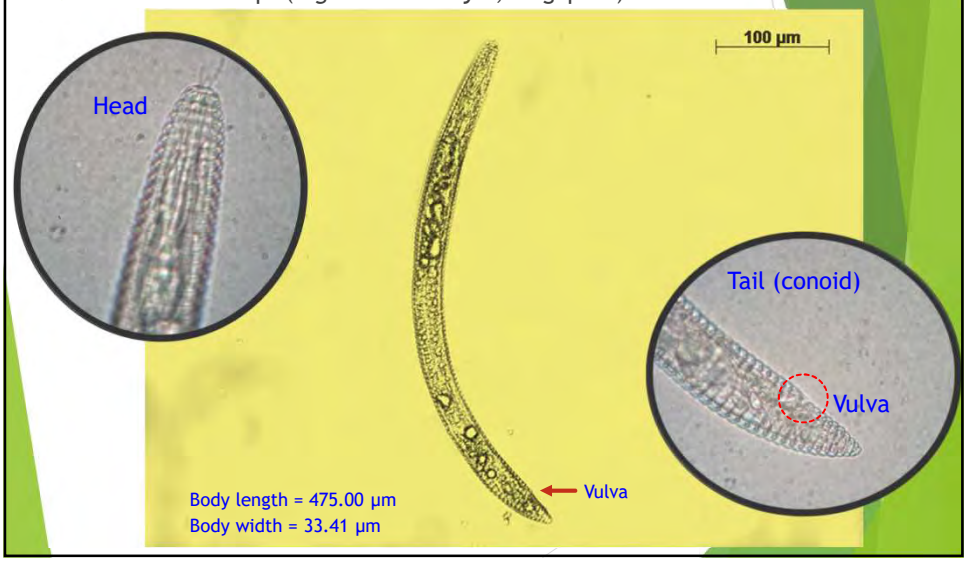
RESULTS : Morphological Identification

- *Rotylenchulus reniformis* (Yard longbean; Cambodia)



RESULTS : Morphological Identification

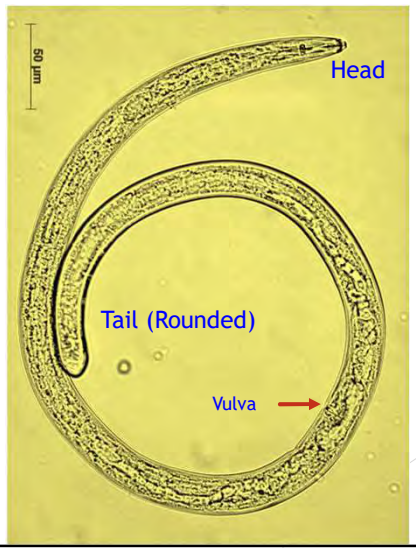
- *Criconemella* sp. (*Digitaria didactyla*; Singapore)



RESULTS : Morphological Identification

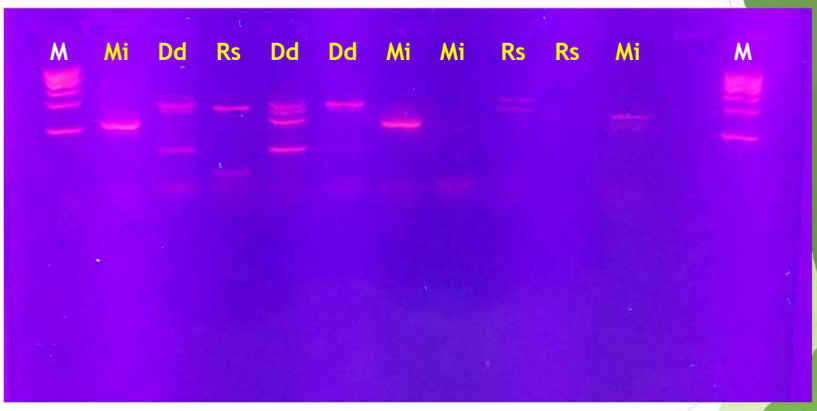
- *Rotylenchus* sp. (Maize; Thailand)

Body length = 883.75 µm
Body width = 33.41 µm



RESULTS : DNA Gel Electrophoresis

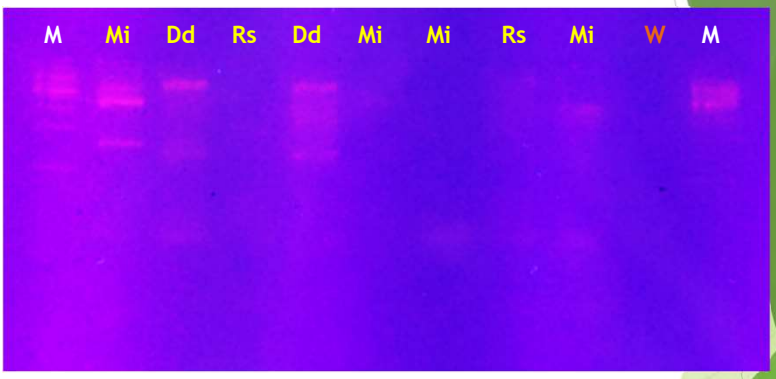
- PCR products from different kind of nematodes



