Attachment Program: Diagnostics of Plant Parasitic Nematodes in Japan

(JAIF Funded Project on Taxonomic Capacity Building to Support Market Access for Agricultural Trade in the ASEAN Region)

Organizer: Ryukoku University (Otsu Campus), Japan

Duration: 7th February to 4th April 2023

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1. BACKGROUND INFORMATION

The spread of plant-parasitic nematodes causes serious impact in agricultural trade mostly to tropical and subtropical regions in the world for which there is a high demand for agricultural products.

There are various species of plant-parasitic nematodes such as the root-knot (*Meloidogyne* spp.), root lesion (*Pratylenchus* spp.) and cyst (*Heterodera & Globodera* spp.) infesting various farms in Japan.

Japanese government and experts have delivered efforts in mitigating the economical and biological impact of injurious nematodes. Conduct of research on the detection, chemical, cultural, social and quarantine policies have been the essential elements for the proper management of these injurious plant parasitic nematodes in agriculture.

Japan has been known to conduct many intensive research in the field of nematology. Various institutions serve to conduct several studies in the field of nematology whether it is for extension, alien species, biological research, industrial, policy, and control of emerging pests.

2. OBJECTIVES OF THE ATTACHMENT PROGRAM ACTIVITIES

The main objective of the attachment program is to enhance the capacity of plant quarantine officers in the diagnosis or detection of plant parasitic nematodes.

3. ACTIVITIES OF THE ATTACHMENT PROGRAM

Various activities in relation to nematodes were scheduled mainly for the specimen collection, field study, visits of farm, university, quarantine and research facilities. See Table 1.

Table 1. Schedule of activities of the attachment program on the Diagnostics of Plant Parasitic Nematodes in Japan

Date			Program
2023 Feb.	7	Tue	Arrival at Kansai Airport / Move to hotel in Seta
	8	Wed	Move to Ryukoku University Seta campus / Greeting / Learn daily necessities: shops, restaurants, etc.
	9	Thu	Learn laboratory equipment/Extract nematodes
	10	Fri	Learn laboratory equipment/Extract nematodes
	11	Sat	
	12	Sun	
	13	Mon	Staining and picking of Root Knot nematodes
	14	Tue	DNA Extraction and PCR of Nematodes
	15	Wed	Extraction Soybean Cyst Nematodes
	16	Thu	Identify nematodes by PCR-RFLP methods
	17	Fri	Strawberry and Tomato Farm Visit
	18	Sat	
	19	Sun	
	20	Mon	DNA Extraction and PCR of Cyst Nematode
	21	Tue	Identify nematodes by PCR-RFLP methods
	22	Wed	Staining and picking of Root Knot nematodes
	23	Thu	National Holiday
	24	Fri	Identify nematodes by PCR-RFLP methods
	25	Sat	
	26	Sun	
	27	Mon	Examine plants infected by root-knot nematodes
	28	Tue	Farm Visit for the use of soil free from *Hirschmanniella* sp.
March	1	Wed	DNA Extraction and PCR/RFLP of Cyst Nematode
	2	Thu	DNA Extraction and PCR/RFLP of Cyst Nematode
	3	Fri	Root lesion nematode extraction
	4	Sat	Tour to suburban agricultural facilities / Extract nematodes
	5	Sun	Move to Tsukuba / Visit to Central Region Agricultural Research Center
	6	Mon	Move to Yokohama / Visit to Plant Quarantine Office
	7	Tue	Move to Tsukuba / Visit to Central Region Agricultural Research Center / Move back to Shiga
	8	Wed	Root lesion nematode extraction

	9	Thu	Visit to accredited farms for biological augmentation
	10	Fri	Examine plants infected by root-knot nematodes
	11	Sat	
	12	Sun	
	13	Mon	Microscopy and counting of Root Knot nematodes
	14	Tue	Inoculation of lotus plants with *Hirschmanniella* sp. infected soil
	15	Wed	Identify nematodes by PCR-RFLP methods
	16	Thu	Replanting of sweet potato through cuttings
	17	Fri	Identify nematodes by DNA sequencing
	18	Sat	
	19	Sun	
	20	Mon	Identify nematodes by DNA sequencing
	21	Tue	National Holiday / Move to Kumamoto
	22	Wed	Visit to Kyushu Okinawa Agricultural Research Center
	23	Thu	Visit to agricultural farm in Kumamoto
	24	Fri	Visit to Kumamoto University / Move back to Shiga
	25	Sat	Make specimen and microscopy of nematodes
	26	Sun	
	27	Mon	Visit to NARA Institute of Science and Technology
	28	Tue	Visit to Kyoto University/ Introduction to Pine Wilt Nematodes
	29	Wed	Forest Visit / Sampling and Extraction of Pine Wilt Nematodes
	30	Thu	Visit FFPRI Kansai branch / Learn about insect-parasitic nematodes
	31	Fri	Lecture and LAMP – PCR detection of Pine Wilt Nematode
April	1	Sat	
	2	Sun	
	3	Mon	Meeting - Overview of the latter half of training / Presentations on the Results of the Attachment Program/Certificate Presentations
	4	Tue	Move to Kansai airport / Go back to each country

