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THESIS

DEVELOPMENT OF THE MOLECULAR MARKERS FOR SPECIES IDENTIFICATION OF ROOT-KNOT NEMATODE INFESTING GUAVA IN THAILAND

KANSIREE JINDAPUNNAPAT

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Kansiree Jindapunnapat 2012: Development of the Molecular Markers for Species Identification of Root-Knot Nematode Infesting Guava in Thailand. Master of Science (Plant Pathology), Major Field: Plant Pathology, Department of Plant Pathology. Thesis Advisor: Mr. Buncha Chinnasri, Ph.D. 84 pages.

Nakhon Pathom and Samut Sakhon provinces are important guava cultivating areas in the central region of Thailand where the guava trees have widely been infected by root-knot nematodes (*Meloidogyne* spp.). Soils were collected from the six orchards as follows: Thaveesak (TV), Khlongjida (KJ), Payoon (PY), Apinya (AP), Namthan (NT), and Khlongton (KT). Tomato plants (cherry cultivar) were planted in the collected soils which had been mixed with sterile soils (3 samples/ orchard). Second-stage juveniles (J2) were hatched and randomly selected at about 20 J2/sample. Consequently, the size and other morphological characteristics of J2 were measured. The results demonstrated that L values of the J2 were lower than the standard value but not a value, c value, and h value which were almost similar to the standards. The examination of the perineal patterns of the adult females of root-knot nematodes reaffirmed that there were two *Meloidogyne* species infecting guava roots. *M. incognita* was found in TV, KJ, and AP orchards whereas *M. enterolobii* (the species never reported in Thailand) existed in NT, PY, and KT orchards. Guava roots infected by *M. enterolobii* showed heavy galls. The females had transparent bodies. The males were present in a high number. To ensure correct identification of the species of nematodes, the molecular techniques based on esterase phenotypes and molecular markers using the following sets of primer marker 1108: 5'-TACCTTTGACCAATCACGCT-3' and C2F3: 5'-GGTCAATGTTCAGAAATTTGTGG-3' were conducted. The results demonstrated that root-knot nematodes collected from NT, PY, and KT orchards had *M. enterolobii* esterase phenotype, VS1-S1, as compared to J1, I1, and F1 phenotypes in *M. javanica*, *M. incognita*, and *M. konaensis*, respectively. The size of DNA bands amplified from nematode samples in NT, PY, and KT orchards was approximately 700 bp, as compared to 1,700 bp in *M. incognita* and *M. javanica*. Development of a molecular marker only specific to the genus *Meloidogyne* was undertaken. The PCR-based technique using the primer set of UNI_16sM and UNI_COIIM yielded an amplicon of approximate 400 bp and 150 bp, respectively, only on *Meloidogyne* samples, but not on *Pratylenchus*, *Rotylenchulus*, *Helicotylenchus*, *Aphelenchus*, and *Steinernema*. For a molecular marker specific to all nematodes in the Phylum Nemata, the PCR product of an approximate 1 kb derived from DNA amplification using the primer Nema_18s was found.

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Student's signature Thesis Advisor's signature

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Kansiree Jindapunnapat

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INTRODUCTION

Root-knot nematode or *Meloidogyne* spp. (Goldi, 1892) is an important plant-parasitic nematode of many economically important crops due to its polyphagous nature. *Meloidogyne* is of Greek origin and means "apple-shaped female." Root-knot nematodes widely distribute throughout the world, especially in the Tropics and Subtropics, because they are highly adapted to be obligate parasite of various crops ranging from tubers, vegetables, fruits, flowers, ornamental plants and field crops. (Sasser and Carter, 1985). Plants infected by root-knot nematodes generally exhibit stunting, wilting under moisture stress, die back, and galled roots. As a result, death, decreased yields or low quality products usually followed. Root-knot nematodes are endoparasitic; moving intercellularly in plant roots and sedentarily feeding in plant vascular vessels. Worldwide yield losses caused by root-knot nematodes were about 5-12% (Sasser and Freckman, 1987). In Germany, 55% of tomato yields were reportedly reduced due to nematode infections while a 75% reduction in cowpea and a 22-84% decrease in green bean were evident in Nigeria and India, respectively (Ali, 1997).

Thailand is a country of limitless varieties of tropical and subtropical fruits. Guava is produced mainly in the central region of Thailand. Root-knot nematodes, *Meloidogyne* spp., are important parasitic nematodes which have been diagnosed in numbers of plants (Sontirat, 1989). Over 80% of the crops including economic field crops, vegetables, and fruits risk have been damaged by a widely distribution of root-knot nematodes in Thailand.

Root-knot nematodes have been widely found attacking chilli in Ubon Ratchathani province and potato in Tak province, which situated in northeast and west region of Thailand, respectively. Severe problems were reported in chilli and potato (Sontirat *et al*., 1999). In 2006, Sukhakul noted that the damage caused by root-knot nematodes on guava was firstly reported at Bangkruai district, Nonthaburi province in 1987 and 1990. The symptoms included the stunting and yellowing of guava trees and leaves, respectively. In 1992 and 1997, the infestation of root-knot nematodes to guava plantations (cultivars: Klom Sali and Pan Sithong) was observed in Sampran district, Nakhon Pathom province. Guava plants infected by the nematodes showed overall decline and loss in production. In 1999, root-knot nematodes (*M. incognita*) were identified as the causal agent of the declination in guava trees in Cha-am district, Petchburi province. Recently, in 2003 and 2004, *M. incognita* also caused severe damage on guava in many areas of Sampran district, Nakhon Pathom province.

Identification of root-knot nematodes could be accomplished using several methods. One of the techniques utilizes the gap between the Cytochrome Oxidase Subunit II (COII) and 16s Ribosomal RNA in the mitochondrial genome (Power and Harris, 1993). Efficient molecular markers have been developed in root knot nematodes by comparing the nucleotides between both regions. Our goal was, therefore, aimed at developing a molecular marker to identify root-knot nematodes in guava in Thailand. Rapid and accurate identification of root-knot nematodes will allow us to detect and diagnose the nematodes in guava seedling stage, thereby preventing a wide spread of nematodes in guava planting areas.

OBJECTIVES

1. Survey and identify root-knot nematodes (*Meloidogyne* spp.) causing root-gall diseases in guava in the central region of Thailand.

2. Select methods and DNA markers which are convenient and efficient in identification of root-knot nematodes (*Meloidogyne* species) in guava in Thailand.

3. Develop markers of the two mitochondria genes Cytochrome Oxidase Subunit II (COII) gene and 16s ribosomal DNA gene specifically targeted to the genus *Meloidogyne*.

LITERATURE REVIEW

1. Root-knot nematodes – *Meloidogyne enterolobii* of Guava

1.1 Histology and crop damage

Guava, scientific name as *Psidium guajava*, is in the Family Myrtaceae which is similar to roseapple (*Eugenia javanica*) and eucalyptus gum (*Eucalyptus citriodora*). Guava is originated in Central America, Peru or Mexico, and spread throughout the world including Asian countries by the Spanish and Portuguese explorers. Guava has become almost a staple fruit in many countries including Thailand and Vietnam (Roy, 2011). Guava is a small-to-middle shrub tree. Thai researchers have developed many guava varieties to be planted in the country in which Klom Sali and Pan Srithong varieties have been extensively cultivated. Nakhon Pathom and Samut Sakhon provinces in the central region are important guava-planting areas with 22,753 rai or 8,987.44 acres of planting, as compared to 43,249 rai or 17,083.36 acres of the whole country areas for guava cultivation. In Southeast Asia, Thailand is a larger producer (Kwee and Chong, 1990). The whole country production in 2009 was 114,186 tons (2,827 kg per rai or 1,116.67 kg per acre) (Office of Agricultural Economics, 2009). In the USA, Hawaii is the major producer with a state-wide production of 46,000 tons (Davis, 1988).

Guava is a satellite fruit because it is successfully adapted to tropical and subtropical areas. Even though climates are cold or dry, guavas could tolerate. The optimal conditions of guava cultivation are at the temperature between 7-16 ℃ and pH 4.5-8.2. Guava contains 112 calories 4.2 g of protein, 1.6 g of fat, and 23.6 g of carbohydrates per serving. Guava also contains over 5 times Ascorbic acid (Vitamin C) than citrus and essential vitamins rich as well as Beta-carotene (Vitamin A), and Folic acid (Vitamin B_9), and the dietary minerals, potassium, copper and manganese (US Department of Agriculture National Nutrient Database, 2008). Fresh guavas were also exported (in the report of Thailand foreign agricultural trade statistic, 2009). In

2008, it had the quantity of 1,567 tons and valued 32,500,000 baht. In 2009, the quantity increased to 2,001 tons and valued 31,621,000 baht. However, most guavas are produced for domestic consumption. One of the reasons is that they have low cost but high medicinal properties. Particularly, the leaves have quercetetin compounds capable to inhibit microorganism to heal wound, diarrhea and dysentery, hold odor and prevent cancer. Guava capsules, a commercial product of the Government Pharmaceutical Organization of Thailand (GPO) containing ground guava leaves, are recommended to administer to the patients with diarrhea at the rate of 3-5 pills (260 mg/ pill) per 6 hrs of time. The results are satisfactory (Dedduang, 2010).

Guava trees have pests (caterpillar, aphid, fruit worm and fruit fly) and important diseases. The fungi, *Fusarium solani* cause wilt disease and bring about gradual decline to undernourishment and death. *Colletotrichum gloeosporioides* causes anthracnose at pre- and postharvest and bring dark colored, sunken and necrotic lesion under high rainfall and humid condition. *Puccinia psidii* cause rust disease with the distortion, defoliation and reduced growth on expanded leaves. Nematodes are widespread and hard to manage due to being as soil bornes. Root lesion disease caused by *Rotylenchulus, Helicotylenchus* and *Tylenchorhynchus* brings about small to large lesion symptoms around the roots. Root knot disease (Fig 1) caused by *Meloidogyne* brings about chlorosis, stunting, premature wilting, nutrient deficiencies, and especially, gall and distorted roots. The guavas are cultivated by grafting stocks or seedlings (Michael and Aaron, 2006; Sontirat, 1998). Gall disease caused by root-knot nematodes (*Meloidogyne incognita, M. javanica, M. arenaria, M. hapla, M. enterolobii* or *M. mayaguensis*) is economically important. *M. incognita* and *M. enterolobii* are pests which mostly found in worldwide guava field including Thailand.

In Brazil, *Meloidogyne mayaguensis* is a major constraint to commercial guava production in commercial and decimated around five thousand hectare in several Brazilian State, causing direct monetary losses of an approximate US$ 61 million (Pereira *et al*., 2009; Gome *et al*., 2009).

In Florida, *Meloidogyne mayaguensis* was found infecting several ornamental plants grown in nurseries and greenhouses and also in guava in 2001 (Brito *et al*., 2004).

In Cuba, guava production has declined during the past quarter century due to increasing pressure from *M. incognita*, *M.arenaria*, *M. hapla*, *M. javanica* (Cuadra and Quincosa, 1982; Rodriguez *et al.*, 1985; Fernandez Diaz Silveira; Ortega Herrera, 1998 and El-Borai and Duncan, 2004).

In Southern Vietnam, guava seedlings showed both above and below ground symptoms caused by *M. enterolobii* such as leaf browning, stunting, leaf drop and death, ground symptoms is galled roots (Iwahori *et al*., 2009).

In Thailand, guavas exhibited decline, leaf drop, yellowing, chlorosis and galled roots caused by *M. incognita* (Sukhakul, 2006). In 2010, Sasnarukkit, studied nematodes population density and root-knot disease rating in guava fields in Samut Sakorn and found significant correlation between the decline in guava yields and galled root levels.

1.2 Root-knot nematodes: *Meloidogyne* spp.

Root-knot nematodes are obligating parasitic of nearly every species of higher plant. They are distributed worldwide and could survive during unfit environmental conditions by developing resting stages so called diapauses or quiescence, causing more complicated control and management than other plant-parasitic nematodes. *Meloidogyne* spp. caused worldwide damage at about 10.7% (i.e. vegetable crops and fruit crops) whereas in economic crops such as field crops and ornamental plants, the damage in both quality and quantity could approximately be 14.0%. There were losses up to 77,689 million dollars per year (Sasser and Freckman, 1987, Sontirat, 1989, Jumroonpong, 1989). Root-knot nematodes, especially, bring about most loss of the product (10-15% from the whole losses product of pests). The most losses occur with

vegetable crops (Valdex, 1979, Sontirat *et al*., 1978, Sontirat, 1981, Sontirat, 1982, Jumroonpong, 1989). Damage levels depend on the reaction of plants to root-knot nematodes and differ greatly according to plant species and cultivars, resulting in reduced growth, yield, lifespan and resistance (Ploeg *et al*., 2007).

Root-knot nematodes have become importantly problematic to crop production due to the 3 following characteristics of the nematodes (Lamberti and Taylor, 1979).

1. Root-knot nematodes are a polyphagous organism in both monocots and dicots, thereby readily spreading to neighboring plants, and adopting the dormancy in order to avoid unfavorable conditions and be inoculative in next seasons.

2. Root-knot nematodes have 2n and 3n (polyploidy) in chromosome number evolutionarily making the nematodes to adapt and survive in harsh environments, and to infect many plants.

3. The reproduction is parthenogenetic. Female are able to produce offsprings without fertilization from males. The meiotic parthenogenesis and mitotic parthenogenesis cause root-knot nematodes to increase rapidly for only 1 month. One adult female produces 300-1,000 eggs Taylor and Sasser (1978) calculated if one female produces 500 eggs and survive 5% in 4 generations, there could be about 390,625 nematodes (noochuay, 1985).

These qualifications render wide dissemination and distribution and high yield losses caused by root-knot nematodes worldwide. Additionally, nematodes are direct and indirect parasites, which could be the carrier of other pathogens; for example, fungi-*Fusarium* sp. and bacteria-*Pseudomonas* sp.

In Thailand, seven species of root-knot nematodes (*Meloidogyne arenaria*, *M. exigua*, *M. graminicola*, *M. incognita*, *M. microcephala*, *M.javanica* and *M. naasi*) have been reported (Boonduang and Pliansichai, 1986; Chunram, 1972; Cliff and Hirschman, 1984; Sontirat, 1981; Toida *et al*., 1996). For worldwide distribution, 4 major species; three tropical species, *M. incognita, M. arenaria* and *M. javanica*, and one temperate species, *M. hapla*, are normally found as these species (Chitwood, 1949) have an extensive host range and are globally distributed. In Thailand, *M. incognita* and *M. javanica* have been mostly found in northeastern part. *M. incognita* usually distribute more widely than *M. javanica*, some areas such as Ubon Ratchathani province, however, only *M. incognita* were reported (Choksan *et al*., 2006).

