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Morphological and Phylogenetic Characteristics of *Ditylenchus dipsaci* among Garlic Plants

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Abstract

Stem and bulb nematode, *Ditylenchus dipsaci* (Kuhn, 1857), is a severe pest on garlic and other vegetables causing yellowing of leaves and rotting of bulbs. The existence of *D. dipsaci* in Indonesia was first reported in Temanggung, Central Java, in 2018. These researches aim to determine the distribution area, identification, and the genetic relationship of *D. dipsaci* which attacked garlic in Central and East Java. The study was conducted by sampling garlic plants attacked by *D. dipsaci* and extraction-isolation by immersion method. Species identification was carried out morphologically, morphometrically, and molecularly using PCR techniques. The results showed that *D. dipsaci* has been spread on garlic plants in Central Java (Magelang, Temanggung, Karanganyar, Tegal, and Brebes) and East Java (Malang and Mojokerto). The varieties that were attacked were Lumbu Kuning, Lumbu Hijau, Sangga Sembalun, and Tawangmangu Baru. The highest population was found in Kaliangkrik, Magelang, with 53.46 nematodes (5 g bulbs)$^{-1}$. Based on morphological and morphometric identification, the species found was *D. dipsaci*. Based on molecular identification, samples from Jabung Mojokerto and Malang Poncokusumo were amplified at ± 600 bp with the D2A / D3B primers in the 28s rDNA region. *D. dipsaci*, found in East Java, was an indigenous Indonesian species based on phylogenetic studies.

Keywords: *Allium sativum* L., Crop protection, Flagship horticulture, Infected area, Pest control, Stem and bulb nematode

1. Introduction

The import value of garlic (*Allium sativum* L.) in Indonesia reaches 559 728 t yr^{-1} or 96.6 % of the consumption of garlic in Indonesia (Ministry of Agriculture - Republic of Indonesia, 2018). Planting imported garlic seeds is one of the government's efforts to achieve garlic self-sufficiency. However, imported garlic seeds can be a carrier media for quarantine pests. *Ditylenchus* spp. is one of the quarantine pests that can be spread in Indonesia through imported seeds. *Ditylenchus* species that have attacked garlic plants are *Ditylenchus dipsaci* Kuhn, 1857 (Pethybridge *et al.*, 2016) and *Ditylenchus destructor* Thorne, 1945 (Yu *et al*., 2012). *D. dipsaci* can cause yield losses in garlic plantations by 15.33 % to 90 % (Abawi and Moktan, 2010; Yavuzaslanoğlu *et al.*, 2015).

Ditylenchus spp. or stem and bulb nematodes mainly attack the host plant's root, tubers, and stems. The infective stage of this nematode is the fourth juvenile. Attacked plants show stunted growth, yellowing and twisting leaves. Infested bulbs are necrotic and tend to rot with dark to a black colour, becoming soft (Yavuzaslanoglu *et al.*, 2015).

D. dipsaci is found in various temperate (Wulandari and Indarti, 2020; Wulandari *et al*., 2021), subtropical to tropical regions such as Europe and the Mediterranean region, North and South America, North and South Africa, Asia (China, India, Iran, Iraq, Israel, Japan, Taiwan, Turkey, Kazakhstan and South Korea) and Oceania (CABI, 2018). *Ditylenchus* spp. can be spread through infected tubers, seeds, crop residues, attached soil, and other plants or weeds as alternative hosts. Planting infected seeds will increase the attack and losses incurred compared to the previous planting season (Sikora and Fernández, 2005). As with plant-parasitic nematodes, such as root-knot nematodes, infection of *Ditylenchus* spp. exhibits poor growth, a loss in quality and yield of the crop (Youssef and El-Nagdi, 2021)

Ditylenchus spp. has been found in garlic plants in Temanggung Regency, Central Java, and is considered *D. dipsaci* based on symptoms of an attack and morphological characters (Indarti *et al.*, 2018). A survey of the *D. dipsaci* distribution area is needed to prevent the spread of nematodes to other regions in Indonesia. This study aims to determine the distribution area, identification, and genetic relationship of *D. dipsaci* which attacked garlic in Central and East Java, Indonesia.

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2. Materials and Methods

2.1. Nematodes sampling and population analysis

Nematode samples were taken by purposive sampling method or by taking symptomatic garlic plants during 2019 at garlic plan in 20 d to 120 d after planting. Sampling locations were Magelang, Temanggung, Karanganyar, Tegal and Brebes Regency (Central Java Province) and Mojokerto and Malang (East Java Province). Nematodes were extracted from bulbs and roots by the water immersion method (Zhang *et al*., 2013). Bulbs and roots were cut into small pieces then immersed in water for 24 h at 18 °C to 25 °C. The water was filtered with 10 mesh sieve to get a clean suspension nematode. The nematodes of each sample were counted for their population.

