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Identification, Distribution and Genetic Diversity of the Golden Potato Cyst Nematode (*Globodera rostochiensis*) in Java Indonesia

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Abstract. Golden potato cyst nematode (*Globodera rostochiensis* (Wollenweber) Behrens) is a nematode species which has worldwide regulatory concern. This nematode causes serious economic problem of potato losses in Indonesia. To study the distribution and genetic diversity of *G. rostochiensis*, 30 soil samples were collected from Java island, Indonesia. Seventeen out of thirty samples were infected by *G. rostochiensis* obtained from Pangalengan West Java, Wonosobo Central Java, Banjarnegara Central Java, Probolinggo East Java and Malang East Java. PCR assay with specific primers for *G. rostochiensis* (PITSr3) produced a single band of 434 base pairs (bp) length from all samples. The internal transcribed spacer (ITS1 and ITS5) regions were subjected to direct sequencing to study the genetic diversity of these populations. Five representative isolates were sequenced and compared with the sequences which available in GenBank. The sequencing data showed that all of the five population represented the same species, *G. rostochiensis*. The identity of *G. rostochiensis* nucleotide sequences ranged from 98% to 100%.

INTRODUCTION

The plant-parasitic nematodes that limit potato production and quality are the potato cyst nematodes (PCN). PCN are the most destructive nematodes around the world [1, 2]. The PCN causes plant damage including chlorosis, stunting, and general poor growth [3]. Barker and Koenning [4] revealed that yield losses incurred in *G. rostochiensis*susceptible potato averaged 38% (12 - 76%), compared to 18.3% (12 - 34%) in resistant potato. Mulyadi*etal.*[5] found that PCN reduced yield ranging from 16.9 to 45.2% when potato was infected by PCN between 2 to 256 cyst in pottled potatoes.

The pale potato cyst nematode (*G. pallida* (Stone) Behrens) and the golden potato cyst nematode (*G. rostochiensis* (Wollenweber) Behrens) are known to parasitize potato in many potato-growing areas in European, Asian and American countries [6]. *G. rostochiensis* has a larger area of distribution than *G. pallida* and it is the most regulated quarantine nematode species in many countries [7]. This species grows in temperate and tropical climates [8]. In Indonesia, *G. rostochiensis* was identified for the first time in the potato-growing area in Malang, East Java, in March 2003 [9]. Furthermore, Mulyadi *etal.*[5] reported that *G. rostochiensis* was found in Central Java (Banjarnegara), East Java (Malang), and North Sumatra (Karo and Simalungun). As the distribution of seed expands, the distribution of *G. rostochiensis* expands as well.

Quarantine pest management requires identification of intra- and inter-specific differences at the species level [10, 11]. Understanding the diversity of *G. rostochiensis* based on the genetic diversity analysis has important

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implications for studying the evolutionary history and genetic structure of populations, and it may provide basic data for its control [12]. DNA sequence variation in the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA cistron can be used to identify many nematode taxa. The objective of this research was to update the distribution of *G. rostochiensis* in Java and to evaluate the genetic diversity of isolates collected from several regions in Java, Indonesia.

MATERIALS AND METHODS

Soil sampling for PCN: Thirty soil samples were collected from potato producing areas in Java, Indonesia from May 2013 to June 2014 (Fig. 1), covering different altitudes ranging from 1583 - 2073 m asl in three provinces.

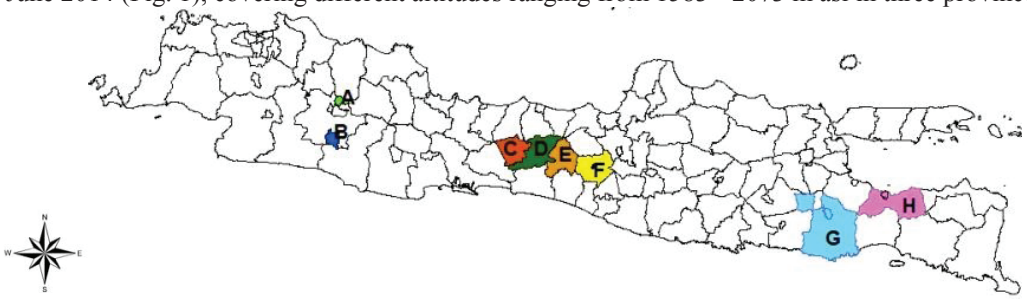

FIGURE 1. Districts in Java, Indonesia where soil samples were collected. The sites ofthecollection were potato producing areas in: A) Lembang; B) Pangalengan (West Java); C) Purbalingga; D) Banjarnegara; E) Wonosobo; F) Magelang (Central Java); G) Malang; and H) Probolinggo (East Java).

Soil samples were collected from the surrounding infected potato plants. In each location, ten sites of 5 x 5-m grid were selected. From each grid, 0 to 20-cm deep soil as much as 250 ml soil/samples were collected and mixed in a bucket to obtain a single composite sample [13]. Each composite sample was thoroughly mixed to obtain a homogenous sample and followed by taking 500 ml of soil for PCN cyst extraction [14, 15].