Taxonomy of root-knot nematodes

Phylum Nematoda; Potts, 1932
Class Secernentea
Order Tylenchida; Thorne, 1949
Superfamily Tylenchoidea; Orley, 1880
Family Meloidogynidae; Skarbilobich, 1959
Sub-Family Meloidogyninae; Skarbilobich, 1959
Genus *Meloidogyne*; Goeldi, 1892
Species *incognita*
javanica
arenaria
hapla

Life cycle (Fig 2)

Normally, root-knot nematodes produce approximately 300-1,000 eggs, and have with oval shape, smooth and clear bodies. The length and width are about 0.08 and 0.04 mm respectively. Eggs are produced into gelatinous matrix, which is glycoprotein matrix produced by rectal gland in female, and found on root surface or

inside galled roots. After embryogenesis, the egg develops to have nuclei and the cells are divided to 32 cells. There is metamorphosis process in the cells to develop into first-stage juveniles (J1) in egg which will be moult to the infective stages (J2) with the length of 0.3-0.4 mm. Hatch of J2 is primarily dependent on temperature and moisture that are activator from roots. J2 reach and invade root tip intercellularly by using stylet to penetrate root cell wall, causing cell break-down by nematode cellulolytic and pectolytic enzymes. Results are extended size of root tip, growth interruption, and temporary dormancy between the cells in the cortex, separation along the middle lamella, and swelled cells. By that time, J2 accumulate food and use oxidoreductive enzymes, such as chorismate mutase, CLAVATA3-like peptides, ubiquitin pathway, cytokinin and nodulation factors, to activate the metabolism of plants, to initiate permanent feeding sites, and to develop themselves into third-stage juveniles (J3), fourth-stage juveniles and adults. Normally, sex determination of root-knot nematodes is female. By contrast, if unsuitable environment, insufficient food or in quiescence state, root-knot nematodes will develop into males (Fig 3C) to survive in adverse situation. In this period the interaction between plants and root-knot nematodes will determine whether plants are resistant or susceptible. The feeding sites are differentiated specialized nurse cells. They, also called as giant cells, are produced when normal cells proliferate hypertrophically and hyperplastically. The giant cells are hyper-active, induced by root-knot nematodes, divide without forming new cell walls or in the absent of cytokinesis (cell wall division does not occur plate vesicle coalescence during Telophase) in mitosis process. They are most likely formed after repeated endomitosis, causing multiple nuclei within a cell in a few days. Gall size depends on host plant and environment. Life cycle of root-knot nematode period normally takes 17-57 days (Karssen and Moens, 2006).

Figure 1 Root gall symptoms characteristic of infection by root-knot nematodes (*Meloidogyne enterolobii*)

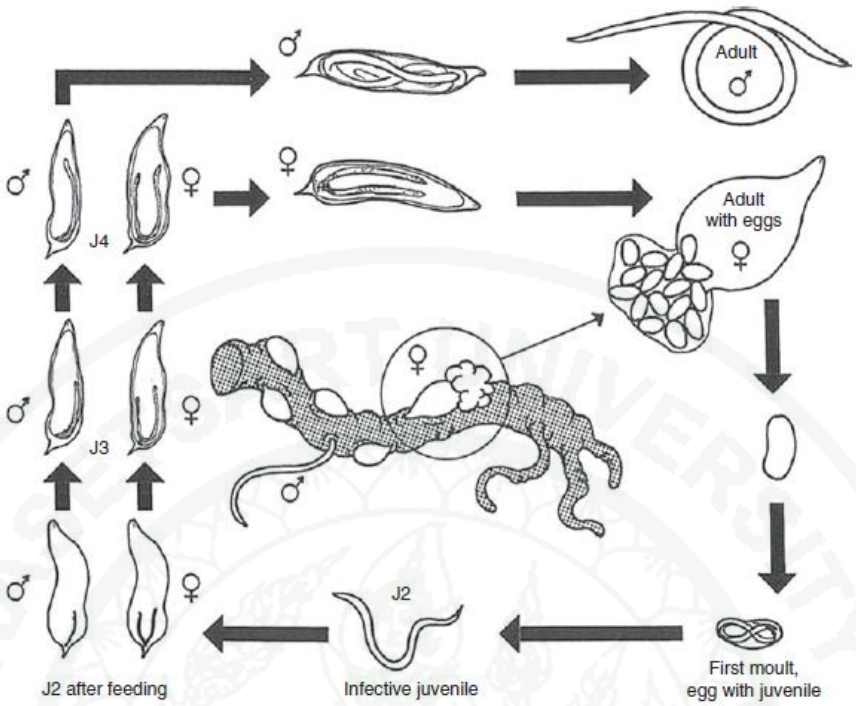

Figure 2 Diagramatic life cycle of root-knot nematode, *Meloidogyne*. J2: second-stage juvenile; J3: third-stage juvenile; J4: fourth-stage juvenile; adult

Source: Karssen and Moens (2006)

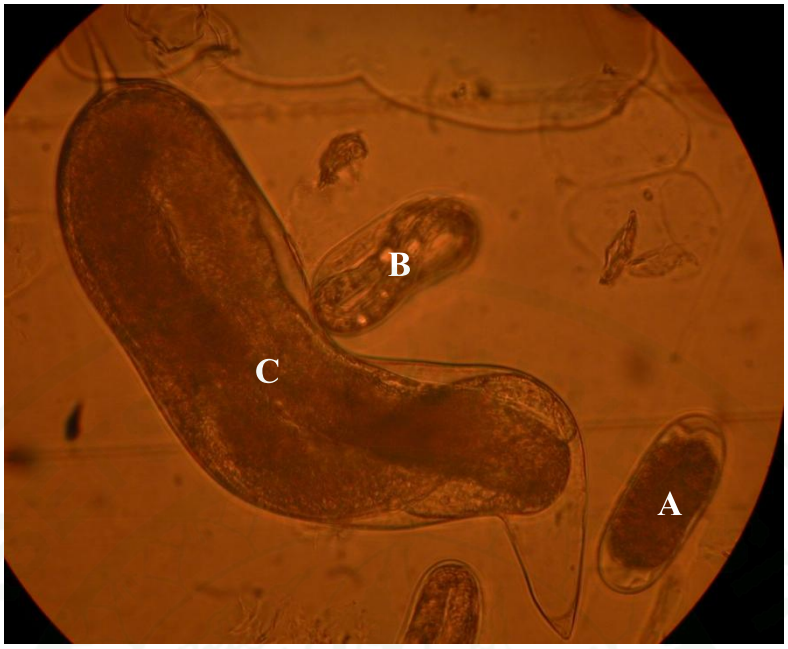

Figure 3 Development stages of root-knot nematode, A: egg B: first-stage juvenile (J1) moults in egg sac C: fourth-stage juvenile (J4) of male *Meloidogyne* sp.

1.3 Plant and root-knot nematodes interaction

Second-stage juveniles (J2) damage plant roots using their stylets, secretions, and pharyngeal glands. Pathogenicity determination (pathogenicity factor and virulence factor is also important for successful nematode penetration). Secretions from root-knot nematodes contain major effectors of parasitism such as β-1,4-endoglucanase (cellulase), pectate lyases and polygalacturonase (pectinase), xylanases (hemicellulase), expansin. All secretions (effectors) are produced by pharyngeal glands, two subventral glands active in the damaging stage. After J2 penetrated to roots and injected the secretions to breakdown the root cells, host plant responds to the pathogen attack by the mechanisms such as physical barriers (cuticle, wax, suberin) and chemical barriers (reactive oxygen species: ROS, phenolic compound, superoxide). Plant responses are dependent on the recognition of the receptor on the cell surface whether they are incompatible interaction (host resistance) or compatible interaction (host susceptibility). In case of host resistance, rapid recognitions between host and pathogen occur and create signal transduction to plant nucleus to activate defense mechanism to invader (pathogen). The plant defense mechanism also comes in the form of structural barrier by depositing callose in plant vascular tissues or developing hypersensitivity reaction (HR) which involves moving-off of plant nucleus from the invasion area, accumulation of toxic substances (brown granules) in the cytoplasm, breaking up of the nucleus, and finally producing cell death to confine and to deprive the nutrient absorption of the pathogens. Chemical barrier has also been reported to assist limiting pathogen progression in plant cells. Production of phytoalexin compounds or pathogenesis-related proteins are such inhibitory compounds.

After root-knot nematodes penetrate and became established in host (plant cells) then J2 undergo metamorphosis until the adult stage. During this period the nematodes also produce secreted enzymes for the purpose of absorbing nutrients and reproduction. The secretions (adult nematodes females) are produced by the pharyngeal gland (one dorsal gland) for feeding site induction and maintenance. Nematode feeding sites (NFS) are formed by multiple rounds of shortened cell cycles or repeated (acytokinetic) mitosis causing sending of signals through signal transduction pathways and producing several transcription factors. Therefore NFS have multiple nuclei within one cell. The differences between giant cell (root-knot nematode) and syncytia have been reported. Through S (synthesis) phase or repeated DNA synthesis or endoreduplication causing the increase in DNA in each nucleus and then the breakdown of the neighboring cell wall. The resultant feeding cells have multiple nuclei similar to those induced by root-knot nematode (Fig 4).

The nematode signals for feeding site induction

1. Chorismate mutase (CM) is the enzyme for converting chorismate to prephenate, which affects plant hormone (auxin: IAA, salicylic acid: SA) that has been implicated in early feeding site development or affects. The production is suppressed by plant defense compounds (systemic defense signaling pathways).

2. CLAVATA3-like peptides work with CLAVATA1 and CLAVATA2 to regulate the balance between cell differentiation and cell division in the shoot apical meristem. The study showed, proliferating division of protoplast cells (hyperplasia) occurred in the presence of CLAVATA 3-like peptides.

3. Ubiquitin pathway proteins are small protein found in cyst nematodes (but absent in root-knot nematodes). They are associated with cell cycle regulation or protein degradation via going through multiple S-phases with no intervening mitosis so that found hypertrophic nuclei are generated.

4. Cytokinins are plant hormones that play an important role in plant developmental pathway and present in the secretion of root-knot nematodes (lower level in cyst nematodes). Zeatins are the major cytokinins found in root-knot nematodes and repeatedly influence the G2 to M phase transition of the cell cycle.

5. Nodulation factors found in the secretion of root-knot nematodes contain a nodulation L (NODL)-like protein. They correlate with developing giant cells and also being important signals in nodulating bacteria.

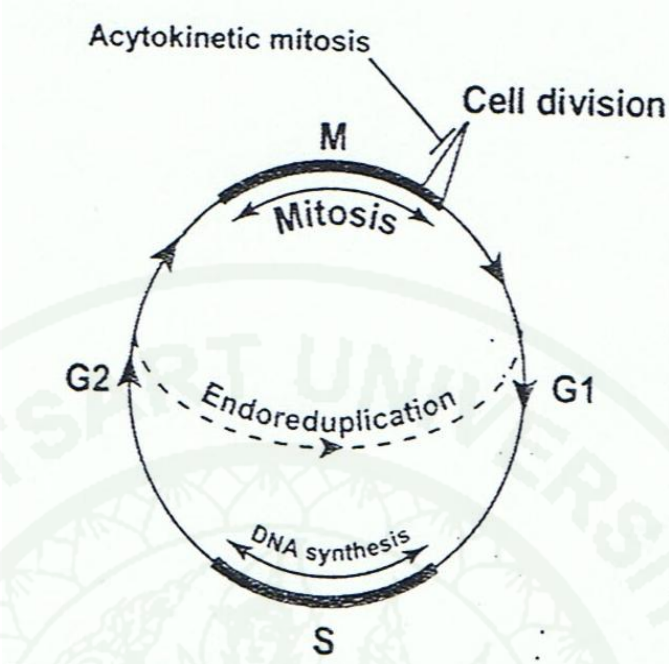

Figure 4 The standard eukaryotic cell cycle consists of four successive stages (G1-S-G2-M). Cyst nematodes activate and repeat the cell cycle in S (synthesis) phase or endoreduplication without mitosis. Root-knot nematodes induce repeated nuclear division but no cell division (acytokinetic mitosis).

Source: Gheysen and Jones (2006)

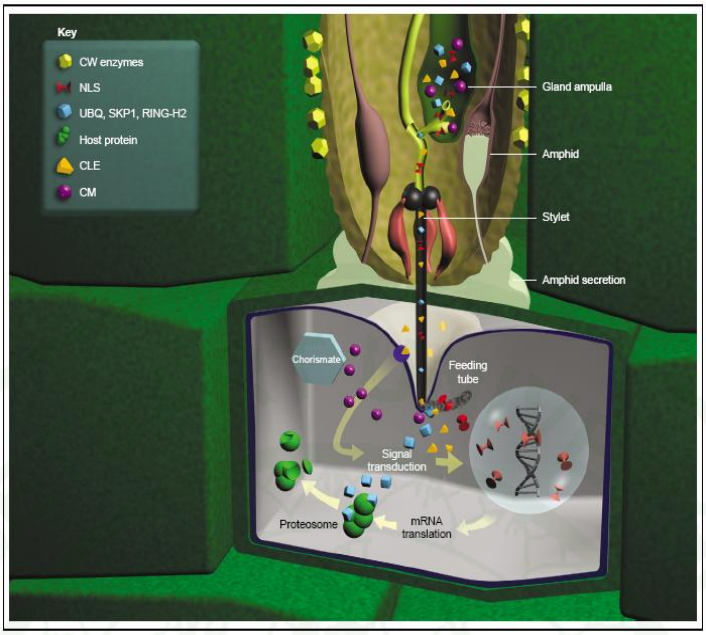

Figure 5 Interaction between a nematode and a host plant cell. The nematode creates signal transductions for nematode feeding site formation (NFS).

Source: Davis and Mitchum (2005)

1.4 General Morphology and Biology of root-knot nematodes

Second-stage juveniles (J2) are a damaging and initially sedentary stage. The length of J2 range approximately between 250-600 µm. They have biological systems (Fig 5) almost similar to general higher animals. Generally, 4 main biological systems are found in root-knot nematodes; (Sontirat, 1998; Eisenback and Hunt, 2009).

1. Digestive system (initial stomata to anus or cloaca) is necessary to help nematodes hatch from egg, penetrate a host root and maintain host parasite relationship. Digestive system is divided into 3 major parts; **Stomodeum** is used to penetrate, damage plant cells, and absorb food. Structurally, stomodeum is composed of lip, stoma, stylet or spear, esophagus and esophageal gland (one dorsal and two subventral glands); **Mesenteron** play an important role in obtaining nutrients and accumulating food (glycogen and lipids). It is composed of made up intestine (extends from the base of the isthmus to the base of metacorpus); **Proctodeum** excretes waste and houses reproductive organs of male consisting of rectum, anus, spicule and rectal gland (producing gelatinous matrix).

