Morphological and Molecular Identification of the Predatory Nematode, *Mononchoides* (Nematoda: Diplogasterida) from Thailand

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Received: March 13, 2020 ; Accepted: January 18, 2021

Abstract

Predatory nematodes; *Mononchoides* are the beneficial nematodes that feed on tiny organisms or other nematodes. *Mononchoides* population collected from guava orchards and para rubber plantations in Thailand was characterized by morphological and molecular techniques. *Mononchoides* species is characterized by a dorsal claw-like tooth; 7-8 µm wide and 3-4 µm long, long body (540-650 µm), didelphic-amphidelphic reproductive system and a relatively short filiform tail (40-50µm long). Genetic analysis of *Mononchoides* based on species-specific primers and sequence on the ribosomal DNA was performed. This molecular diagnosis using Mnc1, Mnc3 and Mn28s primers was confirmed by nucleotide sequences that shared 100% similarity with the known nematode sequences (FJ661080, LC210629, KT884893, LN827618, KJ877211, KF151166, KP067833) in the GenBank database. The *Mononchoides*-specific primers have been developed to offer a rapid species identification approach for further analysis and management.

Keywords: *Mononchoides*; predatory nematodes; specific primer.

1. Introduction

Predatory nematodes belong to the phylum nematode, which is essential for the food web in soil and has the potential for biological control to control and reduce the population of plant-parasitic nematodes. Classification of predatory nematodes is mainly divided into four groups: Mononchida, Dorylaimida, Diplogasteroidea, and Aphelenchoidea. Among these, the mononch nematodes are outstanding due to their behavior that eat other animals as food, especially in preadult and adult stages, and for this reason they have been called "mini tigers" (Ahmad and Jairajpuri; 2010). However,

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DOI:10.14456/tjst.2021.14

the use of mononch as a biological control agent also has limitations as low reproductive rate, long life cycle, cannibalism, difficulty maintaining in culture medium, and sensitivity to environmental changes. Consequently, the possibility to use diplogasterid predators to control plant-parasitic nematodes is higher than the other such groups. Since the diplogasterids have relatively high reproductive and predation rates, they can be easily cultured *in vitro*, rarely cannibalism. They can detect and respond to the attraction of others' nematodes. Moreover, diplogasterids tolerate to unfavorable environments, capable of reducing the quantity of root galling and populations of plant-parasitic nematodes (Fauzia *et al.*, 1998; Osman, 1988; Bilgrami *et al.*, 2005). In 1988, Bilgrami and Jairajpuri found that *Mononchoides longicaudatus* and *M. fortidens* can reduce *Hirschmanniella oyzae*, *Tylenchorhynchus mashhoodi* and *Hopblaimus indicus*. In the same year, Fauzia *et al*. reported that *M. Longicaudatus* could reduce the number of root galls caused by root-knot nematodes in pot experiments and enhance plant growth. Khan and Kim (2005) noted that *Mononchoides fortidens* reduced the root-knot nematode population; *Meloidogyne arenaria* and in the same year Bilgrami *et al*. reported on the feeding behavior of *Mononchoides gaugleri* that *Meloidogyne incognita*, *Heterodera mothi* and *Anguina tritici* juveniles are the best food sources. Furthermore, Bilgrami *et al.* (2008) reported that *M. gaugleri* was effectively reduced the plant-parasitic nematode population in Turfgrass station (New Brunswick, NJ, USA). More importantly, rapidity and accurate resources for detecting genetic diversity enhance the possibility of using these nematodes in biological control.

The diagnosis on the taxonomy of nematodes usually based on morphological characteristics (Calaway & Tarjan, 1973; Griffiths *et al.*, 2006; Koohkan *et al.*, 2014; Shokoohi *et al.*, 2015). However, morphological characters cannot be solely used in placing a new species or distinguish the differences between species due to difficulty to observe and may cause misidentification. Therefore, the current classification of nematodes using more molecular biology techniques provides greater precision, sensitivity, and quantitative accuracy. Introduction of polymerase chain reaction (PCR) to investigate nematodes species contributes to identification techniques even faster. It can be implemented with individual nematodes due to the fewer use of DNA quantities. The classification methods by PCR, such as SCAR PCR, have focused on designing PCR primers to make this validation much more specific (Waite *et al*., 2003; Bhadury *et al*., 2006; Olia *et al.*, 2009; Steel *et al.*, 2011). Therefore, the PCR-based diagnosis techniques seem to offer an attractive alternative option because they are reliable, sensitive, easy to perform and does not require nematological expertise.

