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THESIS

VARIATION OF ROOT-KNOT NEMATODE INFECTING CHILI IN THAILAND

THANAKORN CHANMALEE

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Agricultural Biotechnology)
Graduate School, Kasetsart University
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Thanakorn Chanmalee 2014: Variation of Root-Knot Nematode Infecting Chili in Thailand. Master of Science (Agriculture Biotechnology), Major Field: Agriculture Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Ms. Orawan Chatchawankanpanich, Ph.D. 70 pages.

Fifty soil samples and root knots surrounding infected chili were collected from 10 provinces. They were Chiang Mai, Lamphun, Tak, Khon Kaen, Phetchaburi, Srisaket, Suphan Buri, Ubon Rachatani, Uthai Thani and Songkla. Thirty four samples were classified in tropical species by PCR with 194/195 primers. Nine samples were not classified in tropical species, and it might be *Meloidogyne mayaguensis* according to nucleotide sequences. While, seven samples gave negative result with PCR, so they were unidentified samples. Only tropical species were further used for identification in species level by perineal pattern and PCR with specific primers for *M. arenaria*, *M. incognita* and *M. javanica*. From identification of perineal pattern, twenty eight samples were identified as *M. incognita* and six samples were identified as *M. javanica*. In contrast, twenty seven samples were identified as *M. incognita* and five samples were identified as *M. javanica* by PCR. While, two samples from Chiang Mai (CM6) and Ubon Ratchathani (UB6) gave negative result, so they were not be identified by PCR. In this study, *M. arenaria* was not detected to be a causal agent of root knot disease of chili in Thailand. Samples identified as *M. incognita* and *M. javanica* by both perineal pattern and PCR were used for sequence analysis. Samples identified as *M. incognita* were samples from CM1, CM5, LP1, TK1, TK2, TK5, TK6, TK7, TK8, TK10, TK12, TK13, TK16, TK17, KK1, SK2, SP1, SP2, SP3, SP4, UB3, UB4, UB5, UB7 and UB8. Four samples from CM4, SKH1, SKH2 and SKH3 were identified as *M. javanica*. According to sequence analysis and phylogenetic tree, the genetic variation was not found in population of *M. incognita* and *M. javanica* infecting chili in this study.

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Student's signature Thesis Advisor's signature

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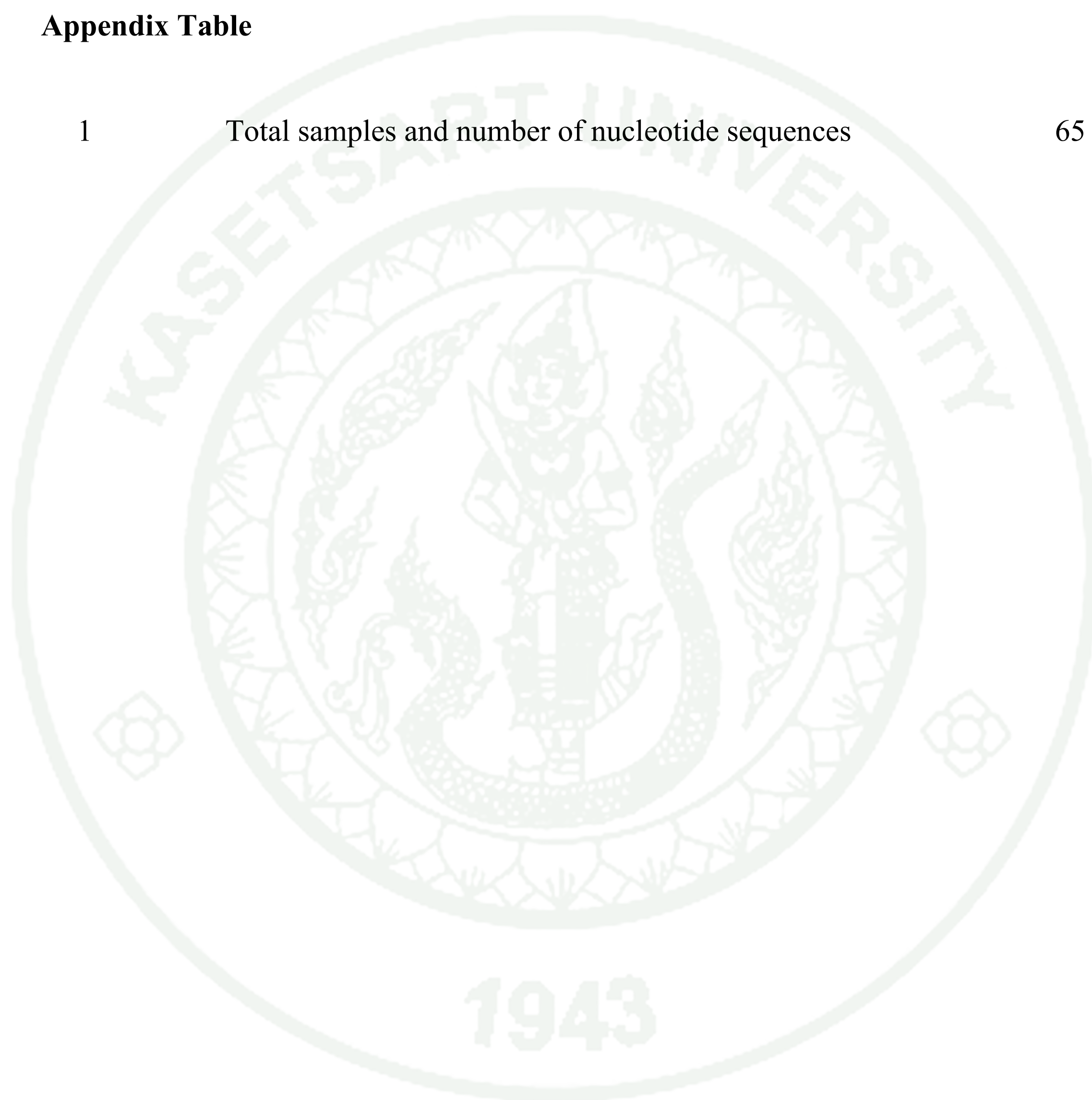

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LIST OF ABBREVIATIONS

bp	=	base pair
°C	=	degree Celsius
$CaCl_2$	=	calcium chloride
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
$CuSO_4$	=	copper sulphate
DNA	=	deoxyribonucleic acid
DNase	=	Deoxyribonuclease
dNTP	=	deoxynucleotide-5'-triphosphate
EDTA	=	ethylenediamine tetraacetic acid
EtOH	=	ethanol
g	=	gram
h	=	hour
kb	=	kilobase pair
l	=	liter
M	=	molar
µg	=	microgram
µl	=	microliter
µM	=	micromolar
mg	=	milligram
mM	=	millimolar
MW	=	molecular weight
$MgCl_2$	=	magnesium chloride
NaCl	=	sodium chloride
ng	=	nanogram
PCR	=	Polymerase chain reaction
RNase	=	ribonuclease
rpm	=	rotation per minute
sec	=	second

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LIST OF ABBREVIATIONS (Continued)

TAE	=	Tris-acetate-EDTA
E. coli	=	*Escherichia coli*

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VARIATION OF ROOT-KNOT NEMATODE INFECTING CHILI IN THAILAND

INTRODUCTION

Chili is an economic vegetable crop in Thailand with high yield and value. Chili is grown all part of Thailand and all seasons. Most is used for domestic consumption. Chili is exported as fresh and processed chili. Thai chili varieties are spicy and aromatic. Chili production is not enough with uncertain quantity. The main problem is from pest including insect pest (trips and white fly) and plant diseases caused by bacteria, fungi, virus and nematode. The outbreak of disease is more severe depending on the weather, soil pH, soil type, human, organic matter in the soil, contamination in compost manure and propagation, seed contamination, soil materials, diseased plant residues and agriculture tools (Tangchitsomkid and Prakob, 2007).

In 2006-2007, the outbreak of root knot disease caused by *Meloidogyne incognita* was reported in chili plantation of Ubon Ratchathani province, Thailand. The area of the outbreak was more than 1,186 acres (3,000 rais). It decreases quality and quantity of chili about 50-100%. Some areas cannot be used for next chili cultivation because of the cumulative of root-knot nematode. Root-knot nematode is rapidly spread in sandy loam to new areas. This problem affected famers in north and northeast of Thailand. A total yield loss was about 50-80 million baths. The spread of nematodes is with irrigation, rain and contamination with agricultural tools. The nematode in infected soils can attach to wheels of the tractor to new areas. In addition, nematode contaminated in plant material and soil can spread to healthy chili seedlings in the field. The problem causes economic impact. In the future, the root knot disease can spread to other areas, if not be controlled correctly (Tangchitsomkid and Prakob, 2007).

Root knot disease can be controlled in many ways, such as planting chili in nematode-free soil, crop rotation, biological control using antagonistic organisms and

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chemical control. Even though, chemical control is effective, but it damages environment, human and animals. The chemical control leaves residues on products and in environment, and nematode can develop resistant to chemical. In addition, the chemical can kill and decrease the beneficial microorganisms in soil. Nowadays, the alternative ways to control root knot disease with more friendly to environment, human and animals are getting more attention. Biological control such as use of nematicide from actinomycete can control root-knot nematode (Ruanpanun *et al.,* 2010). The best way to control root-knot nematode is use of resistant plant to root-knot nematode. It can reduce population of root-knot nematode and not harm to environment, but is specific to root-knot nematode species (Fery and Thies, 2007).

The virulence of root knot disease depends on population and genetic variation of root-knot nematode. Study of genetic variation of root-knot nematode is important for accurate diagnosis and control management. However, the genetic variation of root-knot nematode infecting chili in Thailand is not well investigated. The information on genetic variation of root-knot nematode infecting chili will be useful for epidemic forecast of root knot disease, approaches for control of disease and breeding program for the resistant varieties.

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OBJECTIVE

To study of genetic variation of *Meloidogyne* spp. infecting chili in Thailand

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LITERATURE REVIEW

1. Chili

Chili is a plant of *Solanaceae* family, like tomato, potato and tobacco. The scientific name is *Capsicum* sp. The common name is chili pepper. The origin of chili was in tropical area of America, South America and Middle America. Chili was introduced to Spain since the Columbus era and distributed to other countries. Spanish and Portuguese introduced chili into Thailand since 100 years ago. Chili is spicy and is used for cooking to increase flavor and savor of food, attractive color for eat. Currently, many countries use chili for cooking because it has high nutritional value. Chili has the medicine properties with high vitamin C and ascorbic acid. Ascorbic acid helps expand the blood vessels in the intestines and stomach. It improves food absorption, transport nutrients to body tissue and helps for waste excretion. Thai chili (*C. annuum* and *C. furtescens*) has high vitamin C about 87.0 – 90 mg/100 g and high beta-carotene or vitamin A. In addition, compounds in chili are capsaicin and oleoresin. Capsaicin has a peppery taste and is used in food industry, medicine and therapy. It can reduce the muscle pain of waist, arms, shoulder and other parts of body. Chili also contains other substances that are homocapsaicin, homodihydrocapsaicin, nordihydrocapsaicin and dihydrocapsaicin (Techawongstien, 2006).

Chili can be cultivated in all areas of Thailand. It is a perennial crop, but use as annual crop. It grows well in tropical weather and all year round. Seed germinates at about 20-30 °C. The optimum temperature for growth is 25 °C. The soil pH for planting is about 5 – 6. Now, two types of chili are separated according to fruit size. The first is “big size of fruit” (3-8 cm), and the main cultivations are Chiang Mai, Nakhon Sawan, Uttaradit, Lamphun, Ratchaburi, Nakhon Ratchasima, Chaiyaphum and Loei. The second type is “small size of fruit” (1-3 cm), and the main cultivations are Chiang Mai, Nakhon Sawan, Phetchabun, Mukdahan, Chaiyaphum, Khon Kaen, Sisaket, Nakhon Ratchasima, Ubon Ratchathani and Kanchanaburi (Tapsomboon, 1997).

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2. The economic importance of chili

Nowadays, the chili crop is planted widely with more than 98 countries around the world. In 2012, the total area of chili crop in Thailand is 165,845 acres (419,471.70 rais) and total of yields is 634.64 million tons (Department of Agriculture Extension, 2012).

In 2012, the production areas decreased from 221,406.42 acres (560,000 rais) to 165,845.66 acres (419,471 rais) because of drought. Fifty-two percent of chili production was big fresh chili variety (prig kee nu yai), 40 % was small chili variety (prig kee nu lek) and 7.3% was fresh chili variety (prig kee nu). The main growing areas were Chiang Mai, Chiang Rai, Tak, Khon Khen, Sakhon Nakhon, Nakhon Ratchasima, Ubon Ratchathani, Sisaket, Chaiyaphum, Loei, Nakhon Pathom, Ratchaburi, Surat Thani and Songkla (Department of Agriculture Extension, 2012).

The value of exports in each year was from fresh chili and dried chili. Fresh chili was exported to Malaysia, Japan, Singapore, Taiwan and the Netherlands. Dried chili was exported to United States of America, Australia and Germany. The export of chili and its products during the year 2007 to 2012 decreased. The average value of export was about 512.95 million baht in 2007, but decreased to 153.66 million baht in 2012. The export value of chili decreased every year since 2008 because of insufficient production even for domestic consumption (Figure 1) (National Food Institute, 2012).

The import of chili and its products during 2007 to 2012 was higher than export of chili for both quantity and value. Both fresh and dried chili was imported into Thailand. Most of imported dried chili was from China, India, Myanmar and Indonesia (Figure 1) (National Food Institute, 2012).

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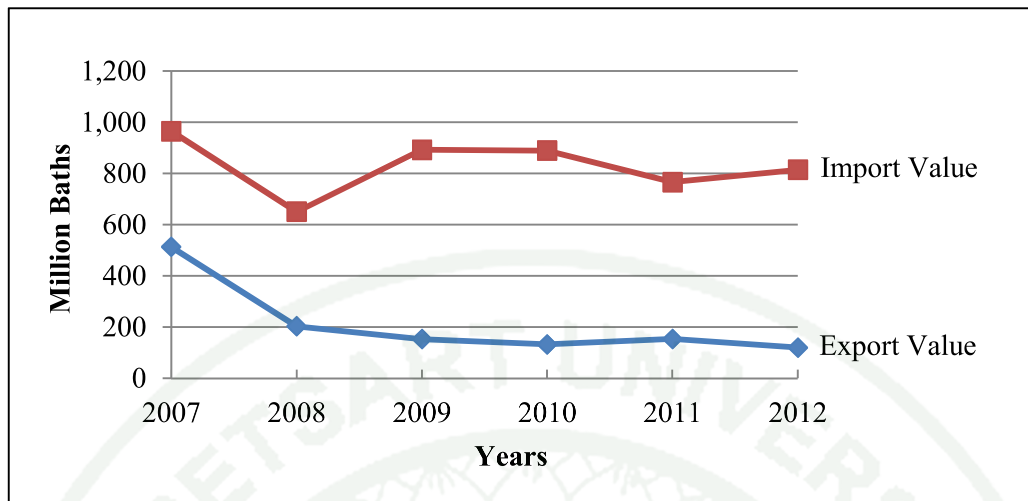

Figure 1 Import and export value of chili and chili products of Thailand from 2007 to 2012.