2.2. Nematodes identification

Identification of nematodes consisted of morphological, morphometric and molecular identification. Morphological identification was carried out by observing esophagus shape, overlapping the esophageal gland with the intestines, the number of lateral lines, tail shape and spiculum (EPPO, 2017; Karssen and Willemsen, 2010). Morphometric characters were c value (body length/tail length) of female, female body length, stylet length, vulva-anus length and spiculum length (EPPO, 2017). The polymerase chain reaction (PCR) technique was used for molecular identification. DNA nematodes were extracted using the modified CTAB method (Devi *et al.*, 2013). The primers used were D2A ACAAGTACCGTGAGGGAAAGTTG) and D3B (TCGGAAGGAACCAGCTACTA) targeting the 28s rDNA region (Douda *et al.*, 2013). PCR was carried out with an initial denaturation stage at 95 °C, 1 min; 30 cycles of denaturation at 95 °C, 15 s, annealing at 57 °C, 15 s and extension at 72 °C, 10 s; final synthesis at 72 °C, 5 min; and the final temperature at 4 °C.

3. Results and Discussion

3.1. Distribution area of Ditylenchus dipsaci

The distribution area of *D. dipsaci* was determined based on the population of *D. dipsaci* in the affected garlic plants. In the field observations, garlic plants attacked by *D. dipsaci* showed yellowing leaves, twisting leaves, and rotting bulbs. These symptoms are in accordance with Sikora and Fernández (2005) which stated that the symptoms of *Ditylenchus* attack on garlic are yellowing leaves and rotting tubers. Garlic bulbs attacked by *Ditylenchus* become brighter, turn dark brown, shrink and eventually show cracks and rot due to additional activity from saprophytic soil organisms (Abawi and Moktan, 2010).

Table 1. *Ditylenchus* spp. population on the garlic bulbs in Central and East Java

Location	Varieties	Altitude (m a.s.l.)	Soil temperature (°C)	Population (5 g bulb)$^{-1}$
Adipuro, Kaliangkrik, Magelang	Lumbu Kuning	1 420 to 1 748	19.5	52.06
Adipuro, Kaliangkrik, Magelang	Tawangmangu Baru	1 408 to 1 766	21.0	53.46
Tuksari, Temanggung	Lumbu Kuning	1 203	25.4	7.60
Paguyangan, Brebes	Sangga Sembalun	1 013	26.0	27.44
Tuwel, Bojong, Tegal	Tawangmangu Baru	975	25.0	9.99
Tuwel, Bojong, Tegal	Lumbu Putih	975	26.0	0.00
Kalisoro, Tawangmangu, Karanganyar	Tawangmangu Baru	1 183	20.0	0.00
Kalisoro, Tawangmangu, Karanganyar	Lumbu Kuning	1 183	19.0	0.00
Kadipekso, Jenawi, Karanganyar	Sangga Sembalun	1 023	25.0	4.61
Segoro Gunung, Ngargoyoso, Karanganyar	Lumbu Kuning	1 085	24.7	6.5
Segoro Gunung, Ngargoyoso, Karanganyar	Tawangmangu Baru	1 087	25.0	6.8
Sajen, Pacet, Mojokerto	Lumbu Hijau	712	22.3	20.81
Padusan, Pacet, Mojokerto	Lumbu Hijau	867	24.1	11.38
Padusan, Pacet, Mojokerto	Lumbu Kuning	853	23.5	0.00
Taji, Jabung, Malang	Lumbu Hijau	1 173	22.3	10.87
Gubugklakah, Poncokusumo, Malang	Lumbu Kuning	1 100	23.8	46.97
Gubugklakah, Poncokusumo, Malang	Lumbu Hijau	1 100	23.8	44.49

m a.s.l. = meter above sea level

D. dipsaci was detected in the center of garlic crops in Magelang, Temanggung, Karanganyar, Tegal and Brebes (Central Java) and Mojokerto and Malang (East Java) (Table 1). The infested varieties of garlic were Lumbu Kuning, Lumbu Hijau, Tawangmangu Baru, and Sangga Sembalun, and all of them were local varieties. Lumbu Kuning is the most widely cultivated variety due to its relatively short growing age (105 d to 116 d).

The highest population of *D. dipsaci* was found in Kaliangkrik, Magelang, Central Java (53.46 nematodes (5 g bulb) $^{-1}$). *D. dipsaci* population that could affect the number and weight of tubers is 14.29 (g of planting medium) $^{-1}$ or 500 nematodes (g tuber peels)$^{-1}$ (Mwaura *et al.*, 2015a). *Ditylenchus* populations in all regions are under the damage rate but have caused damage to bulbs and leaves.