The classification of nematodes was first occurred in Thailand by Dr. Timm in 1962 (Karai, 1966). Nevertheless, the identification or discovery of predatory nematodes in Thailand

has not been reported. As mentioned above, the predatory nematodes effectively control and reduce the damage caused by plant-parasitic nematodes and promote plant growth. In this study, we focus on the classification of predatory nematode; *Mononchoides* from the guava orchards in the central part and rubber plantation in the southern part of Thailand using morphological characters and molecular biological techniques to be used for additional information on the biology of predatory nematodes and the possibility of using them for the further controlling of plant- parasitic nematodes.

2. Methods

Predatory nematode (*Mononchoides* sp.) populations were collected in guava orchards and para rubber plantations in Thailand by randomly distributed over an area. Subsequently, all five cores from each tree were mixed and placed in a plastic bag. Predators were isolated from the soil samples by Cobb's (1918) sieving and a modified Baermann funnel technique. The 300-g soil samples dissolved with 1 L of water were set aside for 5-10 minutes to separate the nematodes from the soil. Then, the supernatants of the resulting soil suspensions were poured through 60-, 150-, and 400- mesh sieves, respectively. Nematodes suspended on the 150- and 400-mesh were collected and placed on the tissue paper. After 48 hours, nematodes were collected from the funnel bottom and counted the number under microscope. Nematodes were killed with hot water (50 °C) and stored at 4 °C with 95% ethanol.

2.1 Morphological Characterisation

Nematodes samples immersed in 95% ethanol transferred to Syracuse watch glasses. Subsequently, picked individual *Mononchoides* into DESS compound (0.25M EDTA pH 7.5, 20% DMSO, NaCl saturated) according to the method of Naem *et al*. (2010) for 5-60 minutes and then transferred nematodes into the sterile distilled water for 5-30 minutes to allow the nematodes recovered in the normal state. The individual nematode was transferred into a drop of water on a slide, covered with a coverslip and sealed with nail polish. The mounted *Mononchoides* were observed under a well- calibrated microscope using the photographic series Digital Camera Canon Power Shot A640. It was connected with the shot shooting program EOS Utility using AxioVision SE64 Rel. 4.9.1 to measure the various parts of the predatory nematode according to the system of De Man Formula (Ferris 2007). After that transferred the individual nematode from each slide into the Eppendorf filled with ddH_2O. The following abbreviations have been used in the text or table: L = overall body length; ratio a = body length / greatest body diameter; ratio b = body length / distance from anterior to esophago-intestinal valve; ratio c = body length / tail length and V = % distance of vulva from anterior.

2.2 Molecular Characterisation

The nematode samples were centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded, then filled with 200 µl of solution I (homogenization buffer: Tris-HCl 50 mM pH 8, NaCl 100 mM, sucrose 200 mM). Stainless beads were then added into each microcentrifuge tube and crushed with a bead grinder for 1 minute. The tubes were filled with 200 µl of solution II (lysis buffer: 1.25% SDS, Tris-HCl 50 mM pH 8, EDTA 50 mM) and then incubated at 65°C for 5 minutes. After that, 128 µL of solution III (5M potassium acetate solution: 5M potassium acetate, glacial acetic acid, dH_2O) plus 200 µL of chloroform were added. The mixture was centrifuged at 13,000 rpm for 5 min, and the upper layer was transferred to a fresh tube, and an equal volume of isopropanol was added and mixed gently. After centrifuging at 13,000 rpm for 5 min, the supernatant was discarded and washed the DNA pellet with 200 µL of cooled 70% ethanol using a pipette. After centrifuging for 60 seconds, the 70% ethanol was discarded, and the resulting DNA pellet was dissolved by added 30 µl ddH_2O containing 10 mg/mL RNaseA. Total DNA was stored at -4 °C for further use, adapted from the methods of Alexander *et al*. (2007).