Source: National Food Institute (2012)

3. Characteristic of root-knot nematode

Root-knot nematode is classified in phylum *Nematoda*, class *Secernentea*, order *Tylenchida*, family *Meloidogynidae*, sub family *Meloidogyninae* and genus *Meloidogyne* (Hirschmann, 1985). It is an invertebrate animal, multicellular, except for the cyst stage. The body shape is eelworm and no appendages. The epidermis has annule lines. The root-knot nematode has 6 different systems including body wall, digestive system, reproductive system, excretory system, muscular system and nervous system. Stylet is special organ of plant parasitic nematodes. Currently, the identification of root-knot nematodes in genus *Meloidogyne* is more than 100 species (Moens *et al.,* 2009). The common root-knot nematodes are *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Taylor and Sasser, 1978).

Root-knot nematode is a high difference between male and female (Figure 2). The second stage juvenile (J2) of both is eelworm shape. The body length is 300-500 micrometer. Head (J2 anterior end) and J2 pharyngeal region are not clear. The J2 tail is conical. The systems of J2 develop complete, except for reproductive system.

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The adult male is long eelworm, about 700 – 2,000 µm. It is free living in soil. The anterior and pharyngeal regions are clear and have spicule and complete systems in the body (Figure 2). The adult female is pear shape (pyriform), and median length is about 0.325 to 0.700 mm. It has a symmetrical bodies and neck. Adult female has two ovaries. The end of body position is round having the reproductive organs (ovary) and anus. They are wrinkled and called the perineal pattern (Figure 2) (Eisenback and Hunt, 2009; Eisenback and Triantaphyllou, 1991; Taylor and Sasser, 1978; Williamson and Hussey, 1996).

4. Life cycle of root-knot nematode

Root-knot nematode has wide host range. Only *Meloidogyne incognita*, *M. javanica* and *M. arenaria* are classified in "Tropical species". They generally live in temperature to a region between 40 °N and 35 °S latitudes (Power and Harris, 1993). Optimum soil temperature for tropical species is 18 – 30 °C and optimum soil temperature for reproduction is 24 – 27 °C. Reproduction of adult female is parthenogenesis. Egg is not fertilizing with male and it can grow to juvenile stage. The female chromosomes are 2n = 32 – 36 (diploid) and 2n = 40 – 46 (triploid). The triploid are generally found. Life cycle of root-knot nematode starts from egg and double chromosome in prophase 1. The next stage is metaphase; the first stage of juvenile in the egg develops to second stage of juvenile. The female produces egg called "egg mass or egg sac" containing eggs about 250 – 525. Eggs are coated by gelatinous matrix (Figure 3) (Eisenback and Hunt, 2009; Hunt and Handoo, 2009).

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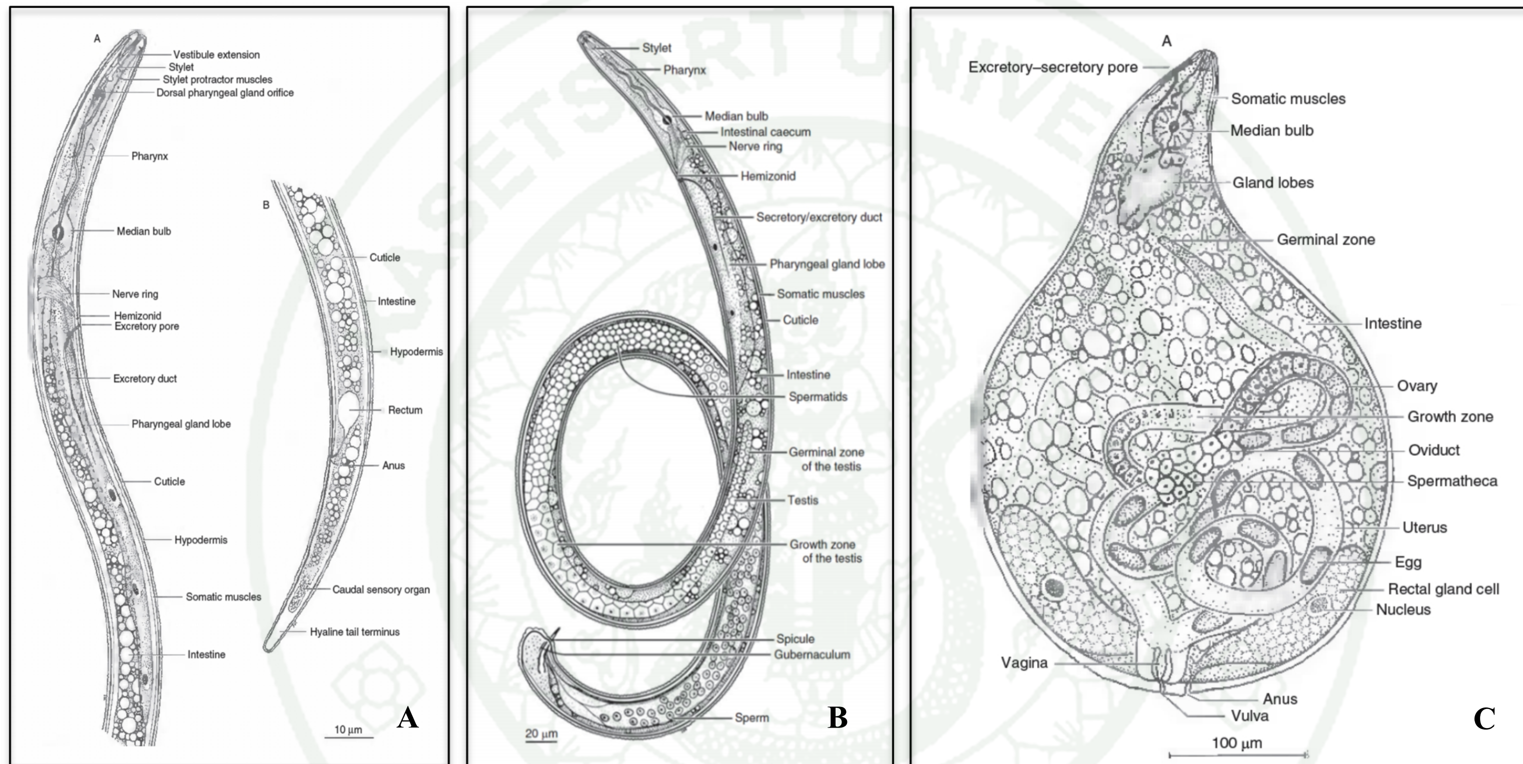

Figure 2 Morphology of root-knot nematode; Second stage juvenile (J2) (A), Morphology of adult Male (B), Morphology of adult female (C)

Source: Eisenback and Hunt (2009)

A first molt of root-knot nematode is inside the egg (Figure 3). The first stage juveniles (J1) develop to second stage juveniles (J2) before hatching. The second stage is juvenile (J2) hatch from eggs by stylet piercing the eggshell. The fresh J2 is infective stage. J2 moves toward roots producing root exudate for initiation of infection. It uses stylet organ to open the plant tissue and embeds head into intercellular of root tissue (cortex) and secretes cell-wall-degrading enzymes. J2 moves vertically in the root to find the feeding site near vascular tissue. Then, J2 becomes sedentary and begins feeding until it develops to adult stage (Figure 4). Nematode induces parenchyma root cells into multinucleate and hypertrophy feeding cells. They have a multinucleate cell. These are "giant cells" or "mother cells" that are sink source for root-knot nematode feeding in order to supply nutrients to nematode until reproduction (Figure 5).

Afterward, J2 enters to sedentary stage and sucks the giant cells. It develops to third stage juvenile (J3). The fourth stage juvenile and adult are developed from molted J3. The molt of female increases the body size. The final molt of female is the pear shape and small head (median length is 0.325 – 0.700 mm). The reproductive stage of female produces egg sac on vulva which locates outside the root. Female lays egg and releases it on the root surface in a gelatinous matrix. Embryogenesis within the egg is followed by the first molt, leading to the J2 (Eisenback and Hunt, 2009; Taylor and Sasser, 1978; Williamson and Hussey, 1996).

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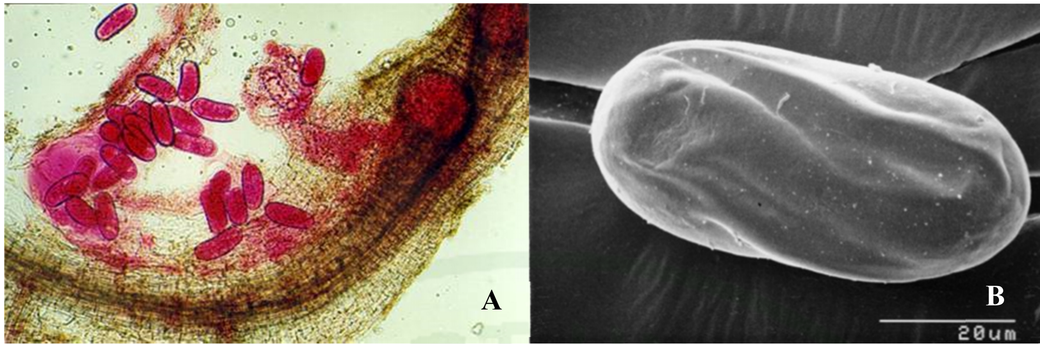

Figure 3 Egg mass of root-knot nematode; Egg mass or egg sac (A) and an egg of root-knot nematode (B)

Source: Mitkowski and Abawi (2003)

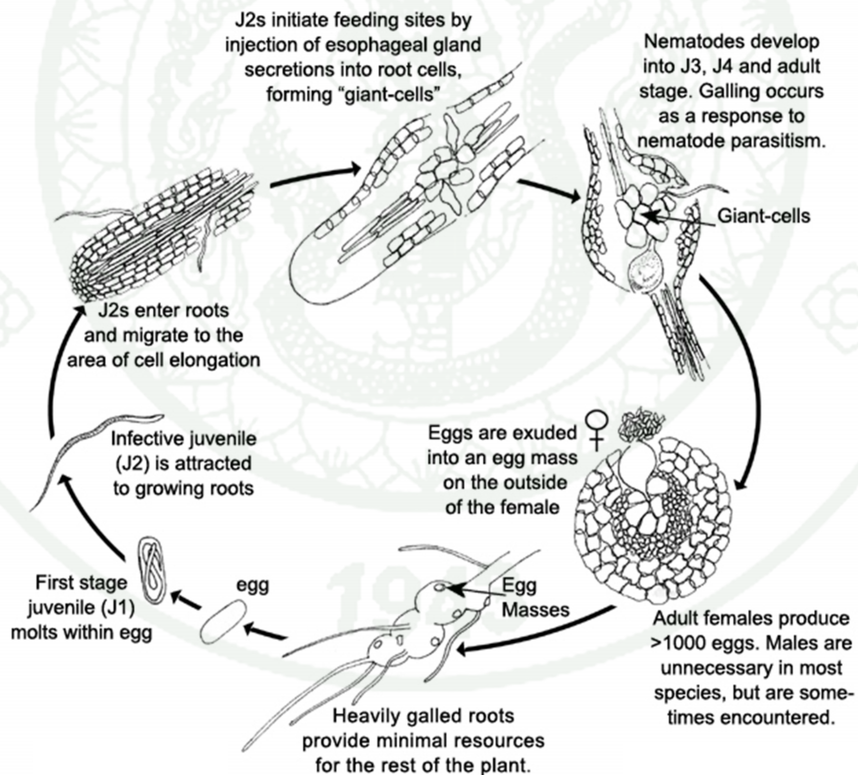

Figure 4 Life cycle of root-knot nematode

Source: Mitkowski and Abawi (2003)

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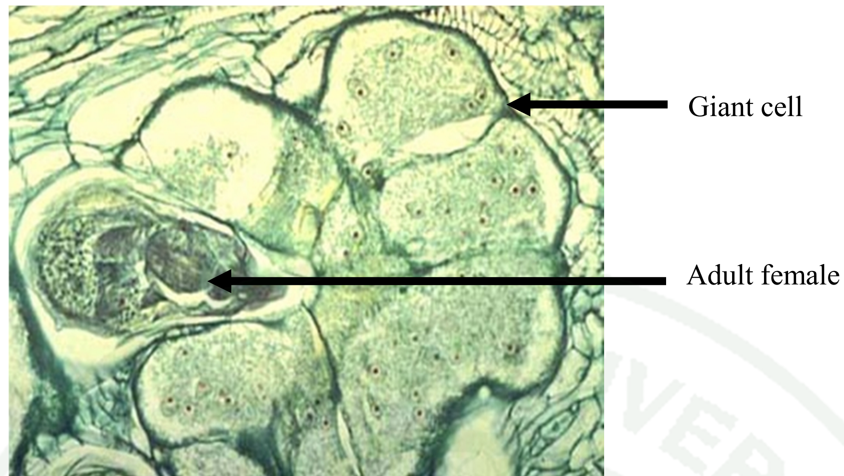

Figure 5 Adult female embedding in the root closely abnormal cell in root tissue called “giant cell”

Source: Mitkowski and Abawi (2003)

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5. Root knot disease of chili

Chili cultivation encounters huge problem from many diseases caused from fungi, bacteria, viruses and nematodes. The root knot disease is one of the most important diseases of chili. It caused by root-knot nematode (*Meloidogyne* spp.). In 2006, the severe outbreak of root-knot nematode in Ubon Ratchathani was reported. It caused damage to quantity and quality about 50-100% of chili yield. Currently, some areas with high epidemic of root knot disease cannot grow chili. Root-knot nematode is rapidly spread in the sandy loam soil. The problem leads to economic impact to chili growers in the Northeast. The quantity and quality of yield are decline. It affected income of farmers (Tangchitsomkid and Prakob, 2007).

