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Morphological and Molecular Identification of *Meloidogyne enterolobii* Populations from Different Chili-cultivated Areas in Ubon Ratchathani Province, Thailand

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Abstract

Root-knot nematodes have caused severe damage to chili grown in Ubon Ratchathani province, Thailand, for a long time. As yield losses caused by these root-knot nematodes have been evaluated and effective control methods have been sought, accurate identification of the species of root-knot nematodes is urgently needed. Thus, this study focused on nematode identification using morphological and molecular techniques. A field survey of damaged chili plants was carried out in 4 districts of Ubon Ratchathani province: Lao Suea Kok (LS), Mueang (AM), Muang Sam Sip (MS) and Det Udom (DA). This survey revealed that many chili plants exhibited symptoms of leaf yellowing, leaf drooping and stunted growth, and numerous root galls were observed when the plants were uprooted, especially the "Superhot" chili variety in AM district. Morphological, morphometric and molecular characterization were studied to identify the causal agent of the disease. Perineal patterns analysis of adult females showed that all had rounded to ovoid shapes, with high to moderately high dorsal arches, and lateral lines. The morphometrics of males and 2nd-stage juveniles (J2) were highly similar to the original description of *Meloidogyne enterolobii*. Molecular identification based on COII-16S rRNA gene regions revealed that *M. enterolobii* was the cause of damage in all sampled locations, and were 99 % similar to *M. enterolobii* populations found in the USA and China. Information regarding the morphological and molecular characters of *M. enterolobii* parasitizing chili plants collected from Ubon Ratchathani province, Thailand, are provided in this study.

Keywords: *Capsicum* spp., *Meloidogyne enterolobii*, Nematode identification, Phylogenetic analysis, Root-knot nematodes

Introduction

Chili (*Capsicum* spp.) belongs to the Solanaceae family and is one of the most important vegetable crops in Thailand, with a total planting area of about 27,000 ha and a total yield of more than 0.28 million tons per year [1]. Yield losses of solanaceous vegetables caused by root-knot nematodes (*Meloidogyne* spp.) have been reported between 12.5 - 80 % in chili, eggplant, tomato and melon [2-4]. In Thailand, *M. incognita* is considered to be a serious pest which can impose yield losses in chili crops in Ubon Ratchathani and Se Sa Ket provinces of 50 - 100 % [5,6]. Previous reports have been published discussing several species of root-knot nematodes (e.g., *M. incognita*, *M. javanica* and *M. enterolobii*) which cause damage to solanaceous vegetables [7,8].

M. enterolobii is one of the most important species of root-knot nematodes listed in EPPO list A2 [9]. This nematode is considered as a severely damaging species due to its high reproduction rate and induction of large galls [10]. Furthermore, *M. enterolobii* has a wide host range viz. sweet potato (*Ipomoea batatas*), coffee (*Coffea arabica*), potato (*Solanum tuberosum*), soybean (*Glycine max*), eggplant (*Solanum melongena*), watermelon (*Citrullis lanatus*), rain tree (*Samanea saman*) and guava (*Psidium guajava*) [9,11,12]. Castagnone-Sereno [10] and Brito *et al.* [13] reported that *M. enterolobii* displays the highest virulence, compared to the other tropical root-knot nematodes, and has the ability to reproduce on crop genotypes carrying resistance to the major species of *Meloidogyne*, such as tomatoes (*Mi 1* gene), potato

(*Mh* gene) and bell pepper (*N* gene). Moreover, *M. enterolobii* alone can inflict up to 65 % losses on crops, which is significantly more damaging than any other root-knot nematode species [14].

To select proper control methods, the correct diagnosis of nematode species is necessary [15]. Traditionally, *Meloidogyne* species are identified based on morphological characteristics and isozyme phenotypes [16-19]. Morphological analysis has been commonly used for *M. enterolobii* identification [20-22]. However, the use of morphological characteristics might not clearly distinguish *Meloidogyne* populations [22]. Another potential method is to analyze molecular markers using the PCR technique, which has become a routine diagnosis for many species of plant-parasitic nematodes due to its accuracy and accessibility [15]. Powers and Harris [23] designed the specific primer C2F3/1108 of root-knot nematodes which amplifies Cytochrome Oxidase Subunit II (COII) and 16S Ribosomal RNA in nematode mitochondrial genome, and generates various DNA fragments in different *Meloidogyne* species. As mentioned previously, identification by employing both molecular and morphological techniques provide more precise, reliable identification [12]. Therefore, the objective of this study was to identify the species of root-knot nematodes collected from chili grown in Ubon Ratchathani province based on their morphological and molecular characterizations.

Materials and methods

Chili root sampling

Mueang Root-knot nematode-infected chili roots were collected from chili plants grown in 12 farms in Lao Suea Kok (LS), Mueang (AM), Muang Sam Sip (MS) and Det Udom (DA) districts of Ubon Ratchathani province (**Figure 1**), located in the northeastern part of Thailand (3 farms per district). Chili plants with symptoms of yellow leaves, stunting, wilting and root galling were randomly selected from 3 spots (3 replications) in 1 farm. The roots were cut and separated from chili stems using pruning shears, put into individual zipper bags, labelled with the farm name and kept in a foam box before transfer to the laboratory.

