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Research Article

Morphological and Molecular Characteristics of *Pratylenchus coffeae* from the Origin of Robusta Coffee Plantation in Malang, East Java

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ABSTRACT

Pratylenchus coffeae is the most important plant-parasitic nematode in Robusta coffee plantations. Information regarding morphology, morphometric and molecular characters of *P. coffeae* has not been reported in Indonesia. This study is aimed to describe those characters of *P. coffeae* that attack Robusta coffee. Root samples were taken from Robusta coffee plantation in Malang, East Java. Nematode extractions was conducted using a mist chamber method. Morphology and morphometric characters were observed from the permanent nematode slides. Single nematode DNA extract was amplified at the D2D3 segment of 28S rRNA and ITS1–5.8S–ITS2 rRNA with universal primers. Amplicon was sequenced and analysed for phylogenetic tree relationships. Female morphological key character of *P. coffeae* observed are: lip with two annulations, four lateral lines, esophageal overlap with intestine ventrally, monodelphic, and truncated tail shape. Male spicules curved ventrally. Female morphometrics are: n=26, L = 556.4 µm, DGO = 2.4 µm, anterior gonad = 174.8 µm, a = 28.5, b = 6.1, b' = 4.1, c = 20.1, c' = 2.3, V = 81.7. A Male is smaller than a female with n=24, L = 505.9 µm, a = 32.3, b = 5.5, b '= 3.9, c = 15.3, c' = 2.8 and T = 40.6. The molecular characters of *P. coffeae* were investigated for two isolates, namely SA1 and SA2. Based on the D2D3 and ITS1-5.8S-ITS2 regions, isolate SA1 has similarity level of 99% and 97% to the *P. coffeae* from NCBI. Similar result was shown by Isolate SA2 with similarity of 100% and 100% respectively. Phylogenetic tree analysis using Maximum Likelihood at the D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2 regions showed that *P. coffeae* in this study was included in one clade with *P. coffeae* from several countries.

Keywords: morphometric, phylogenetic tree, rRNA

INTRODUCTION

Pratylenchus coffeae is the most important plant-parasitic nematode in Robusta coffee plantations in Indonesia. In estimation, this nematode could cause 78% losses in the Robusta plantation with an average of 57% (Wiryadiputra, 1995). The first morphology and morphometric identification of *P. coffeae* were reported by Bally & Reydon (1931) as *Tylenchus coffeae* Zimmerman 1898. It is hard to determine *Pratylenchus* species based only on morphological and morphometric characters (Castillo & Vovlas, 2007). A recent report has successfully identified *P. coffeae* in peanuts in Fujian, China, based on its morphology, morphometry, and molecular characteristics (Liao *et al.*, 2015). Therefore, the identification of *Pratylenchus* species with those approaches needs to be done to ensure the species of *Pratylenchus* in coffee plantations in Malang, East Java.

Molecular identification of nematodes requires attention to the specific location of the gene target for amplification. The amplify location selected based on the speed of evolution of the gene target location. The ribosomal region of RNA (rRNA) that comprises 18S, ITS1, 5.8S, ITS2, and 28S are widely used for identification and molecular nematodes biodiversity analysis because it is fast in detecting the evolutionary process for diversity among genera, species, and sub-species (Blaxter, 2001). Molecular identification of *Pratylenchus* spp. with the amplification target D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2 has been widely reported (Nguyen *et al.*, 2014; Liao *et al.*, 2015). Therefore the same approach is used in this study for molecular identification.

The aim of this study is to identify *Pratylenchus* species that attack Robusta coffee in Malang, East

Java by observing the morphological, morphometric, molecular, and phylogenic relationships of the phylogenic tree compared to *Pratylenchus* isolates from other countries.

MATERIAL AND METHOD

Sample Collection

Root samples were taken from the Robusta coffee plants attacked by phytonematodes in the Sumber Asin Experimental Station, Malang, East Java in July 2018. Root samples were taken from two blocks, that are Sumber Asin 1 (SA1) at coordinates 8°16.199'S 112°42.608'E, altitude 607 m as l, and Sumber Asin 2 (SA2) coordinates 8°16.712'S 112°42.671 'E, altitude 598 m asl.

