NIJON
Nigerian Society of Nematologists

Nigerian Journal of Nematology
Volume 3, 2016
ISSN: 2545-5362
www.nijon.org
pp 13-19

Morphological and Molecular Characterization of *Pratylenchus coffeae* Isolates from Banana Rhizosphere in Peninsular Malaysia

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Abstract
Pratylenchus coffeae, a root-lesion nematode from *Musa* rhizospheres in Peninsular Malaysia, is described and illustrated. Nematodes were recovered from root and soil using the modified Baermann's method and morphological features were measured with the aid of Dino capture camera with graticle. Extraction of Genomic DNA was conducted using Worm lysis buffer and Polymerase chain reaction with specific primers. The species is characterized by a body length range of 423 - 659 µm (mean: 541µm), body width of 19 - 35 µm. Percentage of shaft in relation to stylet ranged between 6 - 12µm and stylet length from 14 - 25 µm. Lip heights and widths of the population examined ranged from 1 - 3 µm and 6 - 7 µm respectively. Similarly, observations on median bulb heights and widths gave ranges of 1 - 2 µm and 6 - 8 µm respectively. Tail of the Malaysia populations examined ranged from 33 - 51 µm length and 14 - 25 µm width respectively while body diameter at vulva ranged from 19 - 50 µm. The sequence comparison of the ITS rDNA expansion region was in the same clades with the *P. coffeae* isolate from Japan (KR102619).

Keywords: Molecular, Morphological, *Musa*, ITS, rDNA, *Pratylenchus*

INTRODUCTION

Root lesion nematode, *Pratylenchus* spp., causes considerable damage to banana plant (*Musa* spp.) wherever they occur. They are among the most economically damaging plant-parasitic nematodes found on a wide range of crops. *Pratylenchus coffeae* (Zimmermann, 1898; Filipjev and Stekhoven, 1941) and *P. goodeyi* (Sher and Allen, 1953) are both major pests of Musa. They also inflict considerable damage on other economic crops. Plant-parasitic nematodes display slight morphological diversity, which are important characters for species differentiation, the likelihood for mistakes in observations and interpretations, as well as numerous other factors, which made accurate and reliable nematode species identification tasking, even for most-qualified nematode taxonomists (Coomans, 2002). Nematode management strategy is species-specific and thus, the identification of nematode species existing in any particular area is imperative. Accomplishment of any successful nematode management approaches and the prevention of the spread of parasitic nematodes locally and internationally, depend on accurate identification to the species level.

The genus *Pratylenchus* is recognizable easily but similarity in species morphology

makes identification difficult. In the course of surveys this may amount to misidentification of a new or less common species as well as a well-known pest, such as *P. coffeae*. Morphometric characters are commonly used in identification, but deficiency of information on character inconsistency can bias identification. Duncan *et al*. (1999) described that body length in *P. coffeae* not only exhibited considerable variation among populations from sources globally but also seasonal fluctuations associated to food availability and/or host, as well as temporal disparity in average female age. Also, the degree of variability in morphometry may vary between species. The general outcome of several studies on intraspecific variation of morphometric characters, such as number of lip annuli, body length, stylet length, length of posterior uterine sac (PUS) and the deMan ratios, not only shows intraspecific variation but also overlapping between species (Castillo and Vovlas, 2007).

Molecular diagnostic tools in combination with phylogenetic analyses have contributed immensely in overcoming such a problem. The 28S rDNA gene has been utilized quite often to characterize different plant-parasitic nematodes populations and species. DNA-based techniques have been employed in the last years to distinguish among species of *Pratylenchus* (Orui, 1996; Al-Banna *et al*., 1997, 2004; Uehara *et al*., 1998; Duncan *et al*., 1999; Waeyenberge *et al*., 2000; Handoo *et al*., 2001; Inserra *et al*., 2001, 2007; De Luca *et al*., 2004; de la Peña *et al*., 2006, 2007; Subbotin *et al*., 2008). Many of these findings also harnessed sequence variation to construct phylogenies. The purpose of this investigation was to characterize a new isolate of *Pratylenchus coffeae* from Malaysia, using morphometric and molecular tools.