M = Marker (500bp) Mi = *Meloidogyne incognita* (Japan) Rs = *Radopholus* sp. (Banana)
Dd = *Ditylenchus dipsaci* (Garlic)

RESULTS : RFLP Gel Electrophoresis

- PCR products from different kind of nematodes



M = Marker (100bp) Mi = *Meloidogyne incognita* (Japan) Rs = *Radopholus* sp. (Banana)
Dd = *Ditylenchus dipsaci* (Garlic) W = Water

DISCUSSION

- Less nematodes from the samples collected at the field
- Soil was very wet (after and during raining). Better to collect nematode during dry day; schedule should be flexible (Lecture during raining)
- Perhaps it better to collect from more locations and crops
- Some nematodes were lost during sieving (some nematodes were waste away and some other were remaining in the sieve
- Tools for collecting sample were small. We might need bigger tools like Hoe to dig deeper.
- Plants/trees were generally healthy, most symptoms are perhaps from other plants pest.
- Successfully extracted DNA from *M. incognita*, *D. dipsaci*, and *Radopholus* sp. but species identification using restriction enzyme (*Hinf* 1) does not give clear DNA pattern.

CONCLUSION

- Fixation is crucial in morphological identification (morphometric) and need to be confirmed species using molecular assay.

Suggestion

- Proceed sequencing and blast in gene bank for final species confirmation.
- Single gel is required for faster loading process and may provide sharper bands result.

Sawasdee khap
Sehingga berjumpa Lagi..

4: PRESENTATION OF GROUP 4

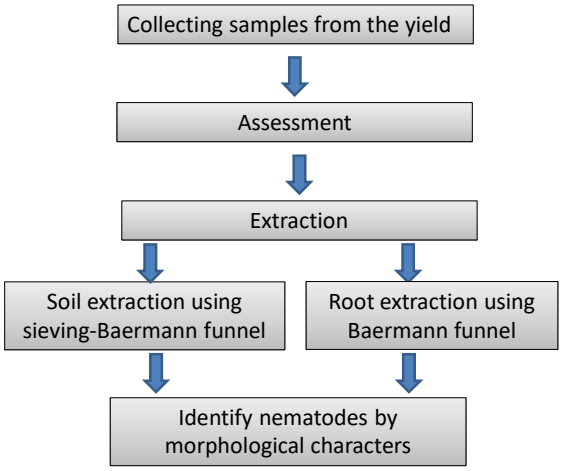
Group Report
By Group 4

Mr. Nit Ti	Cambodia
Ms. Souphatsone Voravong	Laos
Ms. Khine Thanda Moe	Myanmar
Ms. Elmi Muliya	Indonesia

Training Workshop on Diagnostic of Plant Parasitic Nematodes

23rd February to 7 March 2020

Methodology



Methodology

I. A. Samples Collection

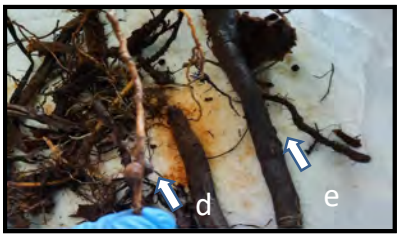
Method of collecting soil samples

- We need to finding symptom of plant part
- Removing grasses and upper soil 25 cm and then take the soil samples about 300 g, placing soil samples into plastic bag
- Label

Sample Collection

No	Location	Collection site	Date of sampling	Crop	Crop Stage
1	Mekarsari	2nd	February 25th 2020	Banana	Generative
2	Mekarsari	3th	February 25th 2020	Papaya	Generative
3	Mekarsari	4th	February 25th 2020	Citrus	Generative
4	Mekarsari	5th	February 25th 2020	Guava	Vegetative
5	Mekarsari	6th	February 25th 2020	Palm oil	Generative
6	Mekarsari	7th	February 25th 2020	Ipomoea (kangkung)	Generative

1. Banana Plant



Symptom (above ground)
1. Toppling (a)
2. Yellowing (leaves) (b)

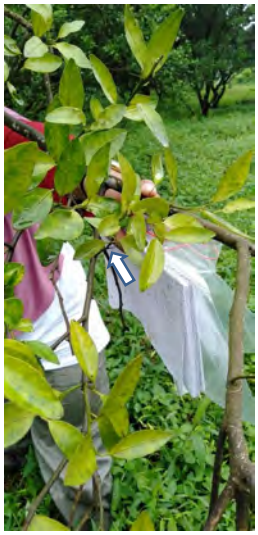
Symptom (below ground)
1. Root lesion (c)
2. Root knot (d)
3. Root rot (e)