Nematode Extraction from the Root and Preparation of Permanent Mound Slides

The nematode was extracted from infected root samples by a mist chamber method modified from EPPO (2013). Modifications were conducted to the weight of each sample (10 g) and incubation duration for 4 days. The target nematode obtained was prepared as permanent mount following Ryss (2017) method. A total of 1 ml of nematode suspension was poured into a 1.5 ml collection tube then incubated for 20 minutes. The suspension volume was reduced slowly until 60 µl. FA 4:1 solution (4% formalin and 1% acetic acid) at 90°C was poured into the nematode suspension until 1 ml. The suspension was incubated using a water bath at 85°C for 1 hour for fixation then incubated at room temperature for 24 hours. FA 4:1 solution suspension was diluted with distilled water for three times. From each dilution was taken 800 µl FA 4:1 and then added 800 µl of distilled water carefully so that the nematodes were not wasted. The nematode suspension was left as 150 µl at the end of the dilution. Two drops of glycerol were dripped and spread evenly on an object glass that has a 1 cm diameter paraffin ring followed by 3 drops of distilled water on it. 150 µl nematode suspension was dripped slowly over the glycerol and distilled water layers to form a mound of water and then incubated for 12 hours. Nematodes that already contain glycerol were picked and transferred to another object-glass with 20 µl glycerol on it. The mounting slide was covered with cover glass and glued with transparent nail polish. Nematode permanent mount slide was labeled and ready to be observed.

Morphology and Morphometric Characterization

Nematode permanent mount slide was observed using the Olympus BX 51 binocular light microscope with 2 MP Indomicro HDMI Camera CCD camera. Morphometric photographs and measurements were done by using ToupView®. Morphometric characterization is based on the de Man formula (Siddiqi, 2000; Nguyen, 2010). Morphometric characteristics measured were: L = body length; widest body diameter; a = body length/widest body diameter; b = body length/distance from the anterior end to the pharyngo–intestinal junction; b' = body length/distance from the anterior end to the posterior end of phyaryngeal gland; c = body length/tail length; c' = tail length/body width in the anus (female) or cloaca (male); distance from anterior to vulva; V = distance of vulva from anterior end x 100/total body length (%); stylet length; lip width; lip height; DGO = distance from the base of the stylet knob to the dorsal pharyngeal gland orifice; tail length; anterior length of female gonads; the distance from the anterior to the middle of the metacorpus; MB = distance from the anterior end to the median bulb; distance from anterior to pharyngo-intestinal junction; pharygeal overlap; distance from anterior to the base of the esophageal gland; post-vulval uterine sac; Body width in the anus (female) or cloaca (male); vulva to anus; o = DGO*100/stylet; spicula length; testis; and T = distance from the cloaca to the anterior part of the testis x 100/body length (%).

Molecular Characterization and Phylogenetic Analysis

DNA extraction. The nematode DNA extraction was using Holterman *et al*. (2006) method. A morphologically confirmed *P. coffeae* was transferred into a 0.2 ml PCR collection tube containing 25 µl of nuclease-free water. A total of 25 µl of the Holterman Worm Lysis Buffer solution containing 200 mM NaCl, 200 mM Tris-HCL pH 8, 1% 2-mercaptoethanol, and 800 µg/ml Proteinase K were added into a 0.2 ml PCR collection tube with *P. coffeae* in it. The mixture was homogenized by vortex machine for 1 minute and then incubated using a PCR machine (GeneAmp® PCR System 9700 from Applied Biosystems) with a temperature setting of 65°C for 90 minutes and 99°C for 5 minutes. Nematode DNA was ready to be amplified or stored at -20°C.

***Target DNA amplification*.** A total of two target DNA regions (LSU D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2) were amplified separately. A mixture for amplification PCR with a composition of 2 µl *P. coffeae* DNA template, 12.5 µl 2x Go Taq® Green Master mix (Promega), 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, and 8.5 µl nuclease-free water were put into a 200 µl microtube for each target DNA region. Primers used in the LSU D2D3 segment of 28S rRNA was forward D2A '5-ACAAGTACCGTGAGGGAAAGTTG-3' and reverse D3B '5-TCGGAAGGAACCAGCTACTA-3' (De Ley *et al.*, 1999) while the ITS1-5.8S-ITS2 rRNA region was forward TW81 '5-GTTTCCGTA GGTGAACCTGC-3' and reverse AB28 '5-ATAT GCTTAAGTTCAGCGGGT–3' (Kaplan *et al.*, 2000). The PCR machine used was the GeneAmp® PCR System 9700 brand from Applied Biosystems. Each DNA target region has the same PCR cycle and annealing temperature and was amplified separately. PCR cycles for each primer pair consisted of 40 cycles. Each cycle consisted of denaturation (94°C, 60 seconds), annealing (55°C, 60 seconds), and extension (72°C, 120 seconds). The cycle began with initial denaturation of 94ºC for 4 minutes and ended with 72ºC of the final extension for 10 minutes. The amplicon was electrophoresis with 1% agarose gel and visualized by UV transilluminator.