4. DAILY ACTIVITIES

A. LABORAOTRY STUDIES

Various activities were conducted during the conduct of the attachment program in Japan. The laboratory techniques conducted and demonstrated in the Applied Nematology Laboratory were the following:

- •DNA Extraction using ISOHAIR
- •Conduct of PCR using Harris and Power Protocol
- •Gel Electrophoresis
- •Restriction Fragment Length Polymorphism (RFLP)
- •Brilliant Blue Staining of RKN infected plants
- •Picking of stained RKN eggs
- •Extraction of Cyst Nematode (Fenwick Funnel)
- •Quantification of Lesion Nematodes
- •Quantification of *Pratylenchus* spp. using Real-Time PCR

Various nematodes are being controlled and studied in Japan. These plant parasitic nematodes infect many varieties of their host crop. The farmers may opt to cultivate resistant varieties and use nematicides to control the spread of PPNs. The detection is deemed necessary to monitor the presence of PPNs. In relation to plant quarantine, the conduct of PPN detection is crucial in the declaration of the freedom of pests in the agricultural trade. Certain small projects were also conducted for the detection of various plant parasitic nematodes in Japan.

A.1. PROJECT 1: Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR - RFLP) and DNA Sequencing for the Detection of Meloidogyne spp. in various crops in Japan.

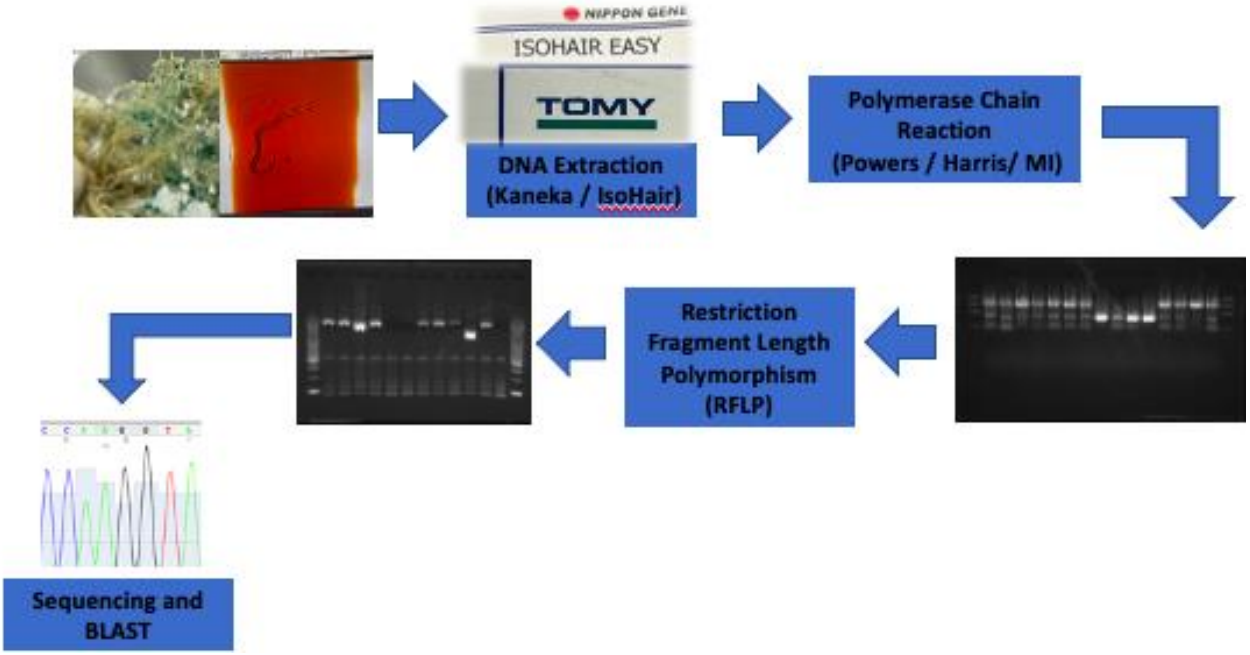

Figure 1. Flow chart of activities on the identification of root-knot nematodes (*Meloidogyne* spp.)

Table 2. Temperature protocol on the DNA extraction using ISOHair Easy.

Step	Temperature (°C)	Time (Minutes)
Step 1	55	30:00
Step 2	95	5:00
Step 3	4.0	Overnight

The PCR reagent kit used was from TAKARA and the primer sequences utilized were the following. Please see Table 3 for the details of the primers in the PCR 1.