2. Papaya plant

Symptom (above ground)
1. Yellowing

Symptom (below ground)
No symptom

3. Citrus plant

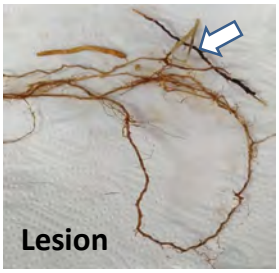


Symptom (above ground):
1. Yellowing
2. Crinkle

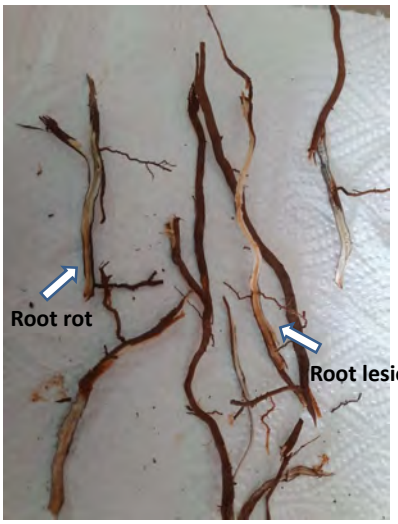
Symptom (below ground):
Root lesion

4. Guava plant

Stunting



5. Palm oil Plant



6. Ipomoea plant (kangkung)

Stunting

Lesion

Gall

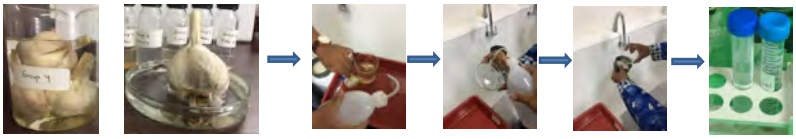
Root knot

B. Nematode extraction

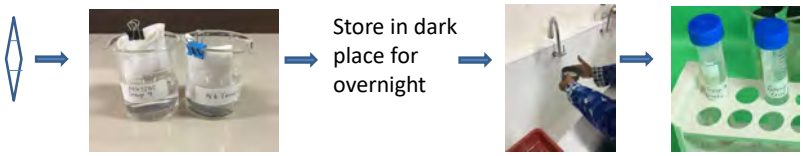
1. Nematode extraction from soil (Sieving-Baermann funnel)

2. Nematode extraction from root

3. Nematode extraction from bulb (garlic)



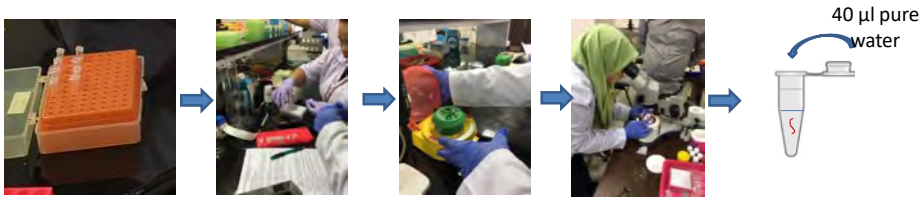
4. Nematode extraction from seed (rice)



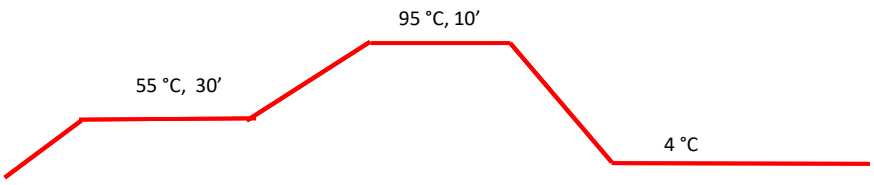
5. Nematode extraction from soil by centrifugation

C. Morphological Identification (heating-killing-mounting)

II. A. DNA Extraction



10 µl ISOHAIR
(Nippon Gene Co, Ltd.)

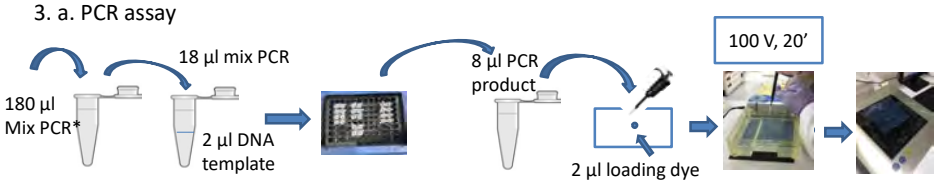


B. Electrophoresis

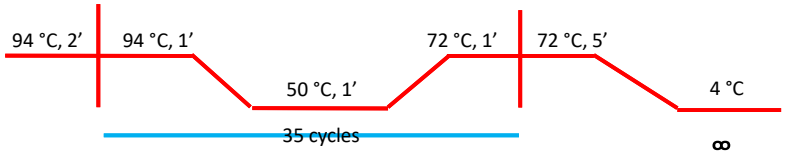
1. a. Preparation of 1.2% agarose gel for PCR assay

b. Preparation of 3 % agarose gel for RFLP assay

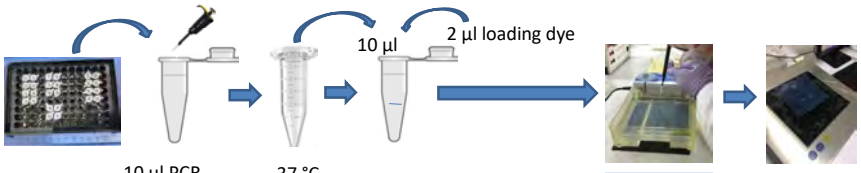
3. a. PCR assay



*100 µl PCR premix+60 µl H_2O+ 10 µl AB28 (F)+10 µl TW81 (R))



b. RFLP assay



Morphological Identification of Plant Parasitic Nematode

Banana Plant (root)