***Analysis of nucleotide sequences and phylogenetic tree construction*.** PCR products were analyzed for their nucleotide sequences by Sanger dideoxy sequencing method. The DNA Sequence Assembler v4.7 trial version was used for contiq. In the contiq process, the ambiguous sequence was also cut. Nucleotide sequences were aligned with BLAST® from the National Center for Biotechnology Information (NCBI). The nucleotides were aligned with reference sequences from NCBI using Bioedit 7.2.6.1. version. MEGA 6.06 was used for phylogenetic tree analysis using the Maximum Likelihood method, bootstrapping 1000 times, and using the model with the lowest BIC (Bayesian Information Criterion) value on the model test results.

RESULT AND DISCUSSION

P. coffeae isolates from Robusta coffee plantations in Malang, East Java were characterized with polyphasic identification. Polyphasic characterization is a combination of phenotype identification method which are morphology and morphometry measurement with genotype identification which is molecular characteristics (Subbotin & Moens, 2006). Therefore, this study explains the morphological, morphometric, and molecular characteristics of *P. coffeae*.

P. coffeae *Morphological Characteristics*

***Female*.** Female has clearly visible stylet with a rounded basal knob. Lips have two thin annulations and slight setoff from the body. Four lateral lines are parallel to form three parallel chords. Metacorpus is oval and clearly visible. The esophageal gland overlap with intestine ventrally, which end in pharyngo-intestinal junction. One ovary leads to anterior (monodelphic). Vulva lips are well-formed and clear with post-vulval uterine sac (PUS) underneath. The large and oval–shaped spermatheca is clearly visible. Truncate–shaped tail tip. (Figure 1). These characteristics are similar to the key characters of *P. coffeae* as reported by Castillo & Vovlas (2007) and Nguyen (2010).

***Male*.** The body is generally straight after obtained permanent fixation. The body shape is generally the same as females except in sexual dimorphism (Nguyen, 2010). Body size is slightly smaller than females. Opportunities to be found for males are almost the same as females. The stylet knob is slightly smaller than the females. Male spicules curved ventrally. The tip of the tail is pointed with a bursa that covers the spicula in a slightly crenate shape (Figure 1). These characters are in accordance with those reported by Castillo & Vovlas (2007).

P. coffeae *Morphometric Characteristics*

The morphometry of *P. coffeae* from Malang, East Java which comprises of 26 females and 24 males has many similarities with *P. coffeae* that reported by Inserra *et al.* (2001) and Nguyen (2010) (Table 1). Measurement of female morphometry was obtained (µm): body length (L) = 556.4 ± 47.2 (487.4-654.4), widest body diameter = 19.6 ± 2.2 (15.8-24.8), distance from anterior to vulva = 454.5 ± 37.8 (392.9-528.6), stylet length = 16 ± 0.6 (14.6-16.7), lip width = 7.6 ± 0.4 (7–8.2), lip height = 2.4 ± 0.3 (2–3), DGO = 2.4 ± 0.4 (1.8–3.2), length tail = 27.9 ± 3.5 (21.1–34.4), anterior length of female gonads = 174.8 ± 24.5 (133–224), distance from anterior to middle metacorpus = 54.1 ± 3.5 (45.2–58.6), MB = 40.1 ± 2.5 (35.1–44.8), distance from anterior to pharyngo-intestinal junction = 92.1 ± 8