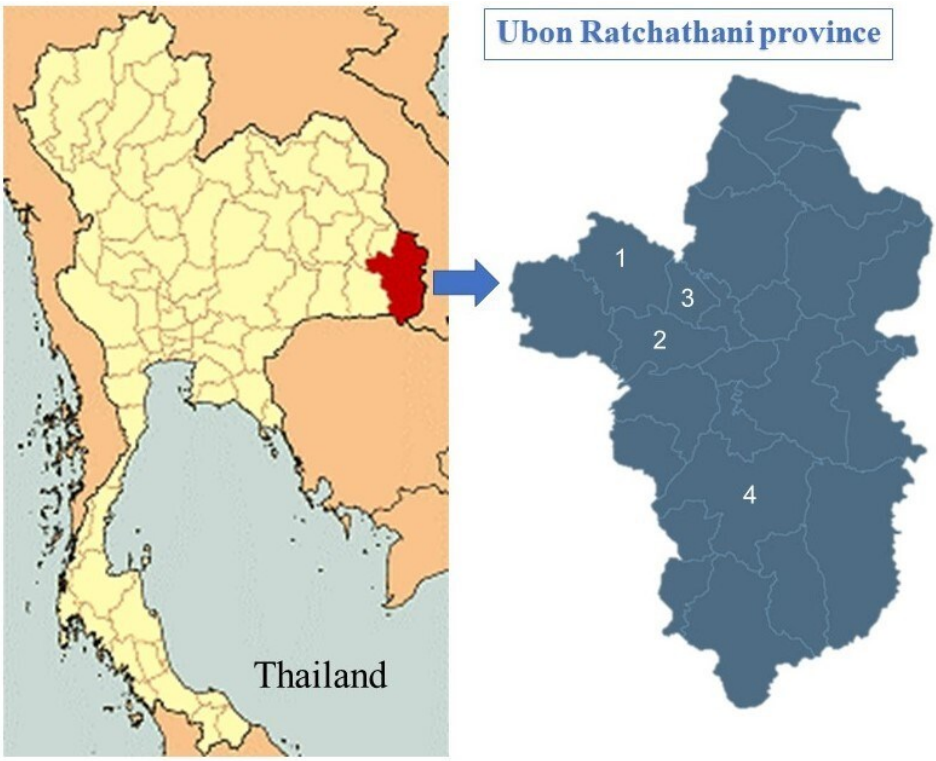

Figure 1 Sampling locations of chili farms from 4 districts, including MS (1), AM (2), LS (3) and DA (4), in Ubon Ratchathani province, Thailand.

Preparation and purification of root-knot nematode (*Meloidogyne* spp.)

Root samples were further cut into approximately 1-inch pieces using pruning shears. One hundred g of roots were randomly selected, and transferred into a 5-inch-diameter pot filled with 400 g sterilized

loamy soil. Subsequently, a 4-week-old water convolvulus (*Ipomoea aquatica* Forssk.) was transplanted into the 5-inch-diameter pot which was maintained in the greenhouse for 2 months. Nematode pure cultures collected from each root sample were prepared using the single egg-mass technique [24] as follows: Water convolvulus roots were washed with tap water until free of soil. Nematode egg masses were teased from water convolvulus roots using forceps under a stereomicroscope and transferred singly onto a 24 micro-well plate containing distilled water (1 egg mass/well). Three days later, J2 hatched from the single egg masses were inoculated onto 4-week-old water convolvulus plants grown in 2-inch diameter pots filled with sterilized soil. The inoculation was conducted by pipetting the suspension of J2 into the 2 holes made around the root zone. Water convolvuluses were maintained for 2 months in the greenhouse. Subsequently, the nematode samples collected from the pots were used for nematode identification.

Morphological identification

Perineal pattern analysis of adult females: Perineal patterns of nematode females were prepared by following the method developed by [25]. Ten adult females selected from water convolvulus roots of each district were cut using a razor to separate the anterior and posterior parts and placed onto a transparent plastic sheet under a stereomicroscope. The perineal patterns were transferred to a drop of 45 % lactic acid to trim off cuticles and clean the pattern. Subsequently, the perineal pattern was transferred to a drop of 4 % glycerine mounted on a glass slide, covered with a coverslip and sealed with nail polish. The slide was labelled and placed under a compound microscope for morphological identification.

Measurements of males and J2: Males extracted from infected roots and J2 hatched from the single egg mass were fixed on glass slides following Sienhorst's method. Males and J2 of each isolate were observed and photographed under a compound microscope (Olympus BX50, Japan) equipped with a Canon digital camera, well-calibrated (10× (1 unit) = 10 µm, 40× (1 unit) = 2.5 µm and 100× (1 unit) = 1 µm) which was installed with Axiovisionle482 software, for image measurement [26]. The following characteristics of the nematodes were measured (body width, stylet length, the dorsal gland orifice (DGO) to the stylet base, pharynx length, tail length, maximum tail width, spicule length, hyaline tail terminus), and calculated according to the De Man Formula [27]: L = mean body length, a = mean body length/maximum body width, b' = mean body length/pharynx length, c = mean body length/tail length, c' = tail length/maximum tail width and %h = (h/tail length) ×100).