Helicotylenchus (a) whole body,
(b) head, (c) tail

Palm oil(root)

a

Criconemoides (a) whole body,
(b) head, (c) tail

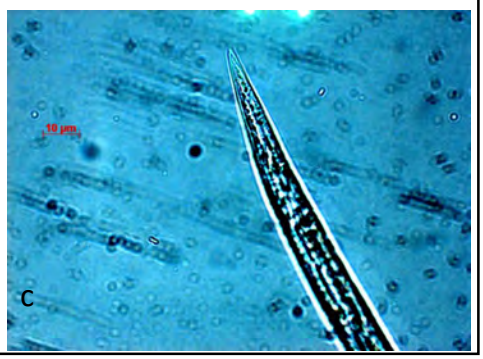
Palm oil (root)

Rotylenchulus (a) whole body, (b) head, (c) tail

Ipomoea (root)

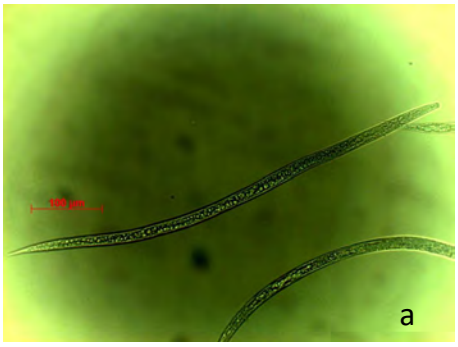
Meloidogyne (whole body)

Rice seed



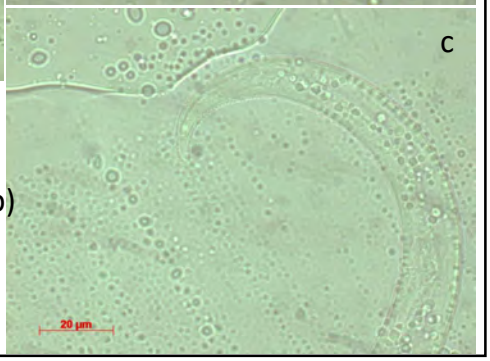
Aphelenchoides (a) whole body, (b) head, (c) tail

Garlic bulb



Ditylenchus (a) whole body, (b) head, (c) tail

Wet soil



Rotylenchulus (a) whole body, (b) head, (c) tail

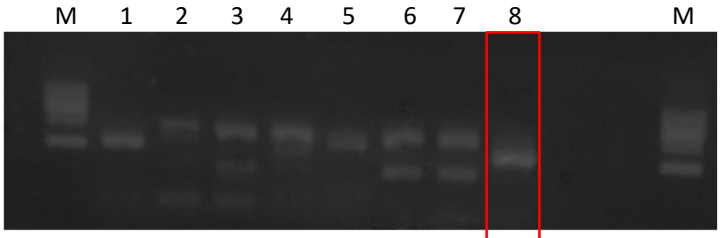
Table 1 Samples Collected

Crop	Crop stage	Symptom	
		Above ground	**Below ground**
Banana	Vegetative	Toppling Yellowing	Root rot Root knot Root lesion
Papaya	Generative	Yellowing	No symptom
Citrus	Generative	Yellowing Crinkle	Root lesion
Guava	Vegetative	Stunting	Root lesion
Palm oil	Generative	Yellowing	Root rot Root lesion
Ipomoea (kangkung)	Generative	Stunting	Gall Root lesion Root knot

Crop	Extraction Technique Used	Nematode species (Genus)
Banana	Sieving-Baermann funnel	*Dorylaimida* sp
	Baermann funnel	*Helicotylenchus* sp
Papaya	Sieving-Baermann funnel	-
Citrus	Sieving-Baermann funnel	Free living
	Baermann funnel	-
Guava	Sieving-Baermann funnel	*Cephalobus* sp
	Baermann funnel	-
Palm oil	Sieving-Baermann funnel	*Rhabditis* sp
	Baermann funnel	*Criconemoides* sp Rotylenchulus sp
Ipomoea (kangkung)	Sieving-Baermann funnel	-
	Baermann funnel	*Meloidogyne* sp
Rice seed		*Aphelenchoides* sp *Mononchida* sp
Garlic bulb		*Ditylenchus* sp
Dry soil	Centrifugation	-
Wet soil	Centrifugation	*Rotylenchulus* sp

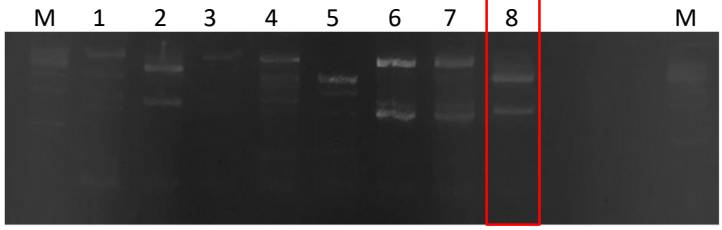
Gel Electrophoresis

1. PCR Assay



M; Marker 500 bp, 1-8: samples (1: garlic, 2: Mi, 3: Garlic, 4: Banana, 5: Banana, 6: Garlic, 7: Garlic, 8: Mi)