For PCR amplification with specific primers, the DNA sequence of the ribosomal RNA region of *Mononchoides* was obtained from the GenBank database. Small subunit ribosomal RNA (18S rRNA) and large subunit ribosomal RNA (28S rRNA) were selectively amplified from genomic DNA by PCR, using primers designed to anneal to conserved positions within the gene. Comparative sequencing nucleotide differences with the other groups of nematodes using alignment program, ClustalW (Thompson *et al*., 1994). In total, three primer pairs; Mnc1, Mnc2, and Mnc3 targeting to 18S region and one primer pair; Mn28s targeting to 28S region of *Mononchoides* have been verified using the program, Primer3 (Rozen & Skaletsky 2000), which will calculate the parameters such as melting temperature and stability to define that might be appropriate as PCR primer sites and synthesized. A standard reaction volume was 25 µL, comprising PCR buffer at 10x concentration, dNTPs at a concentration of 2.5 mM for each nucleotide, 10 µM of each primer and *Taq* DNA polymerase at 1 unit (Fermentas). To each reaction, 1 µL of the extracting nematode DNA template was added, typically containing around 2–10 ng of genomic DNA. The volume was adjusted with ddH_2O to 25 µL per reaction. The optimal thermocycling conditions were found to be: an initial denaturation at 95 °C for 5 minutes, after which 35 cycles of amplification (94 °C for 30 seconds; annealing temperature depends on primers: 56-60 °C for 30 seconds; 72°C for 1 minute); followed by a final extension at 72°C for 5 min. Negative controls were included in all PCR amplifications to test for contaminants in the reagents. Following amplification, PCR products were checked for size and specificity by electrophoresis in 2% (w/v) agarose gels, with 0.5x TAE (Tris-acetate-EDTA) buffer, stained with DNA staining dye (ultraPower (China)) and photographed under ultraviolet (UV) light under

gel document system. The length of the DNA fragment was estimated by comparison with 100 bp-plus DNA ladder (Fermentas). The DNA fragments were sent to Pacific Science Co., Ltd. (62,64 Soi Charansanitwong 49/1, Charansanitwong Road, Bangbumru, Bangplad, Bangkok 10700, Thailand) for sequencing of both sense and anti-sense strands. The sequence data were compared with other nucleotide sequences available through the National Center for Biotechnology Information (NCBI, USA) databases. Multiple sequence alignments and comparisons were performed using the computer package Clustal W, then edited to remove major gaps using Microsoft Word. Figtree program was used to construct phylogenetic trees using the corrected distance and neighbor-joining. One hundred bootstrap re-samplings of the data assessed the stability of the resultant tree topologies.