Morphometric values of J2 or males from each district were statistically analyzed using SPSS software (version 26.0; SPSS Inc., Chicago, IL, USA). Differences among the values were determined by analysis of variance (ANOVA) and the means were compared using the Tukey HSD test ($p \leq 0.05$).

Molecular identification

DNA extraction: An adult female derived from nematode pure cultures was transferred into a PCR tube containing 25 µL of sterilized water. Then, 25 µL of lysis buffer containing 0.2 M NaCl (A&D Technology), 0.2 M Tris-HCL pH 8.0 (A&D Technology), 800 µg/mL of proteinase K (Worthington Biochemical) and 1 % (vol/vol) β-mercaptoethanol (Sigma) were added into the tube. The reaction was incubated under 65 °C for 90 min and 99 °C for 5 min in a PCR machine (Biometra Tgradient Thermoblock PCR Thermocycler). DNA, which had been extracted, was used immediately or stored at –20 °C for later use [28,29].

Polymerase Chain Reaction (PCR): The extracted root-knot nematode DNA was used as the template and the reaction was performed as follows: 1.5 µL each of 10 µM forward and reverse primers (C2F3/1108 and sequence-characterized amplified regions (SCAR) MK7F/MK7R), 3 µL of DNA template, 9 µL of dH2O and 15 µL of 2× PCR master mixed with dye solution i-taq (Intron biotechnology) were used per 1 reaction - in total 30 µL. The PCR condition was performed as follows: C2F3/1108 (denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min, with the final extension at 72 °C for 7 min) [23] and SCAR MK7F/MK7R (denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, with the final extension at 72 °C for 7 min) [30]. Following DNA amplification, 5 µL of DNA products was screened on 1.5 % agarose gels, and a 100 bp plus DNA marker (Biotechrabbit) was included as the standard. The gel was analyzed in the electric field of 100 volts for 30 min and visualized on a UV box.

DNA sequencing and alignment analysis: PCR products were purified and sequenced by an independent lab (Solgen Inc., South Korea). The homology and phylogenetic affinities of the sequences were determined by making comparisons with those from GenBank. The Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software was used to produce a consensus. Nucleotide sequences were aligned using ClustalW. Consequently, the obtained sequences were deposited in NCBI GenBank database under accession numbers MW125621 - MW125625, MW167102 - MW167111 and MW177689 - 177695.

Relationships and genetic diversity of the nucleotide sequences of *Meloidogyne* populations isolated from infected chili were analyzed by selecting the nucleotide sequences of COII and 16S rRNA gene in mitochondrial DNA, using *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. arabica*, *M. exigue*, *M. graminis* and *M. marylandi* as outgroup taxa. The alignments were conducted by making comparisons between GenBank nucleotide sequences with those generated from each primer set using ClustalW. Multiple gene sequence alignments, the genetic distance between populations and phylogenetic reconstruction were performed using MEGA7. Phylogenetic trees were performed via the Maximum Likelihood (ML) methods based on the Gamma distribution (GTR + G) model. For all analyses, 1000 bootstrap replicates were performed to test the node support of the generated tree [30].

Results and discussion

Results

Root-knot symptoms of chili

Damage to chili plants caused by root-knot nematodes in Ubon Ratchathani province was severe, especially harmful to "Superhot" chili variety in AM district (**Figure 2**). Above-ground symptoms of chili infected by root-knot nematodes include yellowing, stunting and declining. When chili plants were uprooted, huge and conspicuous root galls were evident on the entire root systems.

Figure 2 Samples of galled roots of chili collected from 4 locations (districts) in Ubon Ratchathani province (A) AM, (B) MS, (C) DA and (D) LS.

Morphological identification

Adult female perineal patterns: Morphological studies of the perineal patterns of nematode adult females collected from LS, MS, DA and AM districts were conducted (**Figures 3(A) - 3(D)**). The perineal patterns were rounded to ovoid shapes, with high to moderately high dorsal arches and lateral lines, which were the characteristics of *M. enterolobii*.

Measurement of males: Morphometrics of males was shown in the form of average ± standard deviation (**Table 1** and **Figures 3(E) - 3(I)**). The results showed that none of the morphometrics were significantly different in any of the nematode isolates: Body length (1016.4 to 1564.0 µm), body width (21.5 to 33.7 µm), stylet length (19.6 to 26.1 µm), DGO (3.5 to 5.9 µm), pharynx length (162.5 to 284.5 µm) tail length (9.3 to 12.6 µm), width (15.6 to 23.3 µm), spicule length (24.2 to 31.6 µm), a ratio (36.2 to 67.4), b' ratio (5.3 to 8.6), c ratio (109.3 to 156.0) and c' ratio (0.4 to 0.8).

