Research Article

Potato Cyst Nematode in East Java: Newly Infected Areas and Identification[*]

Nematoda Sista Kentang di Jawa Timur: Daerah Sebaran Baru dan Identifikasi

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ABSTRACT

Potato Cyst Nematodes (PCN), *Globodera rostochiensis* has noted to be a devastated pest on potato in Indonesia. It is listed as the A2 pest by Plant Quarantine of Republik Indonesia, and it was also being a highly concerned plant parasitic nematode species worlwide. Therefore, both intensive and extensive surveys should be done to monitor the spread of PCN, especially in East Java as one of the centre of potato plantations in Indonesia. The aim of this study was to study the distribution of PCN in four potato plantations in East Java, i.e. Batu, Magetan, Probolinggo, and Pasuruan which were located between 1,205 to 2,063 m above the sea level. Extraction and isolation of cysts from soil samples was done using Baunacke method, and it was followed by identification of the nematodes using morphological and molecular approaches according to Baldwin and Mundo-Ocampo. The results showed that PCN was found on all sampling sites, i.e. Batu (Sumber Brantas, Jurang Kuali, Tunggangan, Junggo, Brakseng); Magetan (Dadi, Sarangan, Singolangu); Probolinggo (Tukul, Pandansari, Ledokombo, Sumberanom, Wonokerto, Ngadas), Pasuruan (Wonokerto, Tosari, Ledoksari, Ngadiwono). Magetan and Pasuruan were noted as new infested areas in East Java. Both morphological and molecular methods showed that the species found on all sites was *Globodera rostochiensis.*

Keywords: East Java, *Globodera rostochiensis*, identification, newly infected area, potato

INTISARI

Nematoda Sista Kentang (NSK), Globodera rostochiensis *telah tercatat sebagai hama yang menghancurkan tanaman kentang di Indonesia. NSK terdaftar sebagai Organisme Pengganggu Tumbuhan Karantina golongan A2 oleh Badan Karantina Pertanian Republik Indonesia, dan juga merupakan spesies nematoda parasit tanaman yang sangat merugikan di seluruh dunia. Oleh karena itu, baik survei intensif maupun ekstensif harus dilakukan untuk memantau penyebaran NSK, terutama di Jawa Timur sebagai salah satu sentra tanaman kentang di Indonesia. Tujuan dari penelitian ini adalah untuk mempelajari distribusi NSK pada empat daerah sentra penanaman kentang di Jawa Timur, yaitu Batu, Magetan, Probolinggo, dan Pasuruan yang terletak antara 1.205 sampai 2.063 m di atas permukaan laut. Ekstraksi dan isolasi sista NSK dari sampel tanah dilakukan dengan metode Baunacke, dan dilanjutkan dengan identifikasi secara morfologi dan molekuler menurut Baldwin dan Mundo-Ocampo. Hasil penelitian menunjukkan bahwa NSK ditemukan di semua lokasi pengambilan sampel, yaitu Batu (Sumber Brantas, Jurang Kuali, Tunggangan, Junggo, Brakseng); Magetan (Dadi, Sarangan, Singolangu); Probolinggo (Tukul, Pandansari, Ledokombo, Sumberanom, Wonokerto, Ngadas), Pasuruan (Wonokerto, Tosari, Ledoksari, Ngadiwono). Magetan dan Pasuruan tercatat sebagai daerah sebaran baru di Jawa Timur. Hasil identifikasi secara morfologi dan molekuler menunjukkan bahwa spesies yang ditemukan di semua lokasi adalah* Globodera rostochiensis.

Kata kunci: daerah sebaran baru, Globodera rostochiensis*, identifikasi, Jawa Timur, kentang*

INTRODUCTION

Plant parasitic nematode was one important factor reducing the productivity of potato in Indonesia, which only ranged from 13.4 to 15.3 tons/ha (Hadisoeganda, 2006). The most notorious nematode on potato is the Potato Cyst Nematodes (PCN), including *Globodera rostochiensis* and *Globodera pallida.* The nematode causes economic loss on solanaceae crops due to plant chlorosis, stunting, and general poor growth (Luc *et al.,* 1995), while the level of damage caused by nematode can reach 50−75% (Hadisoeganda, 2006).