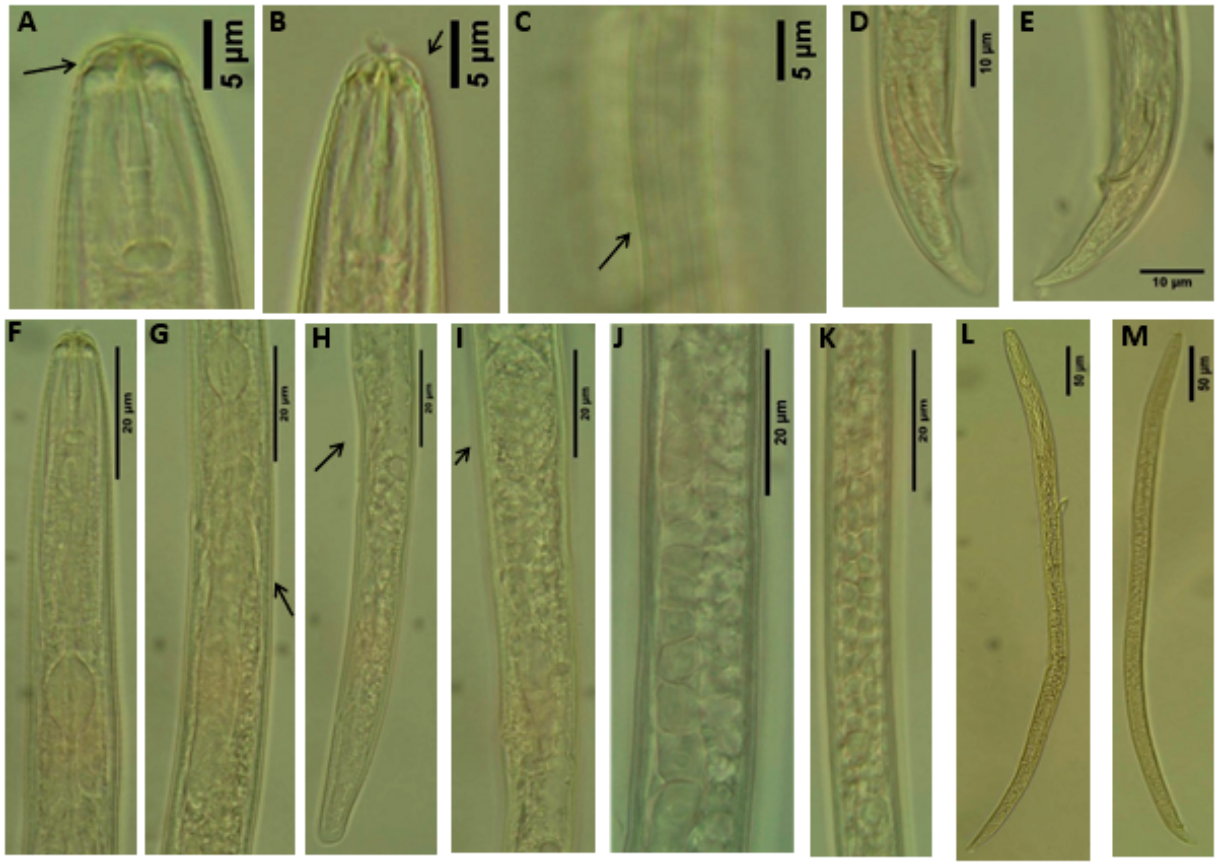

Figure 1. Photo of *Pratylenchus coffeae* isolates from Malang, East Java which was taken with the binocular light microscope; (A) two annulations on the female lips, (B) male lips, (C) lateral field of middle part of female body, (D–E) male spicule and tail, (F) anterior part of female, (G) pharyngo-intestinal junction, (H) post-vulval uterine sac in females, (I) female spermatheca, (J) female anterior genital branch, (K) male anterior genital branch, (L) female body, (M) male body