Table 3. Polymerase Chain Reaction primers for the detection of Root Knot Nematodes.

Primer		Sequence
Power and Harris Primers, 1994	Forward	5'-GGTCAATGTTCAGAAATTTGTGG-3'
	Reverse	5'-TACCTTTGACCAATCACGCT-3'
Harris et al., 1990	Forward	5'-TAAATCAATCTGTTAGTGAA-3'
	Reverse	5'-ATAAACCAGTATTTCAAACT-3'

Table 4. Polymerase Chain Reaction Protocol using Power and Harris, 1994.

Step	Temperature (°C)	Time (Min)
Initial Denaturation	94	2
Denaturation	94	1
Annealiing	48	2
Extension	68	3
Final Extension	72	10
Incubation	10	Overnight

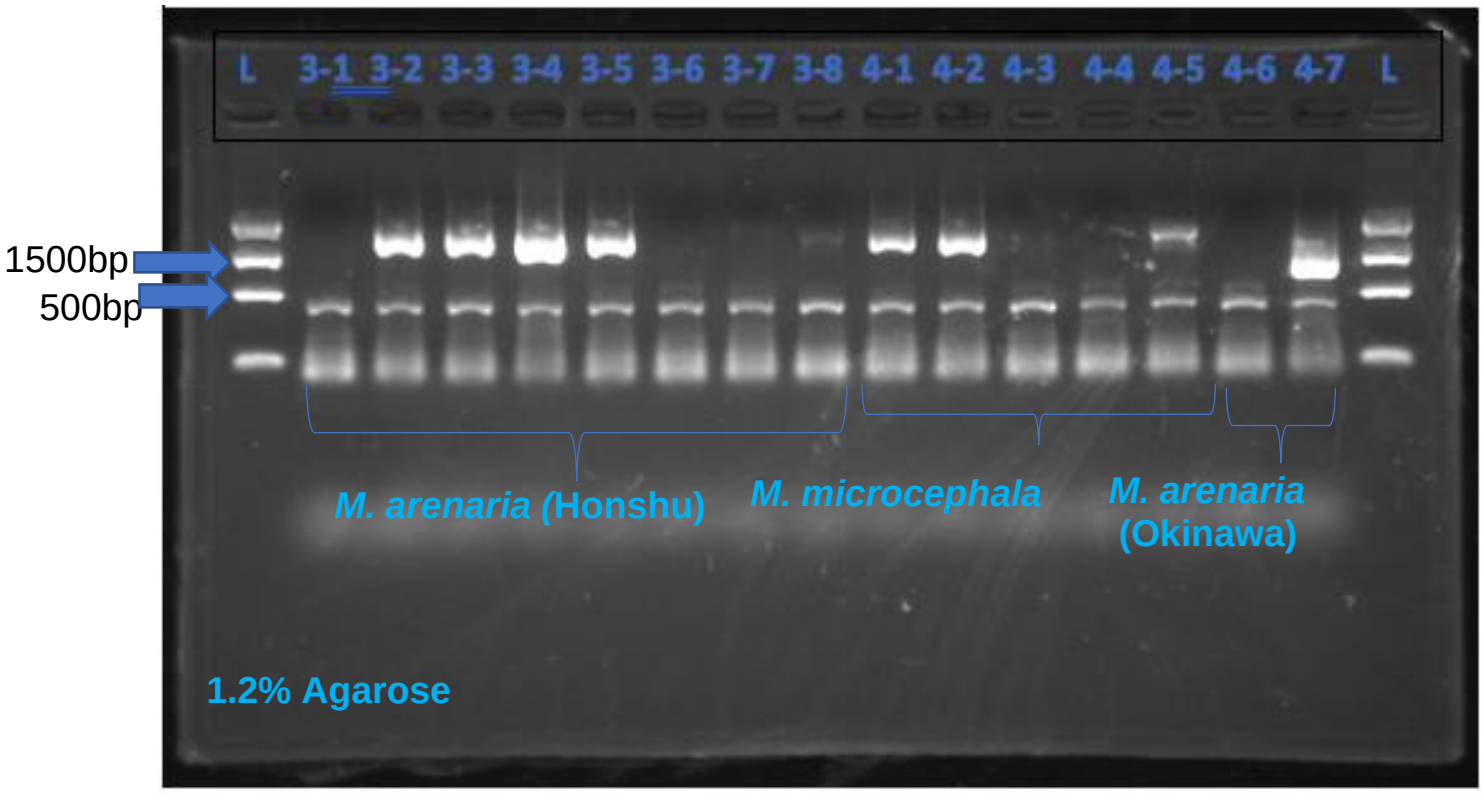

Figure 2. Sample Gel Electrophoresis using Powers Primer

Table 5. Root Knot Nematode species identificaion using PCR and RFLP Analysis

Code	Powers (kb)			Harris (kb)			Species	Remarks
	Expected	PCR	RFLP	Expected	PCR	RFLP		
3-1, 3-2, 3-3, 3-4, 3-5, 3-6, 3-7, 3-8	1.7	1.7	1.7	1.7	1.7	0.9 0.7 ~0.2	*Meloidogyne aeranaria* (Honshu)	Confirmed
4-1, 4-2, 4-3, 4-4, 4-5	1.7	1.7	1.7	1.7	1.7	0.9 0.7 ~0.2	*M. microcephala*	Confirmed
4-6, 4-7, 4-8, 4-9, 4-10	1.1	1.1	1.1	None	None	-	*M. aeranaria* (Okinawa)	Confirmed
4-11, 4-12, 4-13	1.7	1.7	-	1.7	1.7	1.6 ~0.2	*M. javanica*	Confirmed
4-14, 4-15, 4-16, 4-17, 4-18	0.5	0.5	-	*n/a*	-	-	*M. hapla*	-
5-1	0.5	0.5	*n/a*	*n/a*	-	-	*M. chitwoodi*	-
7-2, 7-3	unknown	1.7	*n/a*	*unknown*	*1.7*	~0.2	Unknown	For sequencing

Table 6. DNA Sequence Analysis using BLAST

Sample	Tube Number	BLAST ID	% Identity	BLAST Accession
Mix	29H	*Meloidogyne incognita*	98.94	NC_024097.1
		Meloidogyne javanica	98.69	NC_026556.1
		Meloidogyne arenaria	98.63	NC_026554.1
MC	38H	NO SEQUENCE	N/A	N/A
MY	40H	NO SEQUENCE	N/A	N/A
Camellia	42H	*Meloidogyne incognita*	97.13	NC_024097.1
		Meloidogyne javanica	96.75	NC_026556.1