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The PCN was first discovered in 2003 in Bumiaji, Kota Batu, East Java Province, and the species is confirmed as *Globodera rostochiensis* (Mulyadi *et al.,* 2003; Indarti *et al.,* 2004). Nurjanah *et al.* (2016) mentioned that this species was found in Probolinggo and Batu, but the exact infested locations are still unconfirmed.

The PCN has ability to settle in one area, and difficult to be eradicated completely. The PCN can spread locally through farming activities in the area. Meanwhile, remote deployment might be happened through planting of potato seeds, moving of plants, and usage of infected soil and water (Wang *et al.,* 2001, Ibrahim *et al.,* 2003). The usage of agricultural equipment and vehicles can also be potentially spread the PCN (EPPO, 2004; Brodie, 1993). The importance of PCN as pest on potato need to be cautioned, especially on the potential to be spread into uninfested areas. Therefore, surveilance and monitoring to track movement of PCN should be done. Then, this study was aimed to trace and ascertain the possibility of the massive spread of PCN in East Java area. The molecular technique was also used to support the morphological technique to ensure the correct identification of PCN species (Bulman & Marshall, 1997).

MATERIALS AND METHODS

PCN Cyst Collection

PCN soil sampling. Eighteen soil samples were collected from potato plantation in East Java (Figure. 1), which were determined by altitudes and soil temperature. Soil samples were collected using the purposive sampling method, that was taking soils from rhizosphere of the potato plants allegedly infected by PCN, about 0 to 20 cm deep for as much as 500 ml soil (Shurtleff & Averee, 2000).

Cyst extraction. Mature cyst of PCN were isolated by sieving and extracting 50 ml of soil samples using Baunacke Method (Benzooijen, 2006). Cyst were slightly dried and collected into the tube, then were kept at 4 ºC.

PCN population analysis. Number of PCN cyst in each of the soil samples taken were counted.

Identification

Morphological characterization. Morphological observation was done on juveniles hatched from the cyst, which were then fixed with FAA (Dropkin, 1992). Morphological differentiation between *Globodera rostochiensis* and *G. pallida* was done by observing differences of knob stylet of the second juveniles (20 specimens/sampling location) and perineal pattern of the cyst (10 specimens/sampling location).

Photo micrographs of cyst vulval area and semi permanent fixed larvae were made with an automatic camera Olympus DP 21 attached to a Olympus CX 31 compound microscope; and measurements were made with an ocular micrometer on the same micro-scope. Specimens were morphologically identified with recent taxonomic keys based on combination of second-stage juveniles and cysts characters including body length, body width, stylet length, tail and hyaline part of tail length, Granek's ratio (the distance from anus to the nearest edge of vulval basin divided vulval basin diameter), and the number of cuticular ridges between vulva and anus (Fleming & Powers, 1998).

Molecular characterization. DNA extraction was performed following methods described by Fullaondo *et al.* (1999) with some modifications. Sixty PCN cysts were crushed in Eppendorf tubes using plastic micro-pestles in 300 µl of lysis buffer and 5 µl of proteinase K. The mixture was incubated at 65ºC for

Figure 1. Sampling locations in East Java (source: MapInfo Pro™)

1 hour and then incubated at 95°C for 10 minutes. 300 µl of chloroform and isoamylalcohol (24:1) was added. The homogenate was sentrifuged at 11,000 rpm for 10 min. The supernatant was transferred to a new tube and 1.5 time (supernatant total volume) binding buffer was added with the supernatant, and stored for 20 min at -20 °C. This mixture was sentrifuged at 11,000 rpm for 10 min. The pellet was precipitated with 500 ml 70% ethanol, then sentrifugated at 14,000 rpm for 10 min. The pellet was dried in laminar air flow and dissolved in 20 µl nuclease free water.