Measurement of J2: Morphometric study of forty J2 from each sampling location was conducted and the data were shown in the form of average ± standard deviation (**Table 2** and **Figure 4**). The morphological

characteristics of body length (354.5 to 477.2 µm) and width (13.0 to 16.6 µm), stylet length (10.3 to 14.0 µm), tail width at anus (8.1 to 16.5 µm), a ratio (22.5 to 34.1 µm) and c' ratio (3.6 to 8.2) did not differ among the 4 locations. However, tail length, DGO, c ratio, hyaline tail terminus (h) and h% varied among the nematode populations. MS isolate (average 55.4 µm) showed significantly lower tail length than LS isolates (average: 59.2 µm). In contrast, DGO (average 3.5 µm), c ratio (average 7.5) and h% (average 34.2) of MS isolate were longer than LS isolate (average DGO 3.3 µm), c ratio (7.0) and h% (30.2), but it was not significantly different from DA and AM isolates.

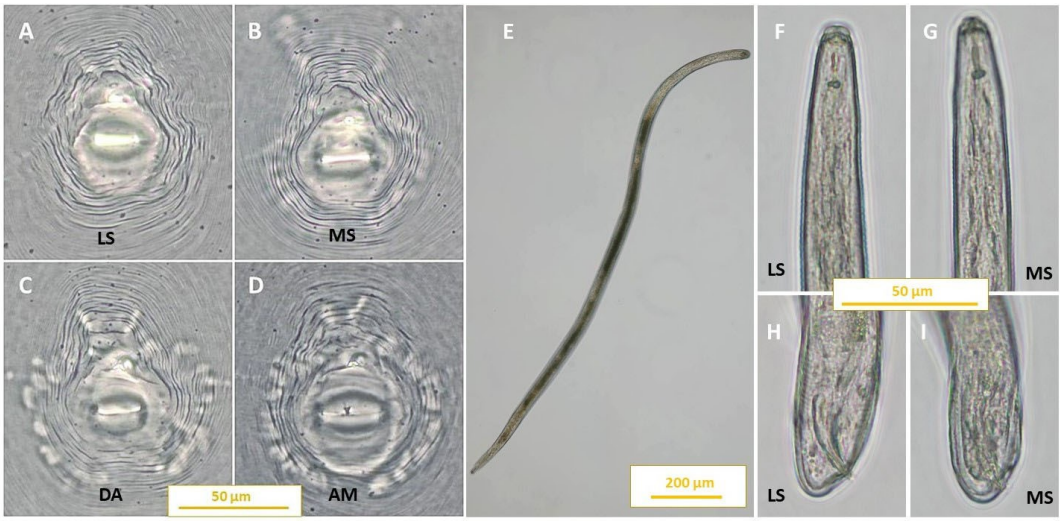

Figure 3 Photomicrographs of perineal patterns and males of *M. enterolobii* collected from chili farms in LS, MS, DA and AM districts, Ubon Ratchathani province. (A and D) perineal patterns, (E) entire body of a male, (F and G) anterior region of males and (H and I) tail of male.

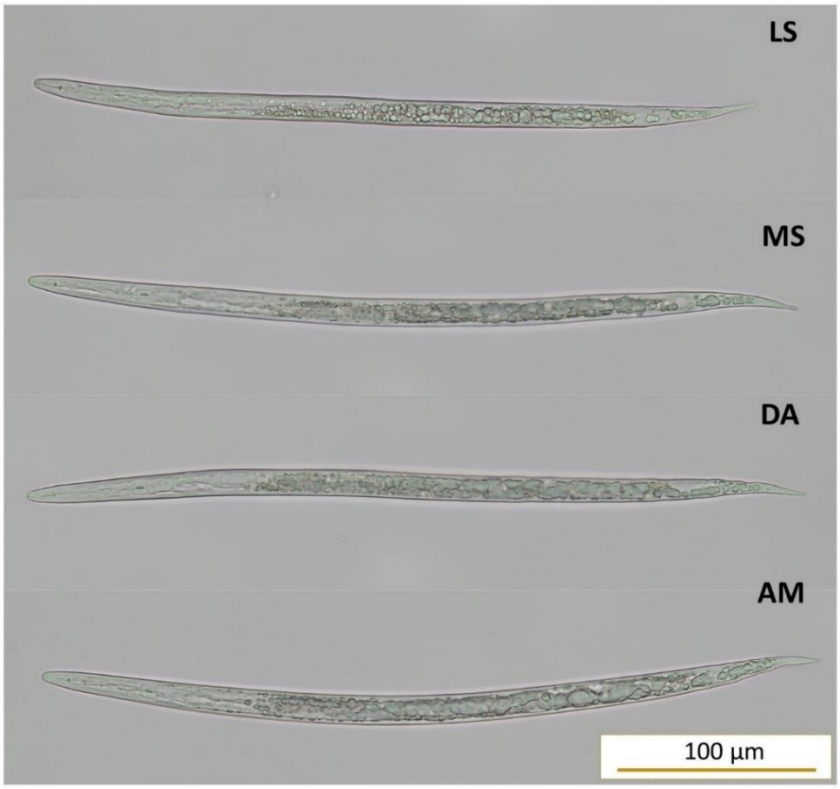

Figure 4 Photomicrographs of J2 of *M. enterolobii* obtained from chili farms in LS, MS, DA and AM districts, Ubon Ratchathani province, Thailand.

Table 1 Morphometrics of males of *Meloidogyne* collected from LS, MS, DA and AM districts (main chili production areas) of Ubon Ratchathani province and their comparison with *M. enterolobii* as previously reported by [37].