MATERIALS AND METHODS

Morphological characterization

Nematodes were individually picked, killed and mounted temporally on slides and observed under the compound light microscope. Characters such as overall body length, body width, stylet length, percent (%) of shaft to stylet, lip height and width, length and width of median bob, diameter of body at vulva, spickle and gubernaculum length, length and width of tail were measured with the aid of Dino lite capture camera, according to synoptic key of Orton-Williams and Siddiqi (1973).

Molecular characterization

Three *Pratylenchus* specimens from banana (*Musa paradisiaca* L.) at Peninsular States, Malaysia, were included for amplification and sequencing of small subunit (18S) rDNA. Extraction of Genomic DNA was done according to Williams *et al*. (1994) using Worm lysis buffer. Individual nematode was picked, washed in 0.1 mol NaOCI solution then rinsed in distilled water and put in 0.5 ml PCR tube containing 15 µl lysis buffer. The samples were placed in -80ºC for 10 minutes. Samples were warmed at room temperature, and thereafter mineral oil added. It was then incubated for 1 hour at 60ºC after which it was heated at 95ºC for 15 minutes, cooled at 4ºC. A measure of 2.5 µl was used as template for PCR amplification. TW81_ GTT TCC GTA GGT GAA CCT GC as forward primer and AB28_ ATA TGC TTA AGT TCA GCG GGT as reverse prime were used at 1.5 µl in 25 µl reaction volume. The amplified DNA was run in agarose gel electrophoresis using 1 kb ladder and visualized using gel doc XR system (Bio-Rad, UK).

Purified DNA containing ITS gene was then sent for sequencing to determine their nucleotides arrangement. Sequencing service was provided by a company, First Based SdnBhd, Malaysia. The purified plasmids were sequenced using the automated DNA sequencer (ABI PRISM®). Results of DNA sequences were analyzed by bioinformatics software **Genostar**. The ITS gene sequences from respective samples were aligned using BioEdit software version 7 (Hall, 2005) The partial length nucleotides sequences of the

Pratylenchus isolates were searched for sequences similarities to other sequences which are available in the NCBI database by using Basic Local Alignment Search Tool (BLAST) algorithm (NCBI, 2017).

RESULTS

Morphological identification of *Pratylenchus coffeae*

Female body rather slender in young females and thicker in older ones. Body width increases from the level of the median pharyngeal bulb towards the vulva and decreases from the level of the vulva towards the tail tip. Cubicula annulation fairly conspicuous. Stylet strong, basal knobs of stylet are round to oblong. Median pharyngeal bulb broadly oval to nearly round (Figure 1b). Glandular lobe of pharyngeal glands overlapping anterior end of the intestine ventrally and slightly. An obliquely truncated tail tip shape was observed with the highest frequency in most of the populations ((Figure 1a).

The morphological characteristic of the *Pratylenchus* females populations collected in Peninsular Malaysia are in Table 1. The body length of the Malaysia females ranged from 423 to 659 µm (mean: 541µm). The females population from Johore had the shortest mean body length (423 µm), while the population from Selangor had the highest mean body length (659µm). The mean body width of the Malaysia females ranged from 19 to 35 µm with female population from Pahang having shorter body length and Selangor having the longest. Percentage of shaft in relation to stylet smaller in Pahang and the highest recorded in Selangor, with the range from 6 to 12 µm. Stylet length ranged from14 to 25 µm with Johor population having shorter stylet compared to the Selangor

