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MOLECULAR AND PATHOTYPE IDENTIFICATION OF POTATO CYST NEMATODES

IDENTIFIKASI MOLEKULER DAN PATOTIPE NEMATODA SISTA KENTANG

Mulyadi, Siwi Indarti*, Bambang Rahayu T.P., & B. Triman

Laboratory of Nematology, Department of Plant Pest and Disease, Faculty of Agriculture, Universitas Gadjah Mada
Jln. Flora 1, Bulaksumur, Sleman, Yogyakarta 55281

**Corresponding author. E-mail: siwiindarti@yahoo.com*

ABSTRACT

In Indonesia, potato cyst nematode (PCN) was first reported in Bumiaji, Kota Batu, East Java by PT Syngenta and was identified as *Globodera rostochiensis*. Based on the surveillances, *G. rostochiensis* were also found in Batur, Banjarnegara, and Kejajar, Wonosobo, and Pangalengan, Bandung. In addition, in Batur, Banjarnegara, another species which was identified as *G. pallida* was found. The aim of this research were to identify the species of PCN using molecular method, pathotype identification, and to study the distributions of PCN especially in Java. The PCN are collected from potato planting areas in Kota Batu, East Java; Wonosobo and Banjarnegara, Central Java; and Pangalengan, Bandung, West Java. PCN were extracted and isolated from soil, and then identified by morphological and molecular analysis. PCN were found in potato planting areas in Kota Batu, East Java; Wonosobo and Banjarnegara, Central Java; and Pangalengan, West Java. Based on the morphological characters, molecular method, and the differential host test, the PCN identified as *G. rostochiensis* are amplified an approximately 434 bp with pathotype Ro2.

Key words: *Globodera* spp., molecular identification, pathotype, PCR, potatoes

INTISARI

Di Indonesia, nematoda sista kentang (NSK) pertama dilaporkan di Bumiaji, Kota Batu, Jawa Timur oleh PT Syngenta yang diidentifikasi sebagai Globodera rostochiensis. *Berdasarkan hasil survei, NSK ditemukan di Batur, Banjarnegara dan Kejajar, Wonosobo, Pangalengan. Spesies* G. pallida j*uga ditemukan Batur, Banjarnegara. Penelitian ini bertujuan untuk mengidentifikasi spesies NSK menggunakan metode molekuler, identifikasi patotipe NSK, dan untuk mengetahui penyebaran NSK khususnya di Pulau Jawa. Sampel NSK dikumpulkan dari lahan pertanaman kentang di Bumiaji, Kota Batu, Jawa Timur; Wonosobo dan Banjarnegara, Jawa Tengah; serta Pangalengan, Bandung, Jawa Tengah. NSK diekstraksi dan diisolasi dari tanah yang selanjutnya diidentifikasi secara morfologi dan analisis molekuler. NSK yang terdapat pada lahan pertanaman kentang ditemukan di Kota Batu, Jawa Timur; Wonosobo dan Banjarnegara, Jawa Tengah; serta Pangalengan, Jawa Tengah. Berdasarkan karakter morfologi, metode secara molekuler, dan uji kesesuaian inang, NSK yang diperoleh teridentifikasi sebagai* G. rostochiensis *yang teramplifikasi pada kisaran 434 bp dengan patotipe Ro2*.

Kata kunci: Globodera *spp., identifikasi molekuler, kentang, patotipe, PCR*

INTRODUCTION

Potatoes are subjected to many pests, including nematodes. In addition to cyst nematodes (*Globodera* spp.), root-knot nematodes (*Meloidogyne* spp.) can cause severe damage to potato production (Scurrah *et al*., 2005). In Indonesia, potato cyst nematode (PCN) was first report in Bumiaji, Kota Batu, East Java by PT. Syngenta. Then, these nematode was identified as *Globodera rostochiensis* (Mulyadi *et al*., 2003; Indarti *et al*., 2004). Based on the surveillances in Central Java, PCN were also found in Batur, Banjarnegara and Kejajar, Wonosobo (Mulyadi *et al*., 2003). *G. rostochiensis* was also distributed in Pangalengan, West Java. In addition, *G. pallida* was found in Batur, Banjarnegara (Lisnawita, 2005).