2. Nervous system helps in nematode movement and is mainly comprised of nerve ring and sensory organs. In male, nervous system assists nematodes to migrate out the root system and find suitable mating, while in female, it facilitates the movement of nematodes' head-ends for correct feeding. Nerve ring which acts as the main operation system is connected to four cords lining along the nematode body. Nervous system is divided into three parts as follows: **Head** which houses important sensory organs so called "amphids" These sensory organs perform as chemo-receptors for nematodes to locate plant roots. **Epidermal** acts as the tacto receptor through the celia positioned on nematode cuticle. In addition, hemizonid and cephalid are also sensory organs located in the excretory pore region. However, there is no real function for these organs that have ever been reported. **Tail** consists of a sensory organ called "phasmid". It is situated in the area between nematode anus and tail tip and probably acts as a tactoreceptor.

3. Secretory-Excretory system is not complete and plays a role in regulating the equilibrium of water in nematode body. This system is located ventrally in cuticle. In J2 and male, pore is located near metacarpus while in female it is located anterior to median bulb or stylet base.

4. Reproductive system is more or less complete for nematodes. Root-knot nematodes are readily reproduced by mitotic parthenogenesis and therefore increase in number in a short period of time. The offspring of root-knot nematodes mostly are females. The reproductive system starts developing from the genital primordium (origin point) when nematodes are at initial second-stage juveniles (J2). Subsequently, it progresses as nematodes molt into 3, 4 and adult stages. Nematode sex is also determined by stress (environment, food, etc.).

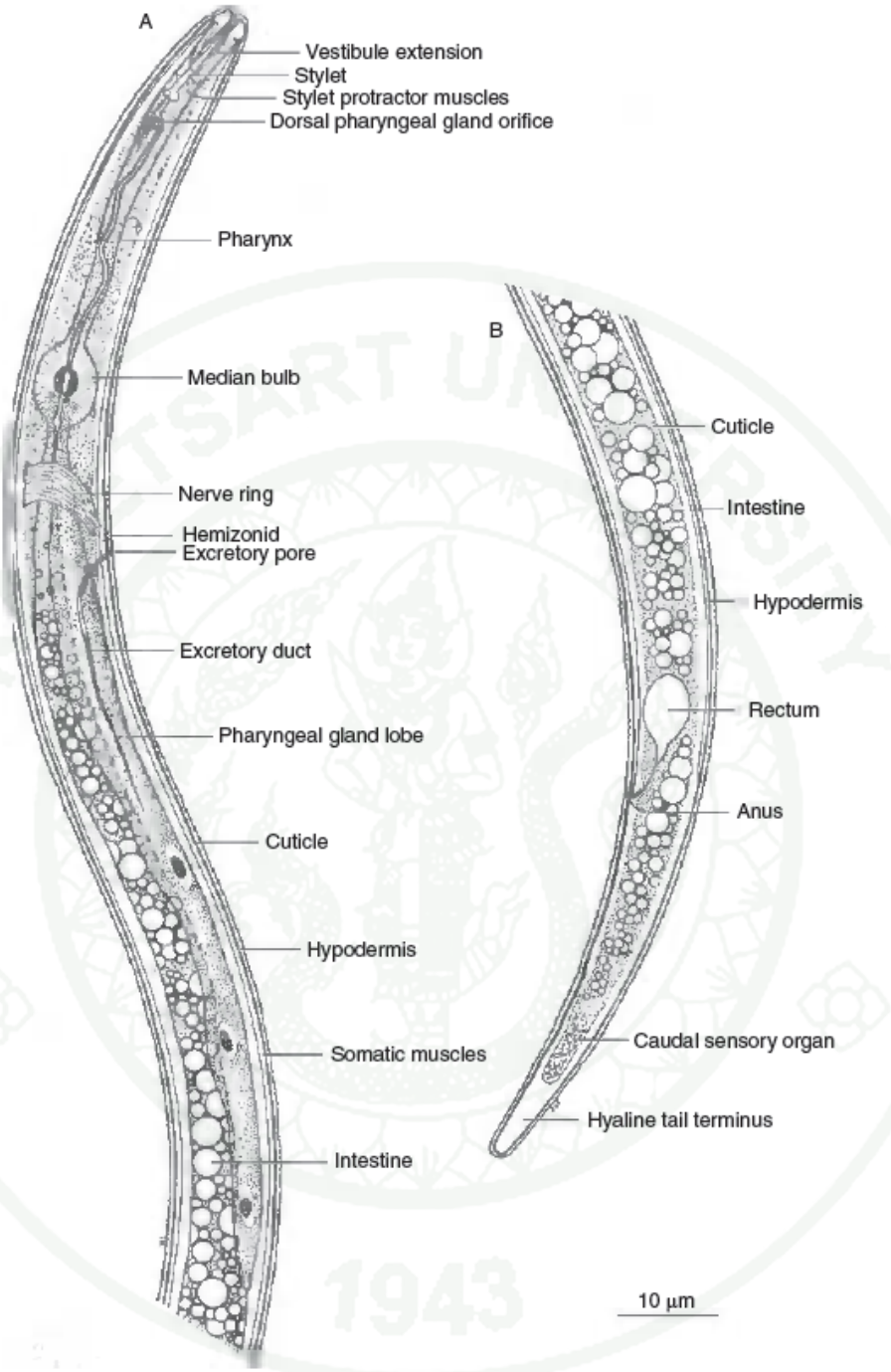

Figure 6 Morphology and important organs of a second-stage juvenile of root-knot nematode; A: anterior region B: posterior region

Source: Eisenback and Hunt (2009)

2. Morphological Identification species of root-knot nematode (*Meloidogyne* sp.)

2.1 Second-stage juvenile identification

Measurements of nematode characters are in the list (Table 1) and should be conducted in at least 20 specimens for each J2, male or female stages using compound microscope. Measurements should be made by appropriate magnification of eyepiece such as using x 100 objective oil immersion for stylet, knob, DGO, x10 objective for body length and width in order to obtain accurate data. The measurements need to be repeated in the form of mean± standard deviation (µm).

Second-stage juvenile description were measured; body form, head shape and form annules, general appearance of stylet (length) and shape knobs (height and width), DGO (dorsal gland orifice), excretory pore position, hemizoid position in relation to excretory pore, overlap of pharyngeal gland, form rectum, tail shape and form of tip, development of hyaline region (Eisenback and Hunt, 2009).

The samples from should be recorded with detailed data using GPS (Global positioning system) each field and specimens should be fixed and preserved in glycerin (Seinhorst solution) to make permanent slides.

Important characteristics for species (*Meloidogyne* sp.) identification.

Meloidogyne incognita

Female

- Body pear-shaped without posterior protuberance
- Stylet length (range) 15-16 µm, knobs rounded and offset

Male

- Head not offset and incomplete head annulations
- Lip is elevated labial disc, without lateral lip
- Stylet length 23-26 µm (mean: 24.5)
- Knobs rounded to oval shaped and offset
- DGO to stylet knobs 2-4 µm

Second-stage juvenile

- Body 350-450 µm long
- Tail slender and 43-65 µm long; hyaline tail part 6-14 µm long and tail tip rounded.

Host: Both monocotyledonous and dicotylendonous plants

Remarks: Inducing usually large galls and distributing together with *Meloidogyne arenaria* and *Meloidogyne javanica*, restricted in the temperate regions to glasshouses.

Meloidogyne javanica

Female

- Body pear-shaped without posterior protuberance
- Stylet length (range) 14-18 µm, knobs ovoid and offset

Male

- Head not offset
- Lip is not elevated labial disc, without lateral lip
- Stylet length 19-23.5 µm (mean: 20.5)
- Knobs well developed ovoid shaped and offset
- DGO to stylet knobs 3-5.5 µm

Second-stage juvenile

- Body 400-560 µm long
- Tail slender and 47-60 µm long; hyaline tail part 9-18 µm long and tail tip finely rounded.

Host: Both monocotyledonous and dicotylendonous plants

Remarks: Inducing usually relatively large galls and distributing together with *Meloidogyne arenaria* and *Meloidogyne javanica*, restricted in the temperate regions to glasshouses.

Meloidogyne hapla

Female

- Body pear-shaped without posterior protuberance
- Stylet length (range) 13-17 µm, knobs relatively small, rounded and set off

Male

- Head clearly offset and head cap rounded
- Lip is not elevated labial disc, with lateral lip
- Stylet length 19-22 µm (mean: 20.5)
- Knobs relatively small, rounded and set off
- DGO to stylet knobs 4-5 µm

Second-stage juvenile

- Body 360-500 µm long
- Tail slender and 48-70 µm long; hyaline tail part often irregularly shaped 12-19 µm long and tail tip finely rounded.

Host: Many dicotylendonous plants including ornamental and food crops, monocotylendonous hosts are rare.

Remarks: Inducing usually relatively large galls, often with secondary roots, often found in the tropical and subtropical regions at higher altitudes.

Meloidogyne arenaria

Female

- Body pear-shaped without posterior protuberance
- Stylet length (range) 13-17 µm, knobs rounded and backwardly sloping

Male

- Head not offset and head cap rounded
- Lip is not elevated labial disc, without lateral lip
- Stylet length 20-27 µm (mean: 23)
- Knobs rounded and backwardly sloping
- DGO to stylet knobs 4-8 µm

Second-stage juvenile

- Body 400-600 µm long
- Tail slender and 45-70 µm long; hyaline tail part 6-15 µm long and tail tip finely rounded and pointed.

Host: Both monocotyledonous and dicotylendonous plants

Remarks: Inducing usually large galls, which sometimes appear as a string of pearls along the root, and distributing together with *Meloidogyne arenaria* and *Meloidogyne javanica*, restricted in the temperate regions to glasshouses. *M. arenaria* is not as common as the other two species.

Meloidogyne enterolobii

Female

- Body pear-shaped without posterior protuberance
- Stylet length (range) 14-17 µm, knobs reniform, indented, offset

Male

- Head not offset
- Lip is not elevated labial disc, not offset and lateral lip absent
- Stylet length 18-25 µm (mean: 20.5)
- Knobs rounded, posteriorly, offset
- DGO to stylet knobs 3-5 µm

Second-stage juvenile

- Body 377-528 µm long
- Tail slender and 43-63 µm long with bluntly pointed tail tip; hyaline tail part 5-15 µm long.

Host: Many dicotylendonous plants including vegetable crop.

Remarks: Widely distributing, emergent pest species with potential to great economic damage, resistant to *Mi1* gene

Table 1 Morphometrics of *Meloidogyne* n. sp. All measurements are in µm and in the form; mean ± standard deviation (range)

Character/stage	Female		Male	J2
	Holotype	Paratypes	Paratypes	Paratypes
n		20	20	20
L	✓	✓	✓	✓
a	✓	✓	✓	✓
c			✓	✓
T			✓	
Max. body diameter	✓	✓	✓	✓
Neck length	✓	✓		
Stylet length	✓	✓	✓	✓
Stylet knob height	✓	✓	✓	✓
Stylet knob width	✓	✓	✓	✓
DGO	✓	✓	✓	✓
Excretory pore to anterior end	✓	✓	✓	✓
Interphasmidial distance		✓		
Vulva length		✓		
Vulva-anus distance		✓		
Tail length			✓	✓
Spicule length (median line)			✓	
Gubernaculum length			✓	
Testis length			✓	
Hyaline tail terminus (h)				✓

Source: adapted from Eisenback and Hunt (2009)

* At least 20 specimens for each J2, Female or Male.
* n = number
* L = mean of body length
* a = body length / body width
* c = body length / tail length

2.2 Perineal Pattern identification

Adult females are pearly white in color and have relatively rounded or pear shaped bodies. In full-grown females, throughout sedentary state until death, the cuticle annulations are visible in the head region and posterior part where a characteristic unique cuticular pattern or perineal pattern (Karssen and Moen, 2006) is mostly need for root-knot nematodes (*Meloidogyne* sp.) species identification. This area comprises the dorsal region; dorsal arch, lateral line, phasmid and tail terminus and the ventral region; vulva, anus, striae, wing and punctuations (Fig 6). The preparation of perineal pattern follows Hartman and Sasser (1985) methods which use lactophenol solution as the fixative solution and the specimen are observed under microscope. Originally, there were 4 major species which had been studied, *M. incognita*, *M. javanica*, *M.arenaria*, *M. hapla*. In 2002, Karssen differentiated the perineal pattern of *Meloidogyne* into 12 species (Fig 7) (Hunt and Handoo, 2009). The distinct description of each root-knot nematode (*Meloidogyne* sp.) is as follows;

Meloidogyne incognita

- Perineal pattern usually with relatively high dorsal arch and without lateral line

Meloidogyne javanica

- Perineal pattern rounded with distinct lateral lines.

Meloidogyne hapla

- Perineal pattern with fine striae, rounded with low dorsal arch, punctuation above the anus, lateral line present.

Meloidogyne arenaria

- Perineal pattern rounded with low dorsal arch, distinct striae and lateral lines sometimes weakly visible.

Meloidogyne enterolobii

- Perineal pattern rounded to ovoid, dorsal arch rounded, striae fine and widely spaced; lateral field absent or with single line occurring at junction of dorsal or ventral arches.

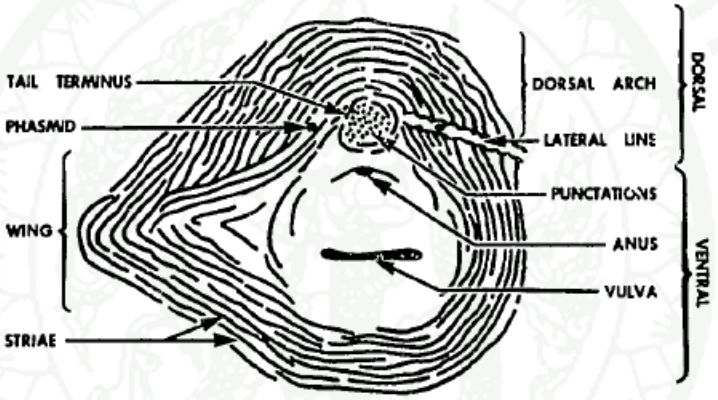

Figure 7 Perineal patterns of root-knot nematode (*Meloidogyne* sp.)