Morphometrics	Chili (This study)				Pacara Earpod Tree [37]
	LS	MS	DA	AM	
N	3	3	3	3	20
L	1373.5 ± 357.8a (1016.4 - 1732.0)	1436.7 ± 112.6a (1350.1 - 1564.0)	1341.5 ± 173.5a (1170.1 - 1517.0)	1351.5 ± 88.6a (1261.6 - 1438.7)	1599.8 (1348.6 - 1913.3)
a	55.5 ± 10.5a (47.3 - 67.4)	49.3 ± 1.9a (47.4 - 51.1)	44.8 ± 8.4a (36.2 - 53.1)	50.5 ± 4.5a (46.0 - 55.0)	37.9 (34.1 - 45.5)
b'	6.6 ± 0.5a (6.1 - 7.0)	7.4 ± 1.1a (6.4 - 8.6)	6.0 ± 0.6a (5.3 - 6.6)	7.0 ± 0.8a (6.2 - 7.8)	-
c	135.6 ± 23.9a (109.3 - 156.0)	128.6 ± 22.1a (110.8 - 153.3)	123.4 ± 1.3a (121.9 - 124.3)	117.8 ± 6.4a (110.7 - 123.0)	131.6 (72.0 - 173.4)
c'	0.5 ± 0.1a (0.4 - 0.6)	0.6 ± 0.2a (0.5 - 0.8)	0.5 ± 0.0a (0.5 - 0.6)	0.5 ± 0.0a (0.5 - 0.5)	-
Body width	24.5 ± 2.7a (21.5 - 26.4)	29.1 ± 1.3a (28.3 - 30.6)	30.4 ± 4.6a (25.2 - 33.7)	27.0 ± 3.8a (24.6 - 31.3)	42.3 (37.0 - 48.3)
Stylet length	21.1 ± 0.6a (20.6 - 21.8)	21.6 ± 1.7a (19.6 - 22.6)	21.8 ± 1.5a (20.7 - 23.5)	23.6 ± 2.3a (21.5 - 26.1)	23.4 (21.2 - 25.5)
DGO	4.2 ± 0.6a (3.8 - 4.9)	4.1 ± 0.6a (3.6 - 4.8)	4.7 ± 1.1a (3.7 - 5.9)	4.7 ± 0.4a (4.3 - 5.0)	4.7 (3.7 - 5.3)
Pharynx length	206.8 ± 40.2a (168.0 - 248.3)	197.5 ± 30.6a (162.5 - 219.2)	225.8 ± 51.3a (189.9 - 284.5)	193.7 ± 17.3a (173.8 - 205.1)	-
Tail length	10.0 ± 0.9a (9.3 - 11.1)	11.3 ± 1.2a (10.2 - 12.6)	10.9 ± 1.3a (9.6 - 12.2)	11.5 ± 0.2a (11.3 - 11.7)	12.5 (8.6 - 20.2)
Tail width	19.7 ± 3.0a (17.5 - 23.1)	21.1 ± 2.8a (15.6 - 21.1)	20.3 ± 1.5a (19.3 - 22.0)	22.3 ± 1.0a (21.4 - 23.3)	-
Spicule length	27.0 ± 0.9a (26.1 - 27.8)	27.3 ± 3.2a (24.6 - 30.8)	27.0 ± 4.0a (24.2 - 31.6)	26.9 ± 3.6a (24.6 - 31.1)	30.4 (27.3 - 32.1)

The value ± standard deviation (min-max) was compared using Tukey HSD test. Similar lower-case letters indicates that means are not significantly different ($p \leq 0.05$).

Molecular identification

Identification of root-knot nematodes based on the PCR technique using C2F3/1108 in all nematode samples (4 locations) generated DNA products of 705 bp (**Figure 5(A)**). This DNA band size matched with *M. enterolobii* reported by [13,31,32] from USA, Vietnam and China, respectively.

The amplification of SCAR MK7F/MK7R marker was used to confirm the species of *M. enterolobii* [40]. The amplified products of all nematode samples were 500 bp (**Figure 5(B)**), which confirms that *M. enterolobii* is the causal agent of chili root-knot symptoms in several areas of Ubon Ratchathani province, Thailand.

The BLAST analysis of the 22 DNA sequences (GenBank no. MW125621 - MW125625, MW167102 - MW167111 and MW177689 - MW177695) of COII and 16S rRNA gene regions provided 99 - 100 % identity match with *M. enterolobii* from China and USA. Regarding nucleotide alignments, the obtained 22 nucleotide sequences were similar among the nematode population (more than 99 %) and low genetic distance between various groups ranged from 0.000 to 0.013 (**Figure 6**). MS4:AM2, MS5:AM2, DA3:AM2, DA4:AM2 and AM1:AM2 populations had the highest genetic distance (0.013). Consequently, nucleotide sequences of several *Meloidogyne* spp., based on COII and 16S rRNA gene regions, were selected for further phylogenetic studies. The results indicated that all of the obtained 22 nucleotide sequences were classified in the same group as *M. enterolobii*, with 100 % probability support, while *M. incognita* and *M. javanica* clustered closely to *M. arenaria* and in a different clade with *M. enterolobii* (**Figure 7**).

