



Research Article

Phytonematodes Community and Polyphasic Character of *Aphelenchoides varicaudatus* on Garlic Plants in Tegal Regency, Central Java

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ABSTRACT

The interception of *Ditylenchus dipsaci*, *D. destructor*, and *Aphelenchoides fragariae*, which are classified as quarantine pests, has been reported to occur on imported garlic bulbs used for consumption at several traditional markets in Bogor. This finding should increase awareness of the chance of garlic contamination in Indonesia's garlic fields. This study aimed to evaluate the nematode community and determine polyphasic characters of *Aphelenchoides* species from garlic plantations. The study was conducted by sampling soil and plants from garlic plantations in Tuwel and Rembul Villages, Bojong District, Tegal Regency, Central Java in December 2018. Nematode extraction was done using a mist chamber for plant materials and sugar flotation – centrifugation methods for soil samples. Parameters measured included nematode species identity, absolute population and prominence indexes. Nematode identification was conducted based on their morphological characters. Further identification based on morphometric and molecular characters (polyphasic) conducted for nematodes species suspected as quarantine pests. Six genera of nematodes identified were *Helicotylenchus*, *Aphelenchoides*, *Rotylenchulus*, *Aphelenchus*, *Criconemoides*, and *Tylenchus*. *Aphelenchoides* sp. had the highest prominence value from plant tissue with a value of 6.32 and is categorized as a quarantine pest genus (*A. fragariae*) in garlic and was further identified to the species level. Further identification based on polyphasic characters showed the *Aphelenchoides* sp. found was *A. varicaudatus*.

Keywords: molecular; morphology; morphometric; prominence value

INTRODUCTION

Indonesia imports more garlic compared to its national production. Within 2007–2018, 96% percentage of national needs were imported, which is approximately reach 446,522.20 kg (BPS, 2019). This high importation cause high risk of quarantine pest to enter Indonesia. Nematode inception reports have shown occurrences of *Ditylenchus dipsaci*, *D. destructor*, and *Aphelenchoides fragariae* on imported garlic bulbs used for consumptions and sold in traditional markets located in Bogor and surrounding areas. These nematodes are quarantine pests (Kementan, 2018) on garlic and threat garlic production of high altitude fields in Indonesia. Damage due to nematodes are estimated to reduce 10–100% of garlic production in various countries (Sturhan & Brzeski, 1991; Abawi *et al.*, 2012; Cheng *et al.*, 2015).

Yield loss in garlic may occur when the fields are contaminated with nematodes. Nematode contaminated seeds are possible threats for crops. In addition, nematodes are able to reproduce on

plants, which posses a larger threat for garlic production. Garlic seed importation by Indonesia has been reported during 2007–2009 with an average volume of 298,323 kg and 2017–2018 with an average volume of 1,000,350 kg (BPS, 2019). Garlic seed importation should be monitored for the possibility of quarantine nematodes to enter Indonesia. Nematode species identification with a comprehensive approach using morphological, morphometric, and molecular techniques, or known as polyphasic characteristics, are considered to provide accurate results. Biochemical and molecular methods and their tools have successfully identified nematodes (Carneiro *et al.*, 2017). Therefore, these methods are hoped to confirm nematode species characteristics accurately.

Nematode contamination in fields sets a threat of yield loss and spreading to other garlic production fields. Correct identification to species levels will provide correct information regarding to nematode species. Therefore, identification of nematode community in garlic productions should be done in fields using polyphasic methods.

MATERIALS AND METHODS

Location and Sampling Techniques

Survey and sampling were done using a purposive sampling method (van Bezooijen, 2006). Garlic plants that were suspected to be infected by nematodes (Indarti *et al.*, 2018) were obtained from a garlic production field in Village of Rembul and Tuwel, Bojong, Tegal.

Nematode Extraction

Nematodes were extracted from 100 mL of soil sample solutions using a floatation and centrifuse method (van Bezooijen, 2006). Nematode extraction from plant parts (10 g of root, bulb, stem, and leaves) was done using a Baermann funnel and modified with a mist chamber (EPP0, 2013). Nematode specimens were prepared using methods from Ryss (2017).

Morphological and Morphometric Identification

Nematode specimens were identified to genus and species using identification keys from Tarjan *et al.* (1977). Identification was done under a binocular microscope (Olympus BX 51). Density of nematode populations from each samples were counted at enlargement of 40×. Morphometric characteristics of nematodes were determined using de Man's formula.