Source: Eisenback *et al*. (1981)

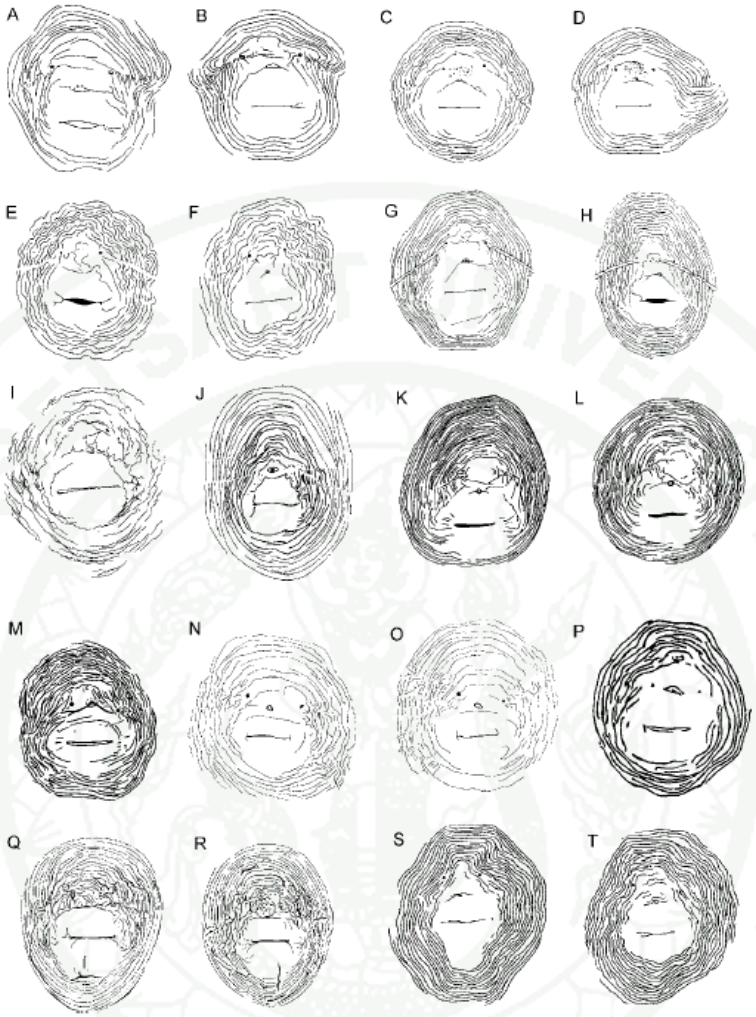

Figure 8 Comparison of perineal patterns among 12 major species of *Meloidogyne.* A, B: *M. arenaria*; C, D: *M. hapla*; E, F: *M. incognita*; G, H: *M. javanica*; I: *M acronea*; J: *M. chitwoodi;* K, L: *M. enterolobii*; M: *M. ethipoica*; N, o: *M. exigua*; P: *M. fallax*; Q, R: *M. graminicola*; S, T: *M. paranaensis* (Drawings not to scale. A-H, after Orton Williams(1972, 1973, 1974, 1975); I, after Page (1985); J, after Jepson (1985); K, L after Ramman and Hirschmann (1988); M, after Whitehead (1968); N, O, courtesy of Janel Machon; P, after Karssen (1996); Q,R, after Mulk (1976); S, T, after Carneiro *et al*.(1996))

Source: Hunt and Handoo (2009)

3. Biological and Molecular identification of root-knot nematode (*Meloidogyne* sp.)

3.1 Biological identification

Esbenshade and Triantaphyllou (1985) developed an identification method by employing the isozyme electrophoresis of esterase [E.C. 3.1.1.1] and malate dehydrogenase [E.C. 1.1.1.37]. This method obliviously separated the species of root-knot nematodes.

Esterase is a hydrolase enzyme that splits the ester into acid and alcohol in a chemical reaction with water namely hydrolysis. Esterase varieties differ depending on surface specificity, protein structures and biological function.

Esterase is a protein or enzyme found in adult females of root-knot nematode and commonly utilized for *Meloidogyne* species identification (Dickson *et al*., 1971). Soluble protein extracted from nematodes were macerated in a buffer solution and separated on polyacrylamide or starch gels by an electrophoresis (PhastSystem). The differences in molecular masses (protein masses) generate differential band patterns between species or populations and may be used as taxonomic markers. When compared with the marker (*Meloidogyne javanica*) (Fig 9), this technique has several advantages for example rapid detection from a single nematode female and no interference from the effects of environmental and developmental variations (Esbenshade and Triantaphyllou, 1985; Subbotin and Moens, 2006).

In Thailand, this method was firstly conducted by DOA and JIRCAS (Toida *et al*., 1996). The phenotyphic band patterns of nematode esterase isozyme are shown in Table 2 and Fig 8 (Karssen and Moens, 2006);

Meloidogyne incognita

Esterase I1 type and malate dehydrogenase N1 type

Meloidogyne javanica

Esterase J3 type and malate dehydrogenase N1 type

Meloidogyne hapla

Esterase H1 type and malate dehydrogenase H1 type

Meloidogyne arenaria

Esterase A1, A2 and A3 type and malate dehydrogenase N1 and N3 type

Meloidogyne enterolobii

Esterase VS1-S1 type and malate dehydrogenase N1a type

Table 2 The results of esterase patterns by Esbenshade and Triantaphyllou (1985).

Species	Host plants	Esterase phenotypes*	Band patterns
M. graminicola	Upland rice	VS1	Broad one band
M. incognita	Tomato	I1	One band
M. Javanica	Tomato	J3	Three band
M. incognita	*Rivinia humilis*	I1	One band
M. incognita	*Acalypha indica*	I1	One band

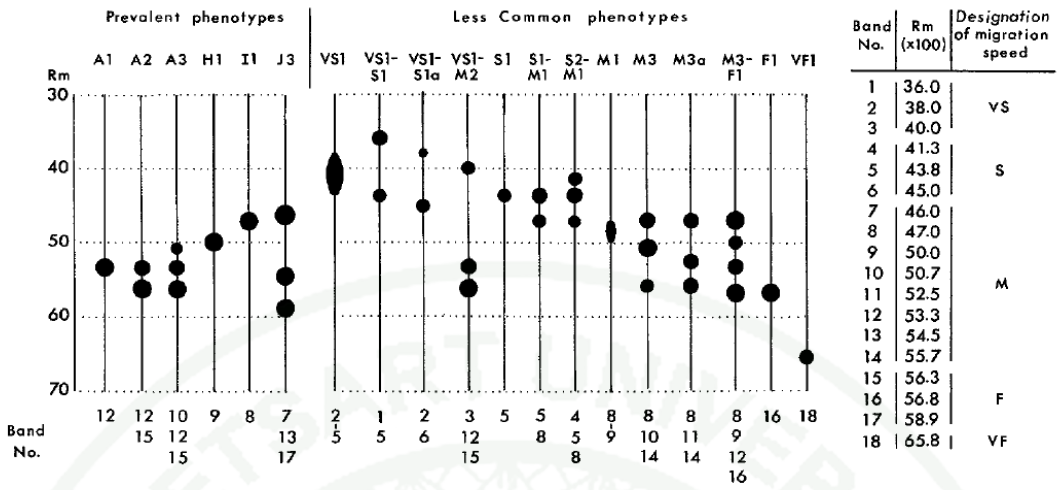

Figure 9 Esterase phenotypes and rate of distance A: *M. arenaria*, H: *M. hapla*, I: *M. incognita*, J: *M. javanica*, VS1-S1: *M. enterolobii* rate of distance of major bands is separate species; VS: very slow, S: slow, M: medium, F: fast, VF: very fast

Source: Esbenshade and Triantaphyllou (1985)

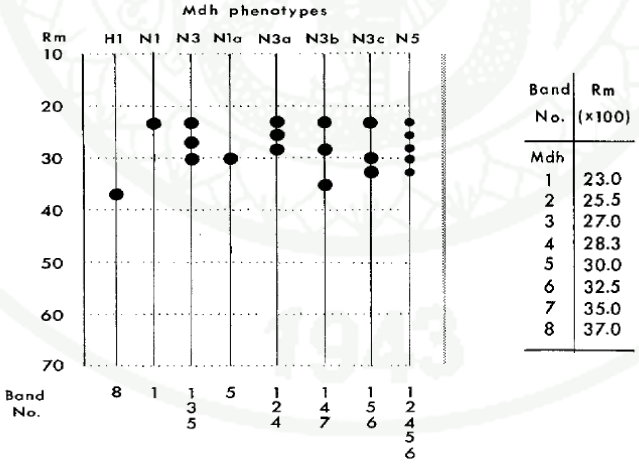

Figure 10 Phenotypes of malate dehydrogenase (Mdh) H1: *M. hapla*, N1: *M. incognita*, *M. javanica* or *M. arenaria*, N3: *M. arenaria* or *M. incognita*, N1a: *M. enterolobii* , *M. chitwood*, *M. graminicola*, *M. naasi*, *M. oryzae* or *M. platani*, N3a: *M. querciana*, N3b: *M. incognita*

Source: Esbenshade and Triantaphyllou (1985)

3.2 Molecular identification

Presently, the molecular technique “Polymerase Chain Reaction (PCR)” is extensively accepted as the means to produce more rapid and accurate nematode identification as compared with morphological techniques such as perineal pattern, second-stage juvenile measurement and protein electrophoresis.

PCR is a technique almost similar to the DNA replication process which uses large numbers of copies of DNA molecule in plant or animal genomes as DNA template. The process needs the following reagents: oligonucleotide primers (short nucleotides, which complementary with DNA template), DNA polymerase (Taq), deoxynucleotide triphosphates (dNTPs), buffer and $MgCl_2$.

PCR procedure consists of the following three steps determined by temperature conditions: denaturation (95 ℃), annealing (55-60 ℃ depend on Tm), extension (72 ℃) for 30-40 cycles. Billion copies of DNA have been amplified and then detected by agarose or polyacrylamide gels and visualized using ethidium bromide under UV radiation. Some other methods for nematode identification using PCR concepts are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and multiplex PCR.

Multiplex PCR is major development in DNA diagnostics and enables the detection of one or several species in a nematode mixture by a single PCR test. That comprises of two primer sets; universal primer set, which amplifies genus level (18s rRNA, 28s rRNA genes) and specific primer set, which amplifies species of nematodes.

Genes widely used for molecular analysis are nuclear ribosomal RNA genes and mitochondrial DNA because of high copy numbers and evolutionary relationships between species (Makedonka *et al.*, 2001). Spaces between the following genes are normally under investigation; internal transcribed spacer 1 and 2 (ITS1 and ITS2) between 18s, 5.8s and 28s rRNA genes on nuclear (Hugall *et al*., 1999), intergenic spacer (IGS) between Cytochrome oxidase subunit II (COII) and Large small subunit RNA gene (16s rRNA) on mitochondrial DNA (Power and Harris, 1993).

In 1991, Okimoto published the map structure of the mitochondrial genome of root-knot nematode (*Meloidogyne*) (Fig 10). It showed the location of 12 coding genes, large and small ribosomal RNA (rRNA) genes and transfer RNA (tRNA) genes. In 1993, Power and Harris reported identification of *Meloidogyne* species by multiplex PCR methods using primer sets to amplify the region between the COII and 16s rRNA genes (1108 and C_2F_3) and complete intergenic spacer (IGS) (tRNA-His). The results showed that three sizes were detected. *M. incognita* and *M. javanica* produced 1.7 kb fragment; *M. arenaria* produced 1.1 kb fragment; *M. hapla* and *M. chitwoodi* produced 0.52 kb fragment. This primer set was also used in another study to differentiate more species of root-knot nematodes (Table 3).

In 1995, Zijlstra *et al*. discriminated *M. hapla*, *M. chitwoodi* and *M. fallax* based on ITS region of nuclear DNA, using primer sets (18s and 26s, F194 and F195, ITS3) (Vrain *et al*., 1992 and Ferris *et al*., 1993) amplified ITS1-5.8S-ITS2 genes by PCR-RFLP. In 1999, Hugall *et al*. discriminated *M. incognita*, *M.javanica* and *M. arenaria.*

In 2005, Floyd *et al*. designed universal primer sets (18s_F and 18s_R) to amplify nematode ribosomal DNA gene (18s rDNA). These primers generated product of about 1 kb fragment in size.

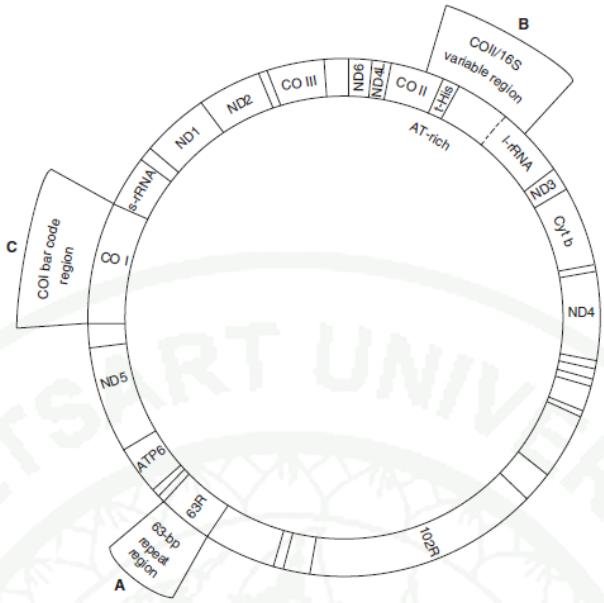

Figure 11 Diagramatic structure of the mitochondrial genome of root-knot nematode (*Meloidogyne*); A) 63 bp repeat region, B) COII and 16s region, C) COI bar code region. Okimoto, 1991

Source: Blok and Power (2009)

Table 3 PCR products amplified by the primer set C2F3 and 1108

PCR product sizes	Root-knot nematode species
1.5-1.6 kb	*M. arabicida, M. arenaria, M. ethiopica, M. incognita and M. javanica*
1.2 kb	*M. paranaensis*
1.1 kb	*M. arenaria, M. floridensis, M. morocciensis, M. thailandica*
750 bp	*M. enterolobii (M. mayaguensis)*
520-540 bp	*M. chitwoodi, M. fallax, M.graminicola, M.graminis, M. hapla, M.haplanaria, M. mali, M.marylandi, M.microtyla, M. naasi, M. oryzae, M. partityla, M. suginamiensis, M. trifoliophila*

Source: Blok and Power (2009)

MATERIALS AND METHODS

Materials

I Experiments at Kasetsart University, Bangkhen campus, Thailand.