		Meloidogyne arenaria	96.59	NC_026554.1
Camellia	43H	*Meloidogyne incognita*	99.19	NC_024097.1
		Meloidogyne javanica	98.94	NC_026556.1
		Meloidogyne arenaria	98.98	NC_026554.1

Weeks were spent on the very intense and very detailed picking and molecular identification of root-knot nematodes. It was started from the roots or incubated RKN isolates and were processed for DNA extraction, series of PCR, RFLP and sequencing analyses. DNA Isolates with very distinct bands or quality were chosen for the succeeding steps. Selected DNA isolates were amongst of the batch that had proceeded to sequencing.

Project 1 Conclusion and Comments

Using the Mi, Powers and Harris PCR RFLP identification protocols, we were able to narrow down the identification of certain root-knot nematodes from a single egg mass and infected roots. There were however, samples that showed unspecific bindings observed in the gel. Probably RKN species not covered by the primers used for detection that needs further tests and analysis. Some were undetermined maybe due to lack of DNA or human error. Gel excision followed by purification or PCR clean up prior to DNA sequencing were used to further verify the homology of the samples from a DNA collection.

A.1. PROJECT 2: Real – Time PCR (RT-PCR) Detection and Quantification of *Pratylenchus* spp. in Chrysanthemum

Japan is known to be one of the major producers and consumption markets of Chrysanthemum for its highly valued aesthetic characteristics. *Pratylenchus penetrans, P. pseudocoffeae and P. kumamotoensis* are all known to infest chrysanthemum plants.The use of RT-PCR has been a detection method for absolute and relative quantification of various samples including nematodes.