Table 2 Morphometrics of J2 of *Meloidogyne* collected from LS, MS, DA and AM districts (main chili production areas) of Ubon Ratchathani province and their comparison with *M. enterolobii* as reported by [37].

Morphometrics	Chili (This study)				Pacara Earpod Tree [37]
	LS	MS	DA	AM	
N	40	40	40	40	30
L	407 ± 24.3a (354.5 - 445.6)	410 ± 25.4a (360.1 - 477.2)	404.8 ± 20.7a (370.0 - 449.5)	409.4 ± 21.2a (364.8 - 449.5)	436.6 (405.0 - 472.9)
a	28.8 ± 2.2a (23.9 - 33.0)	28.8 ± 2.4a (23.4 - 33.0)	28.5 ± 2.6a (22.5 - 34.1)	28.8 ± 2.6a (24 - 32.8)	28.6 (24.0 - 32.5)
c	7.0 ± 0.9b (5.5 - 9.2)	7.5 ± 0.9a (5.7 - 9.2)	7.2 ± 0.8ab (6.0 - 9.3)	7.2 ± 1.1ab (5.4 - 9.3)	7.8 (6.8 - 10.1)
c'	5.8 ± 0.8a (4.4 - 8.2)	5.4 ± 0.8a (3.6-7.1)	5.5 ± 0.6a (4.2 - 6.7)	5.5 ± 0.8a (4.1 - 7.5)	–
Body width	14.2 ± 0.9a (13.0 - 16.6)	14.3±1.0a (13.1 - 16.5)	14.3 ± 1.0a (13 - 16.5)	14.3 ± 1.0a (13.0 - 16.5)	15.3 (13.9 - 17.8)
DGO	3.3 ± 0.3b (2.9 - 4.0)	3.5 ± 0.4a (3.0 - 4.8)	3.6 ± 0.3ab (3.0 - 4.0)	3.4 ± 0.3ab (3.0 - 4.0)	3.4 (2.8 - 4.3)
Stylet length	12.6 ± 0.8a (13.0 - 16.6)	12.6 ± 0.7a (11.2 - 13.8)	12.7 ± 0.8a (10.3 - 14.0)	12.3 ± 0.8a (10.3 - 13.8)	11.7 (10.8 - 13.0)
Tail length	59.2 ± 6.3a (46.0 - 71.9)	55.4 ± 6.0b (41.5 - 67.0)	56.7 ± 5.2ab (44.7 - 67.7)	57.7 ± 7.8ab (44.9 - 75.5)	56.4 (41.5 - 63.4)
Tail diameter at anus	10.3 ± 0.7a (8.1-11.0)	10.3 ± 0.9a (13.1 - 16.5)	10.3 ± 0.6a (8.7 - 11.0)	10.5 ± 0.5a (9.4 - 11.0)	-
h	17.7 ± 2.7ab (10.7 - 23.4)	18.8 ± 3.2a (12.4 - 25.5)	16.6 ± 2.2b (10.1 - 20.7)	18.8 ± 2.4a (14.9 - 23.6)	-
h%	30.2 ± 5.4bc (18.4 - 44.7)	34.2 ± 5.6a (23.5 - 48.3)	29.5 ± 4.5c (20.0 - 39.9)	33.1 ± 5.8ab (21.9 - 45.0)	-

The value ± standard deviation (min-max) was compared using Tukey HSD test. Similar lower-case letters indicates that means are not significantly different ($p \leq 0.05$).

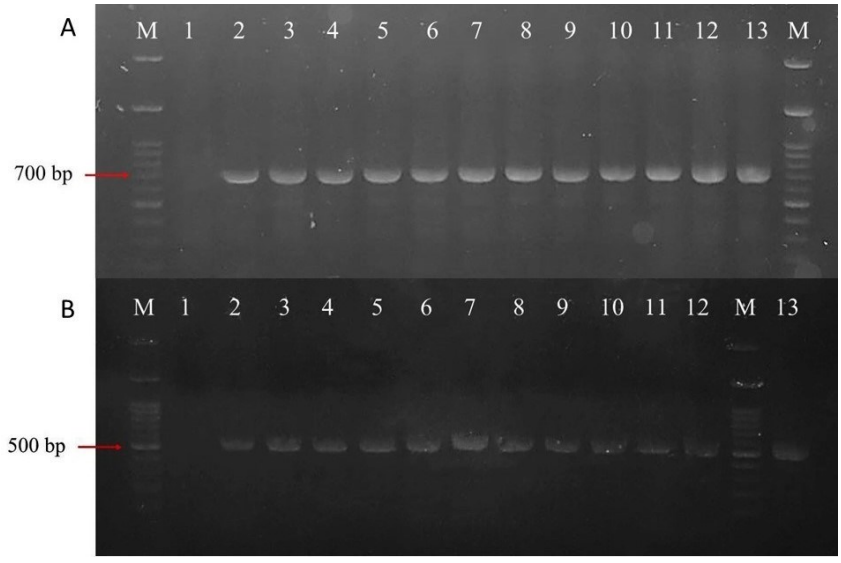

Figure 5 DNA fingerprints of *M. enterolobii* from 4 districts (3 farms in each district) in Ubon Ratchathani province using primer C2F3/1108 (A) and MK7F/MK7R SCAR marker (B). (1) Negative control (distilled water), (2 - 4) AM samples, (5 - 7) MS samples, (8 - 10) DA samples, (11 - 13) LS samples and (M) DNA marker (100 bp+).