1. Nematode population

1.1 Soil Collection

1. Global Positioning System machine or GPS machine
2. Spades
3. Plastic bags
4. Permanent marker or label marker
5. Foam box

1.2 Nematode extraction and measurement of nematode numbers in soil.

1. Sieve mesh size 60, 200, 400 mesh
2. Funnel mesh filter
3. Syracuse watchglass
4. Stereomicroscope
5. Counter

1.3 Nematode sample collection

1. Sterile soil: Sida, Kampoo and Clay
2. 6 inch-d-plastic pots
3. Autoclave
4. 14-day-old tomatoes

2. Morphological analyses

2.1 Morphology of Perineal pattern

1. Stereomicroscope
2. Compound microscope
3. Plastic sheets
4. Scalpel or razor
5. Dissecting needle
6. Forceps
7. Glass slide and Cover slips

2.2 Measurement of second-stage juvenile

2.2.1 Permanent mounts of nematodes

1. Stereomicroscope
2. Incubator
3. Hot water and distilled water
4. Aluminum slide
5. Cover slips
6. Medium glass tube
7. Petri dishes
8. Syracuse watchglass
9. 1000 µl pipettes and tips

2.2.2 Measurement

1. Binocular Microscope
2. Permanent slide
3. Oil emersion
4. Ocular meter

II. Experiment at University of Hawaii at Manoa, USA.

1. Nematode Population

1.1 Nematode multiplication

1. Sterile soil: loamy soil
2. 10- inch-d-pots
3. 14- day-old tomatoes

1.2 Egg mass hatching

1. Forceps
2. Stereomicroscope
3. BP dishes
4. Pencil
5. Permanent marker

1.3 Soil sample elutriation (field soils) and Nematodes collection

1. Semi automatic elutriator machine
2. Standard mesh size 35 mesh and 500 mesh
3. Centrifuge machine
4. Microscope
5. Beakers
6. Eyelash equipment

2. Enzyme analysis: *Meloidogyne* species identification

1. PhastSystem machine and equipment
2. Incubator
3. Stereomicroscope
4. Analytical balance (4 digits)

3. DNA analysis

3.1 Identification of *Meloidogyne* at genus level

3.1.1 Design of universal primers specific to the genus *Meloidogyne*

1. Data Base from Gene Bank (NCBI)
2. ClustalW v.2 Program
3. Oligo Program
4. Vector NTI Program
5. Oligo analyze 3.1 of IDT

3.1.2 DNA Extraction and Polymerase Chain Reaction

1. Stereomicroscope
2. Polymerase Chain Reaction machine
3. Agarose electrophoresis
4. Vortex machine
5. Micro centrifuge machine
6. Pipetman
7. Tube incubator

3.2 *Meloidogyne* species identification

1. Stereomicroscope
2. Polymerase Chain Reaction machine
3. Agarose electrophoresis
4. Vortex machine
5. Micro centrifuge machine
6. Pipetman
7. Tube incubator

Methods

I. Experiments in Kasetsart University, Bangkhen Campus, Thailand.

1. Nematode population

1.1 Soil Collection

Soils were collected from six nematode-infested orchards in Samut Sakhon province (Khlongton orchard (KT) and Nakhon Pratom province (Thaveesak orchard (TV), Kongchida orchard (KJ), Payoon orchard (PY), Apinya orchard (AP), Namthan orchard (NT)). Longitude and latitude in each area were recorded by GPS machine. Four composite soil cores around the root zone of a guava plant were collected at the depth of 8 inches or 20 cm for each sample. The collected soils were then thoroughly mixed. One-fourth of the mixed soils were put into plastic bags. The soils samples were labeled and transferred to the laboratory in a foam box.

1.2 Nematode extraction and measurement of nematode numbers in soil.

Approximate 300 g soil samples were for nematode extraction via Baerman funnel method. Nematodes were extracted from the soil by pouring the supernatants of soil suspensions into series of 60, 200 and 400 mesh sieves. Nematodes suspended on the 200 and 400 mesh sieves were collected and placed on tissue papers in the funnels. Three days later, nematodes at the bottom of the funnels were kept and observed under microscope.

1.3 Nematode sample collection

Field soils were mixed with sterile loamy soils (3 pots/ orchard). Two 14-d-old tomato plants were planted into the mixed soils and allowed to grow for 2 months.

2. Morphological analyses

2.1 Morphology of Perineal pattern

Nematode females were teased from roots (5 females/ orchard) from each orchard using a dissecting needle. Subsequently, they including their egg masses, were transferred into tissue culture wells filled with sterile water. Nematode females were separated from their eggs, and then placed in formalin solution. Eggs in the wells were allowed to hatch into second-stage juveniles. Females were transferred into a drop of lactophenol on a plastic sheet. Then, under microscope, 1/3 posterior end of the females were cut off by a scalpel or razor. Nematode perineal patterns were further trimmed off and cleaned with lactic acid. Perineal patterns were transferred to the middle of a lactophenol drop on a glass slide. A cover slip was placed and sealed with nail polish. Later on, the slides were observed under a compound microscope (Hartman and Sasser, 1985).

2.2 Measurement of second-stage juvenile

2.2.1 Permanent mounts of nematodes

Second-stage juveniles (J2) from single egg masses were hatched and subsequently killed by pouring into water (temperature of 80 °C). Nematode samples were added with FA 4:1 fixatives (10 parts of formalin (40% formaldehyde), 1 part glacial acetic acid, and 89 parts of distilled water) and incubated for 48 hours. Nematodes were transferred into syracuse watch glasses filled with Seinhorst I solution (20 parts of 95% ethanol, 1 part of glycerin, and 79 parts of water). Later on, the watch glasses were placed inside petri dishes containing 95% ethanol. The incubation under 35-40 °C was followed. Twelve hours later, Seinhorst II solution (95 parts of 95% ethanol, and 5 parts of glycerin) was added into the syracuse watch glasses which subsequently were stored under 40 °C until only glycerin remained in the watch glasses. Twenty nematodes were transferred into a drop of glycerin on a slide, covered with a cover slip, and sealed with nail polish.

2.2.2 Measurement

Second-stage juveniles mounted on permanent slides by Sienhorst's method were observed and measured under a microscope which had previously been well calibrated; (10x) 1 unit = 10 µm, (20x) 1 unit = 5 µm, (40x) 1 unit = 2.5 µm, (100x) 1 unit = 1 µm**.** 20 second-stage juveniles were used for one sample.

Ten characteristics of nematodes were measured.

1. L (mean length)
2. a (body length/ body width)
3. c (body length/ tail length)
4. Body width
5. Stylet length
6. DEGO
7. Excretory pore to anterior end
8. Tail length
9. Hyaline tail terminus (h)
10. h% (h/ tail length x 100)

II. Experiments in the University of Hawaii at Manoa, USA.

1. Nematode Population

1.1 Nematode multiplication

Tomato plants (Orange Pixie cultivar) were planted and inoculated by ~ 10 egg masses of each of the following nematode isolates: (Namthan orchard (NT), Payoon orchard (PY) and Khlongton orchard (KT)). Populations of root-knot nematodes were further allowed to increase for 2.5 months. For comparative studies, root-knot nematodes previously identified as *M. incognita*, *M. javanica*, and *M. konaensis* by their esterase patterns and presently maintained on tomato plants at the Magoon greenhouse, the University of Hawaii at Manoa, were used.

1.2 Egg masses hatching

Tomato roots were collected and gently washed free of soil and placed into a plastic flask. Egg masses were teased by a forcep, and transferred to water in BP dishes, and labeled with their names, numbers on the outside BP dishes with a pencil. Second-stage juvenile had been allowed to hatch from eggs for 7 days and then were kept in 1.5 ml microcentrifuge tubes. Nematodes samples were kept under -20 ℃ for further DNA analysis

1.3 Soil sample elutriation (field soils) and Nematode collection

Soil samples were collected from the agricultural orchards on the Island of Oahu, Hawaii. 300 g soil samples were put in the tubes of the semi automatic elutriator machine stationed at the Department of Plant and Environmental Sciences, University of Hawaii. The machine was turned on and nematodes were automatically separated from the soil through a series of 35 mesh and 500 mesh sieves. Nematodes collected on the 500 mesh sieves were washed and transferred into beakers. Later on, nematodes were separated from debris by the centrifugal flotation method. Nematodes in soil samples were counted and observed under a microscope. *Pratylenchus*, *Rotylenchulus*, *Helicotylenchus*, *Aphelenchus* were handpicked by an

eyelash mounted on a bamboo stick, transferred to 1.5 eppendrofs (15 nematodes/genus/tube) and frozen under -20 °C for 48 hours.

2. Enzyme analysis: *Meloidogyne* species identification

Three isolates (NT, PY, KT) of root-knot nematodes (*Meloidogyne*) were obtained from tomato plants (Orange Pixie cultivar) *M. javanica*, *M. incognita* and *M. konaensis* were used as the standard to compare with the three isolates. Females were teased from tomato roots and placed in saline solution (0.16M NaCl) and frozen (-20 ℃). Females were transferred by a forcep to well PhastGel sample stamps containing 0.9 µl extraction solution (20% sucrose and 2% Triton X-100, 0.01% bromophenol blue) and macerated with a small cooper rod. The nematode homogenates were analyzed by automated PhastSystem (Amersham Pharmacia Biotech) electrophoresis using native polyacrylamide gels (Gradient 10-15) and native buffer agarose strips after electrophoresis, the gels were esterase stained (0.1 M Phosphate buffer, 0.0075 g EDTA, Fast Blue RR salt 0.015 g, 0.10 g α-naphthyl acetate in 0.5 ml acetone/gel) and incubated at 37 ℃ for 1 hour and fixed in fixative (10% acetic acid, 10% glycerol) for 1 hour. (Esbenshade and Triantaphyllou, 1985)

3. DNA analysis

3.1 Identification of *Meloidogyne* at genus level

3.1.1 Design universal primers specific to the genus *Meloidogyne*

The nucleotide data basing on (Gene Bank) the two mitochondrial gene; Cytochrome Oxidase subunit II (COII) gene and 16s ribosomal RNA (16s rRNA) gene of root-knot nematodes were searched. The data bases of *Meloidogyne* were aligned and compared with *M. hapla* and *Radopholus similis*, which have complete mitochondrial sequence. The primers were designed by Oligo Program or Vector NTi Program and checked by Oligo analyze 3.1; Analyze, Hairpin, Seif-Dimer, Hetero-Dimer, NCBI Blast and TM Mismatch.

3.1.2 DNA Extraction and Polymerase Chain Reaction

Egg masses were obtained from tomato roots by gently washing the soil from the roots. The egg masses were hatched to small glass dishes filled with water. Twenty J2 of *Meloidogyne* and 15 nematodes of the following genera: *Aphelenchoides* sp., *Helicotylenchus* sp., *Pratylenchus* sp., *Rotylenchulus* sp., (all were isolated from the agriculture farms on the island of Oahu, Hawaii), and *Steinernema* sp. (currently was cultured in the Nematology Lab at the University of Hawaii) were placed in 200 µl of distilled water using eppendorf 1.5 ml and frozen at -20 ˚C, After 48 hr. at -20 ˚C 1 g glass bead were added to the tube and the tube were shacked from vortex. The supernatant was kept to new 1.5 ml microcentrifuge tube and DNA was extracted using the genomic DNA (Promega, USA). DNA template 1.5 µl, forward primer and reverse primer 0.5 µl/ primer (UNI_COIIM, UNI_16sM, Nema_18s following Table 4) Taq 1X master mix (goTaq @ PCR Master Mix, Promega) 10 µl and sterile H_2O 7.5 µl in total 20 µl The cycling conditions following Table 5. Following DNA amplification, the product 10 µl were screened on 1.5% agarose gels for primer Nema_18s and 2.5% agarose gels for primer UNI_COIIM and UNI_16sM, and PCR products were compared with marker (Hyper Ladder IV),

Negative and Positive in electricity 70 Volts for 3 hrs. Consequently, PCR product were stained with ethidium bromide and visualized on an UV box.

3.2 *Meloidogyne* species identification

Egg masses of each (NT, PY, KT) orchard were obtained from tomato roots by gently washing the soil from the roots. The egg masses were hatched in small glass dishes filled with water. The 20 second-stage juveniles of *M. incognita, M. javanica* and *M. enterolobii* were placed in 200 µl of distilled water in a 1.5 ml eppendorf and frozen at -20 ˚C, for 48 hr. 1 g glass beads were added to the tube and vortex. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and DNA was extracted using the genomic DNA (Promega, USA). DNA template 1.5 µl, forward primer and reverse primer 0.5 µl/ primer (1108 and C_2F_3 (Powers and Harris, 1993). following Table 4) Taq 1X master mix (goTaq @ PCR Master Mix, Promega) 10 µl and sterile H_2O 7.5 µl in total 20 µl The cycling conditions following Table 5. Following DNA amplification, 10 µl products were screened on 1.5% agarose gels and PCR products were compared with marker (Hyper Ladder IV), electricity 70 Volts for 3 hrs. Consequently, PCR product were stained with ethidium bromide and visualized on UV box.

Table 4 Nucleotide sequence of Primers

Primer Name	Nucleotide sequences
Nema_18sF	**5'-GGGCGGTATCTGATCGCC-3'**
Nema_18sR	**5'-CGCGAATRGCTCATTACAACAGC-3'**
UNI_COIIFM	**5'-CTGATGTAATTCATGCTTGGG-3'**
UNI_COIIRM	**5'-TCCACAAAT TTCGGAACATTG-3**
UNI_16sFM	**5'-CGTGATTAGTCAAAGGTAGCAAGG-3'**
UNI_16sRM	**5'-GATAAAGAACTCTATTTTACAACG-3'**
1108	**5'-TACCTTTGACCAATCACGCT-3'**
C_2F_3	**5'-GGTCAATGTTCAGAAATTTGTGG-3'**

Table 5 Polymerase Chain Reaction conditions of each primer set

PCR cycle	**Temperature (°C)**	**Time (min)**	
Initial denature	94	5	
Denaturing	94	1	
Annealing	53 (UNI_COIIM)	1	35 cycles
	49 (UNI_16sM)		
	55 (Nema_18s)		
	48 (1108,C_2F_3)		
Extension	72	1	
		2	(1108,C_2F_3)
Final Extension	72	10	
Hold	4	α	

RESULTS AND DISCUSSION

Results

I. Experiment at Kasetsart University, Bangkhen Campus, Thailand.

1. Nematode population

The topography of Samut Sakhon and Nakhon Pathom provinces, Thailand, was clay soil and gutter field (Fig 12). Guavas were heavily damaged by root-knot nematodes. The characteristic of below-ground symptoms mainly includes decayed roots and characteristics of above-ground symptoms are yellowing, stunting, blight leaves and declining (Fig 13). The longitude and latitude of each area were recorded by GPS machine. The area positions were specified by using Google map (Table 4). Khlongjinda orchard (KJ), Payoon orchard (PY), Thaveesak orchard (TV), Namthan orchard (NT) and Apinya orchard (AP) are in Nakhon Pathom province, Khlongton orchard (KT) is in Samut Sakhon province. The distances between each orchard are 1.5, 2.8, 3.7, 0.3 and 10.6 km respectively (Table 6, Fig 14).

The number of second-stage juveniles (J2) of root-knot nematodes (*Meloidogyne*) was measured in 300 grams of soil. By average, 11, 91, 6.67, 28, 16.33 and 42 J2 were found in KJ, PY, TV, NT, AP and KT soils, respectively (Table 7). In the greenhouse at the Department of Plant Pathology, Kasetsart University, Bangkhen, Thailand, tomato plants (Cherry cultivar) had been planted in the soils–collected from the six orchards for 2 months (60 days). As the result, some isolates of root-knot nematodes infected into tomato roots as shown by the following symptoms; small root galls, root rot, stunting and wilting. Seven out of 18 tomato plants were alive but galled. They were AP2, TV2, TV3, PY2, KJ3, NT2 and KT3 (Table 7).

Figure 12 The topography of Samut Sakhon and Nakhon Pathom provinces, Thailand. was clay soil and gutter field.