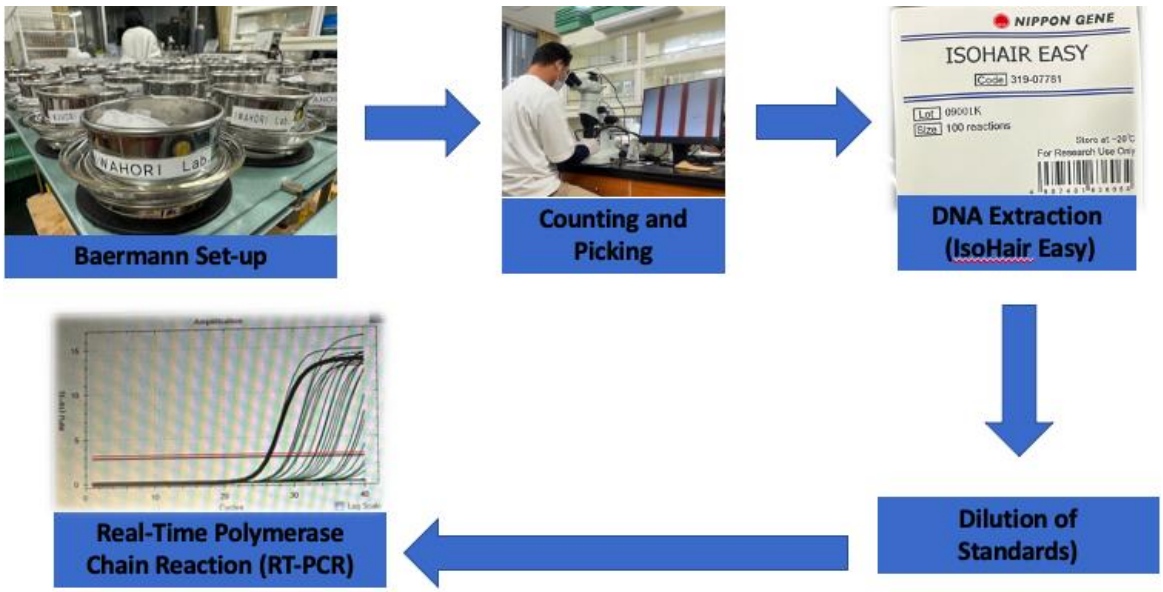

Figure 2. Flow chart of activities on the quantification of root lesion nematodes (*Pratylenchus* spp.) using RT – PCR.

Table 7. Real-Time Polymerase Chain Reaction (RT-PCR) primers for the detection of root lesion nematodes (*Pratylenchus* spp.)

Primer set	Target organism	Sequence
Pkumaf	*P. kumamotoensis*	5'-CGTGAAACCGATGAGATGGAAAC-3'
Pkumar		5'-CAATGGGAGTGC-GGATGAATAC-3'
Pcoff	*P. pseudocoffeae*	5'-TTCCGACCCGTCTTGAAACA-3'
Pcofr		5'-CACATCAGCTCCG-GATGGATA-3'
NEGf	*P. penetrans*	5'-ATTCCGTCCGTGGTTGCTATG-3'
NEGr		5'-GCCGAGTGATCCACCGATAAG-3'

Table 8. Real-Time Polymerase Chain Reaction (RT-PCR) protocol using the above mentioned primers (Table 7) for the detection of root lesion nematodes *(Pratylenchus* spp.)

Stage	°C	Mins	
Initial Denaturation	98	3:00	
Denaturation	98	0:10	39x
Annealing	60	0:30	

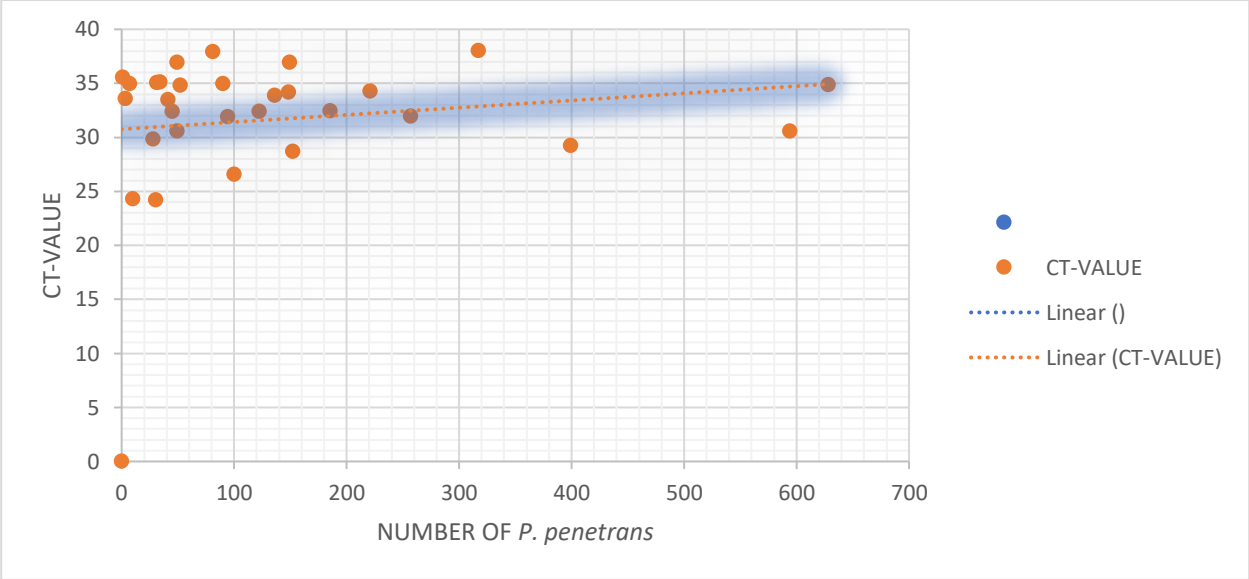

Figure 3. RT-PCR results on the quantification of *Pratylenchus penetrans*

Three very infective root lesion nematodes were observed for its quantity using absolute quantification. *Pratylenchus kumamotoesnsis, P. pseudocoffeae and P. penetrans* were detected and quantified using RT-PCR using their respective primers following the same cycling protocol.