The fragment of ITS region was amplified using the species-specific primers PITSr3 (AGCGCAGA CATGCCGCAA for *G. rostochiensis,* amplicon size 434 bp) and PITSp4 (ACAACAGCAATCGTCGAG for *G. pallida,* amplicon size 265 bp) in combination with common primer ITS5 (GCAAGTAAAAGTC GTAACAAGG) (Bulman & Marshall, 1997; White *et al.,* 1990) in a multiplex reaction. The PCR amplification was done in a 25 µl reaction volume containing beads ready to go, 1 µl primer, 19 µl nuclease free water and 3 µl of DNA template. The amplification temperature profile was: 2 min initial denaturation at 94°C and 7 min final extension at 72°C with the intervening 35 cycles of 30 s at 94°C, 30 s primer annealing at 60°C and 30 s primer extension at 72°C. All PCR products were run in electrophoresis at 50 V for 1 h in 0.8% agarose gels and visualized on UV ilumination.

RESULTS AND DISCUSSION

This research found two new infested areas infected by PCN, i.e. Pasuruan and Magetan. Before 2015, the distribution area of PCN was only in Batu and Probolinggo, but after 2016, the PCN has spread onto those two new locations which was marked with the establishment of cyst in soil (Table 1).

The number of cyst in Pasuruan and Magetan was fairly low, ranging from 1.2 to 6.0 cysts/50 ml soil. This suggests that PCN in those two districts were still in the process of development. Meanwhile, the highest number of cyst was found in Pandansari, Probolinggo (46.0 cysts/50 ml soil), and the lowest number was found in Sumberanom, Probolinggo (0.8 cysts/ 50 ml soil) (Table 2).

Temperature is a limiting factor that regulates the activity of nematodes including ability to extend its distribution range (Kaczmarek *et al.,* 2014). According to Wallace (1964), the lowest temperature that inhibits

the activity of *G. rostochiensis* is 5−10 °C, the temperature optimum for emergence is 21°C, for development is 18−24°C, and for reproduction is 21−27°C. Temperature that inhibits the emergence of *G. rostochiensis* is 30°C, inhibits the activity of PCN is 30−35°C, and hampered its development is 29−32°C. According to Mulyadi (2009) larval activity takes place at 10°C and stops at 40°C. The optimum temperature for the development of *G. rostochiensis* on host plants range between 18−24°C and inhibited at 29−32°C. The optimum temperature for *G. rostochiensis* growth at 20°C (Nurjanah *et al.,* 2016), range between 15.7−23.1°C (Trifonova, 1999). The results showed that the cysts were present in all sampled areas, with soil temperatures ranged between 19−23°C, and the altitude ranged between 1,205 to 2,063 m above the sea level. (Table 2). Thus, soil temperature at all locations seemed to be suitable for the development of *G. rostochiensis.*

Table 1. Distribution of *Globodera rostochiensis* in East Java

Location	*Globodera rostochiensis*	
	Before 2015	2016 to 2017
Batu	+	+
Magetan	-	+
Pasuruan	-	+
Probolinggo	+	+

The regression analysis showed that the number of PCN cysts increased when the soil temperature declined. From the pattern shown on Figure 2, a relationship model between soil temperature and the number of PCN cysts could be made using the following equation:

Total of PCN cyst = 20.545-0.0457 × Soil Temperature

That model could be used to interpret the impact of temperature on the number of cyst. In the range of 19−23°C, an every one degree Celcius of soil temperature decrement could add 0.0457 PCN cyst.

Morphological measurement of second-stage juveniles and cysts are shown in Table 3 and 4. The body of second-stage juveniles were vermiform, head rounded, and slightly set off with prominent cephalic sclerotization. Stylet was short, well developed, basal knobs rounded without forward projection (Figure 3C and 3D). Tail tapered uniformly with hyaline, and ended with a finely rounded to pointed terminus (Figure 3E−J). Second-stage morphological values from the