Figure 13 Guavas were damaged by root-knot nematodes; yellowing, stunting, blight leaves and declining.

Table 6 Longitudes and Latitudes of each orchard by Global Positioning System (GPS) machine and distances between orchards (km).

Name of Orchards	Position (Longitude and Latitude)	Distance
Khlongjinda	13˚ 42.8381'N 100˚ 09.5702'E	1.5
Payoon	13˚ 43.0904'N 100˚ 10.1812'E	2.8
Thaveesak	13˚ 43.2280'N 100˚ 11.6015'E	3.7
Namthan	13˚ 41.9052'N 100˚ 12.3640'E	0.3
Apinya	13˚ 41.8179'N 100˚ 12.3428'E	10.6
Khlongton	13˚ 64.9323'N 100˚ 20.9389'E	

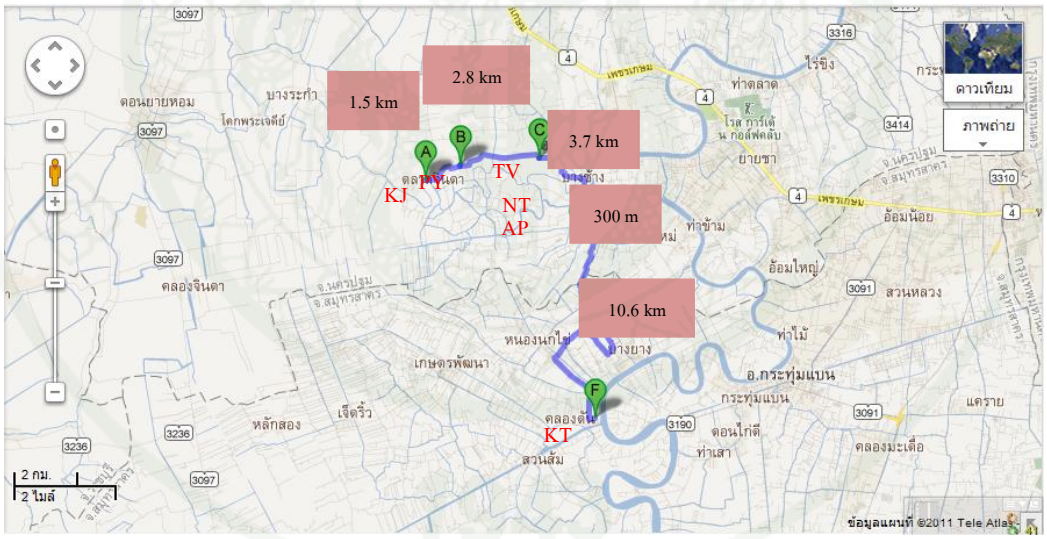

Figure 14 Locations of the orchards in Samut Sakhon and Nakhon Pathom provinces by Google map: A) Khlongjinda orchard (KJ) B) Payoon orchard (PY) C) Thaveesak (TV), D) Namthan orchard (NT), E) Apinya orchard (AP) and F) Khlongton orchard (KT).

Table 7 Total and averaged numbers of root-knot nematodes (*Meloidogyne*) in 300 gram of soil collected from guava orchards; 3 samples/ orchard.

Name of orchard	Sample No.	Total number of root-knot nematodes in 300 g soil	Averaged nematode number in each orchard
Khlongjinda	1	2	11
	2	3	
	3	28	
Payoon	1	2	91
	2	245	
	3	26	
Thaveesak	1	1	6.67
	2	6	
	3	13	
Namthan	1	10	28
	2	46	
	3	28	
Apinya	1	3	16.33
	2	32	
	3	14	
Khlongton	1	12	42
	2	15	
	3	69	

2. Morphological identification

The species of root-knot nematodes were identified using 5 perineal patterns /isolate. In each area, same results were obtained (Table 8). In the area namely Apinya, Thaveesak and Khlongjinda orchards. The perineal patterns with high dorsal arches, no wing and lateral lines were remarkably shown resemble to *M. incognita* (Fig 15 A-D). In, Payoon (Fig 16A), Namthan (Fig 16B) and Khlongton (Fig 16C-D) orchards have not been report in this area is *M. enterolobii*, which is characteristic similar between *M. incognita* and *M. arenaria*, some pattern have moderately high dorsal arch, wing or lateral lines. The perineal patterns with the characteristics more or less similar to *Meloidogyne incognita* and *M. arenaria* were found these characteristic resemble to *M. enterolobii.* In addition, some perineal pattern showed moderately high dorsal arches and wing or lateral line (Fig 16 A-D).

The second-stage juvenile (J2) were measured by a microscope and the data were given in the form of average ± standard deviation (Table 9).

Table 8 Identification of species of root-knot nematodes collected from different guava orchards based on nematodes perineal patterns.

Name of orchard	No. tomato plants	No. females	CODE	Nematode Species
Apinya (AP)	2	1	AP2-1	I
	2	2	AP2-2	I
	2	3	AP2-3	I
	2	4	AP2-4	I
	2	5	AP2-5	I
Thaveesak (TV)	3	1	TV3-1	I
	3	2	TV3-2	I
	3	3	TV3-3	I
	3	4	TV3-4	I
	3	5	TV3-5	I
	2	1	TV2-1	I
	2	2	TV2-2	I
	2	3	TV2-3	I
	2	4	TV2-4	I
	2	5	TV2-5	I
Khlongjinda (KJ)	3	1	KJ3-1	I
	3	2	KJ3-2	I
	3	3	KJ3-3	I
	3	4	KJ3-4	-
	3	5	Kj3-5	-
Namthan (NT)	2	1	NT2-1	-
	2	2	NT2-2	E
	2	3	NT2-3	-
	2	4	NT2-4	E
	2	5	NT2-5	-
Khlongton (KT)	3	1	KT3-1	E
	3	2	KT3-2	E
	3	3	KT3-3	-
	3	4	KT3-4	E
	3	5	KT3-5	-
Payoon (PY)	2	1	PY2-1	E
	2	2	PY2-2	E
	2	3	PY2-3	E
	2	4	PY2-4	-
	2	5	PY2-5	E

* I = *M. incognita*, E = *M. enterolobii* or *M. mayaguensis* and - = Fail or none

Table 9 Morphometrics (mean, standard deviation, and range) of second-stage juveniles of six isolates of *Meloiodgyne* in guava in central region of Thailand.

Mophometrics	Isolate 1 (Apinya) (AP)	Isolate 2 (Thaveesak) (TV)	Isolate 3 (Khlongjinda) (KJ)	Isolate 4 (Namthan) (NT)	Isolate 5 (Payoon) (PY)	Isolate 6 (Khlongton) (KT)
N	5	167	20	20	20	20
L	368.0±35.64 (340.0-430.0)	381.05±27.05 (320.0-445.0)	384.50±35.17 (320.0-430.0)	366±23.63 (335.0-410.0)	376.25±26.70 (330.0-420.0)	367.50±26.33 (310.0-400.0)
a	31.53±5.07 (24.0-36.0)	31.48±6.13 (19.5-45.33)	31.03±6.30 (21.3-43.0)	25.78±4.69 (20.1-39.0)	32.37±5.85 (23.0-41.0)	24.32±4.49 (18.1-35.0)
C	6.68±0.72 (5.76-7.58)	6.78±0.91 (3.12-9.25)	4.57±0.91 (3.29-7.0)	8.66±1.16 (6.96-11.21)	6.45±0.77 (5.3-8.0)	8.97±1.41 (6.61-13.33)

Table 9 (Continued)

Mophometrics	Isolate 1 (Apinya) (AP)	Isolate 2 (Thaveesak) (TV)	Isolate 3 (Khlongjinda) (KJ)	Isolate 4 (Namthan) (NT)	Isolate 5 (Payoon) (PY)	Isolate 6 (Khlongton) (KT)
Body width	12±2.74 (10.0-15.0)	12.62±2.78 (7.5-20.0)	12.78±2.25 (10.0-15.0)	14.50±1.79 (10.0-16.0)	12±2.38 (10.0-15.0)	15.65±3.31 (10.0-12.0)
Stylet length	12.0±1.12 (10.0-12.5)	12.13±1.57 (7.5-15.0)	9.35±1.55 (7.50-12.50)	13.48±1.23 (11.0-16.0)	12.75±1.38 (10.0-15.0)	12.60±1.30 (10.0-14.0)
DGO	4.0±0.02 (3.3-4.5)	4.28±0.62 (2.5-7.0)	4.31±0.78 (3.0-6.0)	2.69±0.49 (2.0-4.0)	4.10±0.52 (3.2-5.0)	2.55±0.71 (1.5-4.0)
Excretory pore to anterior end	62.5±17.41 (42.5-90.0)	60.59±10.16 (37.5-95.0)	77.50±7.86 (60.0-87.5)	65.65±9.02 (33.0-75.0)	65.25±5.19 (55.0-75.0)	61.25±7.95 (40.0-75.0)

Table 9 (Continued)

Mophometrics	Isolate 1 (Apinya) (AP)	Isolate 2 (Thaveesak) (TV)	Isolate 3 (Khlongjinda) (KJ)	Isolate 4 (Namthan) (NT)	Isolate 5 (Payoon) (PY)	Isolate 6 (Khlongton) (KT)
Tail length	55.5±6.94 (47.5-62.5)	56.09±7.63 (33.5-87.0)	59.88±12.74 (37.5-75.0)	42.95±5.74 (33.0-56.0)	59.13±8.20 (45.0-77.5)	41.70±6.02 (30.0-56.0)
h	8.0±2.74 (5.0-12.5)	7.14±2.58 (5.0-15.0)	10.50±2.31 (7.5-13.75)	8.80±2.50 (5.0-15.0)	7.63±1.90 (5.0-12.5)	9.65±2.18 (5.0-13.0)
h%	14.63±5.9 (10.53-25.0)	12.48±4.14 (4.55-25.0)	17.84±3.66 (13.33-25.0)	20.53±5.17 (11.63-30)	12.88±2.30 (8.7-16.7)	23.45±5.66 (11.11-35.14)

* n = number, L= mean length, a= body length/width, c= body/tail length, h= hyaline tail terminus, h% =h/tail length x 1

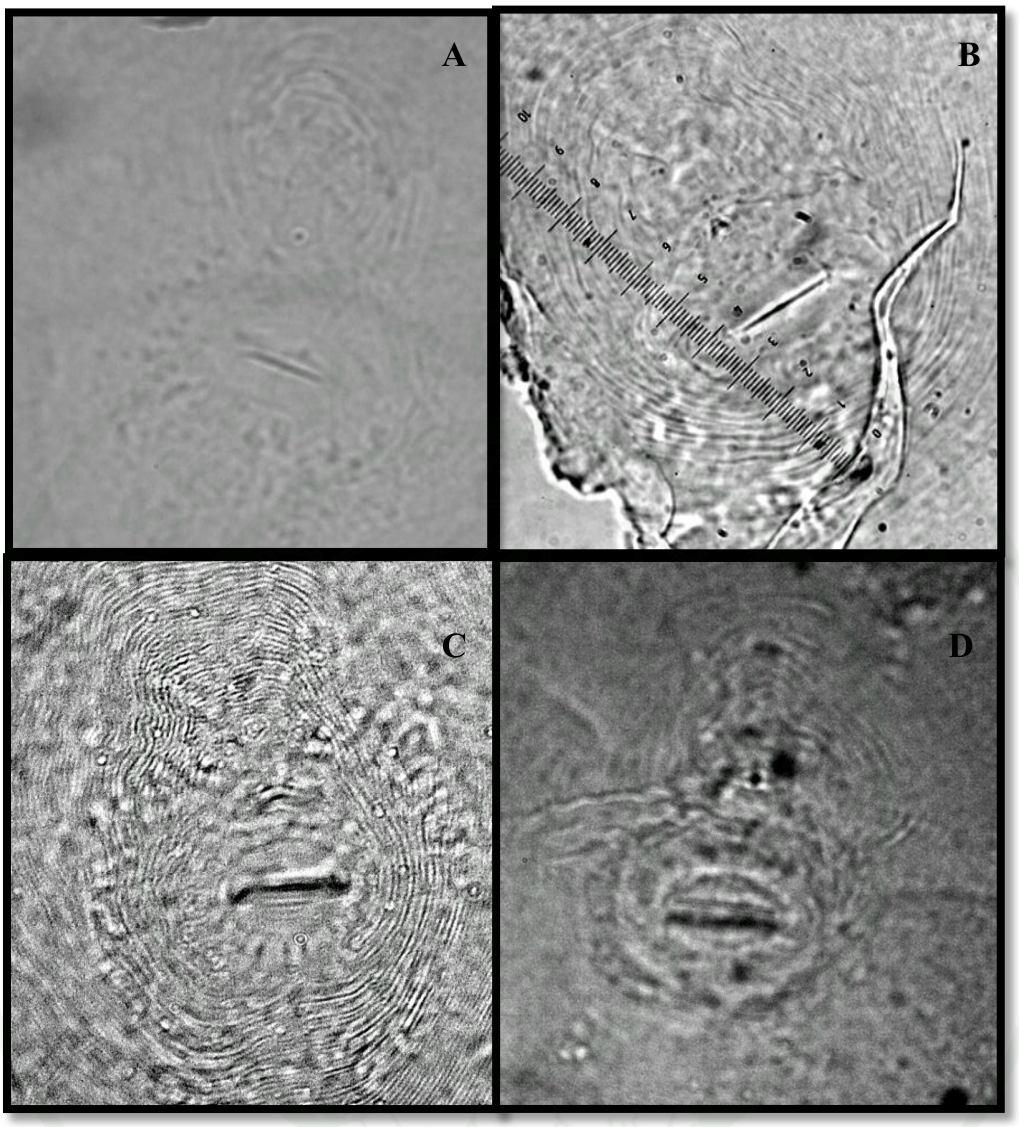

Figure 15 Photographs of perineal patterns of *Meloidogyne incognita* (Thailand populations). A-D) perineal pattern with high dorsal arches and oval shape; *M. incognita* A) Apinya orchard (AP), B-C) Thaveesak orchard (TV), D) Khlongjinda (KJ)