	MS1	MS2	MS3	MS4	MS5	MS6	DA1	DA2	DA3	DA4	DA5	AM1	AM2	AM3	AM4	AM5	AM6	LS1	LS2	LS3	LS4	LS5
MS1	-																					
MS2	0.000	-																				
MS3	0.000	0.000	-																			
MS4	0.001	0.001	0.001	-																		
MS5	0.001	0.001	0.001	0.000	-																	
MS6	0.000	0.000	0.000	0.001	0.001	-																
DA1	0.000	0.000	0.000	0.001	0.001	0.000	-															
DA2	0.000	0.000	0.000	0.001	0.001	0.000	0.000	-														
DA3	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	-													
DA4	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.000	-												
DA5	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	-											
AM1	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.000	0.000	0.001	-										
AM2	0.012	0.012	0.012	0.013	0.013	0.012	0.012	0.012	0.013	0.013	0.012	0.013	-									
AM3	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	-								
AM4	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	-							
AM5	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	-						
AM6	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.000	-					
LS1	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.000	0.000	-				
LS2	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.000	0.000	0.000	-			
LS3	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.000	0.000	0.000	0.000	-		
LS4	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	
LS5	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-

Figure 6 Pairwise comparisons and genetic distance between *M. enterolobii* populations collected from chili fields in LS, MS, DA and AM districts, Ubon Ratchathani province, based on COII and 16S rRNA gene.

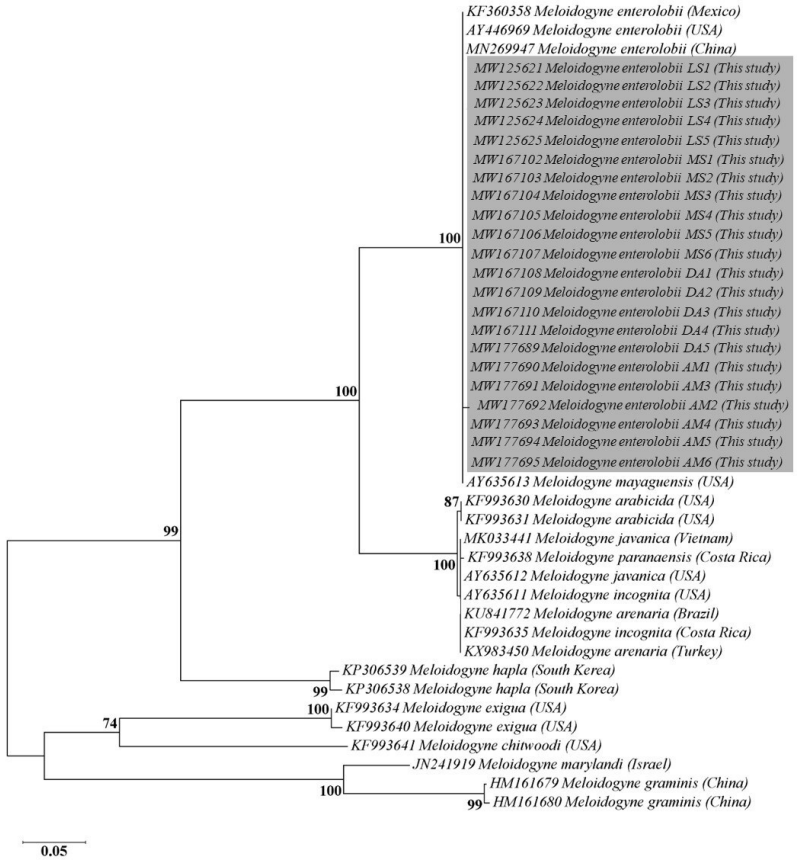

Figure 7 Phylogenetic tree of *M. enterolobii* collected from chili farms in Ubon Ratchathani province, based on COII and 16S rRNA genes, using the Maximum Likelihood method (GTR + G model) with 1000× bootstrap.

Discussion

This study showed that many chili-cultivated areas in Ubon Ratchathani were infected and damaged by root-knot nematodes. The symptoms of damage to chili plants found in this study included plant decline, wilt and chlorosis and abundant and large galls presented in the almost entire root systems. Using morphological and molecular techniques, *M. enterolobii* was identified as the causal agent of the disease. In Thailand, *M. incognita* and *M. javanica* have been mostly identified, using morphological identification, as the causal agents of root galling with the yield losses up to 50 - 100 % in chili plants grown in Ubon Ratchathani and Si Sa Ket provinces [33,34]. *M. enterolobii* has been observed in guava in Nakhon Pathom province, Thailand since 2012 [21]. Previous studies considered management methods to control *M. enterolobii* infecting chili (unidentified source) using *Bacillus* sp. [35] combined with *Streptomyces* sp. and *Trichoderma asperellum* [36]. However, morphological and molecular characterization of *M. enterolobii* have not been described in detail [35,36]. The current study provides information focusing on the morphological and molecular characters of *M. enterolobii* collected in different localities viz. LS, AM, MS and DA districts in Ubon Ratchathani provinces, Thailand.