Table 2. Distribution of PCN cyst in potato producing areas in East Java

Location		Altitude (m asl)	Geographic position	Soil temperature (°C)	Number cyst of PCN in 50 ml soil
Districts/ City	Villages/ Hamlets				
Batu	Sumber Brantas	1,649	S 07º 45' 34.4" E 112º 31' 40.2"	20	8.2
	Jurang Kuali	1,577	S 07º 46' 40.8" E 112º 31' 39"	21	4.0
	Tunggangan	1,771	S 07º 45' 34.4" E 112º 31' 40.2"	19	23.6
	Brakseng	1,868	S 07º 45' 17.5" E 112º 32' 10.8"	19	32.2
	Junggo	1,424	S 07º 40' 11.8" E 111º 13' 41.9"	21	1.6
Magetan	Dadi	1,205	S 07º 52' 46.2" E 111º 13' 59"	23	4.8
	Singolangu	1,407	S 07º 40' 12.1" E 111º 13' 42.3"	22	1.2
	Sarangan	1,497	S 07º 40' 20" E 111º 13' 06.7"	21	4.8
Pasuruan	Tlogosari	1,750	S 07º 53' 20" E 112º 54' 32"	20	2.2
	Ngadiwono	1,800	S 07º 54' 10.4" E 112º 53' 42.9"	19	6.0
	Ledoksari	1,861	S 07º 53' 36" E 112º 54' 18"	19	1.6
	Wonokerto	2,063	S 07º 54' 38" E 112º 54' 16"	19	2.0
Probolinggo	Tukul	1,411	S 07º 57' 18.1" E 113º 05' 45.1"	21	4.6
	Sumberanom	1,479	S 07º 57' 34.4" E 113º 04' 56.9"	21	0.8
	Ledokombo	1,567	S 07º 58' 03.8" E 113º 03' 1.7"	20	8.6
	Pandansari	1,662	S 07º 58' 09.7" E 113º 03' 37.5"	19	46.0
	Wonokerto	1,704	S 07º 55' 14" E 112º 59' 57"	19	8.4
	Ngadas	1,780	S 07º 58' 18.5" E 113º 02' 58.5"	19	10.0
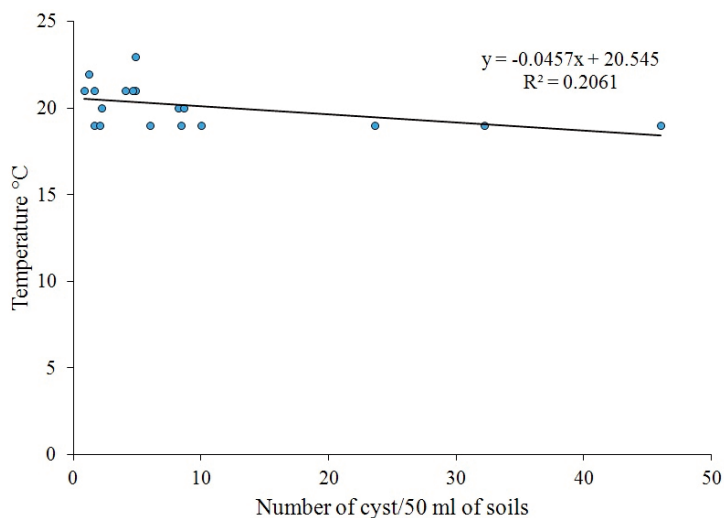
Figure 2. The relationship between the soil temperature and the number of *Globodera rostochiensis* cysts in East Java

analysed populations were very close with observation by Skandar *et al.* (2011).

The first stage in identification of PCN generally involves the spherical with an anterior necklike projections (*Globodera*); perform (elongated-ovoid), the length of the body being about 1.5 times its width, with a protruding neck and no vulval cone (*Punctodera*) from lemon-shape cysts, with both anterior and posterior projections (*Heterodera*). *Cactodera* cysts are less commonly found in agricultural soil samples and range in shape from 'Globodera-like' spheroidal to lemon-shape, with a small posterior cone, the vulva is terminal, becoming a circum-fenestrate opening in the cyst. Identifications of cysts samples are generally made on the basis of cyst shape and fenestration (Fleming & Powers, 1998).