2. RFLP Assay



M; Marker 100 bp, 1-8: samples (1: garlic, 2: Mi, 3: Garlic, 4: Banana, 5: Banana, 6: Garlic, 7: Garlic, 8: Mi)

Conclusion

- Diagnostic of plant infected by nematodes can be assess from above ground and below grown symptoms
- Nematodes can be extracted from soil and plant parts
- Nematodes that we found are plant parasitic nematodes and free living nematodes
- Nematodes can be identified by morphological characters and molecular analysis

Thank You

5: PRESENTATION OF GROUP 5

Training Workshop on Plant Parasitic Nematode

Ariaq, Bekasi, Indonesia, 23rd Feb to 7th Mar 2020

GROUP 5 REPORT

Mr. Happy Cahya Nugrahana – Indonesia
Mr. Mar J. de Guzman – Philippines
Ms. Umashankari Chandra Segaran – Singapore
Mr. Le Nguyen Tuan – Vietnam
Mr. Surasak Saenkhot - Thailand

Bekasi, 6th Mar 2020

Sample Collection




Collection Site	Mekar Sari Fruit Garden
Date of Sampling	25rd February 2020
Crop	Banana
Crop Stage	Harvest

- Infection causes mass destruction of primary roots and poor anchor-age of banana plants.
- Toppling of plants often occurs during windy conditions when bunches are present, hence the general name of 'toppling disease'.

Collection Site	Mekar Sari Fruit Garden
Date of Sampling	25rd February 2020
Crop	Papaya
Crop Stage	Harvest

Leaves are yellowish or show discolored, greenish-yellow bands along the leaf blades

Collection Site	Mekar Sari Fruit Garden
Date of Sampling	25rd February 2020
Crop	Citrus
Crop Stage	Harvest

Yellowing, stunting, dieback, reduced fruit size, and thinning of the canopy

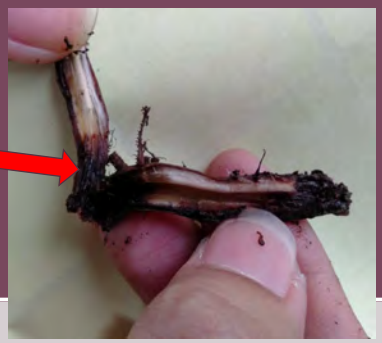
Collection Site	Mekar Sari Fruit Garden
Date of Sampling	25rd February 2020
Crop	Rambutan
Crop Stage	Harvest

Leaf Loss

Collection Site	Mekar Sari Fruit Garden
Date of Sampling	25rd February 2020
Crop	Palm Oil
Crop Stage	Harvest

LESIONS

Collection Site	Mekar Sari Fruit Garden
Date of Sampling	25rd February 2020
Crop	Sweet Potato
Crop Stage	Harvest



Lesion

Jones and Milne (1982) explained that the lateral movement of *R. similis* in the root cortex, as well as secondary colonisation of the vacated cavities by parasitic and saprophytic fungi, enlarges lesions so that the stele may be disrupted.

Galls occur on the primary and secondary roots, whilst distortion of roots and sometimes bifurcation occurs after heavy nematode infections.

Extraction Nematode from Soil Sample
Cobb's Sieving and Baermann funnel methods

Step1

Step2

Step3

Step4

Extraction Nematode from Root Sample
Cobb's Sieving and Baermann funnel methods

Step1

Step2

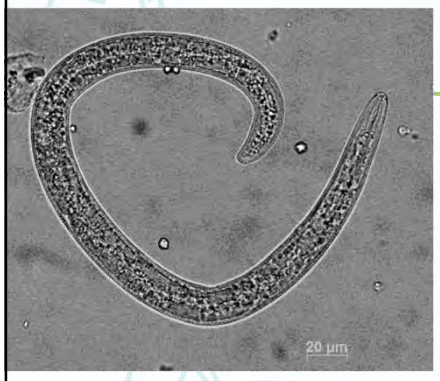
Step4

Step5

Sucrose-Centrifugation Method

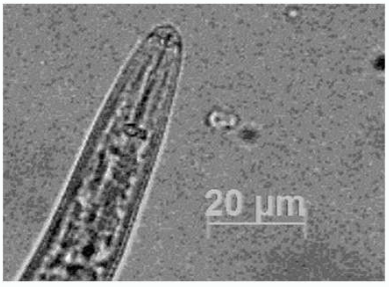
Oil Palm

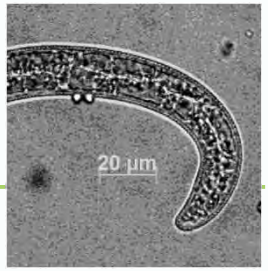
***Helicotylenchus* sp.**



The head region is conoid-rounded, rarely truncate, with a strongly sclerotized labial framework
The stylet is well developed and moderately long (27 µm)