(77.4–106.7), pharygeal overlap = 43.4 ± 8.1 (26.4–57.7), distance from anterior to the base of the esophageal gland = 135.2 ± 10.1 (117–159.4), PUS = 25.6 ± 5.9 (13.3–37.9), body width at anus = 12.5 ± 1.7 (10.5–16) and the distance of the vulva to anus = 71.9 ± 11 (55.6–97.8). Measurement ratio values in females were obtained: a = 28.5 ± 3 (23.4–34.2), b = 6.1 ± 0.6 (4.8–7.8), b '= 4.1 ± 0.4 (3.3–5.2), c = 20.1 ± 2.4 (15–24.1), c '= 2.3 ± 0.3 (1.6–3), o = 15 ± 5.5 (11.2–19.1), V = 81.7 ± 1.2% (79.5–83.9). Measurement of male morphometry characteristics (µm): body length (L) = 505.9 ± 39.5 (441.6–641), widest body diameter = 15.7 ± 0.9 (14.4–18.4), stylet length = 15 ± 0.4 (14.3–15.7), lip width = 6.3 ± 0.3 (5.9–6.9), lip height = 2 ± 0.2 (1.7–2.7), DGO = 2.3 ± 0.3 (1.9–2.8), tail length = 33.9 ± 5.3 (24.8–41), distance from anterior to the middle of metacorpus = 51.7 ± 2.3 (47.3–56.8), MB = 39.4 ± 2.2 (36.3–45.5), distance from anterior to pharyngo-intestinal junction = 91.1 ± 5.7 (79.3–107.3), distance from anterior to the base of the esophagus gland = 131.4 ± 7.8 (114.2–145.6), body width in the cloaca = 12 ± 1.1 (10–14.6), spicula length = 17.4 ± 1.3 (15–19.4), and testis = 205.2 ± 26.3 (163.9–263.2). The measurement ratio values in males were: a = 32.3 ± 2.5 (27.4–39.4), b = 5.5 ± 0.4 (4.8–5.5), b' = 3.9 ± 0.3 (3.4–4.8), c = 15.3 ± 2.9 (12.4–21.6), c' = 2.8 ± 0.4 (2.1–3.5), T = 40.6 ± 4.4% (32.4–52.9).

The morphological characteristics of *P. coffeae* population from Malang has similarities with those reported by Castillo & Vovlas (2007) and Nguyen (2010). The distinctive character of *P. coffeae* by fulfilling PUS is 1–1.5 times body width, which is 25.6 ± 5.9 µm with a body width 19.6 ± 2.2 µm. Lips with two annulation, rounded basal knob stylet, four lines lateral fields forming 3 chords, V values close to 80%, and esophagus overlapping ventrally which are the characteristic of *P. coffeae* (Castillo

Table 1. *Pratylenchus coffeae* morphometry population from Malang compared to population described by Inserra *et al.* (2001) and Nguyen (2010)

Population	In this study		Inserra *et al.* (2001)		Nguyen (2010)	
Host	Robusta Coffee		Coffee		Coffee	
Sex	Female	Male	Female	Male	Female	Male
Species	*P. coffeae*	*P. coffeae*	*P. coffeae*	*P. coffeae*	*P. coffeae*	*P. coffeae*
Sample code	SA	SA	-	-	CH	CH
N	26	24	20	20	15	15
L*	556.4 ± 47.2 (487.4-654.4)	505.9 ± 39.5 (441.6-641)	(520.0-715.0)	(558.5-647.5)	(508-647)	(482.0-673.0)
widest body diameter*	19.6 ± 2.2 (15.8-24.8)	15.7 ± 0.9 (14.4-18.4)	(7.5-25.5)	(18.0-20.5)	(17.6-22.6)	(13.9-20.8)
a	28.5 ± 3 (23.4-34.2)	32.3 ± 2.5 (27.4-39.4)	(23.4-34.0)	(28.7-33.9)	(23.4-35.6)	(26.1-37.5)
b	6.1 ± 0.6 (4.8-7.8)	5.5 ± 0.4 (4.8-5.5)	(5.6-7.2)	(6.0-7.3)	(5.5-7.8)	(6.3-7.1)
b'	4.1 ± 0.4 (3.3-5.2)	3.9 ± 0.3 (3.4-4.8)	-		(3.8-5.2)	(3.9-5.1)
c	20.1 ± 2.4 (15-24.1)	15.3 ± 2.9 (12.4-21.6)	(17.0-31.0)	(19.4-25.4)	(13.1-22.4)	(16.3-21.0)
c'	2.3 ± 0.3 (1.6-3)	2.8 ± 0.4 (2.1-3.5)	-	-	(1.7-2.8)	(2.2-3.1)
distance from anterior to vulva*	454.5 ± 37.8 (392.9-528.6)	-	-	-	-	-
V	81.7 ± 1.2 (79.5-83.9)	-	(76.0-82.5)	-	(78.0-89.6)	-
stylet length*	16 ± 0.6 (14.6-16.7)	15 ± 0.4 (14.3-15.7)	(16.5-17.0)	(14.5-15.5)	(13.8-16.5)	(13.5-15.8)
lip width*	7.6 ± 0.4 (7-8.2)	6.3 ± 0.3 (5.9-6.9)	-	-	(7.3-8.9)	(6.1-8.2)
lip height*	2.4 ± 0.3 (2-3)	2 ± 0.2 (1.7-2.7)	-	-	(1.9-2.9)	(1.3-2.0)
DGO*	2.4 ± 0.4 (1.8-3.2)	2.3 ± 0.3 (1.9-2.8)	(2.5-4.0)	(3.0-4.0)	(2.6-4.2)	(1.9-3.2)
tail length*	27.9 ± 3.5 (21.1-34.4)	33.9 ± 5.3 (24.8-41)	(21.5-36.0)	-	(23.7-38.7)	(23.9-33.4)
female gonad anterior length*	174.8 ± 24.5 (133-224)	-	-	-	(129.7-249.5)	-
distance from anterior to the middle of metacorpus*	54.1 ± 3.5 (45.2-58.6)	51.7 ± 2.3 (47.3-56.8)	-	-	-	-
MB	40.1 ± 2.5 (35.1-44.8)	39.4 ± 2.2 (36.3-45.5)	-	-	-	-
distance from anterior to pharyngo-intestinal junction*	92.1 ± 8 (77.4-106.7)	91.1 ± 5.7 (79.3-107.3)	-	-	-	-
pharyngeal overlap*	43.4 ± 8.1 (26.4-57.7)	-	(34.0-72.5)	-	(29.5-58.7)	-
Distance from anterior to the base of esophagus gland*	135.2 ± 10.1 (117-159.4)	131.4 ± 7.8 (114.2-145.6)	-	-	-	-
post-vulval uterine sac (PUS)*	25.6 ± 5.9 (13.3-37.9)	-	-	-	(24.2-37.9)	-
Body width at anus (female) or cloaca (male) *	12.5 ± 1.7 (10.5-16)	12 ± 1.1 (10-14.6)	(11.5-15.0)	-	-	-
vulva to anus*	71.9 ± 11 (55.6-97.8)	-	70.5-135.0	-	(75.6-105.8)	-
o	15 ± 5.5 (11.2-19.1)	-	-	-	-	-
spicule length*	-	17.4 ± 1.3 (15-19.4)	-	(16.0-18.0)	-	(16.1 - 18.6)
testis*	-	205.2±26.3 (163.9-263.2)	-	(181.0-311.0)	-	(192-288)
T	-	40.6±4.4 (32.4-52.9)	-	-	-	(36.4-52.6)