Data Analysis

Data analysis were done for soil and plant tissue samples. Absolute population (AP) were calculated using the following formula:

$$AP = \left(\frac{v2}{v1} \times n1 \right) \times \frac{100}{v3} \quad (1)$$

AP : nematode population from each 100 g soil sample suspension or 10 g of plant parts.

n1 : average number of nematodes counted from v1.

v1 : is the suspension volume (mL) measured in counting dish.

v2 : total volume (mL) of sample suspension extracted.

v3 : volume (mL) of soil suspense or weigth of plant tissue (g).

Absolute frequency (AF) was calculated from the number of samples that contained a certain nematode species divided by the total number of samples tested. Population density and absolute frequency were used to calculate prominence value (PV) (Norton, 1978; Fourie *et al.*, 2001), using the following formula:

$$PV = \frac{AP \sqrt{AF}}{10} \quad (2)$$

DNA Extraction and Amplification

Nematode DNA was extracted using worm lysis buffer method from an individual nematode (Holterman *et al.*, 2006). Results from DNA extraction was analyzed using a PCR and a universal D2A primer (5'-ACAAGTACCGTGAGG-GAAAGTTG-3') and D3B primer (5'-TCGGAAGGAAC-CAGCTACTA-3') to amplify the D2-D3 gene segment of 28s rRNA (Subbotin *et al.*, 2006). PCR products were sequenced and compared with GenBank, NCBI to determine homology and query cover value.

Sequencing and Nucleotide Analysis

PCR amplification results were sent to 1st Base (Malaysia) to do a nucleotide sequencing. Sequence were analyzed using program basic local alignment search tool (BLAST) on national center for biotechnology information (NCBI). Phylogenetic trees were produced using Mega version 7.0.26 (Kumar *et al.*, 2016) based on the maximum likelihood. Bootstrap were analyzed with 1,000 replications to reconstrute phylogenetic tree.

RESULTS AND DISCUSSION

Symptoms Description

Garlic plants from Tegal showed several symptoms suspected to be caused by nematodes. Symptoms that occurred above ground were yellowing, stunted, falling leaves, and leaf distortion. Symptoms found on organs below ground were fewer numbers and damage on roots (Figure 1). Symptoms found from Tegal samples were similar to damage symptoms on garlic caused by nematodes (Shurtleff & Averre, 2000; Schwartz & Mohan, 2008; Indarti *et al.*, 2018). Garlic plants infected by parasitic nematodes could not stand upright, stunted, had yellow leaves, thickened and short leaves, falling leaves, had yellow or brown spots, softening stems, and death of plants.

Garlic Nematode Community

Nematodes recovered from roots and garlic bulbs are shown in Table 1. Nematodes found in this study were associated with garlic and other vegetables (Firake *et al.*, 2016; Keshari *et al.*, 2018). This indicates that plants and rizhophere of garlic plants in Tegal are not infested by the quarantine pest *Ditylenchus* sp.

Based on population densities, *Aphelenchoides* sp. showed the largest population of 10 nematodes

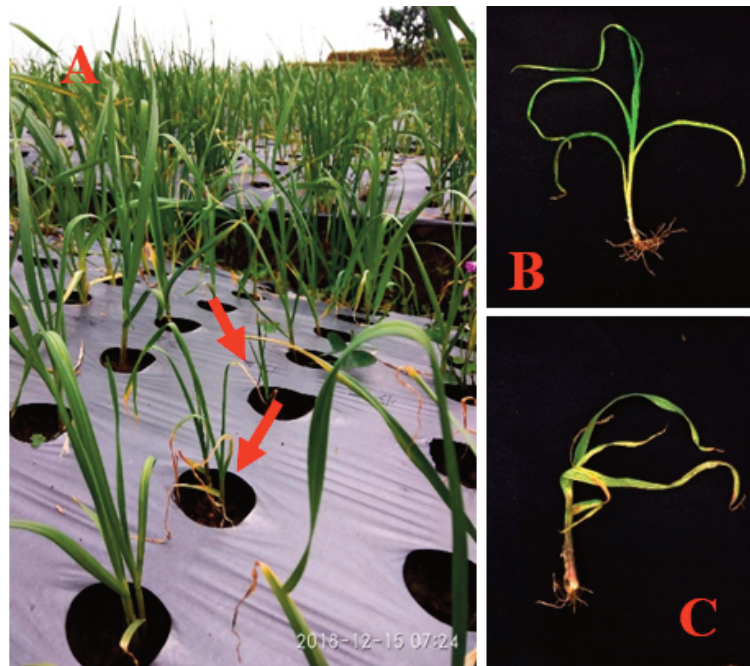


Figure 1. Garlic plants suspected to be infested by nematodes; (A) stunted, (B) discoloration and leaf distortion, (C) shrunk and fragile root systems