Root-knot nematode makes wounds on the root and this is the route for secondary pathogen infection**.** The root knot disease of chili has above and underground symptoms. Symptoms of aboveground are yellowing, stunt and wilt on leave (Figure 6). It decrease yield of chili due to the root and vascular system are destroyed by nematode infection. The underground symptom is swelling of root or gall on the root (Figure 7 and Figure 8).

Soil environment has high impact to the life cycle of root-knot nematode. Many factors promote the root-knot nematode for damaging the host plant. Each of nematode species needs specific conditions for growth such as temperature, humidity, soil type and soil pH. In Thailand, three species of root-knot nematode were found including *M. incognita, M. javanica* and *M. arenaria*. These species exist in specific range of temperature about 25-30 ºC. Sandy loam is suitable for spread of root-knot nematode because it can absorb water. In addition, the root-knot nematode can move with water coat on soil particles. Soil pH is important to life cycle of root-knot nematode. General rang of pH 4.0-8.0 is suitable for living of root-knot nematode and other organisms (Evans and Perry, 2009).

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Figure 6 Chili is infected with root-knot nematode shown stunting, yellowing of leaves and wilts

Figure 7 Root galls caused by root-knot nematode

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Figure 8 Root knot disease underground symptom; root galls (A); galls with brown-egg mass produces the adult female on the root (B)

Source: Mitkowski and Abawi (2003); Tangchitsomkid and Prakob (2007)

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6. Identification of root-knot nematode

Root-knot nematode can be identified by morphological characterization, differential host test and molecular identification. The morphological characterization such as perineal pattern and ratio of body is time consuming, difficult and inaccurate depending on skill of each inspector. Differential host test is labor intensive, time consuming and inaccurate. With the advent of molecular biological techniques, it has been used in combination with morphological characteristic and differential host test to precisely identify root-knot nematode. The molecular identification is rapid and accurate (Hunt and Handoo, 2009).

6.1 Morphological identification

The technique is classified by morphological characteristic of the root-knot nematode. It measures the size of head, stylet pattern, perineal pattern, ratio of body length, location of excretory pore, shape of stylet knob, head shape and lip of each species of nematode (Eisenback *et al.,* 1981; Eisenback and Hunt, 2009).

6.2 Identification by perineal pattern

The perineal pattern is located on bottom of female. It is cuticle encompassing the vulva and anus. The variation of perineal pattern depends on species of nematode. This technique begins by preparation a piece of perineal pattern on slides and observing under the high magnification microscope. Each species of nematode has different patterns (Figure 9 and Figure 10) (Hunt and Handoo, 2009).

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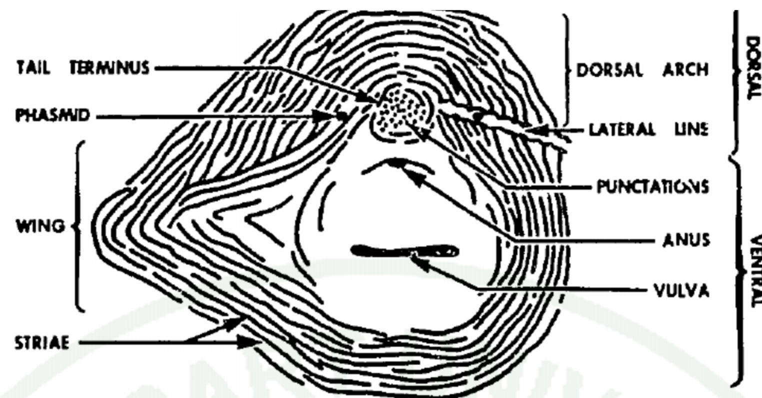

Figure 9 Drawing of perineal pattern of adult female of root-knot nematode

Source: Eisenback *et al.* (1981)

6.3 Identification by size

This technique measures size in each organ such as stylet knob, mouth and head. In addition, the measure includes type of lip and tail, digestive system and ratio of body length and ratio of positions of excretory pore. Measurement begins with preparation of nematode samples on permanent slide for inspecting under the microscope. The samples are J2, adult male and adult female of nematode (Hunt and Handoo, 2009).

6.4 North Carolina differential host test

This technique counts number of root galls in different hosts inoculated by nematodes and then scores hosts into 2 types*, i.e.,* resistant and susceptible host. Six host plant varieties are Cotton - Deltapine 61, Tobacco - NC 95, Pepper - Early California Wonder, Watermelon - Charleston Gray, Peanut – Florunner and Tomato – Rutgers. Each host plant is specific to specific races of root-knot nematode (Table 1) (Hartman and Sasser, 1985).

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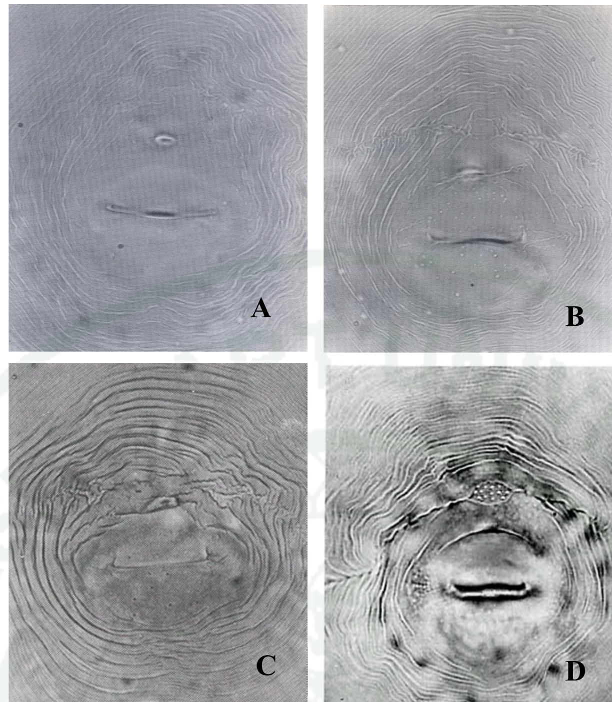

Figure 10 Photomicrographs of perineal patterns of each species of *Meloidogyne*

A: *M. incognita* – Dorsal arch is elongate with more or less flat arch. Striae are smooth or wavy with some forking at lateral line.

B: *M. javanica* - Dorsal arch is round to flat. Lateral incisures is distinct. Few striae extend unbroken from dorsal to ventral sector.

C: *M. arenaria* – Dorsal arch is round to flat. Striae are smooth to wavy and slightly indented at lateral lines. Striae near lateral line are forking and short irregular.

D: *M. hapla* - Perineal pattern is similar hexagon to slightly flattened ovals. Dorsal arch is flat. Lateral lines are not clear and indistinct, although they may be indicated by slight irregularities in the striae or by dorsal and ventral striae that meet at an angle. Striae are smooth to wavy. The tail terminal area is usually marked by punctations.

Source: Eisenback (1985); Taylor and Sasser (1978)

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Table 1 The North Carolina differential host test for *Meloidogyne* sp.

Meloidogyne sp.	Differential hosts [a]					
	Cotton	Tobacco	Pepper	Watermelon	Peanut	Tomato
M. incognita						
Race 1	-	-	+	+	-	+
Race 2	-	+	+	+	-	+
Race 3	+	-	+	+	-	+
Race 4	+	+	+	+	-	+
M. arenaria						
Race 1	-	+	+	+	+	+
Race 2	-	+	-	+	-	+
M. javanica	+	+	-	+	-	+
M. hapla	-	+	+	-	+	+

(-) Indicates a resistant host

(+) Indicates a susceptible host

[a] Cotton - Deltapine 61, Tobacco - NC 95, Pepper - Early California Wonder, Watermelon - Charleston Gray, Peanut – Florunner and Tomato – Rutgers

Source: Hartman and Sasser (1985)

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7. Molecular identification of root-knot nematode

Harris *et al*. (1990) identified species of root-knot nematode in 17 populations by PCR. They were *M. incognita, M. hapla, M. javanica* and *M. arenaria*. The PCR fragment was amplified region of mtDNA about 1.8 kb in length.

Powers and Harris (1993) modified the method of Harris *et al*. (1990) to develop specific primers with annealing sites between cytochrome oxidase subunit II gene and 16S rRNA gene. The specific primers were used in PCR for identification of *M. incognita, M. javanica, M. arenaria, M. hapla* and *M. chitwoodi*.

Xue *et al*. (1993) identified root-knot nematode by AFLP. The primers were Cytol/Cyto2 and Rpmc62/Rpmt63. The PCR product from Cytol/Cyto2 primers was about 2.00 kb and 2.50 kb, specific to *M. incognita*. The PCR product from Rpmc62/Rpmt63 primers was 0.85 kb, specific to *M. incognita* race 4.

Cenis (1993) used RAPD technique to amplify PCR DNA from J2, egg and adult female of root-knot nematode by 11 primers. OPA-1 primer could distinguish four specific species of *Meloidogyne* spp. *(M. incognita, M. javanica, M. arenaria* and *M. hapla)* depends on RAPD pattern.

Hugall *et al*. (1994) found low variation of mtDNA of nematode. The reproductive system of root-knot nematode is parthenogenesis. Egg from adult female can develop to J2 stage by itself, not fertilize from adult male.

Zijlstra *et al*. (1995) indicated that the ITS1 region of rDNA is specific to each of species including *M. incognita, M. javanica, M. chitwood* and *M. hapla.*

Blok *et al.* (1997) could distinguish *M. mayaguensis* from tropical species by different PCR product sizes. The PCR products were amplified from the region between 5S and 18S genes of root-knot nematode.

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Williamson *et al.* (1997) identified species in J2 stage of *M. hapla* and *M. chitwoodi* by SCAR (Specific Sequence Characterized Amplified Region) primers with proteinase K digestion. They found that the DNA extracted from J2 stage was not efficient enough for identification by RAPD.

Zijlstra (1997) found ITS2 of rDNA in nematode. The ITS2 region is the conserved region in rDNA of root-knot nematode. Primers designed from ITS2 region were used to specially identify *M. hapla, M. incognita, M. chitwoodi,* and *M. fallax*.

Zijlstra *et al.* (2000) identified J2 stage of *M. incognita*, *M. javanica* and *M. arenaria* using SCAR primers. Different PCR products could distinguish three species of *Meloidogyne* with 1,200 bp for *M. incognita*, 670 bp for *M. javanica* and 420 bp for *M. arenaria*.

Randig *et al*. (2001) used DNA extracted from single adult female to identify root-knot nematode by RAPD. The primers used for RAPD were primers 2, K01, K06 and K14. RAPD profiles of those species were different. They produced DNA band patterns in the average range of 200–2,000 bp.

Adam *et al*. (2007) identified species of genus *Meloidogyne* using SCAR primer. The SCAR primer can distinguish seven species to four groups according to different PCR product sizes.

Choksan (2007) identified species of root-knot nematode in growing filed in six provinces of northeast Thailand by perineal pattern morphology, differential host test and RAPD-PCR. Two species, *M. incognita* and *M. javanica*, were identified in twenty-four populations.

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Chanmalee and Prakob (2010) used worm lysis buffer (Adam *et al.,* 2007) for DNA extraction of root-knot nematode that collected from disease field in Chiang Mai, Thailand. The SCAR primer was used to identify species of *M. incognita* and *M. javanica* based on different PCR product sizes.

Hu *et al.* (2011) extracted DNA from J2, adult female and root gall for species identification by multiplex PCR using specific primer to *M. incognita, M. javanica* and *M. enterolobii*. Each species of genus *Meloidogyne* showed different PCR product sizes.

Singh *et al.* (2012) identified root-knot nematode that distributed inViti Levu of Fiji by morphological examination and molecular analysis. Total 675 soil samples were collected from infected field by root-knot nematode. Different nematode species gave different PCR product sizes. Common species of root-knot nematode were *M. incognita*, *M. javanica* and *M. arenaria.*

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MATERIALS AND METHODS

Materials

1. PCR thermocycler
2. Electrophoresis gel tank
3. Micropipettes
4. Plates
5. Plastic cylinder
6. Laboratory glassware
7. Pots
8. Water bath
9. Micro tube
10. Incubator
11. Light microscope

Methods

1. Sampling and preparation of root-knot nematode

1.1 Survey and sampling

Fifty samples were collected from chili farm with the outbreak of root knot disease in Thailand (Figure 11) (Table 2). The samples are soil and chili plants with above ground (yellowing, wilting and stunting) and underground symptoms (galls). The soil samples were taken depth about 15-30 cm from soil surface by using hand spoon. Ten spot (50 g per spot) of soil from each chili farm were collected as soil samples by sampling randomly (Singh *et al*., 2012). Total soil samples were 500 g. The soil sample was placed in plastic bag with date, location, and number of samples.

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1.2 Preparation of root-knot nematode population

Sterile soil were added in pot following with infected soils collected from the survey and topped with sterile soil. Sterile soil and infected soil was mixed. Then, 30 days old of healthy chili seedlings were planted in the pot. After planting 30 days, chili plants were removed and washed with tap water. Egg mass on root gall were collected and prepared as inoculum for pure culture of root-knot nematode (Figure 11).

1.3 Preparation of pure culture by single egg mass inoculation

Egg mass from each pure cultures were separated from galls. The egg mass was soaked in sterile water. Eggs hatched to a second stage juvenile (J2) within 1-3 days. Fresh J2 was used for inoculation of chili seedling using 1 egg mass per chili seedling. After 30 days of inoculation, galls on root were observed. Then, egg mass from each pure culture was collected from galls and used for next experiments (Figure 12) (Greco and Vito, 2009).