PCN are widely distributed in Central Java especially in Wonosobo and Banjarnegara. This case occur rapidly than East Java and West Java areas. Growing the potatoes crops as continuously during the season made up the population of PCN are increase. However, in the East Java and West Java have only two seasons during the year. The potato will not be growing up during dry season.

Some act for eradicate of PCN population did not bring out succeed because: 1) the PCN assumed to be established, 2) the potato land in both provinces mostly hillies, 3) social aspects of the farmers especially the farmers skill (they do not have experiences in using fumigant nematicides), and 4) eradication by using fumigant nematicides will be very dangerous to the farmers. In facing the problem caused by PCN, several research activities have been done i.e. bioecology of PCN (life cycle, host plants, pH and soil temperature), resistant respons of several potato cultivars/varieties,

crop rotations, biological control, and nematicides screening (Mulyadi *et al*., 2005). Eventhough, surveillances and monitoring to report the distributions of PCN in potato land is still needed. Based on the experiments by several nematologists, there are two methods for identifying PCN, by morphometrical and molecular approaches (Marshall, 1993; Bulman and Marshall, 1997; Carta and Handoo, 2005; Skantar *et al*, 2007; Quader *et al*., 2008).

PCN pathotype identification have been done based on the differential responses on potato clones (Da Cunha *et al*., 2012). There are five pathotype of *G. rostochiensis* (Ro1, Ro2, Ro3, Ro4, and Ro5) and three pathotype on *G. pallida* (Pa1, Pa2, and Pa3) (Fleming and Power, 1998).

This research was done in the effort to provide more details of PCN species distributions especially in Java. The objectives of this research were: 1) to identify the species of PCN using molecular method, 2) pathotype identification, and 3) to study the distributions of PCN especially in Java. As the supported data, morphological identification and population of PCN, were also observed. Whereas the pathotype identifications of PCN was done by sent the cyst samples to Agri-Food and Biosciences Newforge Lane Institute Belfast, Irlandia.

MATERIALS AND METHODS

Collecting Soil Samples

Soil samples were collected from potato land areas in Bumiaji, Kota Batu, East Java and from Wonosobo and Banjarnegara, Central Java, and Pangalengan, Bandung, West Java. In effort to get numerous numbers and fresh of cysts of PCN, in some locations the soil samples were collected more than one potato planting seasons.

In Bumiaji, East Java soil samples were collected from: 1) Brakseng (±1,700−1,800 m above the sea level); Tunggangan (± 1,600 m a.s.l.); Kembangan (±1,500−1,600 m a.s.l.), Watu Tumpuk (±1,500 m a.s.l.); and Bon XV (±1,200 m a.s.l.). In Wonosobo, Central Java soil samples were collected from Patak Banteng (±800 m a.s.l.) and Kejajar (±1,500 m a.s.l.). Whereas from Banjarnegara were collected from Dieng Wetan and Dieng Kulon (±1.800 m a.s.l.); Dieng "Gapura" (±1,500 m a.s.l.). Karang Tengah (±1,900 m a.s.l.); Batur (1,900 m a.s.l.); Pasurenan (±1,900 m a.s.l.); Karang Bakal (±1,900 m a.s.l.); and Sumberejo (±1,900 m a.s.l.). In West Java, soil samples were taken from Pangalengan, Bandung (± 1,400 m a.s.l.)

Collecting Cysts of PCN

PCN cysts were collected (extracted) from each of the soil sample by using Malcom and Averre III method (2000).

Morphological Identification of PCN

As a supported data, morphological identification to distinguish between *Globodera rostochiensis* and *G. pallida* were also done especially based on the morphological differences of the stylet knob of the larvae/juvenile and on the perenial pattern of the cyst. Besides that, number of cysts of PCN in each of the soil samples taken were also counted.