Nematode population is influenced by abiotic factors such as temperature, humidity, soil texture, and other soil properties (Mulyadi, 2009). Garlic plants were planted at various heights from 712 m to 1 766 m a.s.l. (Table 1).

Nematodes prefer cold and humid climatic conditions in the highland tropics (Sikora and Fernández, 2005). Soil temperatures during the day ranged from 19 °C to 26 °C. *D. dipsaci* has an optimal temperature range of 17 °C to 20 °C, but can still cause significant tuber damage at 26 °C (Mwaura *et al*., 2015b).

3.2. Identification and genetic relationship of Ditylenchus dipsaci

Morphological and morphometric identification were the first steps to identify Ditylenchus species. Morphological character observed: body almost straight when relaxed (Fig. 1A), lip region slightly offset, stylet not long, knobs rounded (Fig. 1B), basal bulb not overlapping intestine (Fig. 1C), and terminus tail tend to tapering-conoid (Fig. 1D). Nematode body lengths ranged from 0.4 mm to 0.6 mm (Table 2), different from the reference (0.8 mm to 1.9 mm). However, all-female body length /tail ratios (c) were identical to D. dipsaci. Stilet length ranged from 7 µm to 13 µm. The length of the nematode spiculum in Kaliangkrik and Gubugklakah ranged between 23 µm to 24 µm and 22 µm to 23 µm. The same species can have different body lengths based on host type and temperature (Hazir et al., 2001). D. dipsaci, maintained in carrot callus, is shorter than nematodes found in garlic plants (Hajihassani et al., 2017). Based on the morphological and morphometric characters, the species of the nematode was Ditylenchus dipsaci.

Morphological and morphometric identification were the first steps to identify *Ditylenchus.* Electrophoresis results showed that two samples from Jabung, Mojokerto, and Poncokusumo, Malang, were amplified at ± 600 bp (Fig 2). The results of the phylogenetic analysis showed that *D. dipsaci* from Jabung, Mojokerto has a close genetic relationship with *D. dipsaci* from Poncokusumo, Malang (Fig. 3). *D. dipsaci* from Malang and Mojokerto have close genetic relations with *D. dipsaci* from various countries but belong to different groups. These results indicated that *D. dipsaci* was genetically different from various countries and was an indigenous Indonesian species.

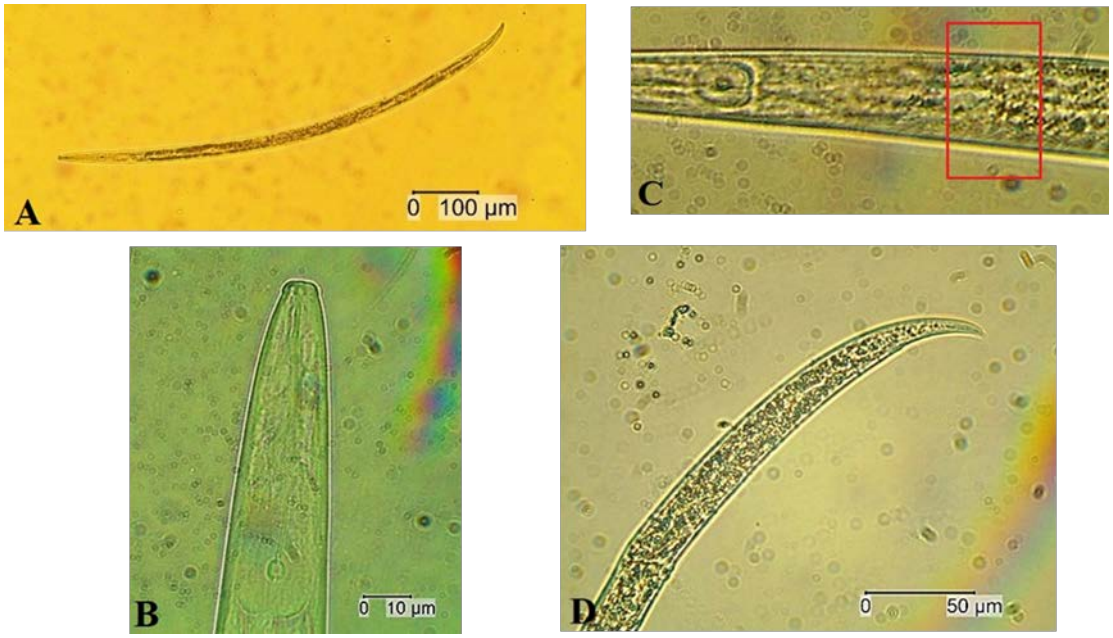

Figure 1. Female body of Ditylenchus dipsaci.: body almost straight when relaxed (A), lip region slightly offset, stylet not long, knobs rounded (B), basal bulb not overlapping intestine (C), and terminus tail tend to tapering-conoid (D).