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Table 2 Location and soil texture which samplings for root-knot nematode were conducted in Thailand during October 2011 - January 2013

Provinces	Number of samples	Sampling time (Month)	Soil texture	Code
Chiang Mai	6	October 2011	Sandy loam	CM1, CM2, CM3, CM4, CM5, CM6
Lamphun	2	November 2011	Sandy loam	LP1, LP2
Tak	18	January 2012	Sandy loam	TK1, TK2, TK3, TK4, TK5, TK6, TK7, TK8, TK9, TK10, TK11, TK12, TK13, TK14, TK15, TK16, TK17, TK18
Khon Kaen	1	February 2012	Sand	KK1
Phetchaburi	1	March 2012	Sand	PB1
Srisaket	2	June 2012	Clay loam	SK1, SK2
Suphan Buri	4	June 2012	Sandy loam	SP1, SP2, SP3, SP4
Ubon Rachatani	12	June 2012	Clay loam	UB1, UB2, UB3, UB4, UB5, UB6, UB7, UB8, UB9, UB10, UB11, UB12
Uthai Thani	1	August 2012	Sand	UT1
Songkla	3	January 2013	Clay loam	SKH1, SKH2, SKH3
Total	50			

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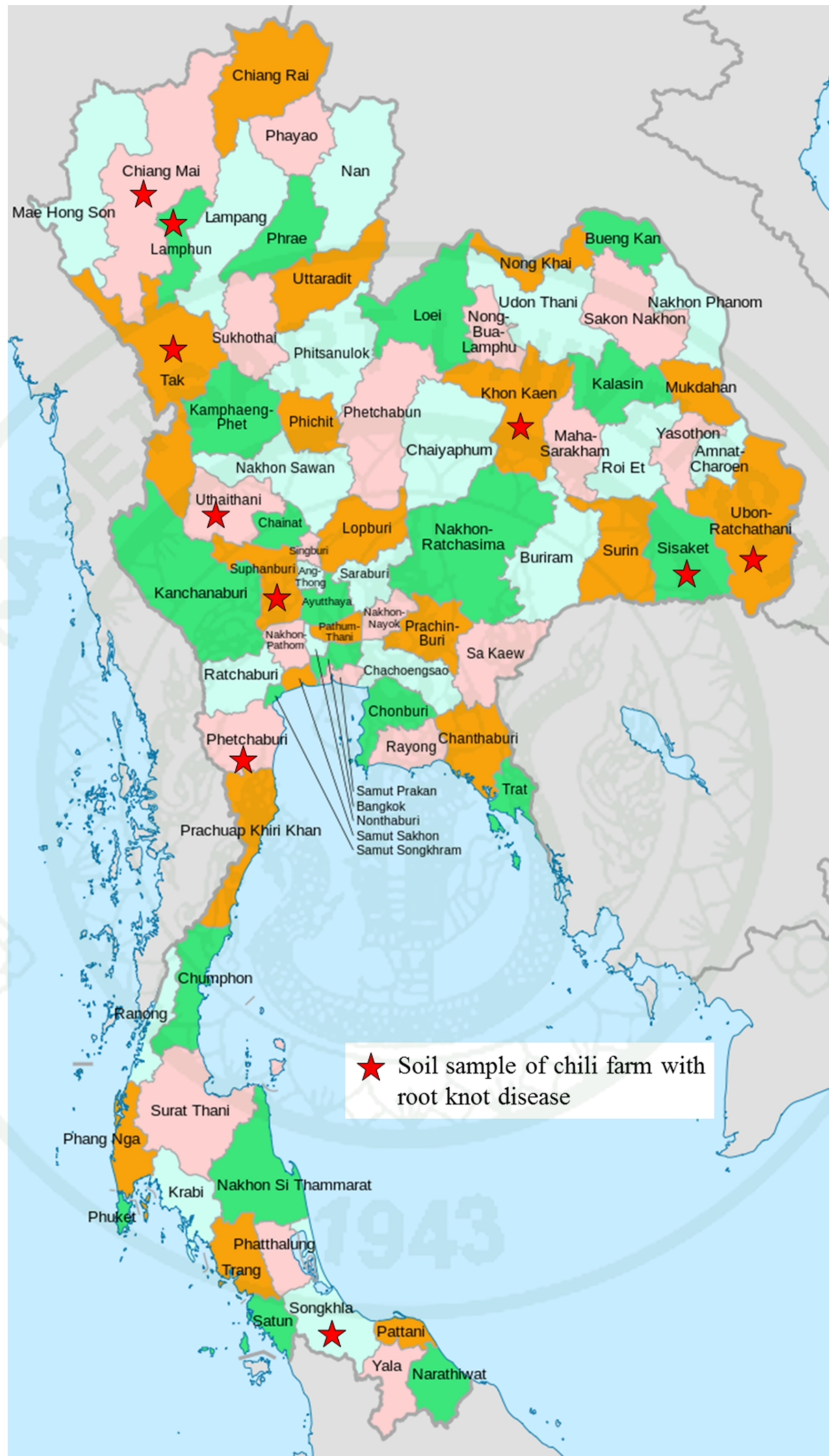

Figure 11 Location where sampling soil samples were conducted

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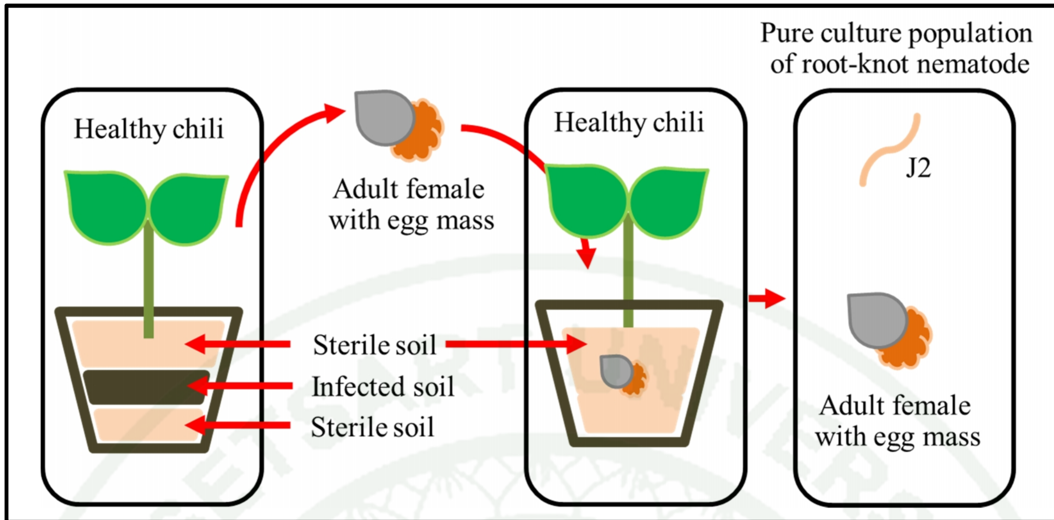

Figure 12 Preparation pure culture population of root-knot nematode in chili plant

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2. Identification of tropical species of root-knot nematode

2.1 DNA extraction of root-knot nematode

Fresh second stage juvenile (J2) was picked up by small needle and placed in hyamine solution (1 mg/ml) for 2 times and transferred to dH_2O about 3 min. Then, the fresh J2 was picked up and placed in 10 µl of worm lysis buffer (WLB) (50 mM KCl, 10 mM Tris (pH 8.2), 2.5 mM $MgCl_2$, 60 µg/ml proteinase K, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin) (Adam *et al.*, 2007) on a glass slide and cut into 2-3 pieces using new sterilized needle under a stereomicroscope. The nematode pieces were transferred by micropipette into a 1.5 ml microtube containing 15 µl of WLB. The tubes were centrifuged at 13,500 rpm for 2 min, and then placed at −80 °C for 15 min. Mineral oil (15 µl) was added to the tube and incubated at 60 °C for 1 h, followed by 90 °C for 10 min. The mineral oil was removed by pipette after the aqueous sample was frozen at −20 °C (Blok *et al.,* 1997).

2.2 Identification of tropical species of root-knot nematode by PCR.

The Initial identification of tropical species of root-knot nematode was performed by PCR using 194/195 primers (Table 3) (Blok *et al.,* 1997). The DNA of J2 was used as template. PCR profile was modified from Adam *et al*. (2007) (Table 4). PCR products of each sample (Table 5) were analyzed by 1% agarose gel electrophoresis.

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Table 3 Specific primers for identification species of root-knot nematode

Code	Sequence	Specificity (PCR product size)	References
194	5'-TTAACTTGCCAGATCGGACG-3'	Tropical species	Blok *et al.*
195	5'-TCTAATGAGCCGTACGC-3'	(720 bp)	(1997)
Far	5'-TCGGCGATAGAGGTAAATGAC-3'	*M. arenaria*	Zijlstra *et al.*
Rar	5'-TCGGCGATAGACACTACAAACT-3'	(670 bp)	(2000)
Fjav	5'-GGTGCGCGATTGAACTGAGC-3'	*M. javanica*	Zijlstra *et al.*
Rjav	5'-CAGGCCCTTCAGTGGAACTATAC-3'	(720 bp)	(2000)
MI-F	5'-GTGAGGATTCAGCTCCCCAG-3'	*M. incognita*	Meng *et al.*
MI-R	5'-ACGAGGAACATACTTCTCCGTCC-3'	(999 bp)	(2004)

Table 4 PCR profiles for primers used for identification of root-knot nematode

	35 cycles				
94 ºC	94 ºC	60 ºC (primer: 194/195) 61 ºC (primer: Far/Rar) 63 ºC (primer: Fjav/Rjav) 63 ºC (primer: MI-F/MI-R)	72 ºC	72 ºC	4 ºC
2 min	30 sec	30 sec	90 sec	7 min	∞

Table 5 Diagnostic key of root-knot nematode by PCR using 194/195 primers giving different sizes of PCR products

PCR product size (bp)	Species
720	Tropical species
780	*M. mayaguensis*
700	*M. hapla*
1,700	*M. fallax* and *M. chitwoodi*

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2.3 Ligation of PCR DNA with plasmid vector

PCR products were ligated into pDrive vector (Qiagen, Germany) (Figure 13). The ligation-reaction mixture was prepared following instruction. The mixture contained 50 ng of pDrive vector, 2X ligation master mix, 3 units of T4 DNA ligase and PCR product (1 µl) in a total volume of 10 µl. The ligation mixture was incubated at 16 °C for 16-18 h, and then transformed into *E. coli* strain DH5α by heat shock transformation.

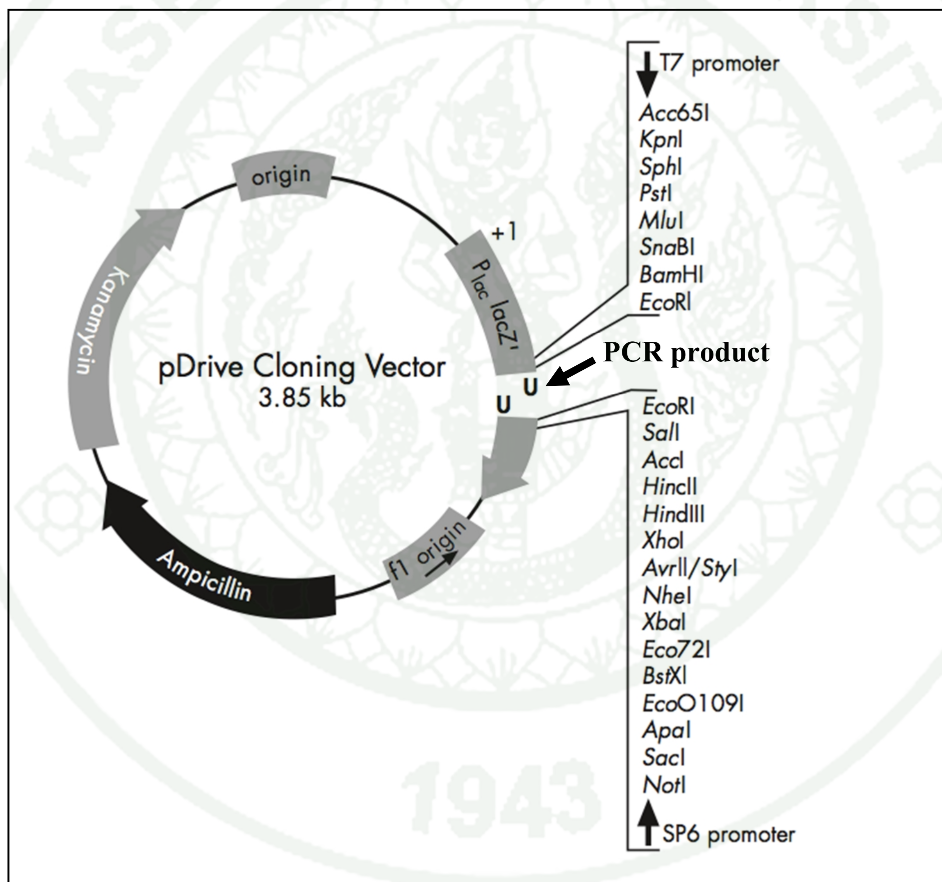

Figure 13 Diagram of cloning of PCR product into pDrive cloning vector

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2.4 Preparation of competent cell of *E. coli* strain DH5α

The single fresh colony of *E. coli* strain DH5α was cultivated in 5 ml of liquid 2X-YT (Yeast Extract Tryptone) medium on shaker at 250 rpm at 37 °C for 16-18 h. One milliliter of fresh culture was grown in 100 ml of liquid SOB (Super Optimal Broth) medium on shaker at 37 °C for 3-4 h. Then, the liquid SOB medium was incubated on ice for 15 min and the bacterial cells were collected by centrifugation at 3,500 rpm for 15 min at 4 °C. Bacterial cells were resuspended in 33.3 ml of cold RF1 (100 mM KCl, 50 mM $MnCl_2$, 50 mM CH_3COOK, 10 mM $CaCl_2$, 15% Glycerol and adjusting pH 5.8 by CH_3COOH) and incubated on ice for 15 min. Bacterial cells were collected by centrifugation at 3,500 rpm for 15 min at 4 °C. Bacterial cells were resuspended in 4 ml of cold RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2.H_2O$, 15% Glycerol and adjusting pH 6.8 by NaOH). A 100 microliters aliquot of competent cells was added into sterile microtube (1.5 ml) and kept at -80 °C until used.

2.5 Heat-shock transformation of the pDrive vector with PCR product into *E. coli* strain DH5α

The ligation mixture was mixed with the competent cells of *E. coli* and chilled on ice for 15 min. Then, the mixture was incubated at 42 °C for 50 sec and immediately chilled on ice for 5 min. 2xYT medium (900 µl) was added, and bacterial cells were cultured on shaker at 37 °C for 1 h. Bacterial cells were spread on solid 2xYT medium containing 100 mg/l of ampicillin, 100 µl of IPTG (100 mM) and 50 µl X-gal (20 mg/ml). Transformed cells were incubated at 37 °C for 12-16 h.