Remarks: *= in µm. Data are shown as average ± standard deviation (minimum-maximum)

& Vovlas, 2007). Lira *et al.* (2014) reported that stylet length from the Brazilian population ranges from 15.8–20.2 µm which has a wider range than that of in this study. Geographical factors and nutrients uptake may create variations in intra-specific morphometry (Castillo & Vovlas, 2007).

Molecular Characteristics of P. coffeae

Molecular characteristics of *P. coffeae* were tested on two isolates, SA1 and SA2. D2D3 segment of 28S rRNA length *P. coffeae* comprises of 759 base pairs (bp) in both isolates, which is similar as reported by Nguyen *et al.* (2014) which is 759 bp on *P. coffeae*. The nucleotide length of the ITS1-5.8S-ITS2 *P. coffeae* region is 952 bp and 941 bp in SA1 and SA2 isolates, respectively. SA1 isolate has 99% and 97% similarities while SA2 100% and 100% at the amplification D2D3 segments of 28S rRNA (Table 2) and ITS1–5.8S–ITS2 (Table 3) are sequential to *P. coffeae* from NCBI. Analysis of the Maximum Likelihood phylogeny tree in the D2D3 segment of 28S rRNA model HKY + G and ITS1-5.8S-ITS2 models K2 + G showed *P. coffeae* in this study included in one clade with *P. coffeae* from several countries. Based on the D2D3 segments of 28S rRNA phylogeny tree, *P. coffeae* SA1 and SA2 belong to a clade with *P. coffeae* from China, Japan, Vietnam and Malaysia with 99% bootstrap value. Phylogeny trees in ITS1-5.8S-ITS2 area of *P. coffeae* SA1 and SA2 are in the same clade as *P. coffeae* from China, Taiwan, Haiti and Vietnam (Figure 2).

Some or all analysis of the D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2 have been used to separate *Pratylenchus* species (Palomares-Rius *et al.*, 2014; Araya *et al.*, 2016), while for *P. coffeae* species have been reported by Nguyen *et al.* (2014) in the D2D3 segment of 28S rRNA region and Liao *et al.* (2015) in the ITS1-5.8S-ITS2 region. Molecular identification using two different amplification regions will ensure the accuracy of *P. coffeae* identification. A study on all these regions showed that with traceability data in NCBI this is identical to *P. coffeae*. *P. coffeae* characterization with a polyphasic approach examining morphological, morphometric and molecular variables is the first report in Indonesia.