Project 2 Conclusion and Comments

It was observed that there is a positive linear correlation between number of nematodes to its Ct or Cq value in the RT-PCR. The higher the number of nematodes which constitutes to higher DNA concentration, then the faster the amplification of the DNA which gives lower Ct or Cq values. However, for the 20g soil, detection using specific *Pratylenchus* spp. primers with very high nematode density were not responsive enough for its correlation to its count, this may be because of human errors during the counting and/or due to inhibitors/contamination associated with the DNA sample.

On the other hand, for the primer set for the detection of *Pratylenchus pseudocoffeae* unfortunately was not responsive in amplifying the standards and the samples as attested from the first attempt and final attempt of the experiment.

A.1. PROJECT 3: Real – Time PCR (RT-PCR) Detection and Quantification of *Pratylenchus* spp. in Chrysanthemum

Pine wilt nematode is an emerging pest of pinewood globally. The injurious nematode is passed on through an insect vector that transfers it to a host pine tree. There were so many studies on this nematode behaviour. To this day, the use of nematicides is being relied on to control its spread but a bit pricey.

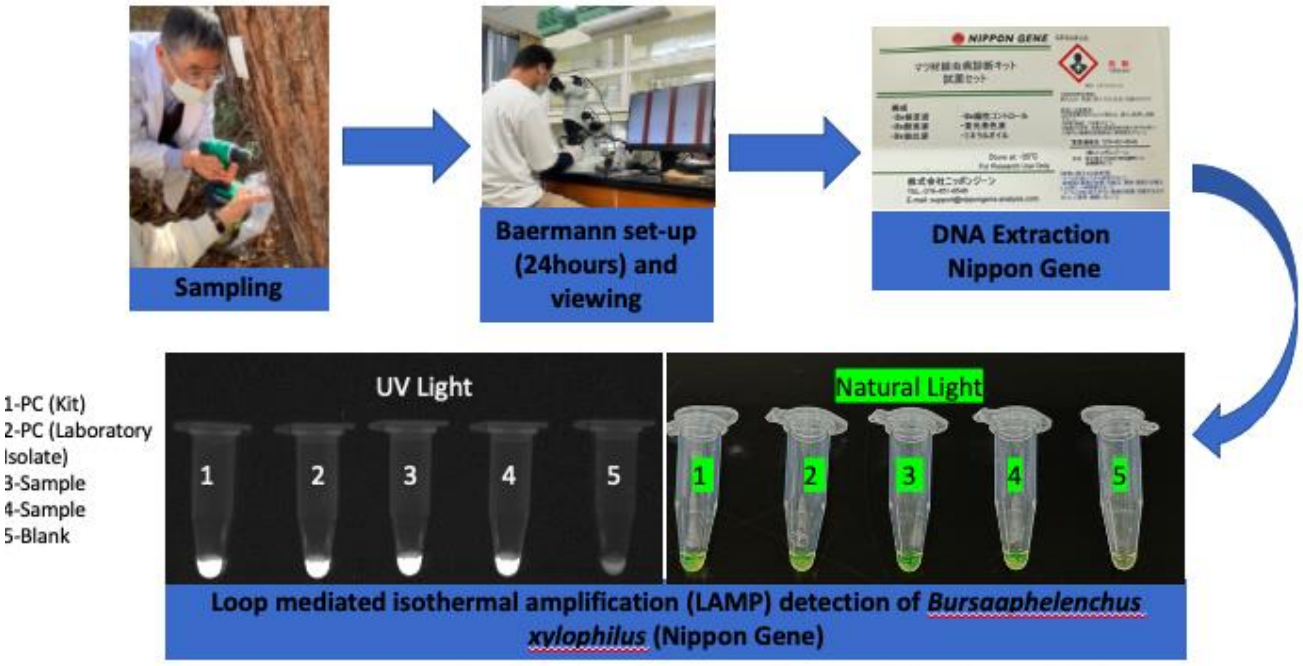

Figure 4. Flow chart of activities on the LAMP – PCR Detection of Pine wilt nematode *(Bursaphelenchus xylophilus)*

Project 3 Conclusion and Comments

The conduct of LAMP detection was successful in determining the presence of the causal pathogen *Bursaphelenchus xylophilus* causing Pine wilt disease. The use of LAMP is a very fast detection method that does not require PCR machine that can be very easily adapted by various parties/users. The method is so sensitive that it is very prone to cross contaminate other samples that may invalidate laboratory results. Right after the last step of LAMP technique, make sure to interpret and document the results to prevent error in analyzing results or place the samples in the freezer (-20°C).