Table 1. Nematode population density from garlic fields located in Tegal

Species	PHI	Population Density			
		Bulb and root	Stem	Leaf	Rhizoperic soil
<i>Helicotylenchus</i> sp.	H	2.22	0	0	61.7
	M	0	0	0	53.8
	I	4.44	0	0	109
<i>Aphelenchoides</i> sp.	H	10	0	0	0
	M	0	0	0	0
	I	2	0	0	0
<i>Rotylenchulus</i> sp.	H	4.44	0	0	3.7
	M	0	0	0	8.15
	I	6.67	0	0	3.7
<i>Aphelenchus</i> sp.	H	2.22	0	0	6.67
	M	0	0	0	2.22
	I	7.78	0	0	8.89
<i>Tylenchus</i> sp.	H	2.22	0	0	4.44
	M	0	0	0	2.22
	I	0	0	0	5.56
<i>Criconemoides</i> sp.	H	0	0	0	0
	M	0	0	0	0
	I	0	0	0	7
<i>Dorylaimus</i> sp.	H	0	0	0	4.44
	M	0	0	0	7
	I	2.2	0	0	4

PHI= Plant Health Index, H= Healthy, M = Mildly infested, I=Infected Plant

in 10 g of bulb and healthy plants (Table 1). *Helicotylenchus* sp. was the most found nematode ca 108.89 nematoda from 100 mL of soil suspension collected around infested plants. The high population

of *Helicotylenchus* sp. is assumed to be caused by the reproduction rate that is affected by its life cycle and suitable environments (Reis *et al.*, 2008). Samples were collected from locations at 975–1045 masl

with soil temperatures of 21–24 °C. This temperature is included as an optimum temperature for nematodes to survive for more than 6 months underground.

Based on prominence values, *Helicotylenchus* sp. was the dominant nematode from soil rhizospheres as much as 97.39 (Table 2). Several reports have demonstrated that nematode population of this species dominates nematode communities on vegetable crops due to their parthenogenetic reproducing ability (Firake *et al.*, 2016; Mathivathani & Subramanian, 2018).

***Aphelenchoides* sp. Morphologic and Morphometric Characteristics**

Female *Aphelenchoides* sp. from this study had round and offset heads, and basal knob shaped stylets. Metacarpus were fully elongate shaped. Valves were located at the body's posterior. Tails were dorsally convex conoid, tapered, and showed a unique mukro on the tip of the tail (Figure 2). Morphological and morphometric characteristic of all *Aphelenchoides* sp. isolates demonstrated similarity with *A. varicaudatus* as described by Huang *et al.* (2012), (Table 3).

The tail characteristics and number of lateral lines of *Aphelenchoides* sp. within this study were

different between *A. fragariae* and *A. besseyi* (Huang *et al.* 2012; EPPO, 2017). The number of lateral lines on *Aphelenchoides* sp. specimen were similar to *A. besseyi* that was 4, while *A. fragariae* only have 2 lines. *A. fragariae* and *A. besseyi* tails were elongate-conoid and conoid, while *Aphelenchoides* sp. tails were convex-conoid. Therefore, characteristics showed by *Aphelenchoides* sp. specimens were similar to morphological characteristics of *A. varicaudatus* as described by Huang *et al.* (2012).

***Aphelenchoides* sp. Molecular Characteristics Based on D2-D3 Sequence on 28S rDNA Gene**

PCR amplification on 28S rDNA gene region D2-D3 using D2A and D3B primer, DNA isolate resulted in a single band \pm 780 bp (Figure 3). Based on the highest identity and query cover score and the lowest E value, *Aphelenchoides* sp. (MN587128) from this study showed similarity to *A. varicaudatus* (Table 4). Phylogenetic analysis showed that *Aphelenchoides* sp. had similarity to *A. varicaudatus* from *Pinus kesiya* (Huang *et al.*, 2012) with 100% homology (Figure 4).