Table 2. Sequence homology of LSU D2D3 segment of 28S rRNA *Pratylenchus coffeae* from Robusta coffee compared with NCBI data

This study		*P. coffeae* from NCBI					
Isolate	Nc	Accession number	Species	Isolate	Qc %	E-value	Identity %
SA1	759	MG906762.1	*P. coffeae*	HN2.2	100%	0	99%
		MG906763.1	*P. coffeae*	HN3.1	99%	0	99%
		KY424279.1	*P. coffeae*	NSEG	99%	0	99%
SA2	759	MG906763.1	*P. coffeae*	HN3.1	100%	0	100%
		KY424279.1	*P. coffeae*	NSEG	100%	0	100%
		KY424276.1	*P. coffeae*	JS43	100%	0	100%

Remarks: Nc =nucleotide, Qc= *Query cover*

Table 3. Homology of regional sequences ITS1-5.8S-ITS2 *Pratylenchus coffeae* from Robusta coffee compared with NCBI data

This study		*P. coffeae* from *NCBI*					
Isolate	Nc	Accession number	Species	Isolate	Qc %	E-value	Identity %
SA1	952	FJ827744.1	*P. coffeae*	Pcof7	99%	0	97%
		JN809834.1	*P. coffeae*	PcHTI1KL3	99%	0	97%
		HQ668117.1	*P. coffeae*	VtN clone 1	99%	0	97%
SA2	941	HQ668117.1	*P. coffeae*	VtN clone 1	100%	0	100%
		JN809834.1	*P. coffeae*	PcHTI1KL3	100%	0	99%
		FJ827744.1	*P. coffeae*	Pcof7	100%	0	99%

Remarks: Nc =nucleotide, Qc= *Query cover*

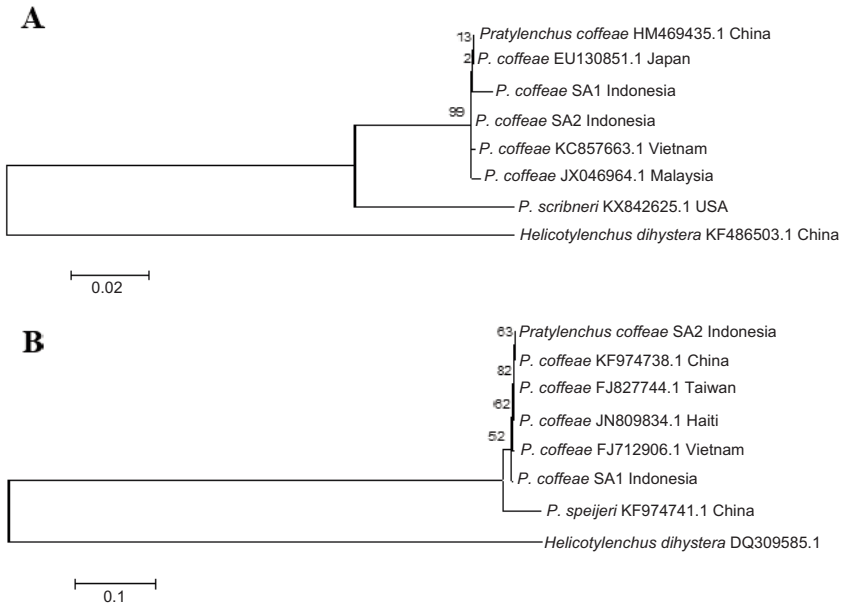

Figure 2. The phylogenetic tree of *Pratylenchus coffeae* by the Maximum Likelihood method; (A) the D2D3 segment of 28S rRNA sequence with HKY + G model (BIC = 3541.9) and 1000 bootstrap replications; (B) sequences ITS1-5.8S-ITS2 with K2 + G model (BIC = 5215.5) and 1000 bootstrap replications; the sequence codes in this study are SA1 and SA2

CONCLUSION

Pratylenchus species in Robusta plantation in Malang, East Java are identified as *P. coffeae.* The morphology and morphometric characteristics of *P. coffeae* in this study are in accordance with the description in key identification, and the molecular characteristics are identical to *P. coffeae* species from China, Haiti, Japan, Malaysia, Taiwan, and Vietnam.

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