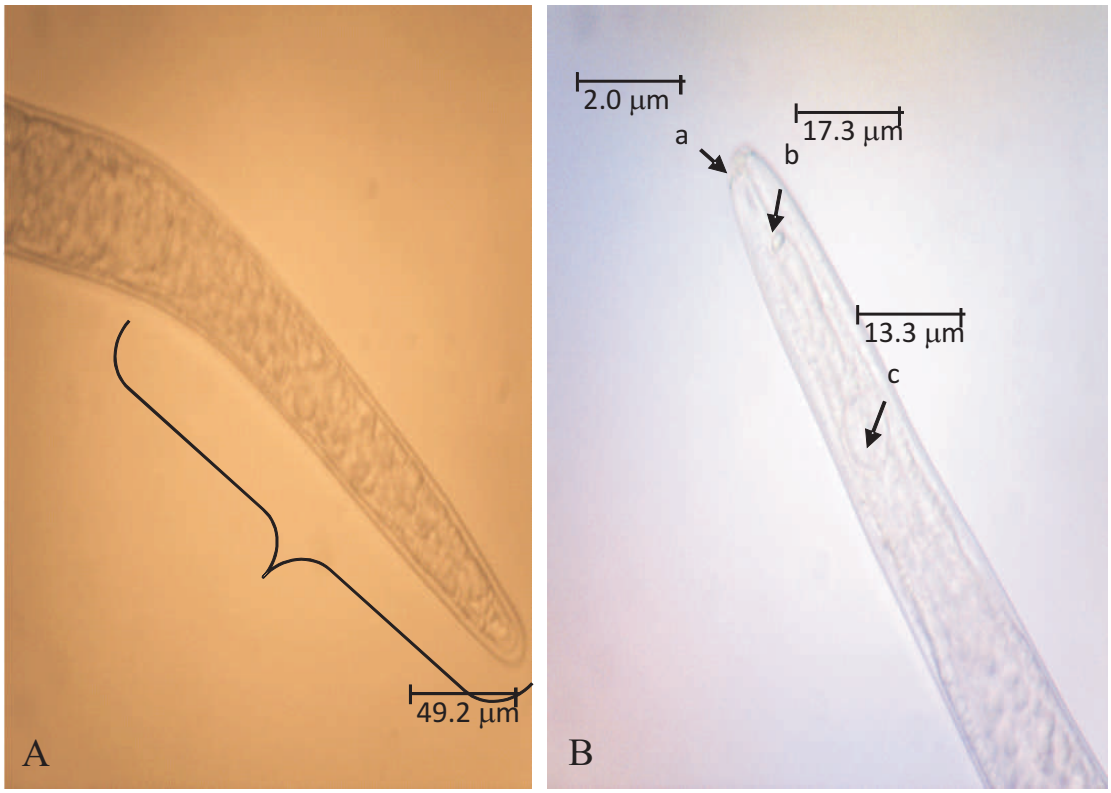

Figure 1: Morpholological features of *Pratylenchus coffeae.* (A) Tail, (B) (a-lip region, b-stylet and c-median bulb.

Table 1: Morphometrics (µm) of the females of the *Pratylenchus coffeae* populations collected in Peninsular Malaysia (n = 7).

	L	W	%SHFT	STYLET	LH	LW	MBH	MBW	TL	TW	DV
JOHOR	467.64±20.9	21.76±0.3	7.20±0.4	15.5±0.5	1.6±0.1	6.5±0.1	20.8±2.1	9.8±0.1	49.2±0.3	14.9±0.1	21.6±0.2
	(423.2-516.6)	(20.9-22.6)	(6.3-8.2)	(14.6-16.8)	(1.4-1.8)	(6.3-6.7)	(18.1-22.4)	(9.5-10.0)	(48.4-50.0)	(14.75.2)	(20.9-21.8)
PERAK	548.68±9.3	24.9±0.9	7.5±0.6	17.6±0.1	2.4±0.2	8.2±0.1	13.3±0.2	10.9±0.1	45.9±2.3	21.1±1.1	23.1±1.7
	(525.7-580.7)	(23.2-28.0)	5.7-8.6	(17.3-17.9)	(1.9-3.2)	(7.9-8.4)	(12.9-13.7)	(10.611.3)	44.8-51.2	18.9-25.1	19.2-28.1
SELANGOR	580.6±79.3	30.3±4.8	12.0±0.8	19.0±6.1	2.5±0.5	7.1±0.1	21.6±2.3	8.0±0.7	51.3±3.36	17.3±1.1	34.2±4.9
	(501.2-659.9)	(25.5-35.1)	(11.3-12.8)	(12.9-25.1)	(2.0-2.9)	(6.9-7.3)	(19.3-23.9)	(8.0-9.3)	(42.7-50.1)	16.2-17.9	20.2-50.
PAHANG	481.9±7.6	19.8±0.7	6.9±0.3	15.2±0.2	2.0±0.2	7.4±0,5	11.4±0.4	10.3±0.4	39.2±2.8	22.1±1.2	20.8±3.5
	(472-492.7)	(19.2-21.3)	(6.5-7.4)	(14.9-15.8)	(1.5-2.3)	(7.2-7.9)	(11.0-13.3)	(9.8-11.2)	(33.2-45.7)	19.3-22.4	20.2-23.6