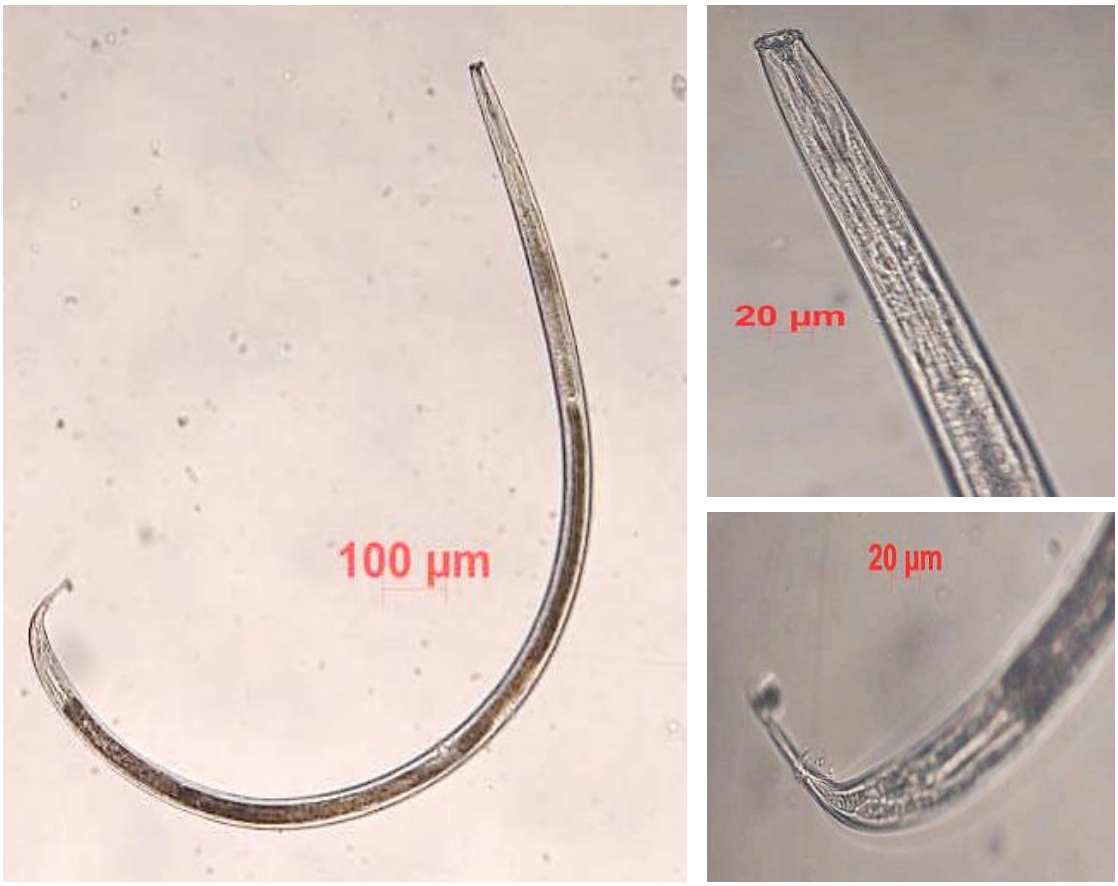

Figure 1 *Mononchoides* sp. A: The female entire body; B: Anterior part; C: Female posterior region.

3. Results and Discussions

3.1 Description

Female: body long, almost straight after fixation. Slightly anteriorly and sharply posteriorly. The cuticle was very thick and clearly annulated. Lip region low, continuous with body contour, comprises six fused lips, each with a small papilla. Stoma wide anteriorly, narrow and slender posteriorly. Cheilostom or the first part of stoma wide, stoma walls strongly cuticularized. Rod- like cheilorhabdion plates. The second part of the stoma overlapping with the gymnostom walls. Gymnostom wide. The anterior part of stegostom bearing a large, massive, claw- like tooth on the dorsal wall with the tip pointing dorsally. Stegostom posteriorly forming a cylindrical tube. The subcentral walls on the left and right have no any tooth, teeth, or denticles. The pharynx is divided into procorpus muscular slightly expanding into muscular metacorpus with valve, median bulb globular and a short, non- muscular, glandular isthmus oblong basal bulb without valves. Nerve ring encircling isthmus in anterior half. Reproductive system amphidelphic with well- developed anteriorly on both left and right side of the intestine. Ovary long. Vulval opening circular, pore-like. Vagina sclerotized. Tail long, filiform.

Male: not found

Type habitat and locality: Deteriorating guava and para rubber collected from the central and southern part of Thailand.

Table 1 Morphometric of *Mononchoides* from the central and southern part of Thailand. All measurements are in µm, and in the form: mean ± s.d. (range).

Characters	*Mononchoides* sp.	
	Guava (*Psidium guajava*)	Para rubber (*Hevea brasiliensis*)
n	6	2
L (overall body length)	650.30±8.00	540.75±2.16
	(730.41-500.07)	(560.28-530.22)
4a (body length / greatest body diameter)	36.68±3.59	35.11±1.70
	(39.30-29.63)	(36.31-33.90)
b (body length / distance from anterior to esophago-intestinal valve)	3.88±0.07	3.71±0.22
	(3.94-3.78)	(3.86-3.55)
c (body length)	13.75±1.14	10.91±1.47
	(15.39-11.92)	(11.95-9.87)
T or V (% distance of vulva from anterior)	55.98±3.04	-
	(62.17-54.25)	
Maximum body diam.	10.78±0.10	10.56±0.01
	(10.94-10.67)	(10.57-10.55)

Table 1 (Continue) Morphometric of *Mononchoides* from the central and southern part of Thailand. All measurements are in µm, and in the form: mean ± s.d. (range).

Characters	*Mononchoides* sp.	
	Guava (*Psidium guajava*)	Para rubber (*Hevea brasiliensis*)
Stoma diam.	8.6±0.08	7.8±0.05
	(9.8-7.6)	(8.1-7.4)
Stoma length	3.5±0.11	4.5±0.03
	(4.8-2.2)	(4.7-4.3)
Corpus length	70.46±2.47	60.39±0.04
	(110.62-4.99)	(60.42-60.36)
Median bulb diam.	12.1±0.24	13.3±0.02
	(15-8.7)	(13.4-13.1)
Basal bulb diam.	1.34±0.31	1.47±0.10
	(1.71-0.99)	(1.54-1.40)
Nerve ring from anterior end	101.4±1.58	91.8±1.29
	(117.4-80.6)	(102.9-82.6)
Anterior to base of esophageal glands distance	16.85±1.95	14.78±0.29
	(18.69-13.12)	(14.98-14.57)
Vulva from anterior end	360.37±3.06	-
	(400.26-310.13)	
Vulva from anus distance	240.19±4.80	-
	(260.39-140.74)	
Length of anterior gonad branch	60.99±0.76	-
	(70.89-50.96)	
Length of posterior gonad branch	60.88±0.91	-
	(70.93-60.07)	
Testis length	-	-
Anal body diam.	12.5±0.22	1.1±0.16
	(16.0-10.00)	(12.1-9.9)
Tail length	40.74±0.31	50.5±0.48
	(51.1-42.0)	(53.9-47.1)