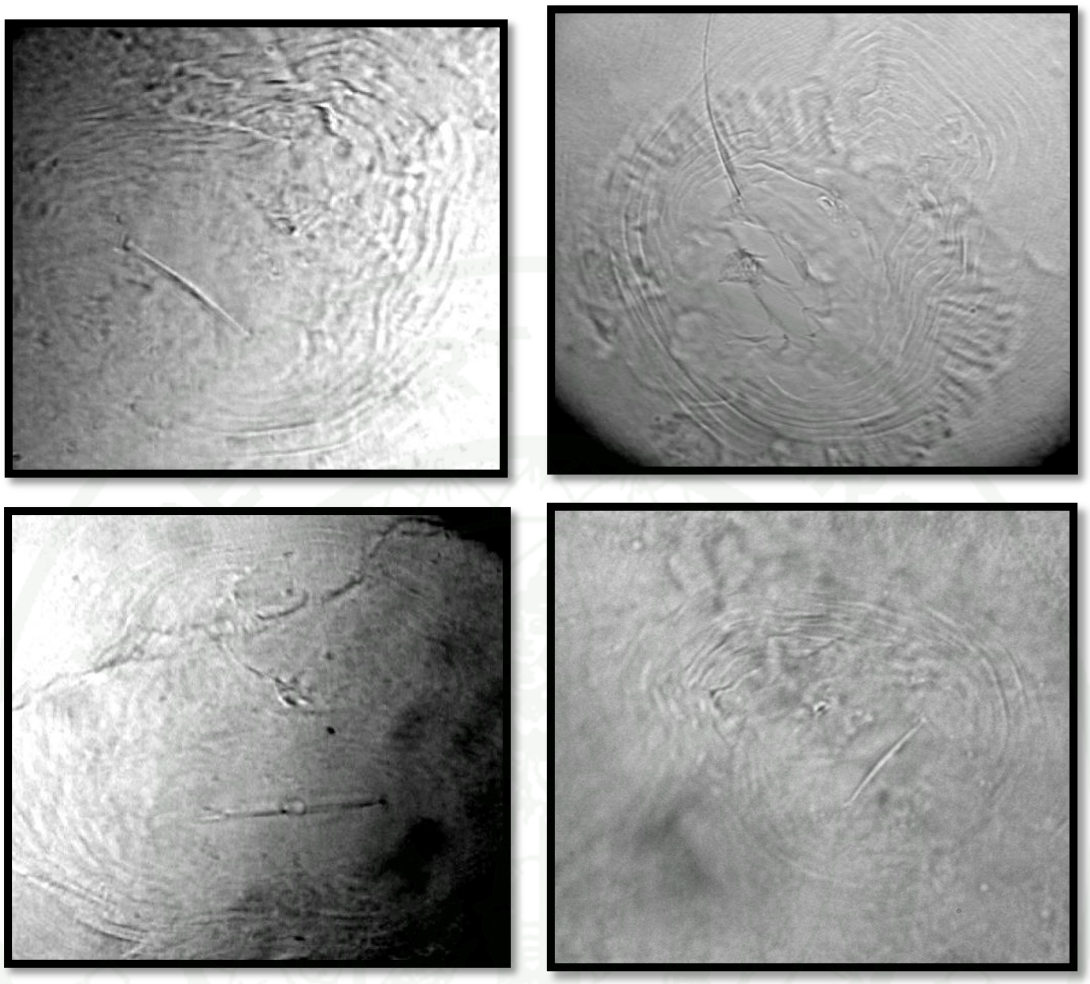

Figure 16 Photographs of perineal patterns of *M. enterolobii* (Thailand populations). A-D) perineal patterns with lateral lines**,** moderately high arches and wing; identify as *M. enterolobii*.A) Payoon orchard (PY), B) Namthan orchard (NT), C-D) Khlongton orchard (KT).

Experiments at the University of Hawaii at Manoa, USA.

1. Nematode Population

In Magoon greenhouse, Hawaii, USA, Tomato plants (Orange Pixie cultivar) were inoculated with ~10 egg masses of three isolates (NT, PY and KT). Two and a half months later, tomato roots showed big galls. Most of nematode females produced eggs inside the galls. Males were present abundantly, female bodies were transparent (Fig 17 A-C).

2. Enzyme analysis: *Meloidogyne* species identification

Nematode proteins were analyzed (esterase isozyme) by using a Phast System machine (electrophoresis technique). Nematode isolates were collected from the guava planting orchards of 6.5 km apart (between NT and PY) and 16.6 km apart (between NT and KT) (Fig. 18). However, each isolate and specimen (20 nematodes/isolate) exhibited a similar VS1-S1 phenotype. This phenotype comprised two bands (*M. enterolobii* phenotype) (Fig. 19). The faster band was as the same size as the only band of *M. incognita* (I1) phenotype and also similar to slowest band of *M. javanica* phenotype. The size of both bands of *M. enterolobii* phenotype was smaller than that of the only band found in *M. konaensis* (F1) phenotype (Fig. 20).

Figure 17 The root symptoms characteristic of *M. enterolobii* damage: A) Namthan orchard (NT), B) Payoon orchard (PY), C) Khlongton orchard (KT)

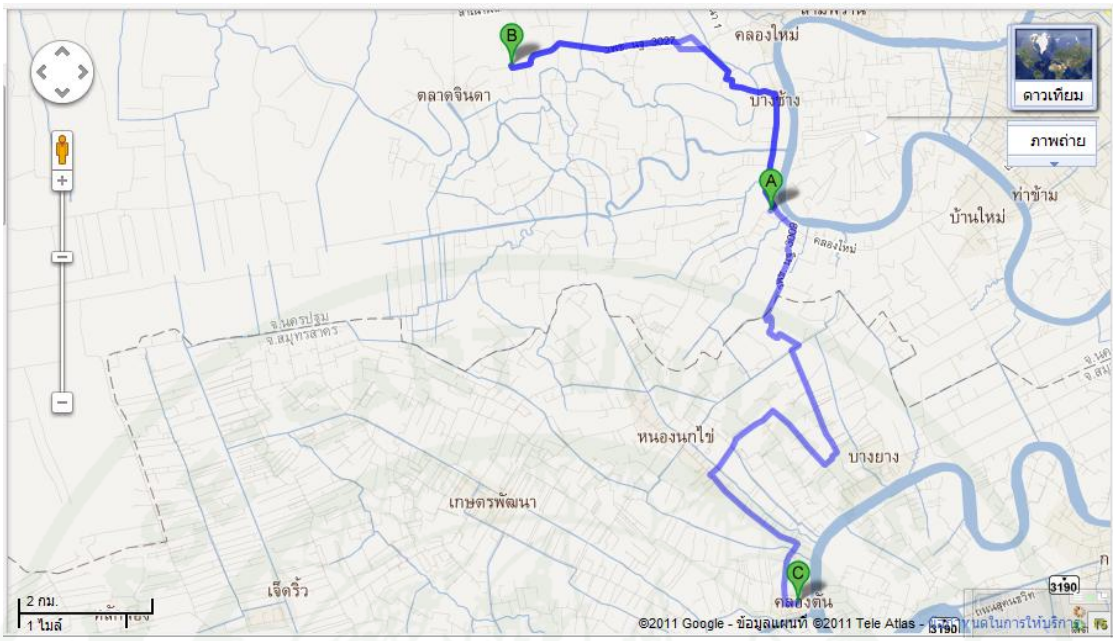

Figure 18 The location of guava orchards A) Namthan orchard (NT) 13˚41.9052’N 100˚12.3640'E, B) Payoon orchard (PY) 13˚43.0904’N 100˚10.1812’E, C) Khlongton orchard (KT) 13˚64.9323’N 100˚20.9389'E

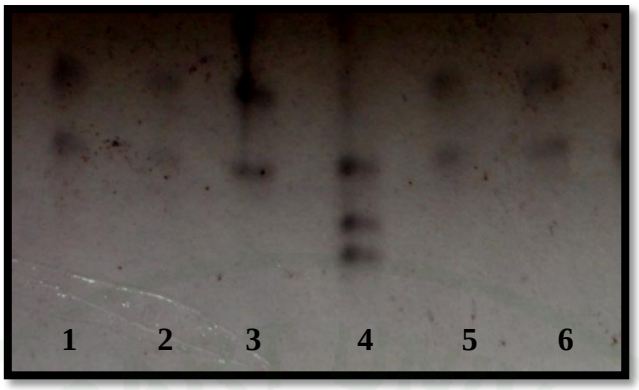

Figure 19 Characteristic of esterase phenotypes (VS1-S1) found in the three nematode isolates from guava in the central region of Thailand. Lane 1, 2, 3, 5 and 6 were single *M. enterolobii* females and Lane 4 was single *M. javanica* female

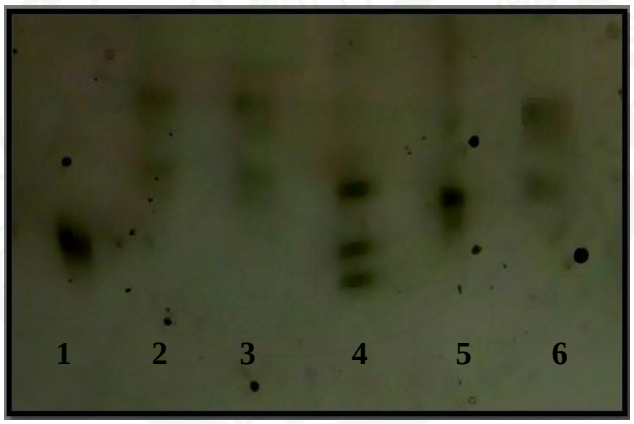

Figure 20 Characteristic of *M. enterolobii* found in the three nematode isolates compared with *M. javanica* (marker), *M. incognita* (slow band) and *M. konaensis* (fast band): Lane 1; *M. konaensis*, Lane 2; *M. enterolobii* isolate 3 (KT), Lane 3; *M. enterolobii* isolate 2 (PY), Lane 4; *M. javanica*, Lane 5; *M. incognita* and Lane 6; *M. enterolobii* isolate 1 (NT).

3. DNA analysis

Meloidogyne identification

In this study, the universal primers were designed from two mitochondria genes; Cytochrome Oxidase subunit II (COII) gene and 16s ribosomal RNA (16s rRNA) gene (Fig 21). UNI_COIIM was designed for COII gene; UNI_COIIFM: 5'-CTGATGTAATTCATGCTTGGG-3' and UNI_COIIRM: 5'-TCCACAAATTTCG GAACATTG-3. This primer yielded a PCR product of size 150 bp in size as shown in the lane no. 2-4 (Fig 22C). UNI_16sM was designed for 16s rRNA gene; UNI_16sFM: 5'-CGTGATTAGTCAAAGGTAGCAAGG-3', and UNI_16sRM: 5'-GATAAAGAACTCTATTTTACAACG-3'. This primer amplified a 400 bp PCR fragment as shown in the lane no. 2-4 (Fig 22B). The primer Nema_18s was designed to be specific to all nematodes in the Phylum Nemata. The resultant DNA fragment of about 1 kb was obtained in the lane 2 to lane 9 which were positive controls, *Meloidogyne incognita*, *M. javanica*, *Aphelenchoides* sp., *Helicotylenchus* sp., *Pratylenchus* sp., *Rotylenchulus* sp., and *Steinernema* sp., respectively (Fig 22A).

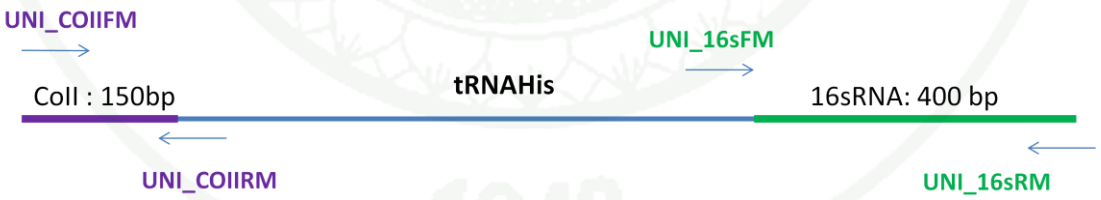

Figure 21 Schematic position of specific primers for amplifying to the Cytochrome Oxidase II region (UNI_COIIM, primer) and the 16s ribosomal RNA region (UNI_16sM, primer) on the mitochondria of *Meloidogyne*.

Meloidogyne Species identification

The primers, 1108 and C_2F_3, were designed to specifically identify the species of *Meloidogyne* spp. The samples included *M. incognita* and *M. javanica*, which had previously been standardized by the esterase enzyme technique before, and *Meloidogyne* samples from the three isolates (NT, PY, and KT orchards). The results demonstrated that DNA amplicons of about 1,700 bp were only obtained in *M. incognita* (lane no. 4) and *M. javanica* (lane no. 3). However, in NT, PY, and KT isolates of *Meloidogyne*, PCR products of approximate 700 bp (lane no. 5-7) were evident (Fig 23).

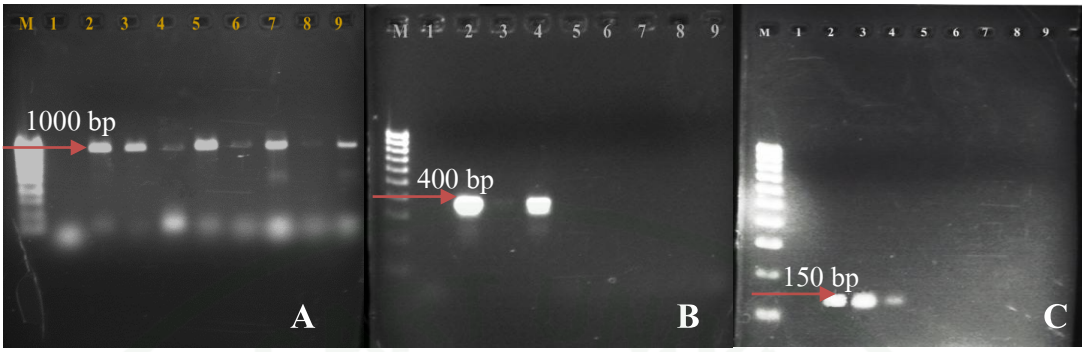

Figure 22 Amplification by each primer A) Nema_18s, 1kb, B) UNI_16sM, 400 bp, C) UNI_COIIM, 150 bp 1) Negative control 2) Positive control 3) *Meloidogyne incognita* 4) *M. javanica* 5) *Aphelenchoides* sp. 6) *Helicotylenchus* sp. 7) *Pratylenchus* sp. 8) *Rotylenchulus* sp. 9) *Steinernema* sp.

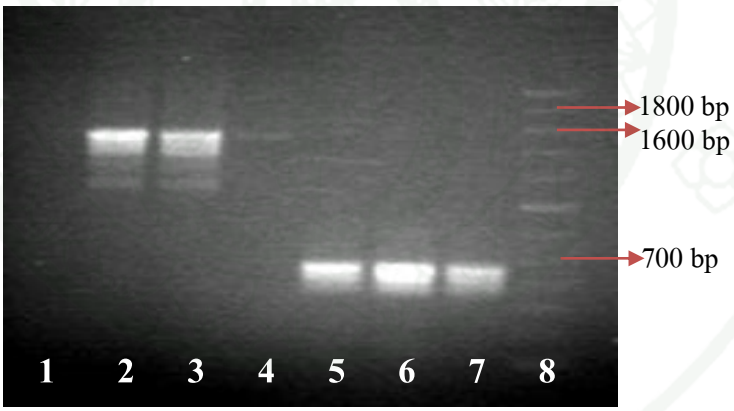

Figure 23 Identification of three isolates of root-knot nematodes (*Meloidogyne* sp.) by the primer sets (1108 and C2F3); 1) Negative control, 2) Positive control, 3) *Meloidogyne javanica*, 4) *Meloidogyne incognita*, 5) *Meloidogyne enterolobii* (NT), 6) *Meloidogyne enterolobii* (PY), 7) *Meloidogyne enterolobii* (KT), 8) marker

Discussion

Root-knot nematodes (*Meloidogyne* spp.) in guava in the central region of Thailand were studied in this research. The nematodes have long been reported as the causal agent of declining disease (i.e. yellowing, stunting, blight leaves) and reduction in guava yields in this region (Sukhakul, 2006 and Sasnarukkit *et al*., 2010). In this research, same symptoms were observed and the root-knot nematodes found in guava in this area were identified as *M. enterolobii*.