Molecular Analysis of PCN

DNA preparation. Eighty nematode cysts were collected (from each soil sample) and put in mortar containing DNA extraction buffer (CTAB 2%; NaCl 1.4 M; EDTA 100mM, Tris-Cl 50 mM pH 8, and mercaptoethanol 1%), then the cysts were ground and added with 250−400 µl CTAB and mix thoroughly. The DNA containing solution in eppendorf was incubated at 65ºC for 30 minutes with shaking every 10 minutes. An equal volume of Chloroform Isoamylic Alcohol 24:1 (CIAA) were added and mix thoroughly by shaking the tube (or vortex) for one minute. DNA containing solution in eppendorf was centrifugated at 12,000 rpm for 10 minutes. The supernatant then were transferred into sterilized eppendorf and the interface debris were left. The supernatant in the tube were added and mixed with 2 times volume of cold absolute ethanol. DNA containing solution were incubated overnight at -20°C. The DNA containing solution then were centrifugated at 12,000 rpm for 15 minutes and the supernatant were discharged, then the DNA pellet were collected. The pellet were rinsed by adding 500−1,000 µl of 70% cold ethanol and recentrifugated at 12,000 rpm for 10 minutes. The supernatant were discharged and DNA pellet were air dried (or vacuum). DNA pellet were dissolved in 20−30 µl aquabidest. The DNA pellet were then cleaned by using microclean. Then the DNA of the nematode cysts were examined by using electrophoresis.

Polymerase chain reaction. PCR reaction to distinguish between *G. rostochiensis* and *G. pallida* were carried out using primers PITSr3 (5`-AGCGCA GACATGCCGCAA-3`) and PITSp4 (5`-ACAACA GCAATCGTCGAG-3`) in combination with primer ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) (White *et al*., 1990; Bulman and Marshall, 1997) in a multiplex reaction (Garcia *et al*., 2009). All PCR amplification was performed in a total volume of 25 µl containing 12,5 µl MMR (Mega Mix Royal), 2 µl primer ITS5, 2 µl primer PITSr3, 2 µl primer PITSp4, 2 µl DNA sample, and 4,5 µl dDH_2O. Reaction steps consisted of an initial denaturation at 94°C for 2 minutes, followed by 35 (specific primers) denaturation cycles at 94 °C for 30 s, annealing at 60 °C (specific primers) for 30 s, an extension cycle at 72 °C for 30 s,

and a final one at 72 °C for 5 minutes (Skantar *et al*., 2007). PCR was carried out using a thermal cycler (BioRad MyCycler thermal cycler). PCR products were separated by electrophoresis in Tris-EDTA buffer with 1% agarose gel at 90 V for 45 minute and then visualized under UV light.

Pathotype Identification

Pathotype identification of PCN was done by sent the cyst samples to Agri-Food and Biosciences Newforqe Lane Institute Belfast, Irlandia. Some clones of Solanum, including *Solanum andigena* CPC1673, *S. desire*, *S. curtzianum* 60.21.19, and *S. vernei* 58.1642/4 were used as specific hosts plants for "host differential tests".

RESULTS AND DISCUSSION

Collecting Soil Samples and Cysts of PCN

The population of cysts in the soil samples which have taken from the potato land in East Java, Central Java, and West Java were listed in Table 1. In East Java, the highest population of PCN was found in Brakseng. This area was growing up with same crop continuously, but at the other areas predominantly rotated with carrot. The distribution of PCN in East Java at 2008th similar with the data result collected by Mulyadi *et al*. (2003). At the same time, in Central Java the PCN were found in several areas in Wonosobo and Banjarnegara, significantly different with the data collected in 2003 (only found in Karang Tengah and Kejajar). Perhaps, these fenomena caused by: 1) started in 2007 in East Java mostly potato land areas were rotated with carrot (non host of PCN), 2) in Central Java the potato planted continuously during the year, and 3) plenty of the farmers in Central Java bought the potato seeds from the other farmers which may from PCN infected areas.

PCN Identifications Based on Morphological Characters

The correct identification of a species should be based on the congruence of several methods. Morphological and molecular methods enable the rapid and reliable identification of different species (Garcia *et al*., 2009). The identification of Globodera to species based on morphology can be difficult because of the variability of key characteristics. Therefore the use of a combination of cyst and second stage juvenile characteristics is recommended for reliable identification (Anonymous, 2009).