Specific primer designs for *Mononchoides* were developed by selecting the highest identity percentage of the known nucleotide sequences in GenBank, comparing with different nematodes genera and choosing different regions to design DNA primers with the GC content between 40-65 percent and temperature melting (Tm) at 50-60 degrees Celsius. The forward primers (F) were selected from the initial part of nucleotide sequences and the end position was chosen as a reverse primer (R). Primers Mnc1, Mnc2 and Mnc3 were designed from the 18s ribosomal RNA gene and at the 28s ribosomal RNA gene was Mn28s primer, as shown in Table 2.

Table 2 Species-specific primers for the predatory nematode, *Mononchoides* used for PCR and sequencing analysis in this study.

Primers	Senses	Nucleotide sequences (5'-3')	%GC	Tm (°C)
Mnc1	Forward	CCA TCG GAC CTG GTG CAC	66.67	60.9
	Reverse	CCC CTG CCA GGG CTA TAC	66.67	60.1
Mnc2	Forward	AAC AAG TCG GTT TTC CGT ACG	47.62	56.9
	Reverse	ACA CGG ACA AAG CTA TCC GTG	52.38	58.9
Mnc3	Forward	TAG CTG ATC GCA TGG TCT TG	50.0	56.2
	Reverse	CTT GGA TGT GGT AGC CGT TT	50.0	56.5
Mn28s	Forward	CAC GCC TGA TCT CAG GAT GTT G	54.55	59.7
	Reverse	CTC ATC GCA GAA GTG CCA ACA C	57.89	59.8

Genetic analysis of *Mononchoides* using Mnc1 primers produced a faint banding pattern of approximately 900-1500 bp but provided the amplicon sizes of *Mononchoides*, *Helicotylenchus* and *Meloidogyne enterolobii* clearly different. The Mnc3 primers showed the amplified DNA size at 750 bp and were slightly different between isolates. The amplification results of Mn28s primers represented the fragment size at 650 bp, similar in the band size to *Iotonchus* and *Mylonchulus* that belong to those predatory nematode groups. At the same time, the amplified DNA patterns of *Mononchoides* were quite different with *Helicotylenchus* and *M. enterolobii* in the group of plant-parasitic nematodes. No amplicons were synthesized from Mnc2 primers. PCR products of eight populations were successfully sequenced and analyzed by comparing with known nematode sequences held online at NCBI (http://www.ncbi.nlm.nih.gov/) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The results showed that the positive sequences gave the best fits with their statistics at 100% identity and expected value of 0.2-1.8. Phylogenetic tree showing relationship using species-specific primers (18S rDNA) of *Mononchoides* are in the same clade with closely related species.

Phylogenetic relationships within the *Mononchoides* populations as inferred from the Maximum Likelihood (ML) analysis are given in figure 2.