2.6 Plasmid extraction from *E. coli* by alkaline lysis method

The plasmid DNA was extracted from white bacterial colony by alkaline lysis method (Sambrook *et al.,* 1989). Bacterial cells (1 ml) were collected by centrifugation at 12,500 rpm for 2 min, and the supernatant was discarded from bacterial cells. The bacterial pellet was resuspended in 100 µl of Solution I (50 mM

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glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) and mixed by vortexing. The cell suspension was chilled on ice for 5 min, and then added with 200 µl of Solution II (0.2 N NaOH, 1% SDS). The mixture was mixed by inverting tube, and tube was chilled on ice for 5 min. Solution III (3 M CH_3COOK) (150 µl) was added and mixed by inverting tube and tube was chilled on ice for 5 min. The tube was spun at 12,000 rpm for 5 min at room temperature, and the 400 µl of supernatant was transferred into a new microtube. An equal volume of phenol: chloroform: isoamyl alcohol mixture (25:4:1 by v/v) was added and mixed well by vortexing. The tube was spun at 12,000 rpm for 5 min at room temperature. The clear aqueous phase was transferred into a new microtube, 2.5 volume of 100% ethanol and 0.1 volume of 3 M CH_3COONa, pH 5.2 were added and mixed by inverting. The plasmid DNA was collected by centrifugation at 12,000 rpm for 10 min. The solution was discarded and the pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min at 4 °C. The pellet was air dried and resuspended in 50 µl of dH_2O containing 20 µg/ml of RNase.

2.7 Verification of PCR fragment inserted into plasmid vector by restriction enzyme digestion

Recombinant plasmid DNA was digested by digestion with *Eco*RI enzyme (Promega, USA) to release PCR fragment from plasmid vector. The reaction contained 10X buffer number 4, *Eco*RI (20 u/µl) and plasmid 500 ng. The reaction was incubated at 37 °C for 3 h. Digested plasmid was analyzed by 1% agarose gel electrophoresis. Recombinant plasmid DNA containing PCR fragment was sequenced by Pacific Science Co., Ltd. DNA sequences were edited and analyzed by DNA Star software (DNASTAR, Inc., USA) for preparing nucleotide information to construct phylogenetic tree.

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3. Identification of species of root-knot nematode.

3.1 Morphological identification by perineal pattern

Character frequently used for *Meloidogyne* species identification is the morphology of the perineal pattern, which locates in the posterior body region of adult female. This area comprises the vulva–anus area (perineal), tail terminus, phasmids, lateral lines and surrounding cuticular striae (Hunt and Handoo, 2009).

3.1.1 Root staining by sodium-hypochlorite with acid-fuchin method

Root galls were washed with water and place them in a 150 ml beaker. Large root systems were cut into sections. A 50 ml of tap water was added into the beaker with 10 ml of 5.25% NaOCl to clear the root tissue. The root were soaked in the solution for 4 min and agitated occasionally. The solution was rinsed. The roots were washed with running tap water for 45 sec and then immersed them in water for 15 min to remove any residual. Then, the water was drained. The roots were transferred to a glass beaker with 30-50 ml of tap water. One milliliter of stock acid-fuchin stain solution was added into the glass beaker and boil for about 30 sec on a hotplate. Stock acid-fuchin solution was prepared by dissolving 3.5 g of acid-fuchin in 250 ml of acetic acid and 750 ml of dH_2O. After the solution was cooled to room temperature, drained the stain solution, and rinsed the roots in running tap water. The roots were destained by boiling in 30 ml of glycerin acidified with a drop of 5 N HCl. The galls were distributed in a small amount of glycerin on a petri dish plate and picked adult female from the gall. All roots were stored in acidified glycerin before use to perineal pattern identification (Byrd *et al*., 1983).

3.1.2 Perineal pattern identification

Adult female staining with acid-fuchin was transferred to a drop of 45% lactic acid in a plastic petri dish. Adult female body was pushed out in the lactic acid solution. Razor blade fragment was embed into the plastic and cut off the posterior of

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the nematode with a paper-cutter action. Body tissue was removed gently from the posterior section with a needle. The posterior was trimmed the cuticle into a square with the perineal pattern in the center. The perineal pattern piece was transferred to a microscope slide in a drop of glycerin, applied a coverslip and sealed. The perineal pattern was examined under light microscope (Hooper, 1970).

3.2 Identification of species of root-knot nematode by PCR with specific primer

Root-knot nematode identified in tropical species was further used for species identification by PCR using specific primers to *M. arenaria, M. incognita* and *M. javanica* (Table 3). PCR profile was modified from Adam *et al.* (2007) (Table 4). PCR products of each species were analyzed by 1% agarose gel electrophoresis (Table 3) and cloned into pDrive vector (Qiagen, Germany). The procedures were performed as same as step 2.1-2.7. Recombinant plasmid vectors containing PCR fragment were sequenced by Pacific Science Co., Ltd. DNA sequences were edited by DNA Star software (DNASTAR, Inc., USA) for construct of phylogenetic tree.

4. Sequence analysis and phylogenetic tree analysis.

The nucleotide sequences of PCR fragment of root-knot nematodes analyzed by perineal pattern and PCR were edited and used for sequence analysis among them and also with other nucleotide sequences of *Meloidogyne* spp. available in GenBank (Table 6). In addition, the nucleotide sequences were aligned for construction of phylogenetic tree by Clustal W method by DNA Star software (DNASTAR, Inc., USA).

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Table 6 List of the *Meloidogyne* species from GenBank has been used phylogenetic tree analysis with DNA sequences in this study

Species name	Isolate	Host plant	Accession number	Source
M. javanica	ZCf	Tomato	JN005834	Hu *et al*., 2011
M. javanica	GNs	Towel gourd	JN005835	Hu *et al*., 2011
M. javanica	GNk	Towel gourd	JN005836	Hu *et al*., 2011
M. javanica	YZy1	Tobacco	JN005837	Hu *et al*., 2011
M. javanica	SMj	Unknown	JN005838	Hu *et al*., 2011
M. javanica	ZH8	Peanut	JN005839	Hu *et al*., 2011
M. incognita	GLs	Towel gourd	JN005840	Hu *et al*., 2011
M. incognita	JS2	Unknown	JN005841	Hu *et al*., 2011
M. incognita	GNq	Eggplant	JN005842	Hu *et al*., 2011
M. incognita	SSs	Towel gourd	JN005843	Hu *et al*., 2011
M. incognita	HZ1	Tomato	JN005844	Hu *et al*., 2011
M. incognita	ZCd	White gourd	JN005845	Hu *et al*., 2011
M. incognita	-	*Tetrastigma hemsleyanum*	KC496012	Unpublished
M. incognita	-	*Tetrastigma hemsleyanum*	KF481971	Unpublished

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RESULTS AND DISCUSSION

Results

1. Identification of tropical species of root-knot nematode

A second stage juvenile of root-knot nematode from pure culture populations from 50 samples was used to identify for tropical species by PCR using 194/195 primers. The primers were designed from Intergenic Spacer 2 (IGS2) that is a region between 5S rDNA and 18s rDNA of root-knot nematode producing PCR fragment with ~720 bp in size (Figure 14) (Blok *et al*., 1997). The different PCR product sizes can be used to distinguish the tropical species such as *M. incognita*, *M. javanica* and *M. arenaria* from temperate species such as *M. mayagiensis, M. hapla, M. fallax* and *M. chitwood* (Table 5) (Adam *et al.,* 2007).

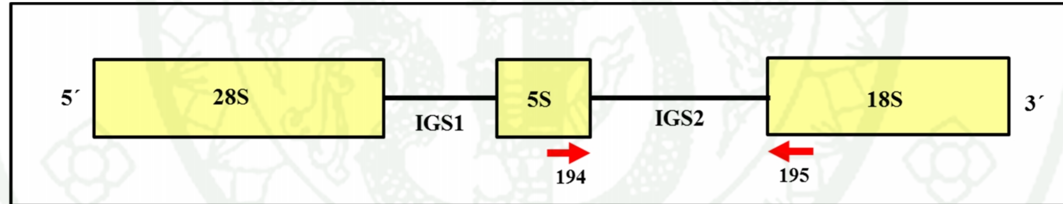

Figure 14 Intergenic Spacer 2 (IGS2) between 5S and 18S genes with location of 194/195 primer

Forty three samples were positive with 194/195 primers while seven samples were negative (Figure 15). In order to confirm the results from PCR, all PCR fragments were cloned into pDrive vector and sequenced for sequence analysis. From sequence analysis, thirty four samples were confirmed to be tropical species with nucleotide sequences between 710-720 bp in length (Table 7). Other nine samples were not tropical species with nucleotide sequences more than 720 bp and it might be *M. mayaguensis* according to nucleotide sequences compared with other published sequences in GenBank (Table 7).

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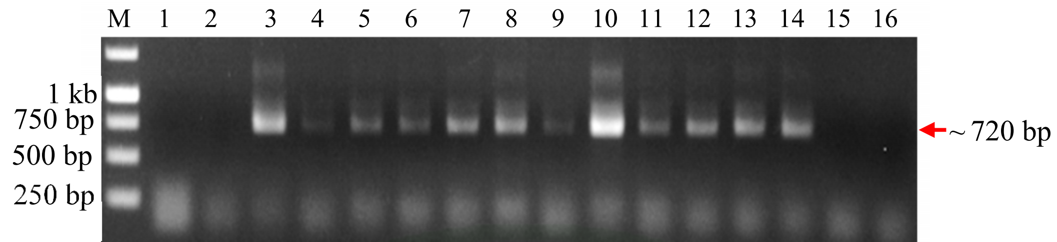

Figure 15 PCR fragments approximately 720 bp obtained from PCR of second stage juveniles (J2) of root-knot nematode with 194/195 primers

M: 1 kb Marker (Fermentas, USA)

1: Negative control (dH_2O)	9: KK1
2: TK3	10: LP2
3: TK4	11: UB5
4: TK5	12: UB6
5: TK6	13: UB7
6: TK7	14: UB8
7: CM5	15: UB9
8: SK2	16: UB10

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Table 7 Identification of tropical species of root-knot nematode by PCR with 194/195 primers

Provinces	Total Samples	PCR Positive		PCR
		Tropical[1]	Not tropical[2]	Negative
Chiang Mai	6	4	2	-
Khon Kaen	1	1	0	-
Lamphun	2	1	1	-
Phetchaburi	1	0	1	-
Songkla	3	3	0	-
Srisaket	2	1	1	-
Suphan Buri	4	4	0	-
Tak	18	14	2	2
Ubon Rachathani	12	6	1	5
Uthai Thani	1	0	1	-
Total	50	34	9	7

[1] Samples gave PCR result with 714 - 720 bp in length

[2] Samples gave PCR result with more than 720 bp in length

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2. Identification of *Meloidogyne* at species level by perineal pattern and PCR

Adult female was selected from pure culture of each tropical population for morphological identification by perineal pattern. Two species of genus *Meloidogyne* were identified. They were *M. incognita* and *M. javanica* (Table 7). Twenty eight samples of tropical species of root-knot nematode populations from thirty four soil samples (Table 8) were identified as *M. incognita* with high dorsal arch and elongated with more or less flattened dorsal arch around perineal pattern. Striae line was smooth to wavy with some forking at lateral line but lateral line was not clear (Figure 16, a-g). Perineal pattern of CM1, UB7 and UB8 showed wavy striae line with some forking at lateral line but lateral line was not clear (Figure 16, a-c). Perineal pattern of TK1 and TK18 showed round shape and more flattened dorsal arch around perineal pattern (Figure 16, d and e). Perineal pattern of CM5 and KK1 showed high dorsal arch and elongated with more or less flattened dorsal arch around perineal pattern (Figure 16, f-g). The others six samples from Chiang Mai (CM6), Lamphun (LP1), Tak (TK4) and Songkla (SKH1, SKH2, SKH3) were identified as *M. javanica* by perineal pattern with vary round to flatten in dorsal arch and clearly incisures distinct of lateral line (Figure 16, h-i).

Species identification by PCR using specific primers was conducted. The primers were designed from RAPD markers that were specific to *M. arenaria*, *M. incognita* and *M. javanica* of root-knot nematode (Adam *et al*., 2007; Zijlstra *et al.,* 2000). Primers MI-F/MI-R, Fjav/Rjav and Far/Rar were specific to *M. incognita*, *M. javanica* and *M. arenaria*, respectively (Table 3).

Among root-knot nematode population of samples collected from chili fields of Thailand, twenty seven samples of root-knot nematode were identified as *M. incognita* by PCR with MI-F/MI-R primers (Figure 17 and Table 8). While, other five samples of root-knot nematode infecting chili from Chiang Mai (CM4), Songkla (SKH1, SKH2, SKH3) and Tak (TK11) were identified as *M. javanica* by PCR with Fjav/Rjav primers (Figure 18 and Table 8). However, two samples from Chiang Mai

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(CM6) and Ubon Ratchathani (UB6) were negative by PCR with all specific primers (Table 8), so they were unidentified.

The conformity results between identification by perineal pattern and PCR method were from twenty five samples classified as *M. incognita* and from four samples classified as *M. javanica* (Table 8). While identification of other five samples, *i.e.*, CM6, TK9, TK11, TK14 and UB6 was contradict between identification by perineal pattern and PCR, for example root-knot nematode from TK9 and TK14 samples was identified as *M. javanica* by perineal pattern, but as *M. incognita* by PCR (Table 8). Therefore, twenty five samples of *M. incognita* and four samples of *M. javanica* were further used for phylogenetic tree analysis.