In this study, the PCN in potatoes area (Table. 2) was identified based on morphological characters using stylet of the larvae/juveniles and perenial pattern of PCN cysts. Result revealed that *G. rostochiensis* have been founded in all of the soil samples from East, Central Java, and West Java except in Watu Tumpuk and Bon XV areas (Table 2). However, the *G. pallida* have not founded in all of the location.

Molecular Identification of PCN

A total of 14 of the PCN sample collected from 16 areas in West Java, Central Java, and East Java (Table. 3) were identified as *G. rostochiensis* using the PCR primers ITS5 and PITSr3. This primers amplified an approximately 434 bp DNA fragment for all the sample, respectively (Fig. 1−5.) However, PITSp4 primer for identified *G. pallida* nematode species did not display amplification. *G. rostochiensis* was determined as the most common PCN nematode species in the West Java, Central Java, and East Java but *G. pallida* was not detected.

Table 1. Number of cysts of PCN in East, Central Java, and West Java

Locations			Number of cysts per 20 g of soil			
			1*)	2*)	3*)	4*)
East Java	Batu	Brakseng	14.30	10.30	-	-
		Tunggangan	13.15	6.00	-	-
		Kembangan	2.25	-	-	-
		Watu Tumpuk	0	-	-	-
		Bon XV	0	-	-	-
Central Java	Wonosobo	Patak Banteng	2.00	22.60	19.30	4.60
		Kejajar	5.00	3.30	0.30	
	Banjarnegara	Dieng Wetan	46.30	-	-	-
		Dieng Kulon	1.30	-	-	-
		Karang Tengah	44.40	44.00	-	-
		Karang Bakal	6.00	-	-	-
		Batur	10.00	-	-	-
		Dieng Gapura	18.30	-	-	-
		Pasurenan	14.00	4.30	0.30	-
		Sumberejo	0.30	16.30	-	-
West Java		Pangalengan	-	-	-	13.67

Note: 1*); 2*); 3*); and 4*) are soil sampling at first, second, third, and fourth.

Table 2. PCN species in East Java, Central Java, and West Java based on morphological characters

Locations			*G. rostochiensis*	*G. pallida*
East Java	Batu	Brakseng	+	-
		Tunggangan	+	-
		Kembangan	+	-
		Watu Tumpuk	-	-
		Bon XV	-	-
Central Java	Wonosobo	Patak Banteng	+	-
		Kejajar	+	-
	Banjarnegara	Dieng Wetan	+	-
		Dieng Kulon	+	-
		Karang Tengah	+	-
		Karang Bakal	+	+
		Dieng Gapura	+	-
		Batur	+	-
		Pasurenan	+	-
		Sumberejo	+	-
West Java		Pangalengan	+	-

Note : (+) identified as *G. rostochiensis* and (-) not identified.

Table 3. PCN species in East, Central Java, and West Java based on molecular identification

Locations			*G. rostochiensis*	*G. pallida*
East Java	Brakseng		+	-
	Tunggangan		+	-
	Kembangan		+	-
	Watu Tumpuk		-	-
	Bon XV		-	-
Central Java	Patak Banteng	(*)	+	-
	Kejajar	(*)	+	-
	Dieng Wetan	(**)	+	-
	Dieng Kulon	(**)	+	-
	Karang Tengah	(**)	+	-
	Karang Bakal	(**)	+	-
	Dieng Gapura	(**)	+	-
	Batur	(**)	+	-
	Pasurenan	(**)	+	-
	Sumberejo	(**)	+	-
West Java	Pangalengan		+	