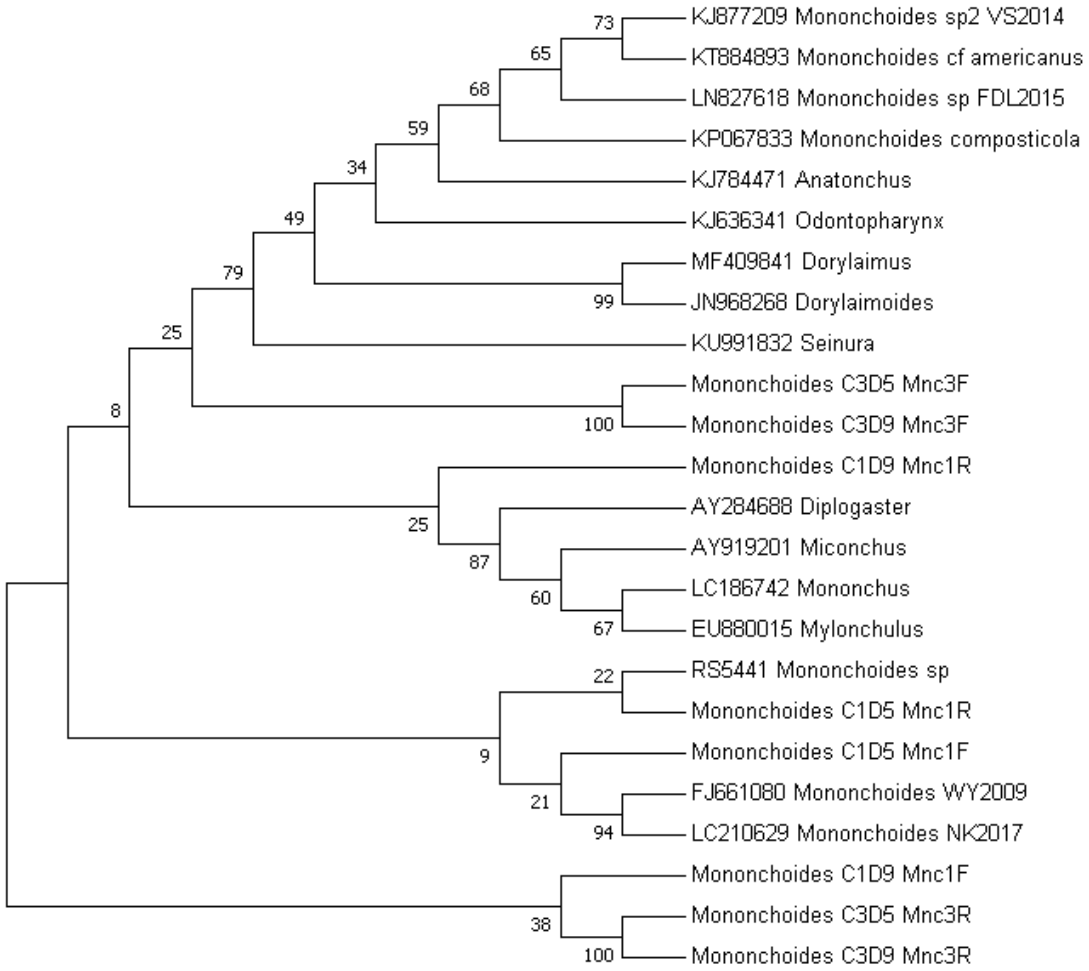

Figure 2 Phylogenetic relationship of *Mononchoides* with most similar sequences of known nematodes inferred from 18s ribosomal DNA gene by using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model. Numbers above the nodes indicate bootstrap values (100 replicates).

4. Conclusion

Predatory nematodes feed on tiny organisms or other nematodes as food, not pests, and are beneficial in maintaining the ecological balance of nature. At present, several researchers have focused on identifying new species of predatory nematodes in different areas worldwide to receive more information on their biology and the possibility of using them to control plant-parasitic nematodes. Unfortunately, the specific surveys of predatory nematodes

have not been performed in Thailand, so the benefits of these nematodes are still unknown.

Concerning the diagnostic of *Mononchoides*, the morphological characters as a dorsal claw-like tooth, longitudinal ridge on the cuticle, gubernaculum, spicule and tail length are commonly used to distinguish from others (Steel *et al*. (2011); Shokoohi *et al*. (2015)). Most of the morphometric values are resemble the report of Steel *et al*. (2011) and Shokoohi *et al*. (2015). However, some features are significantly different as body length and tail length are shorter, which might be due to their habitat and intraspecies variant among populations. Relevant to the genetic diagnosis, this study characterized DNA sequences on ribosomal DNA on *Mononchoides* populations from Thailand. Our results agreed with others reports representing several genetic variations among species and isolates (Troccoli *et al*., 2015). Due to the variations among populations of the beneficial nematodes, additional information for species diagnostic should be valuable. Moreover, the design of specific primers for species identification needs to be improved due to insufficient data available. However, the sequences analysis confirmed that the tested nematodes were *Mononchoides* with 100% similarity with the known sequences (FJ661080, LC210629, KT884893, LN827618, KJ877211, KF151166, KP067833) held online at the GenBank database.

In this present study, *Mononchoides* were successfully extracted and amplified using a polymerase chain reaction by species-specific primers provides a rapid molecular identification approach independent of morphological characteristics. Therefore, this development of *Mononchoides*-specific primers might be helpful for routine identification in nematology laboratories.

5. Acknowledgement

This work was partially supported by the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand" "Center for Advanced Studies for Agriculture and Food, KU Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand" (CASAF, NRU-KU, Thailand).

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