B. FIELD STUDIES

Strawberry and Tomato Farm

Figure 5. Farm visit at Moriyama, Shiga

It was observed in the farm of Takebayashi-san that in order to prevent nematode infestation in his farm is to elevate the growing substrate/farming soil. Continuous monitoring of strawberry plants, bees, application of miticides (if still effective) and other farming practices (fertilization, harvesting, light supply, irrigation).

Quarantine Facility in Yokohama

Figure 6. Yokohama Plant Quarantine visit and presentation

The quarantine officers were able to demonstrate their procedures in plant quarantine and showed us their very operational nematode laboratory and the rest of their facilities that studies detection, host range, and control of nematodes of quarantine concern. They also provide trainings to technical personnel especially to plant quarantine officers that needs reiterations in that specific field.

Research and Quarantine Facility at NARO, Tsukuba

Figure 7. Facility visit at NARO in Tsukuba

They were able to conduct studies of alien species (nematodes). So that once these alien species become reported or existing in Japan, they already have the protocols for the mitigating measures of such. They were so generous enough to tour us in their high-level biosafety laboratory for alien species and totally fully equipped and sophisticated.

Bioaugmentation Farm for Accreditation

We went to a chemical trial and accreditation company. They make use of biofertilizers (for augmentation). And so the plants they were cropping have better quantity and qualities that fits the Japanese market demand or preference.

Research and Extension Facility at NARO – Kyushu Okinawa

Figure 8. NARO – KARC Facility tour and presentation in Kumamoto

The Philippines and Indonesia were able to present our job descriptions as plant quarantine officers. The staff from the facility presented and gave us some tour in their laboratory and demonstrated how they handle clients seeking for knowledge with nematodes and all other extension activities.

Industrial and Farm Visit

Figure 9. Visit in a Shochu company farm

Kirishima is the biggest shochu company in Japan and made from fermented and distilled sweet potatoes. There is a spread of nematodes and infesting many farm lands in the SE Kyushu area or in Miyazaki. There are specifications in the production of sweet potatoes in making shochu. It should not be too big or too small to maintain its other chemical properties such as its starch content. And a certain sweet potato variety is being utilized/propagated for the production of shochu so once nematodes impede in the production then it might pose a serious price change in the production and selling price for the consumers. So the style of the wise farmer that we have visited is to cover its farm, obtain very clean mother materials, avoidance to expose farming inputs outside, mass produce through the sweet potato vegetative properties (because it's very easy to do) and plant in their farm and maintain for fertilization, pest management, and control of environmental conditions.

Visit at Nara Institute of Science and Technology

Figure 10. Visit at University facility studying nematodes

They study clover and beet cyst nematodes through molecule signaling. They are also using model plants to do some proof-of-concept studies. They are also studying parasitic plants (how they recognize hosts, haustoria, how they inhibit the growth and development of their hosts), use of mycorrhiza (a symbiotic fungi) studying disease response, cell fusion when (during infection) cyst nematodes releasing ISC or an effector protein from the stylet of a cyst nematode that opens up one plant cell (plasmodesmata, cell wall, main cell) from one cell to another.

Visit at Kyoto University

Figure 11. Visit at Kyoto University

Kyoto University is one of the imperial universities in Japan. The team were able to have a short visit to a Nematologist, Dr. Yuuko Taiochi who studies Pine wilt nematode. It was demonstrated that the Japanese varieties of Pine wood (such as the Red Pine) were susceptible to the injurious nematode. There were various species of *Bursaphelenchus* and the identification to the species level is very crucial as it may determine which is the one of quarantine concern.

Visit at Forestry and Forest Product Research Institute


Figure 12. Visit at FPRRI.

The team were able to meet Dr. Natsumi Kanzaki who also studied Pine wilt nematodes and keeps strains that are aggressive enough to infect an ordinary pine wood. In his laboratory, we were able to see nematodes with association to insects (symbiotic relationship with insects) but not necessarily tree causing diseases. He also shared to us that all the insects would have carried at least one species of nematode in a very symbiotic manner.

C. SPECIMEN COLLECTION STUDY

Figure 13. Specimen collection processing activities

Samples for observation of root-knot nematodes were obtained from various countries such as Cambodia, Myanmar, Israel and different prefectures in Japan. These samples had gone staining, picking, DNA extraction, PCR, RFLP and sequencing during the attachment program.

Lesion nematodes (*Pratylenchus* spp.) obtained from chrysanthemum experiment and had undergone DNA extraction and RT-PCR analysis for computation

Pine wood samples were obtained at the Ryukoku University. These samples were subjected to LAMP for the detection of Pine Wilt Nematode (PWN) caused by *Bursaphelenchus xylophilus*.