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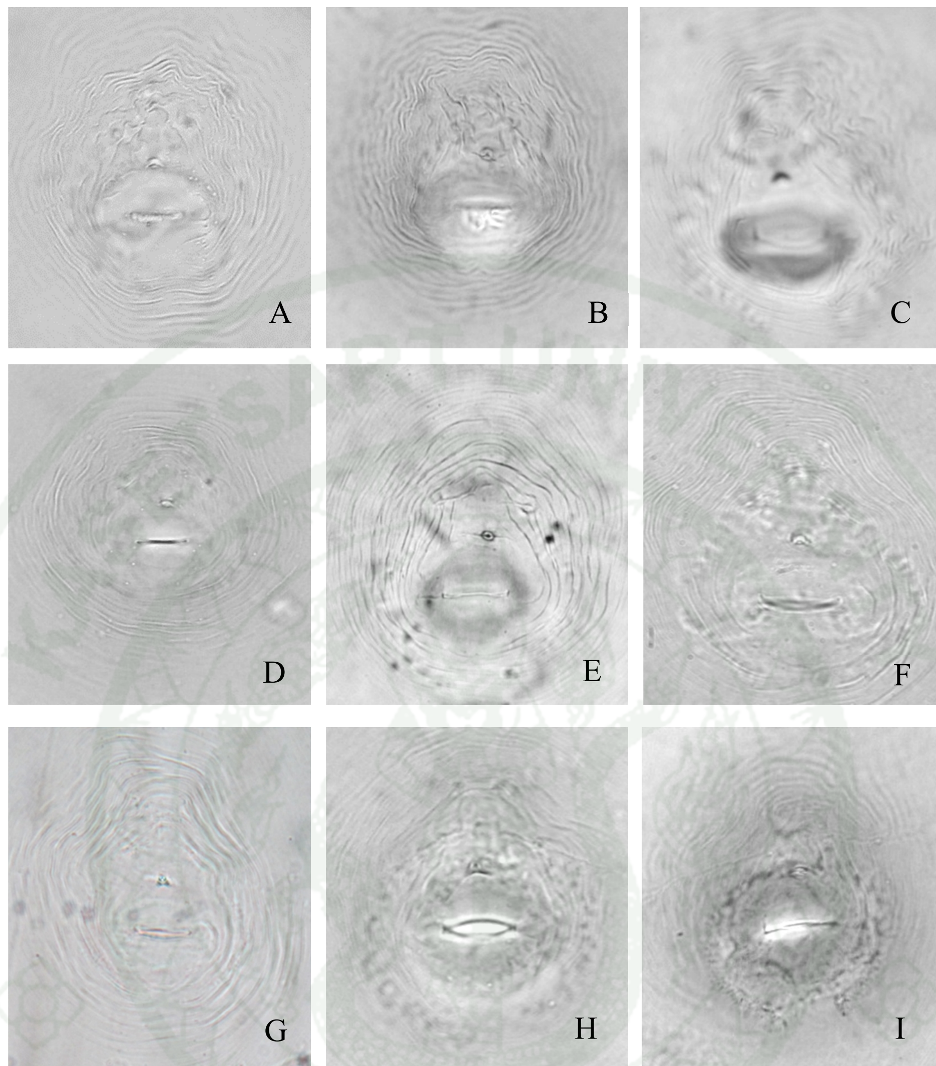

Figure 16 Micrograph of perineal patterns of *Meloidogyne incognita* and *M. javanica*; *M. incognita* (A: CM1; B: UB7; C: UB8; D: TK1; E: TK18; F: CM5; G: KK1) and *M. javanica* (H: SKH1; I: SKH2); A-G: frequent variability observed among perineal patterns of *M. incognita*; H-I: frequent variability observed in perineal patterns of *M. javanica*

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Table 8 Identification of root-knot nematode at species level by perineal pattern and PCR

Provinces	Code	Perineal pattern		PCR	
		M. incognita	*M. javanica*	*M. incognita*	*M. javanica*
Chiang Mai	CM1	√		√	-
	CM4	-	√	-	√
	CM5	√	-	√	-
	CM6	√	-	–	-
Lamphun	LP1	√	-	√	-
Tak	TK1	√	-	√	-
	TK2	√	-	√	-
	TK5	√	-	√	-
	TK6	√	-	√	-
	TK7	√	-	√	-
	TK8	√	-	√	-
	TK9	-	√	√	-
	TK10	√	-	√	-
	TK11	√	-	-	√
	TK12	√	-	√	-
	TK13	√	-	√	-
	TK14	-	√	√	-
	TK16	√	-	√	-
	TK17	√	-	√	-
Khon Kaen	KK1	√	-	√	-
Srisaket	SK2	√	-	√	-
Suphan Buri	SP1	√	-	√	-
Ubon Rachathani	SP2	√	-	√	-
	SP3	√	-	√	-
	SP4	√	-	√	-
	UB3	√	-	√	-
	UB4	√	-	√	-
	UB5	√	-	√	-
	UB6	√	-	-	-
	UB7	√	-	√	-
	UB8	√	-	√	-
Songkla	SKH1	-	√	-	√
	SKH2	-	√	-	√
	SKH3	-	√	-	√

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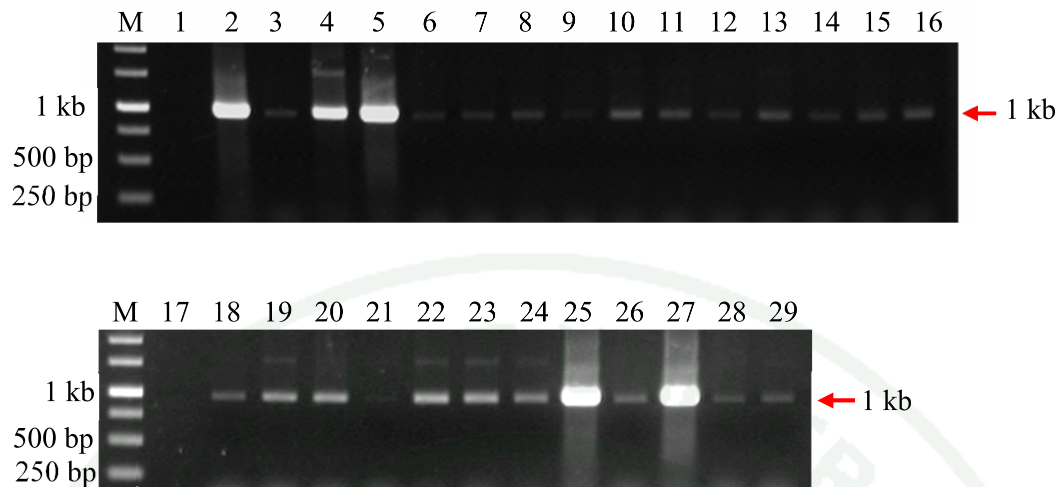

Figure 17 PCR fragments approximately 999 bp obtained from second stage juveniles (J2) from PCR with MI-F/MI-R primers

M: 1 kb Marker (Fermentas, USA)

1: Negative control	17: Negative control
2: CM1	18: TK14
3: CM5	19: TK16
4: SK2	20: TK17
5: KK1	21: UB3
6: LP1	22: UB4
7: TK1	23: UB5
8: TK2	24: UB7
9: TK5	25: UB8
10: TK6	26: SP1
11: TK7	27: SP2
12: TK8	28: SP3
13: TK9	29: SP4
14: TK10	
15: TK12	
16: TK13	

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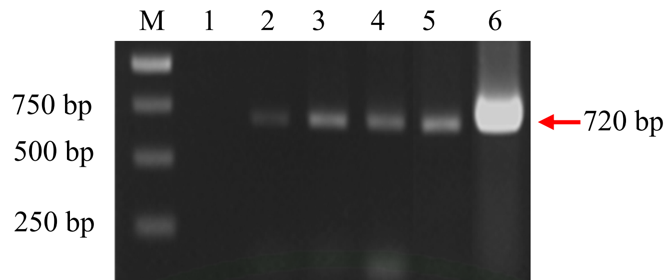

Figure 18 PCR fragments approximately 720 bp obtained from second stage juveniles (J2) from PCR with Fjav/Rjav primers

M: 1 kb Marker (Fermentas, USA)

1: Negative control

2: SKH1

3: SKH2

4: SKH3

5: CM4

6: TK11

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3. Sequence analysis and construction of phylogenetic tree of root-knot nematode

PCR fragments derived from genome of *M. incognita* (~990 bp) and *M. javanica* (~680 bp) were further cloned and sequenced for sequence analysis. Samples identified as *M. incognita* were samples from CM1, CM5, LP1, TK1, TK2, TK5, TK6, TK7, TK8, TK10, TK12, TK13, TK16, TK17, KK1, SK2, SP1, SP2, SP3, SP4, UB3, UB4, UB5, UB7 and UB8 (Table 8). Four samples from CM4, SKH1, SKH2 and SKH3 were identified as *M. javanica* (Table 8).

The nucleotide sequences of PCR fragments derived from genome of *M. incognita* from 25 samples and *M. javanica* from 4 samples (Table 8) were used for sequence analysis and aligned for construction of phylogenetic tree by ClustalW method. Description number below phylogenetic tree, the number is a scale indicating the number of “Nucleotide Substitutions” per 100 residues for DNA sequences. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. The values computed are the mean number of differences per site and fall between 0-1. Zero represents complete identity and 1 represent no identity. The phylogenetic tree scale uses these values multiplied by 100.

The nucleotide sequences of PCR fragments from 25 samples of *M. incognita* had a high identities about >98% (Table 9). This was the same with the nucleotide sequences of PCR fragments from 4 samples of *M. javanica* (Table 10). From construction of phylogenetic tree, the nucleotide sequences from 25 samples of *M. incognita* were grouped together and the nucleotide substitution value of phylogenetic tree was 1.8 (Figure 19). The nucleotide sequences from 4 samples of *M. javanica* were also grouped together and the nucleotide substitution value of phylogenetic tree was 0.4 (Figure 20).

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The nucleotide sequences from 25 samples of *M. incognita* were aligned with 8 nucleotide sequence of *M. incognita* from China (JN005840, JN005841, JN005842, JN005843, JN005844, JN005845, KF481971 and KC496012; Table 6) (Figure 21). They shared a high nucleotide identity >98% (Table 11) and grouped together in phylogenetic tree. The nucleotide substitution value of phylogenetic tree was 1.8 (Figure 21).

The nucleotide sequences from 4 samples of *M. javanica* were aligned with 6 nucleotide sequences of *M. javanica* from China (JN005834, JN005835, JN005836, JN005837, JN005838 and JN005839; Table 6) (Figure 22). They shared a high nucleotide identity >99% (Table 12) and grouped together in phylogenetic tree. The nucleotide substitution value of phylogenetic tree was 0.4 (Figure 22).

From phylogenetic tree analysis, there was no variation among populations of *M. incognita* and *M. javanica* that caused root knot disease of chili in Thailand.

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Table 9 Nucleotide identity of PCR fragment derived from twenty five samples of *Meloidogyne incognita*

	Nucleotide identity (%)																								
Samples	CM1	CM5	KK1	LP1	SK2	SP1	SP2	SP3	SP4	TK1	TK2	TK5	TK6	TK7	TK8	TK10	TK12	TK13	TK16	TK17	UB3	UB4	UB5	UB7	UB8
CM1		99.4	99.4	99.5	99.8	98.9	99.2	99.6	100	99.6	100	99.3	99.6	99.7	100	99	99	99.3	99	99.6	99	99.4	100	99	99.7
CM5			99.4	99.4	99.7	99	99.1	99.6	100	99.4	100	99.3	99.6	99.5	99	99	98.8	99.2	99	99.5	99	99.4	99	99	99.7
KK1				99.4	99.7	99	99.1	99.6	100	99.4	100	99.3	99.6	99.5	99	99	98.8	99.2	99	99.5	99	99.4	99	99	99.7
LP1					99.7	99	99.1	99.6	100	99.5	100	99.3	99.6	99.6	99	99	98.8	99.2	99	99.5	99	99.4	99	99	99.7
SK2						99.3	99.4	99.9	100	99.8	100	99.6	99.9	99.9	100	99.3	99.2	99.5	99	99.8	100	99.7	100	100	100
SP1							98.6	99.2	99	99	99	99	99.2	99.1	99	98.5	98.5	98.7	99	99.1	99	99	99	99	99.3
SP2								99.3	99	99.2	99	99	99.3	99.3	99	98.6	98.5	98.8	99	99.2	99	99.1	99	99	99.4
SP3									100	99.6	100	99.5	99.8	99.7	100	99.2	99.1	99.4	99	99.7	100	99.6	100	100	99.9
SP4										99.6	100	99.5	99.8	99.7	100	99.2	99.1	99.4	99	99.7	100	99.6	100	100	99.9
TK1											100	99.3	99.6	99.7	100	99	99	99.3	99	99.6	99	99.4	100	99	99.7
TK2												99.4	99.7	99.6	99	99.1	99	99.3	99	99.6	99	99.5	100	99	99.8
TK5													99.5	99.4	99	98.9	98.7	99.1	99	99.4	99	99.3	99	99	99.6
TK6														99.7	100	99.2	99.1	99.4	99	99.7	100	99.6	100	100	99.9
TK7															100	99.1	99.1	99.4	99	99.7	99	99.5	100	100	99.8
TK8																98.9	98.8	99.2	99	99.5	99	99.3	99	99	99.6
TK10																	98.4	98.7	99	99.1	99	99	99	99	99.3
TK12																		98.6	98	99	99	98.8	99	99	99.2
TK13																			99	99.3	99	99.2	99	99	99.5
TK16																				99.1	99	99	99	99	99.3
Tk17																					99	99.5	100	99	99.8
UB3																						99.3	99	99	99.6
UB4																							99	99	99.7
UB5																								99	99.7
UB7																									99.6
UB8																									

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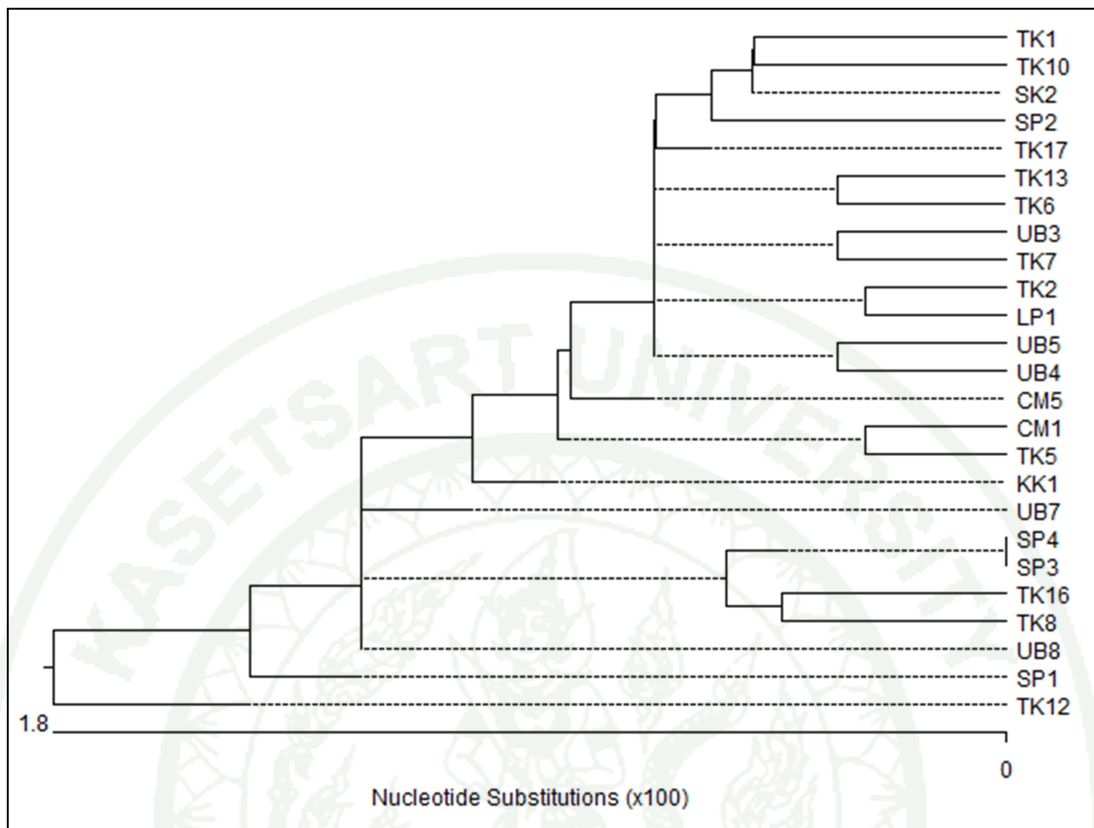