Morphological and morphometric characters can be employed to distinguish *M. enterolobii* from other species, i.e., perineal pattern character, stylet morphology of males and the morphometrics of the head and hyaline tail in the J2 [14,37]. The current study revealed perineal patterns of adult females that were similar to *M. enterolobii* infecting guava in Thailand, as previously identified by [21]. Brito *et al*. [13] described perineal patterns of *M. enterolobii* with high dorsal arches, which were identical to *M. incognita*. Therefore, it is difficult to differentiate *M. enterolobii* from *M. incognita* and *M. arenaria* by relying on the morphology of perineal patterns [21]. In this study, morphometrics (including body length and width, and stylet length) of J2 did not differ between the 4 locations. Although some morphometrics showed variations in the morphological characters among the populations, these values overlapped with those specimens of *M. enterolobii* infecting guava in Thailand, except the hyaline tail terminus is slightly longer [21]. In addition, the comparisons of our specimens with those of the original population of *M. enterolobii* in China described by [37] showed that the average body length (404.8 to 410.0 µm) and average width (14.2 to 14.3 µm) and average c ratio (7.0 to 7.5) of our samples was smaller than the J2 parasitized pacara earpod tree (average body length 436.6 µm, width 15.3 µm and c ratio 7.8), while stylet length, DGO and tail length were closely similar. For the males, all morphometric values did not differ among all studied locations: With average body length 1341.5 to 1436.7 µm, body width 24.5 to 30.4 µm, stylet length 21.1 to 23.6 µm, tail length 10.0 to 11.5 µm and spicule length 26.9 to 27.3 µm. Although the size of males was smaller in the chili samples (this study) than in the *M. enterolobii* original description (average body length 1599.8 µm and width 42.3 µm) (**Table 1**), stylet (23.4 µm) and DGO (4.7 µm) were closely similar [37]. The length of DGO to the stylet base served to identify *M. enterolobii* males (3.7 to 6.2 µm) from *M. incognita* (2.0 to 4.0 µm) [38], however, the DGO values of males in this study corresponded closely to *M. enterolobii*. Therefore, these morphometrics revealed that *M. enterolobii* are present in many chili-cultivated areas of Ubon Ratchatani province. Unfortunately, the use of morphological characteristics does not clearly distinguish *M. enterolobii* [22]. Therefore, molecular characterization was conducted, using PCR and phylogenetic tree analyses, to confirm the morphological results.

This study employed 2 specific primer sets viz. C2F3/1108 and SCAR MK7F/MK7R to identify *M. enterolobii* which generated DNA products of 705 and 520 bp, respectively. This DNA band size matched *M. enterolobii* previously reported in the USA, Vietnam and China [13,30-32]. In Thailand, several root-knot species damaging chili plants were reported, particularly *M. incognita* and *M. javanica* [33]. However, different sizes of DNA products were revealed (1700 bp for *M. incognita* and *M. javanica*) when C2F3/1108 specific primers were used to distinguish root-knot species [23]. The SCAR MK7F/MK7R designed and developed by [30] proved to be species-specific for *M. enterolobii*, as compared with other common *Meloidogyne* species. This primer set has been successfully used to detect *M. enterolobii* in several crops, such as cotton and soybean in the USA [39] and African nightshades in Kenya [40].

The BLAST analysis of the 22 DNA sequences of COII and 16S rRNA gene regions provided a 99 - 100 % identity match with *M. enterolobii* populations previously reported in different hosts, i.e., rain tree (MT648504) in Thailand [12], mulberry (MN269947) in China [41], watermelon (KF360359) in Mexico [42] and soybean (MN809527) and sweet potato (ON320401) in the USA [43,44]. In addition, pairwise sequence alignment showed 99 % similarity to *M. enterolobii* isolates studied and low genetic diversity (0.000 - 0.013). This reveals that there is no variability in the COII and 16S rRNA sequences among the nematode populations in this study. Similar results were reported by [41], although they studied a different gene (COI) of *M. enterolobii* in mulberry. Furthermore, the phylogenetic tree, based on COII-16S rRNA genes, classified the nematodes in the same group as *M. enterolobii* with 100 % probability support and clearly displayed as being in a different clade with *M. incognita* and *M. javanica*. These results are

consistent with the findings reported by [22] and [45], using phylogenetic tree based COII and 16S rRNA gene to identify and demonstrate the relationships among *M. enterolobii* populations in potato, guava and vegetable crops and weeds.

Conclusions

Morphological features of J2s, males and female perineal patterns, and molecular characterization using PCR based on amplification of the COII-16S rRNA and SCAR marker of root-knot nematodes collected from 12 chili farms in 4 districts (3 farms per district) of Ubon Ratchathani province revealed *M. enterolobii* as the sole nematode species causing damage to chili plants. Further studies are needed to elucidate the impact of *M. enterolobii* on yields of chili in nature and its control.

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