The body 984 µm long, typically arcate, and somewhat spiralinform or C-shaped when dead or relaxed by gentle heat

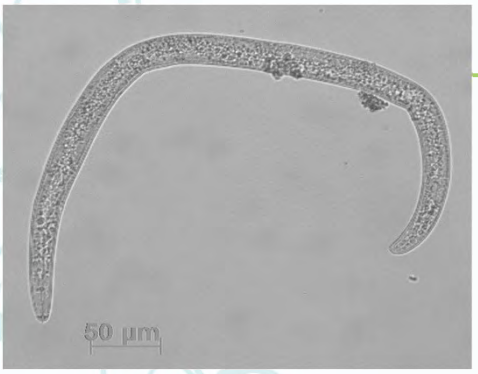




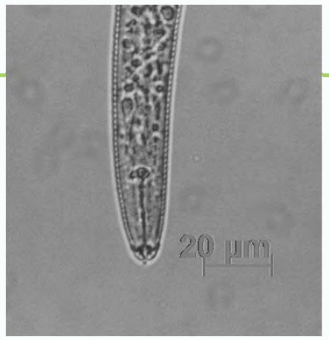
The tip is usually bluntly conical but is occasionally rounded

Rambutan

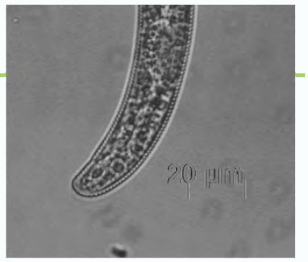
***Helicotylenchus* sp.**



The body 1064 µm long, typically arcuate, and somewhat spiralinform or C-shaped when dead or relaxed by gentle heat



The head region is conoid-rounded, rarely truncate, with a strongly sclerotized labial framework
The stylet is well developed and moderately long (29 µm)



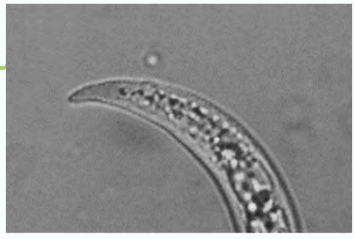
The tip is usually bluntly conical but is occasionally rounded

Citrus

***Rotylenchulus* sp.**

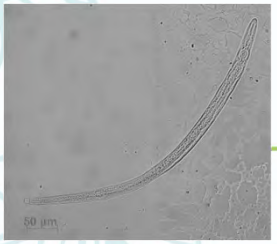
The body 375,56 µm long, and slender. The body assumes an open spiral or C shape when killed by head

The head is rounded, and not set off from the body.
The is short (15 µm) and lightly sclerotized and has rounded knobs.



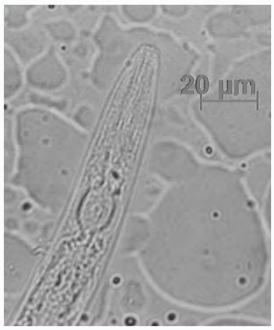
The tail is conoid, pointed

***Aphelenchus* sp.**



The body almost cylindrical, slender, and elongate (988,26 µm long)

The lip region is bluntly rounded to flattened and offset from the body, with a weak cephalic framework.
The nematodes lie almost straight when killed by heat
The stylety is very short (12 µm), with slight thickenings at the base, but without distinct basal knobs.

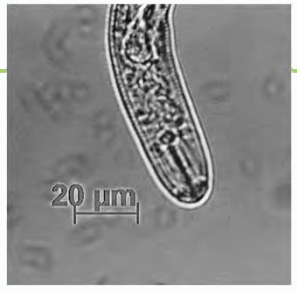


The tail is cylindrical and bluntly rounded

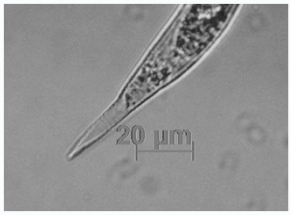
Dry Soil

***Globodera* sp.**

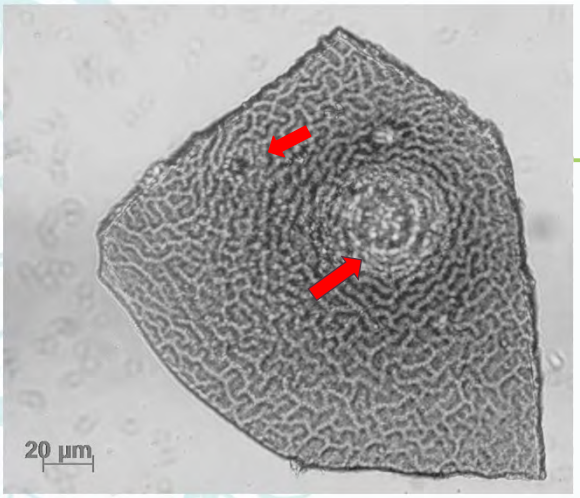
The body 519,24 µm long, and vermivorm



The head region is rounded
The stylet is typically short (21 µm), and the basal knob are rounded