(*): Wonosobo; (**) Banjarnegara; and (+) identified as *G. rostochiensis*

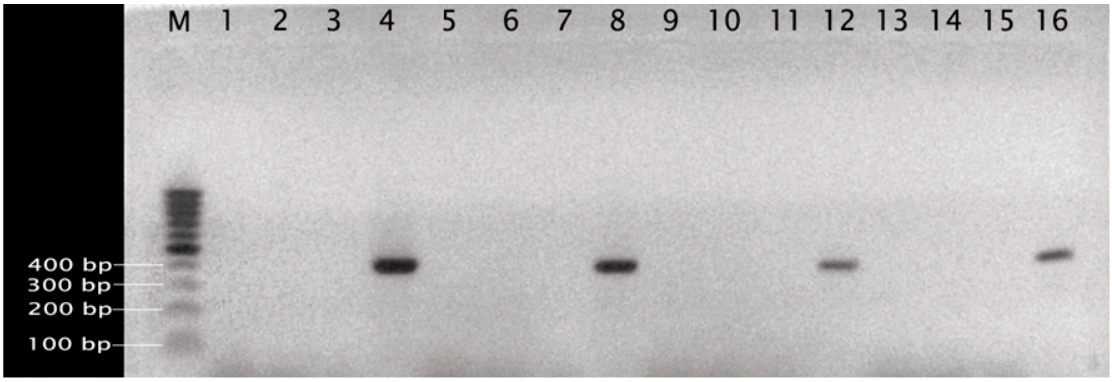

Figure 1. Molecular differentation of Indonesian potato cyst nematodes [molecular weight markers (M) = 100 bp; lane 4, 8, 12, and 16 PCN from Kejajar, Wonosobo identified as *Globodera rostochiensis*]

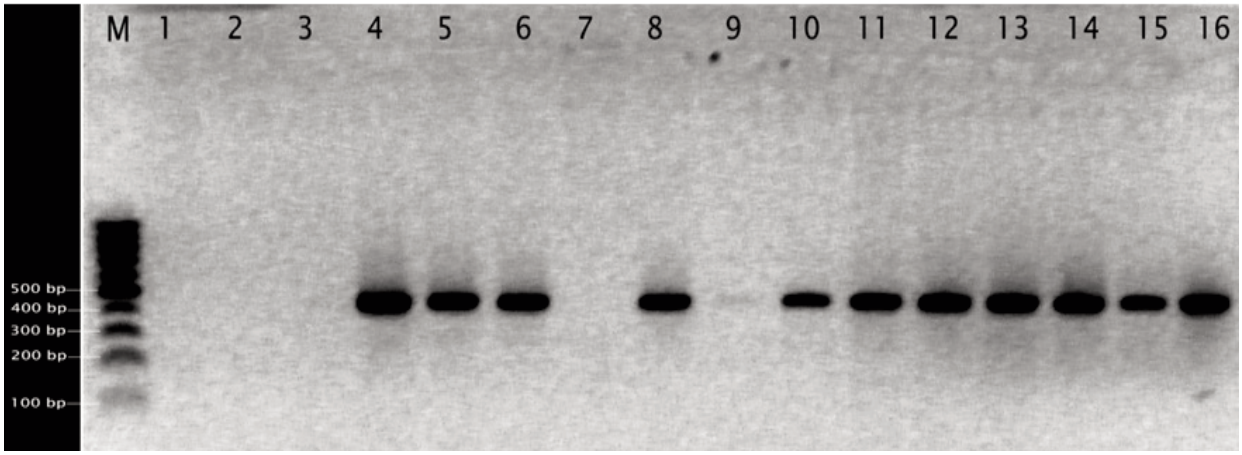

Figure 2. Molecular differentation of Indonesian potato cyst nematodes [molecular weight markers (M) = 100 bp; lane 4 (PCN from Pasurenan); lane 5, 6, 8, and 10 (PCN from Patak, Banteng); lane 11, 12, 13, and 14 (PCN from Batur); lane 15 and 16 (PCN from Dieng Wetan); all were identified as *Globodera rostochiensis*]