Cysts of PCN were light brown to dark brown in color, spherical to subspherical with a protruding neck (Figure 3A). Anus prominent and visible in all specimens, often with a V-shape mark (Figure 4F). Vulval region intact or fenestrated with a single circum-fenestrate opening occupying all part of the

Table 3. Morphological characters of *Globodera* sp. second-stage juveniles

Characters	Juveniles							
	Batu		Magetan		Pasuruan		Probolinggo	
	n	Mean	n	Mean	n	Mean	n	Mean
Body length (µm)	100	463.35 ± 5.61	60	457.79 ± 3.57	80	451.66 ± 3.00	120	441.90 ± 2.63
Body width (µm)	100	22.45 ± 0.24	60	21.34 ± 0.23	80	21.95 ± 0.22	120	19.99 ± 0.22
Stylet length (µm)	100	20.54 ± 0.07	60	20.53 ± 0.10	80	20.59 ± 0.08	120	20.72 ± 0.06
Tail length (µm)	100	49.91 ± 0.36	60	49.21 ± 0.49	80	48.57 ± 0.32	120	47.74 ± 0.28
Hyaline tail terminal length (µm)	100	26.91 ± 0.36	60	27.92 ± 0.34	80	26.54 ± 0.31	120	26.98 ± 0.26

Table 4. Morphological characters of *Globodera* sp. cysts

Characters	Cysts							
	Batu		Magetan		Pasuruan		Probolinggo	
	n	Mean	n	Mean	n	Mean	n	Mean
Distance of anus-vulva (µm)	50	75.78 ± 2.89	30	49.21 ± 2.86	40	74.60 ± 2.76	60	72.88 ± 2.82
Fenestra length (µm)	50	19.59 ± 0.74	30	14.00 ± 0.69	40	18.70 ± 0.60	60	19.16 ± 0.61
Cuticular ridges	50	14.34 ± 0.43	30	13.40 ± 0.38	40	14.83 ± 0.42	60	16.05 ± 0.44
Granek's ratio	50	3.98 ± 0.15	30	3.50 ± 0.08	40	4.07 ± 0.15	60	3.87 ± 0.14

vulval basin. Vulval cone could not be found. Bullae-like structures were noted in few specimens (Figure 4C and D). Cyst wall pattern was ridge-like with heavy punctuations (Figure 4A and E). Cysts morphological values from the analyzed populations were very close with taxonomic keys (Fleming & Powers, 1998).

On average, the population from Probolinggo has the shortest second-stage juveniles (441.90 ± 2.63 µm), while the population of Batu has the longest (463.35 ± 5.61 µm). The population of Batu has the broadest body (22.45 ± 0.24 µm) compared to the population of Probolinggo (19.99 ± 0.22 µm). The stylet of *Globodera* sp. is slighty, stout, and heavily sclerotized, with well-developed, rounded basal knobs that slope backward (except in second-stage juveniles of *G. pallida,* in wich the knobs project forward and the stylet is about 23 µm, and the basal knobs are more rounded and the stylet is typically shorter about 21 µm for *G. rostochiensis*) (Shurtleff & Averee, 2000). Specimens from Probolinggo have the longest stylet (20.72 ± 0.06 µm), and the lowest were from Magetan (20.53 ± 0.10 µm). The results of this study indicate that the length of the stylet is smaller than the literature. Knob stylet of all samples is rounded. The juvenile tail tapers to pointed tip with a clear terminus (hyaline) (Figure 3E). The specimens from Batu have the longest tail (49.91 ± 0.36 µm).; and the shortest are from Probolinggo (47.74 ± 0.28 µm). Magetan has the longest hyaline tail terminal length (27.92 ± 0.34

µm), and the shortest are from Pasuruan (26.54 ± 0.31 µm). Granek's ratio (the distance form the anus to the nearest edge of fenestra, divided by the length of the fenestra) and a number of cuticular ridges between anus and fenestra can be used for identification. Fleming and Powers mentioned that *G. rostochiensis* had Granek's ratio > 3 and number of cuticular ridges between anus and fenestra are 12−31, and *G. pallida* had Granek's ratio < 3 and number of cuticular ridges between anus and fenestra are 8−20. Furthermore, the population of Magetan has the lowest mean value of Granek's ratio (3.50) while the population of Pasuruan has the highest (4.07). Finally, the population of Magetan has the lowest mean number of cuticular ridges between vulva-anus (13.40), while the population of Probolinggo has the highest value (16.05).