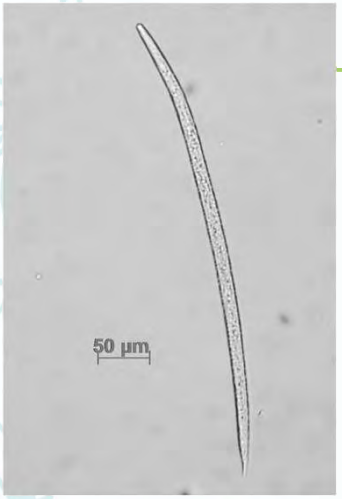
Tail tapering to a finely rounded tip



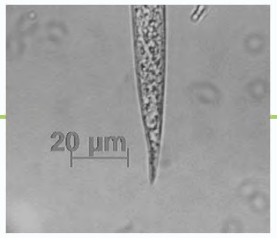
Graneks Ratio= 3,62

Garlic

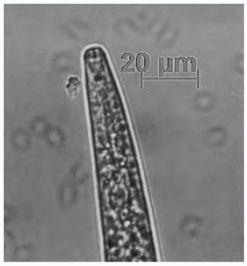
***Ditylenchus* sp.**



The body is long (1241,11 µm), lies straight or slightly curved when relaxed by heat.



The tail is elongate-conoid, has a sharply pointed terminus



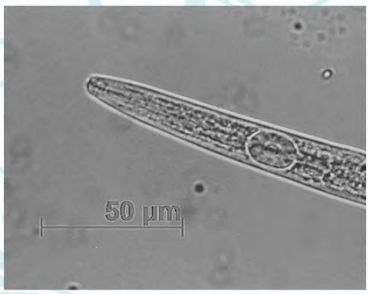
The lips are low, flattened anteriorly, without obvious annules, and not offset from the body, with weak cephalic sclerotization.
The stylet is small (11 µm) and slender, with distinct, well developed basal knobs.

Sweet Potato

***Aphelenchus* sp.**

The body almost cylindrical, slender, and elongate (1048,57 µm long)

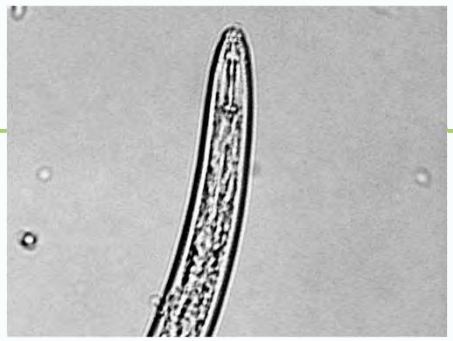
The tail is cylindrical and bluntly rounded



The lip region is bluntly rounded to flattened and offset from the body, with a weak cephalic framework.
The nematodes lie almost straight when killed by heat
The stylety is very short (11 µm), with slight thickenings at the base, but without distinct basal knobs.

***Helicotylenchus* sp.**

The body 1081 µm long, typically arcuate, and somewhat spiralinform or C-shaped when dead or relaxed by gentle heat



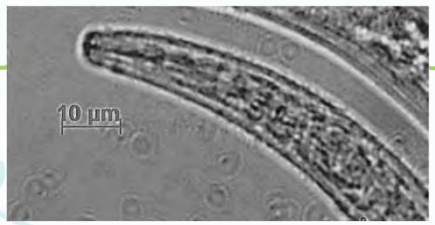
The head region is conoid-rounded, rarely truncate, with a strongly sclerotized labial framework
The stylet is well developed and moderately long (27 µm)

The tip is usually bluntly conical but is occasionally rounded

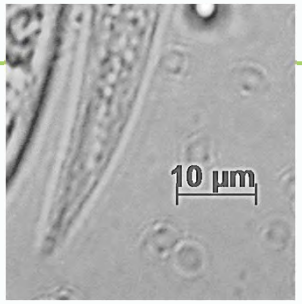
Rice

***Aphelenchoides* sp.**

The body is elongate (964,73 µm long) and very slender



The head is flattened to rounded anteriorly, with a weak cephalic framework, and is slightly offset
The stylet is slender and very short (13 µm), with a needle-like anterior portion and small to minute but distinct basal knobs.

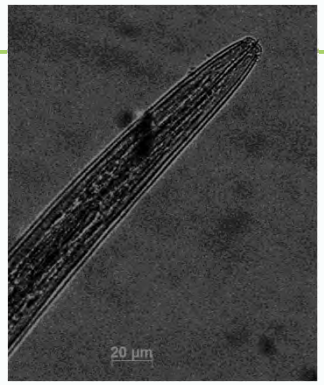


The tail are conoid with mucro (minute spikes) at the tip

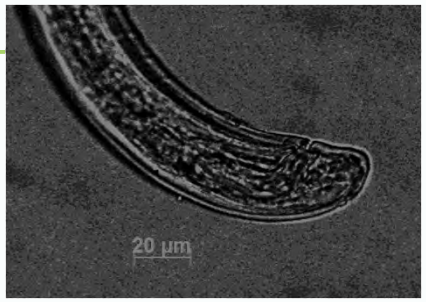
Papaya

***Longidorus* sp.**

The body are very long and slender (1085,94 µm)



The head is not offset
The stylet is very long (204 µm) and formed in two part: the odontostylet and the odontphore which is not sclerotized