Table 2. Nematode population density from garlic fields located in Tegal

Species	PHI	Prominence Value			
		Bulb and root	Stem	Leaf	Rhizoperic soil
<i>Helicotylenchus</i> sp.	H	0.99	0	0	55.16
	M	0	0	0	53.78
	I	1.99	0	0	97.39
<i>Aphelenchoides</i> sp.	H	6.32	0	0	0
	M	0	0	0	0
	I	0.89	0	0	0
<i>Rotylenchulus</i> sp.	H	1.99	0	0	2.87
	M	0	0	0	6.31
	I	2.98	0	0	2.87
<i>Aphelenchus</i> sp.	H	1.41	0	0	4.22
	M	0	0	0	1.41
	I	4.92	0	0	5.62
<i>Tylenchus</i> sp.	H	0.99	0	0	2.81
	M	0	0	0	0.99
	I	0	0	0	4.97
<i>Criconemoides</i> sp.	H	0	0	0	0
	M	0	0	0	0
	I	0	0	0	3.13
<i>Dorylaimus</i> sp.	H	0	0	0	2.81
	M	0	0	0	3.13
	I	0.99	0	0	1.79

PHI= Plant Health Index, H= Healthy, M = Mildly infested, I=Infected Plant

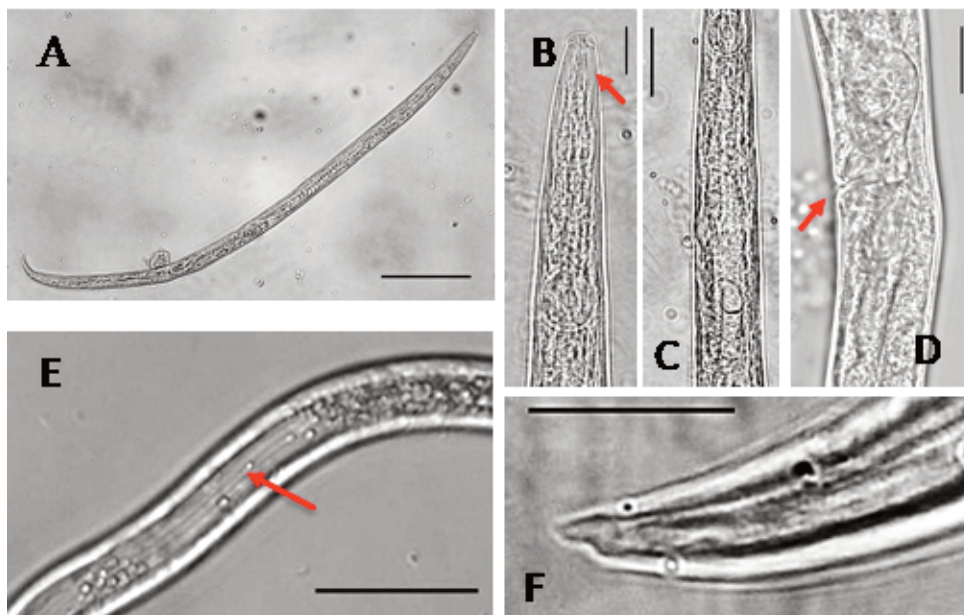


Figure 2. *Aphelenchoides varicaudatus* morphology; (A) female adults, (B) stilet, (C) median bulb and overlapping esophagus with intestine, (D) valve, (E) lateral with 4 lines, (F) mukro (scale: A = 100 μ m; B, D, F = 10 μ m; C = 20 μ m; E = 50 μ m)

Table 3. *Aphelenchoides varicaudatus* morphometric comparison to the literature

Morphometric characteristic (μ m)	Specimen in this study	Huang <i>et al.</i> (2012)
Number of specimen (females)	7	20
Body length	567.35 \pm 74.74 ^a (494.51–704.59)	780 \pm 56.7 (634–900)
Value of body length divided by the largest body diameter	28.71 \pm 3.98 (24.29–35.8)	30.4 \pm 1.68 (26.5–33.2)
Body length divided by distances between head to valve between oesophagus intestine	10.51 \pm 1.14 (9.5–12.23)	9.4 \pm 0.38 (8.8–10.2)
Value of body length divided by tail length	15.47 \pm 1.26 (13.81–16.9)	16.11 \pm 0.58 (15.0–17.1)
Value of tail length divided by tail diameter located on cloaca	3.47 \pm 0.2 (3.24–3.77)	3.8 \pm 0.26 (3.3–4.3)
Distance from valve from gead tip (%)	69.72 \pm 1.15 (68.32–71.2)	69.2 \pm 0.75 (68.2–71.2)
Valve-Anus	151.94 \pm 17.13 (130.67–175.34)	185 \pm 15.3 (155–222)
Stilet	12.39 \pm 1.01 (11.17–14.01)	14.2 \pm 0.5 (13.5–15)
Tail	36.63 \pm 2.96 (32.61–42.34)	49 \pm 2.9 (43–53)

^a: numbers on table are mean \pm standard deviation (minimum and maximum value)