Vales are Mean ± standard error (range); L: Body length, W: Body width, SHFT: % Shaft from stylet, LH: Lip height, LW: Lip width, MBH: Medium bulb height, MBW: Median bulb width, TL: Tail length, TW: Tail width, DV: Diameter of body at vulva

population. Lip heights and widths of the population examined have ranges of (1 to 3 µm) lip height and (6 to7 µm) lip width. Similarly, observations on median bulb height and widths gave ranges of 1 to 2 µm and 6 to 8 µm respectively. Tail of the Malaysia female populations examined ranged from 33 to 51 µm length and 14 to 25 µm, respectively. Diameter of body at vulva ranged 19 to 50 µm.

Molecular identification of *Pratylenchus*

Three representative samples were sequenced in both orientations using TW81 forward and AB28 reverse primers. These sequences were further aligned and edited using BioEdit program which resulted to sequences of about 1000 bp of nucleotides (Figure 2). Search for sequence similarity and confirmation by using BLAST program (NCBI, 2017) suggested and confirmed that these 1000 nucleotide sequences are the partial sequence of the ITS gene of the *Pratylenthus coffeae.* Nucleotide sequences similarity of the ITS gene of local isolates with the reference isolates of *Pratylenchus coffeae* (LC030392) was 95 and 98 % respectively. The phylogenetic analysis of the isolates 18s rDNA gene grouped all the isolates in the species *Pratylenthus coffeae*.

Three isolates grouped together with *Pratylenthus coffeae* from Japan and formed the major clade of specie (clade 1). Clade 2-4 contained isolates with species similar or closely related to *Pratylenthus coffeae*. The molecular phylogenetic analysis confirmed the isolates to be *Pratylenthus coffeae* isolate with scores of 95% to 98% in similarity. The phylogenic analysis and accession numbers shown in Figure 3 and Table 2.