The tail being dorsally convex and buntly rounded to conoid and no bursa

***Rotylenchus* sp.**

The body is long (1236,41 µm)

The tail is conoid enveloped by prominent bursa

The head is hemispherical and offset
The stylet is long (42 µm) and well developed with baal knob

Banana

***Helicotylenchus* sp.**

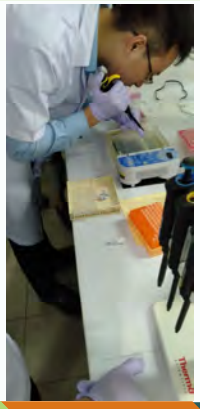
The body 1081 µm long, typically arcuate, and somewhat spiralinform or C-shaped when dead or relaxed by gentle heat

The head region is conoid-rounded, rarely truncate, with a strongly sclerotized labial framework
The stylet is well developed and moderately long (27 µm)

The tip is usually bluntly conical but is occasionally rounded

DNA Extraction and Gel Electrophoresis

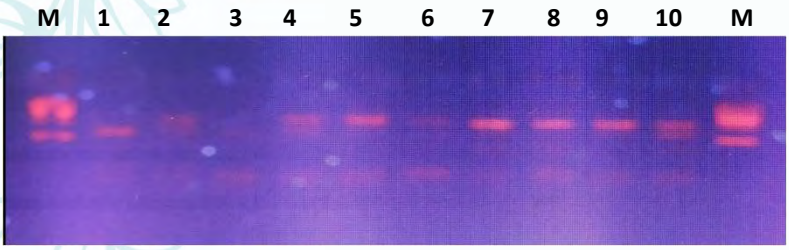
ISOHAIR



DNA Extraction and Gel Electrophoresis

Universal Primer	Sequence	Reference
TW81	GTTTCCGTAGGTGAACCTGC	Tanha Maafi et al. (2003)
AB28	ATATGCTTAAGTTCAGCGGGT	
PCR CYCLING CONDITIONS		
Initial denaturation	94°C - 2 min	
Denaturation	94°C - 1 min	35 cycles
Annealing	50°C - 1 min	
Extension	72°C - 3 min	
Final Extension	72°C - 5 min	

RESULTS OF GEL ELECTROPHORESIS



Based on the results, the DNA extraction and PCR amplification were SUCCESSFUL. The amplified PCR products of all nematode samples showed the same amplicon size.

Lane	Description
M	DNA Marker
1	Banana DNA Extract 1
2	Banana DNA Extract 2
3	Banana DNA Extract 3
4	Banana DNA Extract 4
5	Banana DNA Extract 5
6	Garlic DNA Extract 1
7	Garlic DNA Extract 2
8	Garlic DNA Extract 3
9	Garlic DNA Extract 4
10	Garlic DNA Extract 5

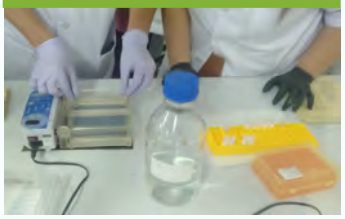
RFLP Method

Step1

Step2

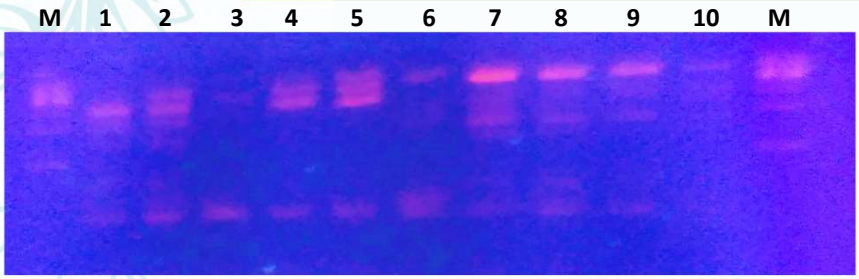
Step3

Step4



Step5

RFLP Analysis



Lane	Description
M	DNA Marker
1	Banana PCR product 1
2	Banana PCR product 2
3	Banana PCR product 3
4	Banana PCR product 4
5	Banana PCR product 5
6	Garlic PCR product 1
7	Garlic PCR product 2
8	Garlic PCR product 3
9	Garlic PCR product 4
10	Garlic PCR product 5

PCR products were used for analysis of restriction fragment polymorphisms. PCR product (8µl) was digested overnight in 10µl reactions with restriction enzyme HinF1 in the supplied reaction buffer. Digested DNA was run on a 3% agarose gel buffered with 1X TBE, stained and photographed as described above.

For banana, samples 1, 2, 4, and 5 do have similar fragments and sizes, except sample 3 due to pipetting error. On the other hand, for garlic, samples 6 to 10 showed similar fragments and sizes.

CONCLUSION

Sample collection is very important for any experiment. In this case, samples were collected at extreme wet conditions. Theoretically, infected soil and root samples will have lots of nematode present but since the soil and roots were wet, there is a possibility where the nematodes may have been washed off. Even though the samples that we collected showed signs of nematode infection, we could not find the targeted nematodes.