The observation on the characteristics of root symptoms showed that *M. enterolobii* induced more prominent galls on tomato (Orange Pixie cultivar) than *M. incognita* and *M. javanica*. In addition, most nematode females produced eggs within the root galls and males were abundantly produced. The females' bodies of *M. enterolobii* were transparent as compared to "milky" appearance observed in the females of *M. incognita* and *M. javanica*. Normally, *Meloidogyne* spp. reproduce pathenogenically and their offspring are mostly females. Under stress conditions, however, the nematode population changes into more males. In this study, it demonstrated that *M. enterolobii* could grow and reproduce on "Orange Pixie cultivar" tomatoes with abundant male offspring. Therefore, it is postulated that "Orange Pixie cultivar" might not be a good host or be tolerate to some extent to *M. enterolobii.* Furthermore, the difference in the characteristics of gall symptoms on Cherry and Orange Pixie tomato cultivars (inducing small and big galls respectively) might be due to the difference in environmental conditions ("Cherry" were grown in Thailand but "Orange Pixie" in USA) or the genetic backgrounds of the two tomato cultivars.

Perineal patterns of the three isolates of *M. enterolobii* were analyzed and compared with the three isolates of *M. incognita* found in Nakhon Pathom and Samut Sakhon provinces. The results illustrated that perineal patterns of *M. enterolobii* were in oval or round shape, some with or without lateral lines, whereas those of *M. incognita* had high dorsal arches and were without lateral lines. Perineal patterns of *M. enterolobii* have been reported to be highly variable among populations as well (Brito *et al.*,2004). In this study, it showed that the perineal patterns of *M. enterolobii*

were more similar to *M. arenaria* than *M. incognita*. In 2004, Brito reported that perineal patterns of *M. enterolobii* were in round or dorsally and ventrally ovoid shape. The patterns also had high trapezoidal dorsal arches which are similar to *M. incognita*. Therefore, it is difficult to differentiate *M. enterolobii* (or *M. mayaguensis*) from *M. incognita* by only observing the morphology of perineal patterns. The previous reports of the occurrence of *M. incognita* in guava in this area, therefore, are in urgent need to be reconsidered since only perineal patterns of nematodes were used for identification. In this study, the identification based on the examination of the nematode esterase enzyme and the space between two mitochondrial genes was employed. As the result, *M. enterolobii* were found in NT, PY, and KT orchards. However, nematode samples collected from KJ, TV, and AP orchards in which only perineal patterns of the nematodes were determined, *M. incognita* were the only species present in these areas. These six guava orchards are located in proximity; it is likely that *M. enterolobii* are also present in KJ, TV, and AP orchards.

The measurement of the second-stage juveniles of the three isolates of *M. enterolobii* was conducted and their morphometric values were compared with the original description (Rammah and Hirschmann, 1988) and the Florida population (Brito *et al*., 2004). Nematode length (L) ranged from 335-410 µm for NT, 330-420 µm for PY, and 310-400 µm for KT. These were significantly lower than those of the original description and the Florida population (Table 8). The measurement of the second-stage juveniles of *M. incognita* and *M. enterolobii* was also made. The results showed a significant difference in the length (L), body length/ width (a), body/ tail length (c), hyaline tail terminus (h) and h/ tail length x 100 (h%) between the two species.

Table 10 Morphometrics (mean, standard deviation, and range) of second-stage juveniles of three isolates of *Meloidogyne enterolobii* from Thailand**.**

Character	Isolate 1 (Namthan)	Isolate 2 (Payoon)	Isolate 3 (Khlongton)	Florida population	Original description
L (mean length)	366±23.63 (335.0-410.0)	376.25±26.70 (330.0-420.0)	367.50±26.33 (310.0-400.0)	450.58±19.45 (377-491.5)	453.6±28.4 (390.4-528.0)
a (body length/width)	25.78±4.69 (20.1-39.0)	32.37±5.85 (23.0-41.0)	24.32±4.49 (18.1-35.0)	29.33±1.35 (25.0-33.1)	30.9±1.9 (26.4-34.7)
c (body/tail length)	8.66±1.16 (6.96-11.21)	6.45±0.77 (5.3-8.0)	8.97±1.41 (6.61-13.33)	8.4±0.4 (7.4-9.9)	8.3±0.4 (7.0-9.2)
Body width	14.50±1.79 (10.0-16.0)	12±2.38 (10.0-15.0)	15.65±3.31 (10.0-12.0)	15.4±0.5 (14.5-16.1)	14.7±0.5 (13.8-15.8)
stylet length	13.48±1.23 (11.0-16.0)	12.75±1.38 (10.0-15.0)	12.60±1.30 (10.0-14.0)	10.88±0.28 (10.2-11.5)	11.6±0.3 (11.1-12.2)
DGO	2.69±0.49 (2.0-4.0)	4.10±0.52 (3.2-5.0)	2.55±0.71 (1.5-4.0)	3.45±0.3 (2.0-4.7)	3.9±0.2 (3.3-4.3)
Excretory pore to anterior end	65.65±9.02 (33.0-75.0)	65.25±5.19 (55.0-75.0)	61.25±7.95 (40.0-75.0)	92.5±7.38 (83.3-99.9)	87.6±3.3 (79.9-97.9)
tail length	42.95±5.74 (33.0-56.0)	59.13±8.20 (45.0-77.5)	41.70±6.02 (30.0-56.0)	53.93±3 (43.1-61.2)	54.4±3.6 (49.2-62.9)
Hyaline tail terminus(h)	8.80±2.50 (5.0-15.0)	7.63±1.90 (5.0-12.5)	9.65±2.18 (5.0-13.0)	11.03±1.93 (5.0-14.7)	-
h%(h/tail length x 100)	20.53±5.17 (11.63-30)	12.88±2.30 (8.7-16.7)	23.45±5.66 (11.11-35.14)	-	-

The patterns of the esterase isozyme of the three isolates of *M. enterolobii* were of phenotype VS1-S1. This phenotype was reported for other *M. enterolobii* or *M. mayaguensis* elsewhere (Zhuo *et al*., 2010; Brito *et al*., 2004; Carneiro *et al*., 2001; Fargette *et al*., 1996 and Rammah and Hirschmann, 1988). VS1-S1 phenotype comprised two bands. The faster band was in the same size as the only band of *M. incognita* (I1) phenotype and also similar to the slowest band of *M. javanica* phenotype. The size of both bands of *M. enterolobii* phenotype was smaller than that of the only band found in *M. konaensis* (F1) phenotype (Esbenshade and Triantaphyllou, 1985, Eisenback *et al*., 1994). When one female was used, the esterase phenotype comprised more slightly fainted or fainted bands, making the detection more difficult and unclear, as compared to those of *M. javanica, M. incognita* and *M. konaensis*. This characteristic of esterase phenotype may be due to low amount of the isozyme obtained from the uniquely transparent females of *M. enterolobii* freshly retrieved from tomato roots. Even through, there are some drawbacks associated with the esterase band pattern of *M. enterolobii*, this method is still convenient, rapid, and reliable.

Development of a molecular marker only specific to root-knot nematodes in guava *Meloidogyne* was performed as well. *Pratylenchus, Rotylenchulus, Helicotylenchus,* and *Aphelenchus* collected from guava fields and the entomo-pathogenic nematode *Steinernema* were used to compare with root-knot nematodes. The 16s ribosomal RNA (16s rRNA) gene and cytochrome oxidase II (COII) gene were aligned and characterized for intraspecific genetic variations in *Meloidogyne*. UNI_16sM and UNI_COIIM were developed with lengths of 400 bp and 150 bp respectively. The primer UNI_16sM and UNI_COIIM were designed to detect specific *Meloidogyne* The primer set UNI_16sM and UNI_COIIM were developed to specifically amplify the portions of mitochondrial genes in *Meloidogyne*. The resultant bands following using these two primer sets were 400 bp and 150 bp in size, respectively. UNI_16sM and UNI_COIIM were only specific to *Meloidogyne* but not to *Pratylenchus*, *Rotylenchulus*, *Helicotylenchus*, *Aphelenchus* and *Steinernema*. In addition, UNI_16sM and UNI_COIIM are able to prime DNA that is damaged, incomplete, or in low concentration.

The identification of the species of *Meloidogyne* spp. was based on the protocol and the primer set (1108 and C2F3) developed by Power and Harris (1993). This primer set has been widely used for species identification of root-knot nematodes for more than 15 years (Blok and Power, 2009). In this study, DNA products of approximate 700 bp were obtained following the amplification of DNA extracted from the three nematode isolates with the primer 1108 and C2F3. The result from this study was coincided with the studies conducted by Brito *et al*. (2004) (705 bp in Florida sample), Zhuo *et al*.(2010) (705 bp in China sample), and Iwahori *et al*. (2009) (705 bp in Vietnam sample). However, in the study by Blok *et al*.(2002), the size of the DNA product of *M. enterolobii* was 750 bp. This might indicate the existence of haplotypes (mitochondrial sequences with at least one confirmed nucleotide substitution) among geographical populations of *M. enterolobii* (Brito *et al*., 2004).

CONCLUSION

In this study, *Meloidogyne enterolobii* or *M. mayaguensis* was firstly reported in guava in Thailand. Nakhon Pathom and Samut Sakhon provinces are important guava cultivating areas in the central region of Thailand. Guava production from these two provinces constitutes approximately 52.61% of the whole country production. In these areas, root-knot nematodes (*Meloidogyne* spp.) are widely distributed as well. Surveys of six guava orchards (i.e. Thaveesak orchard (TV), Khlongjinda orchard (KJ), Payoon orchard (PY), Apinya orchard (AP), Namthan orchard (NT), and Khlongton orchard (KT)) were made. Three soil samples per orchard were randomly taken from guava trees exhibiting nematode-infected symptoms (e.g. stunting, wilting and yellowing). The average number of second-stage juveniles found in 300 grams of soil of each orchard was as follows: 6.67, 11, 91, 16.33, 28, and 42 J2 in TV, KJ, PY, AP, NT, and KT respectively.

In the greenhouse at the Department of Plant Pathology, Kasetsart University, Bangkhen campus, tomato plants (Cherry cultivar) were grown in 15-cm-diameter pots filled with mixed soils (sterile soil + nematode-infested soil) (5 pots per sample). Two months later, egg masses of root-knot nematodes were retrieved from tomato roots. Subsequently, eggs were allowed to hatch into second-stage juveniles (J2) in sterile water. The measurement of the size of J2 was followed. The results showed thatL value (mean length) was smaller than the standard value (original description) but a value (body length/width), c value (body length/tail length) and h value (hyaline tail terminus) close the to standard values.

The perineal patterns of the adult females of root-knot nematodes were observed and two species of *Meloidogyne* were found. *M. incognita* was found in TV, KJ and AP orchard, but *M. enterolobii* existed in NT, PY and KT orchards. The perineal patterns of *M. enterolobii* were variable with moderately high to high dorsal arches, a roundly or oval shape and with or without a lateral line. They were also similar to those of *M. incognita* and *M. arenaria*. Therefore, they are difficult to

separate using only perineal patterns. However, *Meloidogyne enterolobii* has not been reported in Thailand.

The characteristics of root symptoms induced by root-knot nematodes *Meloidogyne enterolobii* on tomatoes (Orange Pixie) at the Magoon greenhouse, Hawaii, USA were observed. The plants were heavily galled. Adult females had transparent bodies, and many males were present. These results were not observed in *M. incognita*. Therefore, tomatoes (Orange Pixie) might be tolerant to *M. enterolobii* because the tomatoes could still survive under heavy infection.

The esterase phenotype of *M. enterolobii* was determined and compared to that of *M. incognita, M. konaensis* (coffee root-knot nematode) and *M. javanica* (standard marker). The results indicated that the three isolates were *M. enterolobii* because of two major bands (VS1-S1 phenotype), which is characteristic of *M.enterolobii*. *M. javanica, M. incognita* and *M. konaensis* have J1, I1 and F1 phenotype respectively.

Molecular identification of root-knot nematodes (*M. enterolobii*), using a primer set derived from mitochondria DNA of cytochrome oxidase subunit II and 16s ribosomal DNA 1108: 5'-TACCTTTGACCAATCACGCT-3' and C2F3 : 5'-GGTC AATGTTCAGAAATTTGTGG-3' was carried out. A band of approximate 700 bp was found in the three isolates of *M. enterolobii* as compared to a band of approximate 1,700 bp observed in *M. incognita* and *M. javanica.* These results reiterated that *M. enterolobii* was present in guava. From these studies, it is the first time that *M. enterolobii* is repeated in Thailand.

Development of a molecular marker only specific to root-knot nematodes in guava renders a rapid and correct identification and detection of the nematodes at the guava seedling stage. *Meloidogyne* was compared with *Pratylenchus, Rotylenchulus, Helicotylenchus, Aphelenchus* (all were collected from guava fields) and the entomo-pathogenic nematode *Steinernema*. The 16s ribosomal RNA (16s rRNA) gene and

cytochrome oxidase II (COII) gene were used to develop a specific molecular marker. These genes were aligned and characterized for intraspecific genetic variation in *Meloidogyne*. The primer UNI_16sM and UNI_COIIM were designed from a portion of the conserve region of the mitochondria to ally specific detect *Meloidogyne.* A band of 400 bp and 150 bp was obtained, respectively, following using these two primers.

The primer Nema_18s was designed to be specific to the Phylum Nematoda and amplified a 1 kb fragment. Primer Nema_18s was compared with Primer UNI_16sM and UNI_COIIM, and produced similar results in detecting *Meloidogyne*. Nema_18s could amplify the other genera in the Phylum Nemata whereas UNI_16sM and UNI_COIIM cannot amplify those genera. Therefore, UNI_16sM and UNI_COIIM are only specific to *Meloidogyne* and do not detect other genera found in guava in Thailand. UNI_16sM and UNI_COIIM are able to prime DNA that is damaged, incomplete, or in low concentrations. UNI_16sM and UNI_COIIM are specific and efficient with *Meloidogyne* and to be used to differentiate *Meloidogyne* from other nematode genera found in guava in Thailand.

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