The vulva is near the terminus and anus, not on a terminal valvular cone (Figure 4E and F). It lies between finely papillated crescentic areas occupying most of the vulval slit and surrounded by a single, circular fenestra. In most species, all the eggs are retained within the mature cyst, but a few species also lay eggs in an external gelatinous matrix (Shurtleff & Averee, 2000).

Eggs of *Globodera* sp. (Figure 3B) remain viable in the soil for a long period and encysted eggs are stimulated to hatch by root exudates. Juveniles (Figure 3C) become active at 10°C and maximum root invasion takes place at 16°C. The mature, enlarged females
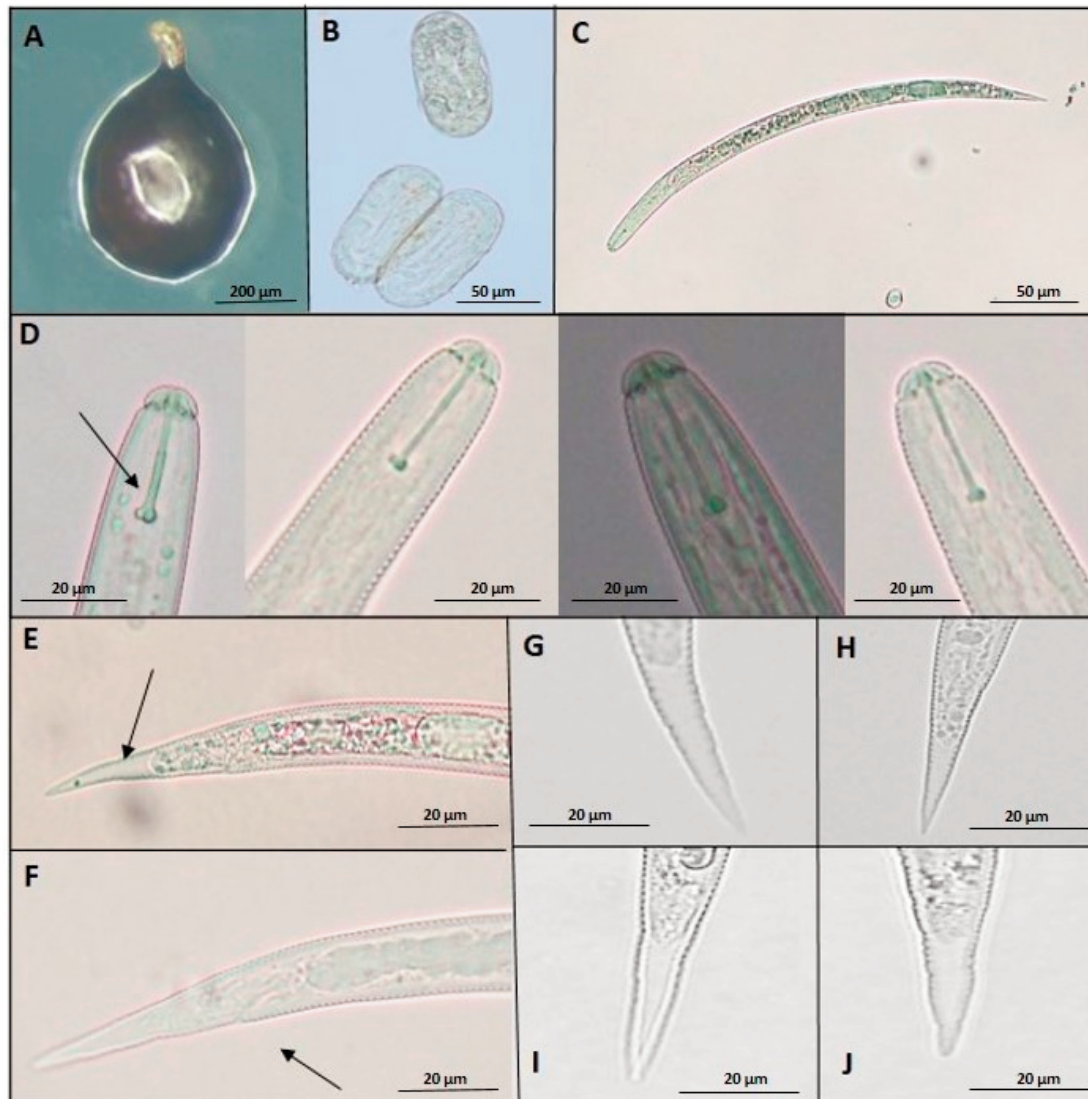
Figure 3. Photomicrographs of cyst, eggs, and second-stage juveniles of *Globodera rostochiensis*: cyst (60× magnification) (A); eggs (400×magnification) (B); whole juvenile (100× magnification) (C); stylet variations of *G. rostochiensis* from: Batu, Magetan, Pasuruan, Probolinggo, narrow show anterior regions showing stylet and knob (D); tail showing hyaline (E); arrow showing anal (F); tail variations of *G. rostochiensis* from: Batu (G), Magetan (H), Pasuruan (I), Probolinggo (J); D−J photographed at 400× of magnification