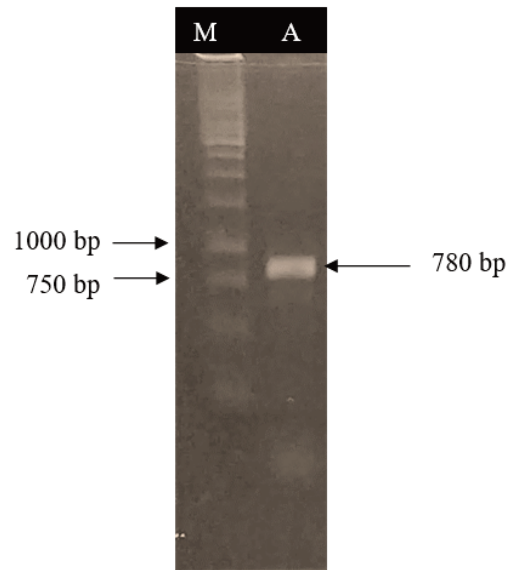


Figure 3. *Aphelenchoides* sp. DNA amplification results using D2A-D3B primers. M= marker 1 kb (Thermoscientific); A= *Aphelenchoides* sp.

Table 4. *Aphelenchoides* sp. from garlic fields located in Tegal with various *Aphelenchoides* species on GenBank

Isolate	Species reference	Access number	Query Cover (%)	Identity (%)	E value
<i>Aphelenchoides</i> sp.	<i>A. varicaudatus</i>	HQ283353.1	100	99.70	0.0
	<i>Aphelenchoides</i> sp.	KT692709.1	100	87.08	0.0
	<i>A. xui</i>	FJ643488.1	100	82.61	4e-163
	<i>A. fragariae</i>	MK292123.1	100	77.59	8e-101
	<i>A. ritzemabosi</i>	KX119136.1	47	85.76	1e-88

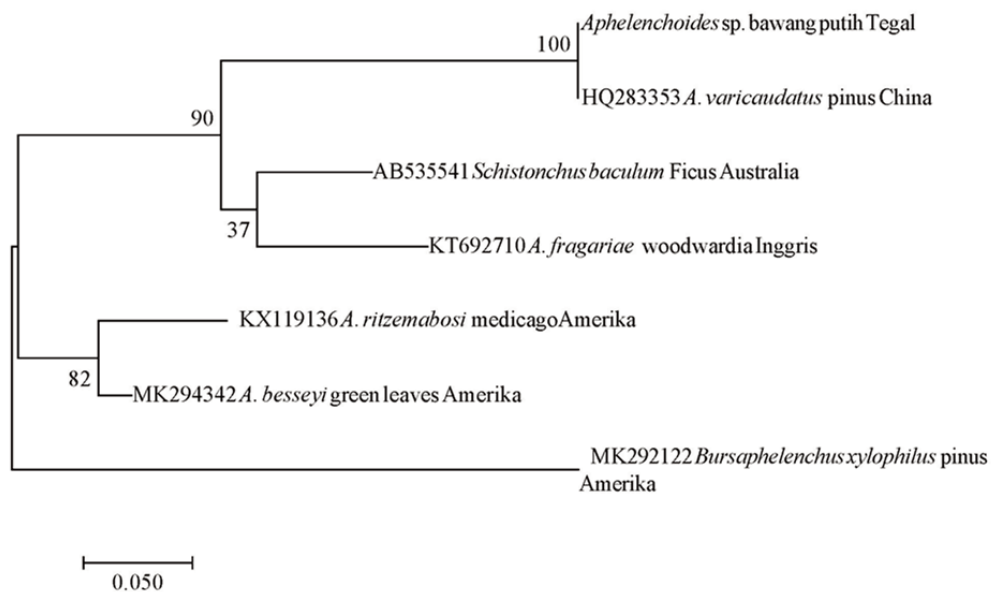


Figure 4. Phylogenetic tree of *Aphelenchoides varicaudatus* from garlic fields in Tegal with *A. varicaudatus* from crop host collected from other countries in Genbank constructed using MEGA 7.0.26 and Maximum-likelihood method

CONCLUSION

Parasitic nematodes collected from garlic samples were *Aphelenchoides* sp., *Helicotylenchus* sp., *Rotylenchulus* sp., *Aphelenchus* sp., *Tylenchus* sp., *Criconemoides* sp., while non-parasitic nematodes recovered was *Dorylaimus* sp. *Aphelenchoides* sp. had the highest prominence values from plant tissue ca. 6.32 and one species of this genus is a quarantine pest. Further identification based on the polyphasic characteristics show that the *Aphelenchoides* sp. specimen was *A. varicaudatus*.

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