D. OTHER ACTIVITIES

- Other activities include assistance to thesis students in the staining, quantification, extraction and preservation of galls of RKN infected crops.
- Processing and disposal of quarantine laboratory samples and infected soil.
- Extraction and quantification of Cyst Nematode (Fenwick method)
- Assistance to students through exposure of lotus seedlings to *Hirschmanniella* spp. infected soil

5. SUMMARY OF THE ATTACHMENT

The attachment program was very successful enough to enhance their knowledge and laboratory experience in conducting technical activities in relation to intense training in the field of nematology. Participants were able to inoculate, manage, detect and quantify various root-knot nematodes (*Meloidogyne* spp.), lesion nematodes *(Pratylenchus* spp.) and Pine wilt nematode (*Bursaphelenchus xylophilus*). Quarantine measures were also enhanced since there were exposure to different Institutions (NARO, private companies, farm and universities).

6. RECOMMENDATION FOR FUTURE ACTIVITIES

Continuous intense nematode training for other ASEAN crop protectionists and quarantine officers to further increase their knowledge and awareness to new technologies and new pest reports (and detection). Monitoring of staff performance or country performance in the field of nematology should be done in order to assess their improvement after the attachment program. To study more of the other quarantine nematodes of concern that have big economic impact globally to increase awareness and build rapid phytosanitary measures that will facilitate smooth and safer trade of agricultural commodities between trading countries

7. ACKNOWLEDGEMENTS

Foremost, I would like to acknowledge my sincere gratitude to Professor Iwahori for the continuous support of this two-month attachment program in Japan, his patience, enthusiasm and immense knowledge. His guidance helped me in all the time of conduct of experiment with nematodes. I could not have imagined.

My sincere thanks also go to Dr. Soetikno and Dr. Pinili and the rest of the selection committee for offering me this attachment program at the Ryukoku University.

I thank my fellow labmates in the University for the stimulating activities, tiresome times, technical language translation we were helping each one another to succeed in each and everyone's experiment and for all the fan that we have had in the last two months. I also thank my friends to the other universities and institutions for sharing their knowledge in nematode studies and practical techniques.

8. REFERENCES

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9. ANNEXES

N/A

10. PICTURES

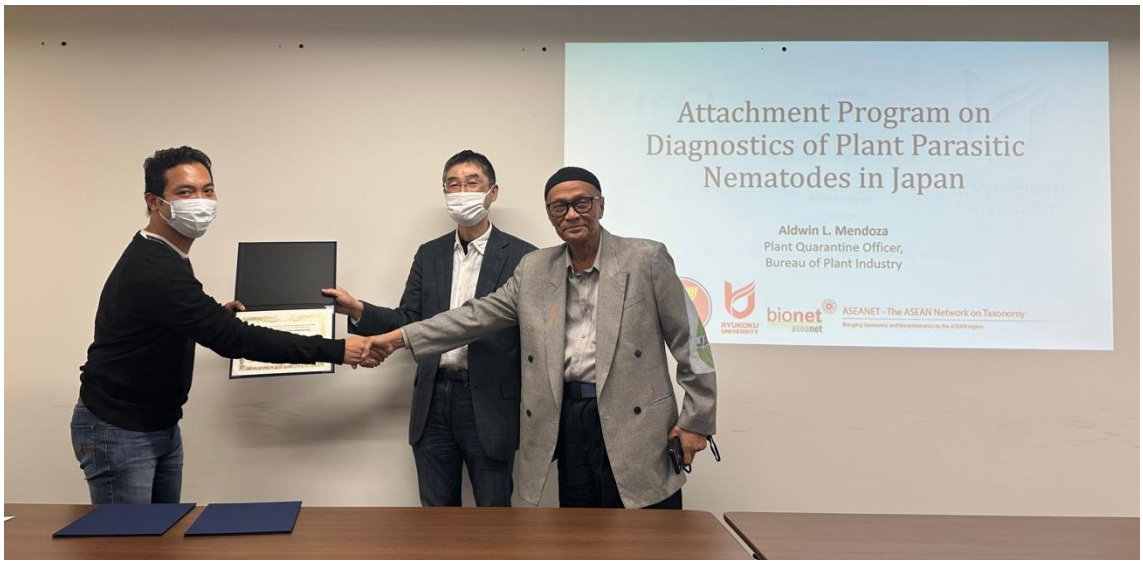

Figure 14. Receiving of certificate of participation by Mr. Aldwin (Kim-San) Mendoza.

Figure 15. Receiving of certificate of participation by Mr. Happy from Indonesia.

Figure 16. Group photo of participants, coordinators and labmates at the Applied Nematology Laboratory.

Figure 17. Group photo of participants and ASEANET-JAIF coordinators