rupture the root tissue but remain attached to the root by their heads and protruding necks which stay inserted in the root tissue. The fertilized females become large and subspherical and go through a sequence of the color change prior to dying and becoming cysts. PCN generally complete one generation during a growing season. Eggs will remain viable in cysts for over 20 years in soils under severe environmental stress. They withstand temperatures of extreme cold (-15°C) and soil desiccation for long periods. A large portion of eggs will hatch only if they are stimulated by potato root exudates (Luc *et al.*, 1995).

However, essential morphological and morphometrical character, such as the juvenile stylet length,

the stylet knobs anteriorly flattened to rounded, without forwarding projections, the number of cuticular ridges and Granek's ratio indicated that the specimens were *G. rostochiensis.* When compared with *G. pallida,* the stylet of the second-stage juvenile was slightly shorter. The stylet knob is rounded. Cysts have a smaller Granek's ratio and more cuticular ridges between the anus and vulva.

Molecular identification with multiplex PCR determined all samples from four locations as *G. rostochiensis* (Figure 5). Using two sets of primers in the same reaction enables species identification in only one step without subsequent analysis. The PCR primers for *G. rostochiensis* using ITS5 and PITSr3.
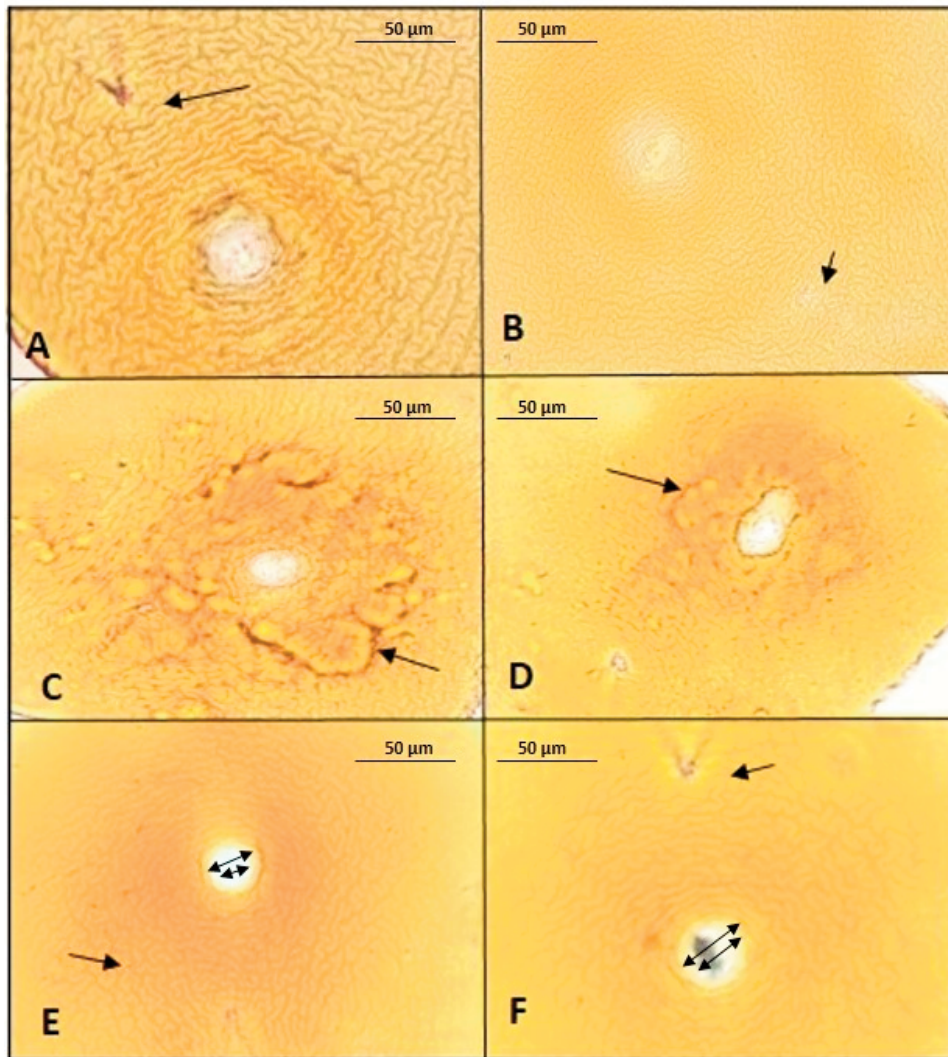