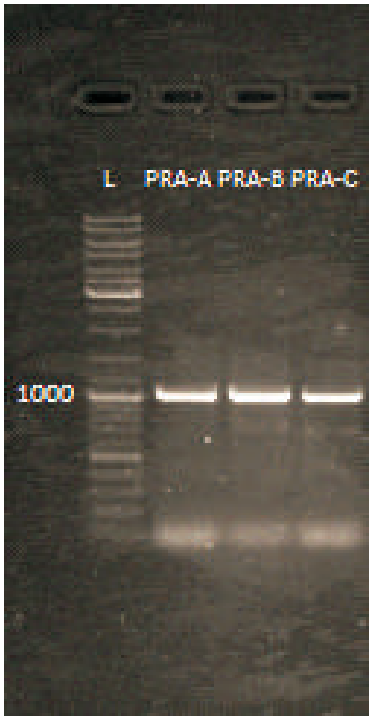

Figure 2: Three representatives isolates of *Pratylenchus coffeae*

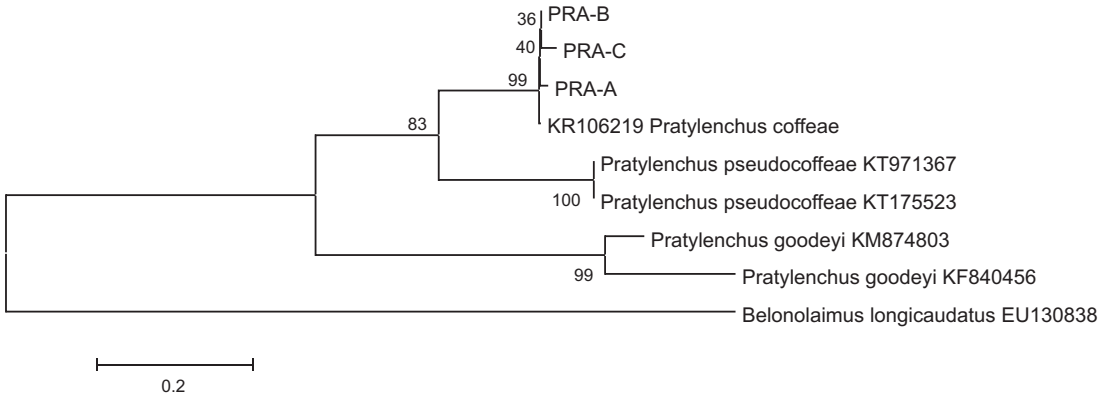

Figure 3: Neighbour-Joining tree of three representative isolates of *P. coffeae* (PRA-A, B and C) populations from Malaysia with their corresponding similar isolates obtained from the Genbank. A bootstrap value > 50% is given in the appropriate clade.

Table 2: Accession Numbers of three representative isolates of *Pratylenchus coffeae* (PRA-A, B and C) with their corresponding matches from the Genbank

Isolate Code	Accession number obtained	Corresponding accession number from Genbank
PRA- A	KX011054	JN809840
PRA-B	KX011055	JN809836
PRA-C	KX011056	KR106213

Figure 3: Neighbour-Joining tree of three representative isolates of *P. coffeae* (PRA-A, B and C) populations from Malaysia with their corresponding similar isolates obtained from the Genbank. A bootstrap value > 50% is given in the appropriate clade.

DISCUSSION

Root-lesion nematodes are considered to be amongst the most significant and damaging migratory endoparasites. Nematode management strategy is species-specific and thus, the identification of nematode species existing in any particular area is imperative. Accomplishment of any successful nematode management approaches and the prevention of the spread of parasitic nematodes locally and internationally, depend on accurate identification to the species level. Our studies clearly revealed that morphological variations among some species of *Pratylenchus*, are often unclear and relying on such characters only runs the risk of wrong identification. Several authors have demonstrated the diagnostic difficulties for discrimination of *Pratylenchus* species (Castillo and Vovlas, 2007; Inserra *et al*., 2007; De Luca *et al*., 2010). In this study, we characterized *P. coffeae* isolates from banana rhizhosphere. Our results when compared to the findings of researchers who worked on *P. coffeae* in other regions of the world showed slight variations. Castillo and Vovlas (2007) reported the works of Sher and Allen (1953), Bajaj and Bhatti (1984), Ryss (1988) and Inserra *et al*. (2001) on *P. coffeae*a showing higher body length of 590, 620, 620, and 600 µm, respectively. While shorter length of 530 and 510 µm were reported by Loof (1960) and Mizukubo (1992). However, *P. coffeae* female populations from Malaysia

examined showed average length of the range 467-580 µm. Similarly, stylet length of the present study ranged from 15-19 µm, which is within the range of earlier works reported. But the position of the vulva was about 50 µm, which is slightly lower than reported elsewhere in the world. Duncan *et al*. (1999) observed that variations in morphometry of *P. coffeae* not only revealed significant difference among populations from sources globally but also seasonal variations associated to presence of host and/or food availability, as well as temporal disparity in average female age. Thus, the degree of variability in morphometry may differ between species. The general outcome of several studies on intraspecific variation of morphometric characters, such as number of lip, body length, stylet length, length of posterior uterine sac (PUS) and the deMan ratios, not only show intraspecific variation but also overlapping between species (Castillo and Vovlas, 2007). The sequence similarity and its confirmation using the BLAST program (NCBI, 2017) suggested and confirmed that the 1000 nucleotide sequences obtained were the partial sequence of the *P. coffeae* ITS gene. Nucleotide sequences similarity of the ITS gene of local isolates with the reference isolate of *P. coffeae* (LC030392) had a similarity level of 98%. The findings were in agreement with those of earlier research findings, though there were minor variations, which were largely attributed to climatic and soil factors.

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NIJON Nigerian Society of Nematologists
Nigerian Journal of Nematology
Volume 3, 2016
ISSN: 2545-5362
www.nijon.org
pp 13-19

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