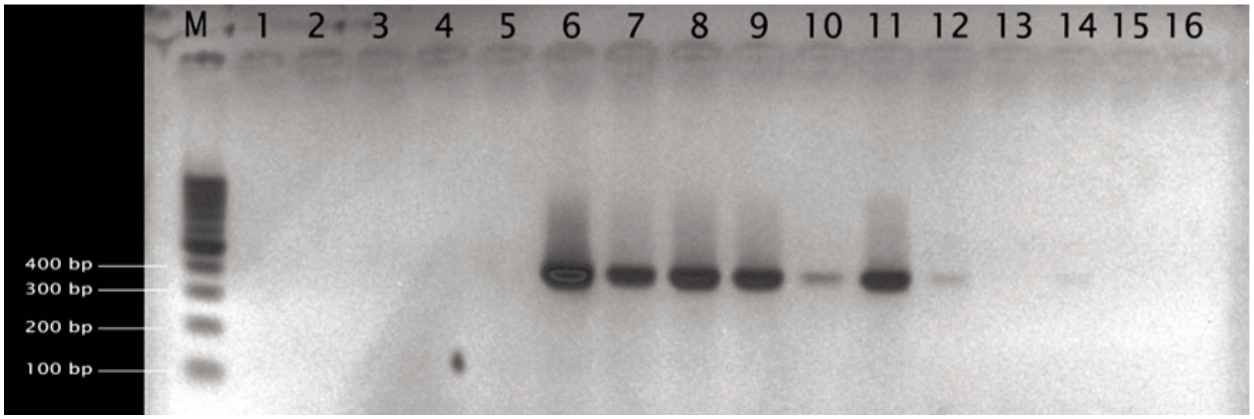

Figure 3. Molecular identification of Indonesian potato cyst nematodes [molecular weight markers (M) = 100 bp; lane 6 (PCN from Dieng Kulon); lane 7 and 8 (PCN from Dieng. Gapura); lane 9 and 10 (PCN from Karang Bakal); lane 11 and 12 (PCN from Sumberejo); all were identified as *Globodera rostochiensis*]

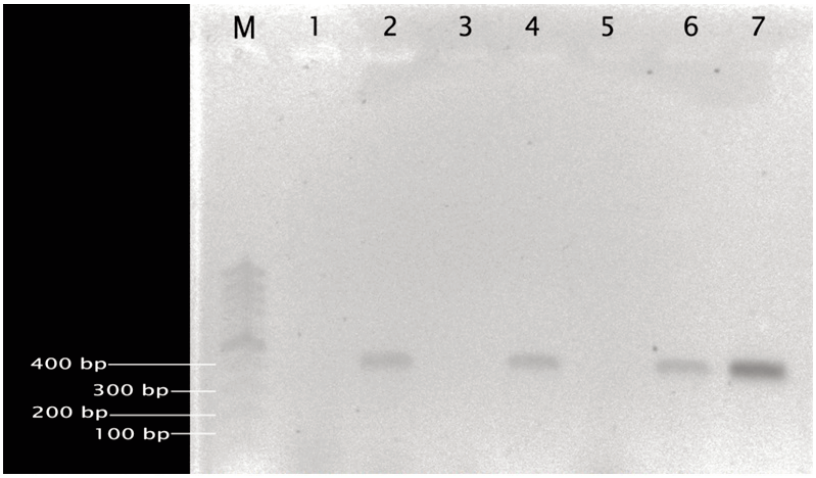

Figure 4. Molecular identification of potato cyst nematodes from Indonesia [molecular weight markers (M) = 100 bp; lane 2 (PCN from Karang Tengah, Central Java); lane 4 (PCN from Tunggangan, East Java); lane 6 (PCN from Kembangan, East Java); lane 7 (PCN from Brakseng, East Java); all were identified as *Globodera rostochiensis*

Figure 5. Molecular idenfication of potato cyst nematodes in Indonesia [molecular weight markers (M) = 100 bp; lane 13 (PCN from Brakseng) identified as *Globodera rostochiensis*]

Pathotype of PCN

Pathotype identification showed all PCN's cysts from Batu (East Java), Wonosobo (Central Java), and Pangalengan (West Java) have survive ability and good reproduction on *Solanum andigena* CPC 1673 and *S. desire*. It's indicated these PCN are *Globodera rostochiensis* with pathotype Ro2. There are no isolates of PCN could multiplication on all *Solanum* clones indicated that *G. pallida* was not found.

CONCLUSIONS

1. Potato cyst nematode in Indonesia were found in potato planting areas in Kota Batu, East Java; Wonosobo and Banjarnegara, Central Java; and Pangalengan, West Java.
2. Based on the morphological characters and molecular method, the potato cyst nematode found in Indonesia was identified as *Globodera rostochiensis* are amplified on approximately 434 bp.
3. Based on differential host test on some *Solanum* clones was indicated the pathotype of PCN in Indonesia is Ro2.

ACKNOWLEDGEMENTS

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