Figure 4. Photomicrographs of terminal areas of *Globodera rostochiensis* cysts: anal-vulval regions with arrows showing anal areas (A and B); bullae-like structures (C and D); cyst wall pattern (E); anal areas with V-shape (F); double arrows show vulva (E and F); A−F photographed at 400× magnification
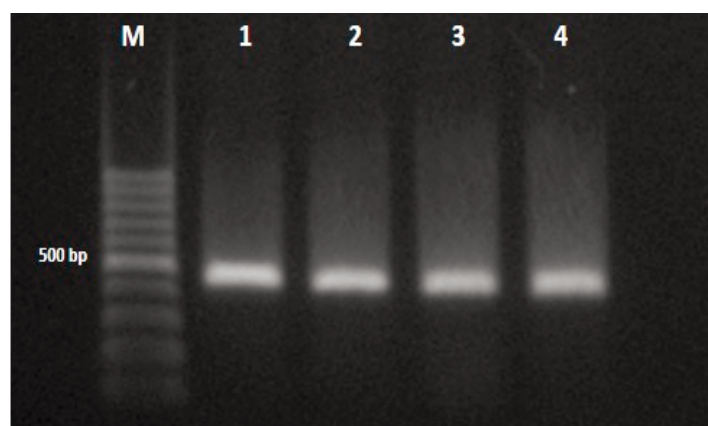

Figure 5. Molecular identification of PCN in East Java: molecular weight marker, 100 bp (M); line: Batu (1); Probolinggo (2); Magetan (3); Pasuruan (4); all were identified as *Globodera rostochiensis*

This primer amplified an approximately 434 bp DNA fragment for all the sample. However, PITSp4 primer for identified *G. pallida* nematode species did not display amplification. The PCR-based methods used in the present study shall play an important role for future surveys and routine identification *G. rostochiensis,* in order to contain the nematode to its current distribution area. In addition, the use of species-specific primers in PCR multiplex reactions, which allows the simultaneous identification of both species in the same sample, will eventually help to detect *G. pallida.*

CONCLUSION

PCN were found in potato planting areas in East Java: Batu, Pasuruan, Probolinggo, and Magetan. Pasuruan and Magetan are new area distributions of *G. rostochiensis* in East Java. Based on the morphological and molecular characters, the potato cyst nematode was determined as *G. rostochiensis.*

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