Table 2. Morphological and morphometrical characteristics of *Ditylenchus dipsaci*

	D. dipsaci (EPPO, 2017)	*D. dipsaci* Kaliangkrik, Magelang	*D. dipsaci* Paguyangan, Brebes	*D. dipsaci* Pacet, Mojokerto	*D. dipsaci* Jabung, Mojokerto	*D. dipsaci* Poncokusumo, Malang
c (body length/tail length) of female	11 to 20	12 to17	11 to 14	11 to 13	13 to 16	11 to 20
Female body length (mm)	(1.0-) 1.1 (-1.7)	(0.45-) 0.52 (-0.59)	(0.50-) 0.55 (-0.62)	(0.34-) 0.46 (-0.75)	(0,3–) 0,4 (–0,5)	(0.31-) 0.41 (-0.64)
Stylet length (µm)	10 to 12	10	10 to 13	9 to 10	7 to10	8 to 10
Vulva-anus (tail length)	1.75 to 2.25	2.22 to 2.86	2.20 to 3.42	2.00 to 3.50	2.25 to 3.01	2.00 to 3.67
Spiculum length (µm)	23 to 28	23 to 24	-	-	-	22 to 23
Form of the tail terminus	Pointed	Pointed	Pointed	Pointed	Pointed	Pointed
Posterior bulb	Not overlapping	Not overlapping	Not overlapping	Not overlapping	Not overlapping	Not overlapping

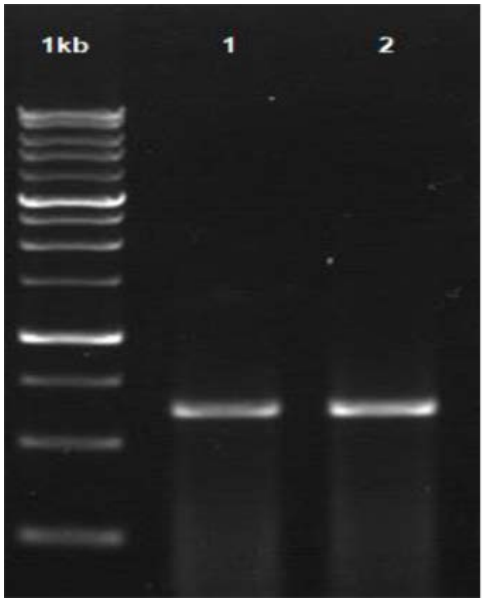

Figure 2. Gel electrophoresis micrograph showing amplicons obtained from PCR reactions; Samples from Jabung Mojokerto (1) and Malang Poncokusumo (2) were amplified at ± 600 bp with the D2A / D3B primers in the 28s rDNA region

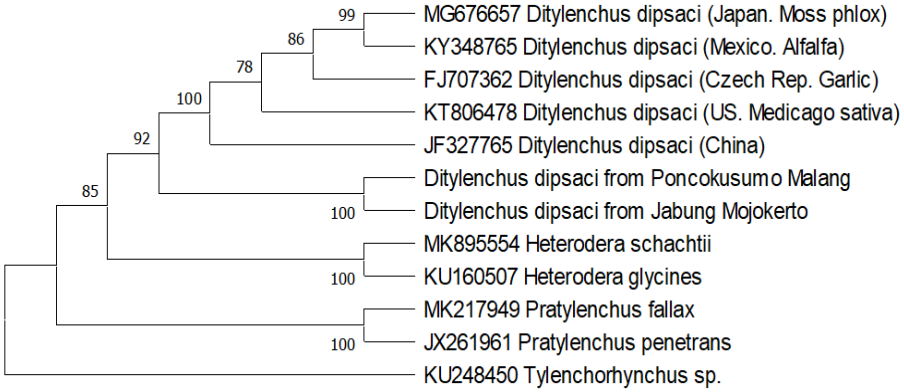

Figure 3. Phylogenetic tree test samples compared to several other nematode species that have been published in the NCBI Database. The method used is the Neighbor-joining (NJ) Kimura 2-parameter model 1.000 bootstraps.

4. Conclusion

D. dipsaci distribution areas in garlic plants in Central and East Java include Magelang, Temanggung, Karanganyar, Tegal, Brebes, Malang, and Mojokerto Regencies. Based on morphological and morphometric identification, the *Ditylenchus* species found was D. *dipsaci* in all affected areas. *D. dipsaci* which attacked garlic in East Java, was an indigenous Indonesian species.

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