Figure 19 Phylogenetic tree based on nucleotide sequence alignment of PCR fragment derived from *Meloidogyne incognita* from Chiang Mai (CM1, CM5), Lamphun (LP1), Tak (TK1, TK2, TK5, TK6, TK7, TK8, TK10, TK12, TK13, TK16, TK17), KhonKaen (KK1), Srisaket (SK2), Suphanburi (SP1, SP2, SP3, SP4) and Ubonratchatani (UB3, UB4, UB5, UB7, UB8)

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Table 10 Nucleotide identity of PCR fragment derived from four samples of *Meloidogyne javanica*

Samples	Nucleotide identity			
	CM4	SKH1	SKH2	SKH3
CM4		99.7	99.7	99.2
SKH1			99.4	98.9
SKH2				98.9
SKH3				

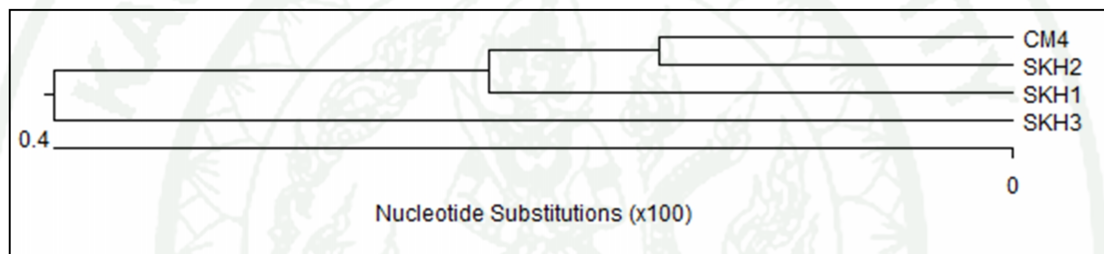

Figure 20 Phylogenetic tree based on nucleotide sequence alignment of PCR fragment derived from *Meloidogyne javanica* from Chiang Mai (CM4) and Songkla (SKH1, SKH2, SKH3)

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Table 11 Nucleotide identity of PCR fragment derived from four samples of *Meloidogyne incognita* with available DNA sequences in GenBank

	Nucleotide identity (%)																																
Sample[a]	1	2	3	4	5	6	7	8	CM1	CM5	KK1	LP1	SK2	SP1	SP2	SP3	SP4	TK1	TK2	TK5	TK6	TK7	TK8	TK10	TK 12	TK 13	TK 16	TK 17	UB3	UB4	UB5	UB7	UB8
1		99.7	99.6	99.7	99.7	99.7	99.7	99.7	99.5	99.4	99.4	99.4	99.7	99.3	99.1	99.6	99.6	99.5	99.5	99.3	99.6	99.6	99.4	99	98.8	99.2	99	99.5	99.3	99.4	99.4	99.3	99.7
2			99.9	100	100	100	100	100	99.8	99.7	99.7	99.7	100	99.6	99.4	99.9	99.9	99.8	99.8	99.6	99.9	99.9	99.7	99.3	99.2	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
3				99.9	99.9	99.9	99.9	99.9	99.7	99.6	99.6	99.6	99.9	99.5	99.3	99.8	99.8	99.7	99.7	99.5	99.8	99.8	99.6	99.2	99.1	99.4	99.2	99.7	99.5	99.6	99.6	99.5	99.9
4					100	100	100	100	99.8	99.7	99.7	99.7	100	99.6	99.4	99.9	99.9	99.8	99.8	99.6	99.9	99.9	99.7	99.3	99.2	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
5						100	100	100	99.8	99.7	99.7	99.7	100	99.6	99.4	99.9	99.9	99.8	99.8	99.6	99.9	99.9	99.7	99.3	99.2	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
6							100	100	99.8	99.7	99.7	99.7	100	99.6	99.4	99.9	99.9	99.8	99.8	99.6	99.9	99.9	99.7	99.3	99.2	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
7								100	99.8	99.7	99.7	99.7	100	99.6	99.4	99.9	99.9	99.8	99.8	99.6	99.9	99.9	99.7	99.3	99.2	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
8									99.8	99.7	99.7	99.7	100	99.6	99.4	99.9	99.9	99.8	99.8	99.6	99.9	99.9	99.7	99.3	99.2	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
CM1										99.5	99.5	99.5	99.8	99.4	99.2	99.7	99.7	99.6	99.6	99.5	99.7	99.7	99.5	99.1	99	99.3	99.1	99.6	99.4	99.5	99.5	99.4	99.8
CM5											99.4	99.4	99.7	99.3	99.1	99.6	99.6	99.5	99.5	99.3	99.6	99.6	99.4	99	98.8	99.2	99	99.5	99.3	99.4	99.4	99.3	99.7
KK1												99.4	99.7	99.3	99.1	99.6	99.6	99.5	99.5	99.3	99.6	99.6	99.4	99	98.8	99.2	99	99.5	99.3	99.4	99.4	99.3	99.7
LP1													99.7	99.3	99.1	99.6	99.6	99.3	99.5	99.3	99.6	99.6	99.3	99	98.7	99.2	99	99.5	99.3	99.4	99.4	99.3	99.7
SK2														99.6	99.4	99.9	99.9	99.6	99.8	99.6	99.9	99.9	99.6	99.3	99.1	99.5	99.3	99.8	99.6	99.7	99.7	99.6	100
SP1															99	99.5	99.5	99.4	99.4	99.2	99.5	99.5	99.3	98.8	99	99.1	98.8	99.4	99.2	99.3	99.3	99.2	99.6
SP2																99.3	99.3	99	99.2	99	99.3	99.3	99	98.6	98.4	98.9	98.6	99.2	99	99.1	99.1	99	99.4
SP3																	100	99.7	99.7	99.5	99.8	99.8	99.6	99.2	99.1	99.4	99.2	99.7	99.5	99.6	99.6	99.5	99.9
SP4																		99.7	99.7	99.5	99.8	99.8	99.6	99.2	99.1	99.4	99.2	99.7	99.5	99.6	99.6	99.5	99.9
TK1																			99.6	99.4	99.7	99.5	99.4	99.1	98.7	99.1	98.9	99.4	99.4	99.5	99.3	99.2	99.8
TK2																				99.4	99.7	99.7	99.5	99.1	99	99.3	99.1	99.6	99.4	99.5	99.5	99.4	99.8
TK5																					99.5	99.5	99.3	98.8	98.7	99.1	98.8	99.4	99.2	99.3	99.3	99.2	99.6
TK6																						99.8	99.6	99.2	99.1	99.4	99.2	99.7	99.5	99.6	99.6	99.5	99.9
TK7																							99.5	99.2	99	99.4	99.2	99.7	99.5	99.6	99.6	99.5	99.9
TK8																								99	98.8	99.1	99.1	99.4	99.3	99.4	99.3	99.2	99.7
TK10																									98.4	98.7	98.5	99.1	98.8	99	99	98.8	99.3
TK12																										98.5	98.3	98.9	98.7	98.8	98.7	98.6	99.2
TK13																											98.7	99.3	99.1	99.2	99.2	99.1	99.5
TK16																												99.1	98.8	99	99	98.9	99.3

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Table 11 (Continued)

	Nucleotide identity (%)																																
Sample[a]	1	2	3	4	5	6	7	8	CM1	CM5	KK1	LP1	SK2	SP1	SP2	SP3	SP4	TK1	TK2	TK5	TK6	TK7	TK8	TK10	TK 12	TK 13	TK 16	TK 17	UB3	UB4	UB5	UB7	UB8
TK17																													99.4	99.5	99.5	99.4	99.8
UB3																													-	99.3	99.3	99.2	99.6
UB4																													0.7	-	99.4	99.3	99.7
UB5																													0.7	0.6	-	99.3	99.7
UB7																													0.8	0.7	0.7	-	99.6
UB8																													0.4	0.3	0.3	0.4	-

[a] Nucleotide sequence of *M. incognita* from China available in GenBank

1 - JN005840, 2 - JN005841, 3 - JN005842, 4 - JN005843, 5 - JN005844, 6- JN005845, 7 - KC496012 and 8 - KF481971

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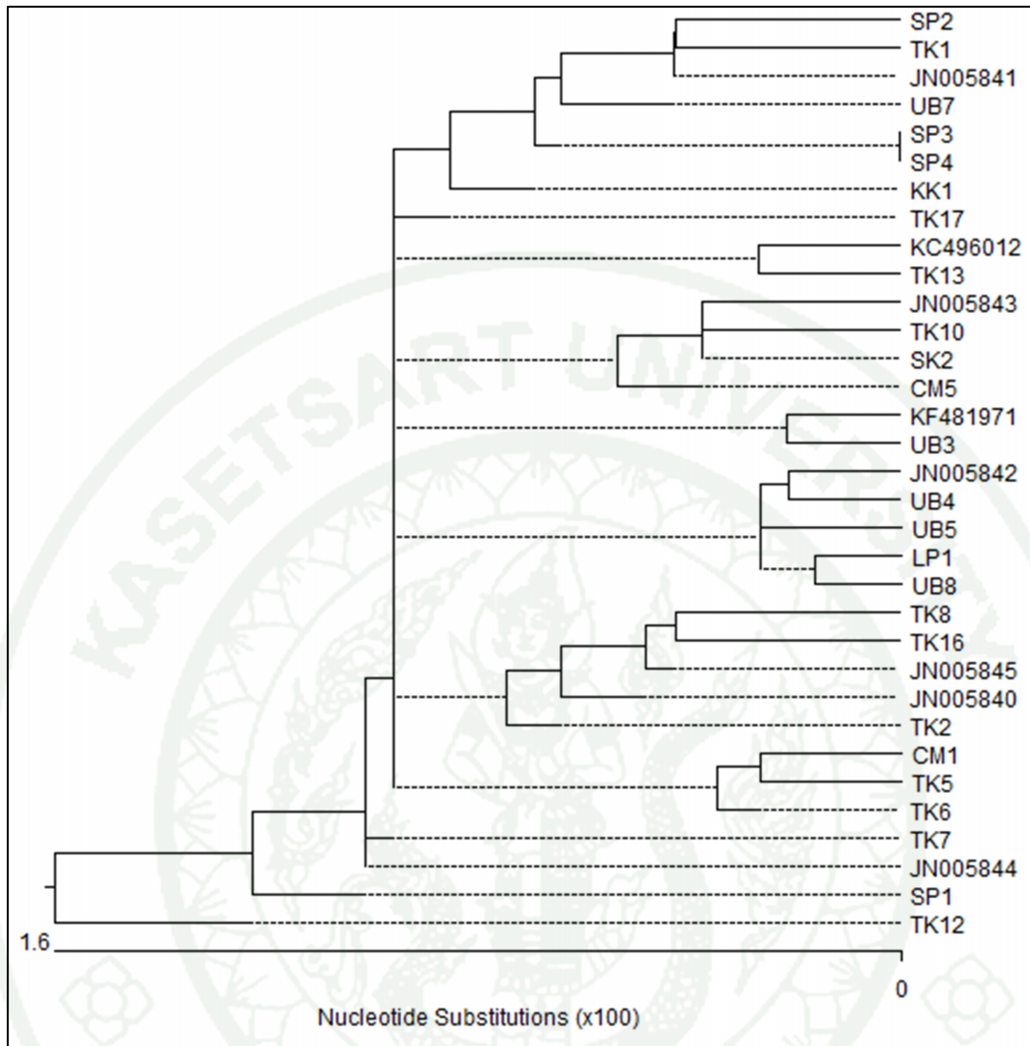

Figure 21 Phylogenetic tree based on nucleotide sequence alignment of PCR fragment derived from *Meloidogyne incognita* from Chiang Mai (CM1, CM5), Lamphun (LP1), Tak (TK1, TK2, TK5, TK6, TK7, TK8, TK10, TK12, TK13, TK16, TK17), KhonKaen (KK1), Srisaket (SK2), Suphanburi (SP1, SP2, SP3, SP4) and Ubonratchatani (UB3, UB4, UB5, UB7, UB8) with other nucleotide sequences of *M. incognita* available in GenBank (Table 6)

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Table 12 Nucleotide identity of PCR fragment derived from four samples of *Meloidogyne javanica* with available accession DNA sequences in GenBank

Sample [a]	Nucleotide identity									
	1	2	3	4	5	6	CM4	SKH1	SKH2	SKH3
1		100	100	100	99.7	99.5	100	99.7	99.7	99.4
2			100	100	99.7	99.5	100	99.7	99.7	99.4
3				100	99.7	99.5	100	99.7	99.7	99.4
4					99.7	99.5	100	99.7	99.7	99.4
5						99.2	99.7	99.4	99.4	99.1
6							99.5	99.2	99.2	98.9
CM4								99.7	99.7	99.4
SKH1									99.4	99.1
SKH2										99.1
SKH3										

[a] Nucleotide sequence of *M. javanica* from China available in GenBank

1 - JN005834, 2 - JN005837, 3 - JN005838, 4 - JN005836, 5 – JN005835 and 6 – JN005839

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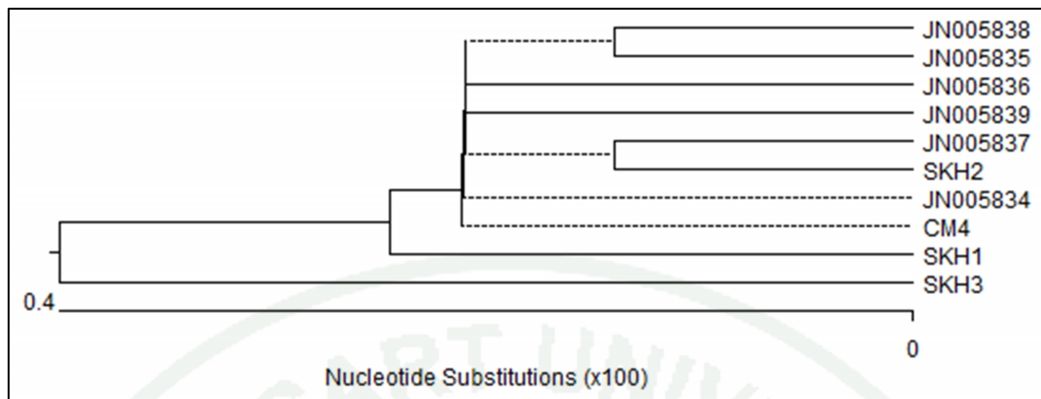

Figure 22 Phylogenetic tree based on nucleotide sequence alignment of PCR fragment derived from *Meloidogyne javanica* from Chiang Mai (CM4) and Songkla (SKH1, SKH2, SKH3) with other nucleotide sequences of *M. javanica* available in GenBank (Table 6)

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Discussion

Root knot disease caused by root-knot nematode (*Meloidogyne* spp.) is an important problem for more than 90 crop species including economic crops such as chili, potato and tomato. Chili is grown as monocrop in Thailand to produce in the mass quantities for industries for local and foreign markets. So, famers have repeatedly used the same land with epidemic of root knot disease of chili production and inefficient methods to control disease. This led to multiplication of root-knot nematode to new production areas.

In this study, 50 samples of soil were collected from the rhizosphere of chilis that had the epidemic of root knot disease. Farmers repeatedly grew chilis and used chili seedlings contaminated with root-knot nematode. Pure culture of root-knot nematode was prepared and multiplied by infection of new chili seedlings. Chilis infected with root-knot nematode develop severe symptoms of root knot disease.

All samples were initially identified by PCR using 194/195 primers. Thirty four samples were identified to be in tropical species group and the other nine samples were not classified as tropical species group. Nucleotide sequences of nine samples were compared with other published sequences in GenBank by BLAST search, they were closely to *M. mayaguensis* (Adam *et al*., 2007). While seven samples gave negative result, so they were unidentified. The difference among these samples might due to diversity of root-knot nematode population infecting chili. The unidentified species are possibly a new race of root-knot nematode and has to be identified.

Adult female from thirty four samples of tropical root-knot nematode from each pure culture population was used to species identification by perineal pattern according to diagnostic key of Eisenback (1985) and Taylor and Sasser (1978). Twenty seven samples were identified as *M. incognita* and five samples were identified as *M. javanica.* While, two samples from Chiang Mai (CM6) and Ubon

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Ratchatani (UB6) gave negative result, and they were identified as unidentified samples.

Among root-knot nematode samples were used to species identification by PCR according to diagnostic key of Adam *et al*. (2007), twenty seven samples of root-knot nematode were identified as *M. incognita* by PCR with MI-F/MI-R primers. While, other five samples of root-knot nematode infecting chili from Chiang Mai (CM4), Songkla (SKH1, SKH2, SKH3) and Tak (TK11) were identified as *M. javanica* by PCR with Fjav/Rjav primers. However, two samples from Chiang Mai (CM6) and Ubon Ratchathani (UB6) were negative by PCR with all specific primers (Table 8), so they were unidentified.

Only samples of root-knot nematode with the conformity of identification results between perineal pattern and PCR were used for sequence analysis. There were twenty five samples of *M. incognita* and four samples of *M. javanica*. However, identification of the others five samples gave unconformity results between perineal pattern and PCR. Two samples (CM6 and UB6) were identified as *M. incognita* by perineal pattern, but they were unidentified samples by PCR. One sample (TK11) was identified as *M. incognita* by perineal pattern, but it was identified as *M. javanica* by PCR method. Two samples (TK9, TK14) were identified as *M. javanica* by perineal pattern, but they were identified as *M. incognita* by PCR method. Those samples with unconformity results did not be used for sequence analysis.

The unconformity between identification by perineal pattern and PCR method might be due to inaccurate identification by perineal pattern, even to an expert (Onkendi and Moleleki, 2013). It contrasted to molecular diagnostic such as PCR that is more sensitive and accurate to distinguish the species of *Meloidogyne* (Blok, 2005). Thus, root-knot nematode should be identified by several techniques to reassure as accurate identification. Each technique has either advantage or disadvantage. Perineal pattern is fast, low cost, and suitable for initial identification, however it is

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not totally accuracte and requires expertise with special skill. In this case of PCR method, it is fast, but very expensive (Singh *et al.*, 2012).

The root-knot nematode identified as *M. incognita* or *M. javanica* were used for sequences analysis and construction of phylogenetic tree. From sequence analysis, there was no variation among populations of *M. incognita* and *M. javanica* found to infect chili in Thailand and no variation with other *M. incognita* and *M. javanica* from China, as well. It might be related to the mode of reproduction of *Meloidogyne* spp. via mitotic parthenogenesis as these results in clonal progenies (Correa *et al.*, 2013). Meiotic parthenogenesis in *Meloidogyne* is facultative in that a single population can reproduce by cross-fertilization when males are present or by meiotic parthenogenesis when males are absent (Muniz *et al.*, 2008; Triantaphyllou, 1985).

In the future, it should be essential to increase the number of samples to confirm the data on the genetic variation of *M. incognita* and *M. javanica* infecting chili in Thailand. In addition, climate change is another factor to increase the root-knot nematode population and epidemic of root-knot nematode to new production areas. Therefore, study of genetic variation of root-knot nematode is important to monitor the emergence of new species of root-knot nematode.

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CONCLUSION AND RECOMMENDATIONS

Conclusion

PCR technique can be used for easy and fast identification of root-knot nematode as compared with identification by perineal pattern. Most of root-knot nematode infecting chili in Thailand is *M. incognita*, and few are *M. javanica*. Root-knot nematode infecting chili in Thailand has no variation among them.

Recommendations

1. To increase the number of samples to confirm the data on the genetic diversity.

2. To increase root-knot nematode samples (replication) for identification because it is a good representative of population.

3. DNA extraction from second stage juvenile using (J2) worm lysis buffer should be done under stereomicroscope within 1-3 min because DNA will be degraded. Worm lysis buffere has to be stored on ice during the whole process of DNA extraction. DNA extracted by this technique can be stored at -20 ºC for a year without degradation.

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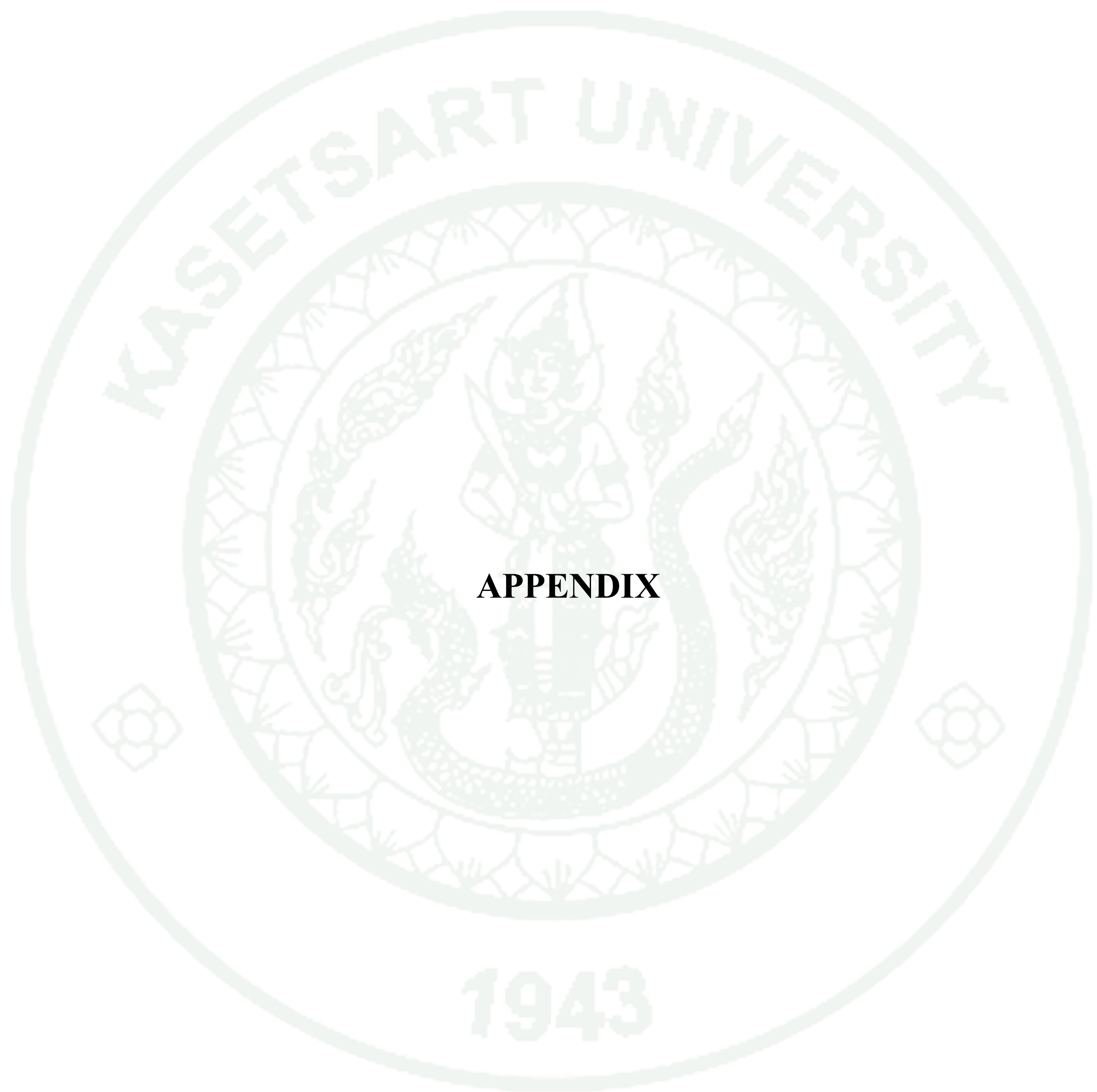

APPENDIX

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Appendix Table 1 Total samples and number of nucleotide sequences

No.	Regions	Code	DNA sequence amplification by 194/195 primers (bp)	DNA sequence amplification by specific primers		Note
				M. javanica (bp)	*M. incognita* (bp)	
1	Sanpatong, Chiang Mai	CM1	713	—	958	
2	Doilor, Chiang Mai	CM2	781	—	—	not tropical
3	Sansai, Chiang Mai	CM3	784	—	—	not tropical
4	Doilor, Chiang Mai	CM4	711	663	—	
5	Doilor, Chiang Mai	CM5	715	—	955	
6	Doilor, Chiang Mai	CM6	716	—	—	unknown
7	Mueang, Srisaket	SK1	736	—	—	not tropical
8	Mueang, Srisaket	SK2	713	—	955	
9	Mueang, Khon Kean	KK1	716	—	955	
10	Mueang, Lumphun	LP1	714	—	954	

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Appendix Table 1 (Continued)

No.	Regions	Code	DNA sequence amplification by 194/195 primers (bp)	DNA sequence amplification by specific primers		Note
				M. javanica (bp)	*M. incognita* (bp)	
11	Mueang, Lumphun	LP2	780	—	—	not tropical
12	Mueang, Uthai Thani	UT1	781	—	—	not tropical
13	Mueang, Phetchaburi	PB1	—	—	—	unknown
14	PhopPhra, Tak	TK1	713	—	959	
15	PhopPhra, Tak	TK2	713	—	955	
16	PhopPhra, Tak	TK3	—	—	—	unknown
17	PhopPhra, Tak	TK4	—	—	—	unknown
18	PhopPhra, Tak	TK5	713	—	954	
19	PhopPhra, Tak	TK6	712	—	955	
20	PhopPhra, Tak	TK7	713	—	955	

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Appendix Table 1 (Continued)

No.	Regions	Code	DNA sequence amplification by 194/195 primers (bp)	DNA sequence amplification by specific primers		Note
				M. javanica (bp)	*M. incognita* (bp)	
21	PhopPhra, Tak	TK8	713	—	957	
22	PhopPhra, Tak	TK9	713	—	956	
23	PhopPhra, Tak	TK10	713		956	
24	PhopPhra, Tak	TK11	714	659	—	
25	PhopPhra, Tak	TK12	711	—	957	
26	PhopPhra, Tak	TK13	713	—	954	
27	PhopPhra, Tak	TK14	713	—	954	
28	PhopPhra,Tak	TK15	781	—	—	not tropical
29	PhopPhra, Tak	TK16	713	—	955	
30	PhopPhra, Tak	TK17	718	—	955	

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Appendix Table 1 (Continued)

No.	Regions	Code	DNA sequence amplification by 194/195 primers (bp)	DNA sequence amplification by specific primers		Note
				M. javanica (bp)	*M. incognita* (bp)	
31	PhopPhra, Tak	TK18	781	—	—	not tropical
32	Mueang, Ubon Ratchatani	UB1	781	—	—	not tropical
33	Mueang, Ubon Ratchatani	UB2	—	—	—	unknown
34	Mueang, Ubon Ratchatani	UB3	713	—	954	
35	Mueang, Ubon Ratchatani	UB4	713	—	955	
36	Muang Samsip, Suphan Buri	UB5	712	—	954	
37	Muang Samsip, Ubon Rachatani	UB6	713	—	—	unknown
38	Muang Samsip, Ubon Rachatani	UB7	718	—	955	
39	Khueang Nai,Ubon Rachatani	UB8	736	—	955	
40	TanSum, Ubon Rachatani	UB9	—	—	—	unknown

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Appendix Table 1 (Continued)

No.	Regions	Code	DNA sequence amplification by 194/195 primers (bp)	DNA sequence amplification by specific primers		Note
				M. javanica (bp)	*M. incognita* (bp)	
41	Buntharik, Ubon Rachatani Ratchathani	UB10	—	—	—	unknown
42	LaoSuea Kok, Ubon Ratchathani	UB11	—	—	—	unknown
43	Phibunmangsahan ,Ubon Rachatani	UB12	—	—	—	unknown
44	Nong Ya Sai, Suphan Buri	SP1	712	—	956	
45	Nong Ya, Suphan Buri	SP2	713	—	955	
46	Nong Ya Sai, Suphan Buri	SP3	712	—	956	
47	Nong Ya Sai, Suphan Buri	SP4	712	—	955	
48	Hatyai, Songkla	SKH1	713	665	—	
49	Hatyai, Songkla	SKH2	713	659	—	
50	Hatyai, Songkla	SKH3	714	664	—	

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