Analysis of physical and chemical properties of soils: The soil samples were sent to Soil Laboratory at Universitas Gadjah Mada (Yogyakarta 55281, Indonesia) for analysis of physical and chemical properties using procedures established in this laboratory. The analysis of physical properties included soil type and soil texture. The analysis of chemical properties included pH-H_2O, pH-KCl, C-N ratio and organic matter.

Cyst extraction: Mature cysts of PCN were isolated by flotation from the samples. Cysts were slightly dried and collected into the tube, then kept at 4 ºC.

DNA extraction: DNA extraction was performed following methods described by Fullaondo *et al.* [16] and Subbotin *et al.* [17] with some modifications. Twenty PCN cysts were crushed in Eppendorf tubes using plastic micro-pestles in 150 µl lysis buffer (125 mM KCl; 25 mM Tris-HCl, pH 8.0; 3.75 mM $MgCl_2$; 2.5 mM DTT; 1.125% Tween 20 and 0.025% gelatin) and 5 µl proteinase K (60 µg/ml). The mixture was incubated at 65°C for 1 h and then incubated at 95°C for 10 min. One volume (150 µl) of chloroform and isoamylalcohol (24: 1) was added. The homogenate was centrifuged at 11.000 rpm for 10 min. The supernatant was transferred to a new tube and 1/10 time (supernatant total volume) sodium acetate (3M NaOAc, pH 5.2) was added with the supernatant, and followed by dilution of absolute ethanol 2.5 times (250 µl) of supernatant total volume, and stored for 20 min at -20°C. This mixture was centrifuged at 11.000 rpm for 10 min. The pellet was precipitated with 500 ml ethanol, then centrifugated at 14.000 rpm for 10 min. The pellet was dried in a vacuum pump for 10 min and dissolved in 20 µl double distilled water.

Polymerase chain reaction: The fragment of ITS region was amplified using the species-specific primers PITSr3 (AGCGCAGACATGCCGCAA for *G. rostochiensis*, amplicon size 434 bp) and PITSp4 (ACAACAGCAATCGTCGAG for *G. pallida*, amplicon size 265 bp) [18] in combination with common primer ITS5 (GGAAGTAAAAGTCGTAACAAGG) [19] in a multiplex reaction. The PCR amplification was done in a 25µl 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. Possible contamination was checked by including negative controls (no DNA) in all amplifications. All PCR

products were run in electrophoresis at 75 V for 45 min in 1.6% agarose gels and subesequently were stained with ethidium bromide and visualized on UV illumination.

Genetic diversity of *G. rostochiensis* on ITS region: *G. rostochiensis* genetic diversity analysis was performed using DNA sequencing techniques. PCR product of *G. rostochiensis* isolate from each district which represented the area of West, Central, and East Java were sequenced. Fragments were sequenced using Applied Biosystem 3130 Genetic Analyzer. The sequence of isolates was first edited in Bioedit. The nucleotides were compared with equivalent sequences of *G. rostochiensis*. The eleven reference sequences of these entries from Genebank were aligned with the sequenced population samples from Java, using the ClustalW multiple alignment functions which available in BioEdit. The phylogenetic analysis was conducted by using sequence id matrix by using Bioedit program.

RESULTS AND DISCUSSION

Physical and chemical properties of soils: The soil type was andosol, soil texture was sand to dust, pH H_2O ranged from 4.53 – 5.70, pH KCl ranged from 4.03 – 5.38. The value of organic matter content ranged from 2.19 - 12.39 %, C/N ratios ranged between 9 and 77. The soil type in Banjarnegara Central Java, Malang and Probolinggo East Java was sand to clay with much sand composition. In that three locations were infected by *G. rostochiensis*. As Wallace [20] in Germany, there was a heavy infestation of *G. rostochiensis* in the sand soil, because the movement of zooparasitis higher on the sand soil than on the clay soil. In this sudy, *G. rostochiensis* were absence in Lembang West Java, Purbalingga and Magelang Central Java. The soil type in Lembang West Java and Magelang Central Java was clay to sand, which had low moisture. As Wallace [20] in moisture soil, the capability of egg and juvenile were decreased. In this study, nitrogen content in soil sample was not influenced to cyst presence. *G. rostochiensis* was not found in Lembang West Java soil sample with high nitrogen content (0.62%). Moreover, *G. rostochiensis* were found in Banjarnegara Central Java and Probolinggo East Java have low nitrogen content (0.07 and 0.14%). Haverkort [21] stated that in the infected soil there was no nitrogen content between 30 – 100 cm depth. In this study, we can interpret that soil type was influenced population density and distribution of *G. rostochiensis*, but pH and nitrogen content were not influenced.

Molecular identification of PCN: PCR analysis by using species-specific primers showed a DNA fragment size of 434 bp in all cyst isolates from West Java, Central Java and East Java, indicating that the species of PCN was *G. rostochiensis*. This amplification did not generate DNA fragment size of 265 bp that is specific genome segments for *G. pallida*. These findings suggest that all locations were infected only by one species of PCN, *G. rostochiensis*. Bulman and Marshall [19] stated that species-specific PCR primers differing at the 3' end, were designed from ITS 1 sequences (PITSr3 and PITSp4) in combination with primer ITS5 accurately distinguished Rol Lincoln and Pa2/3.

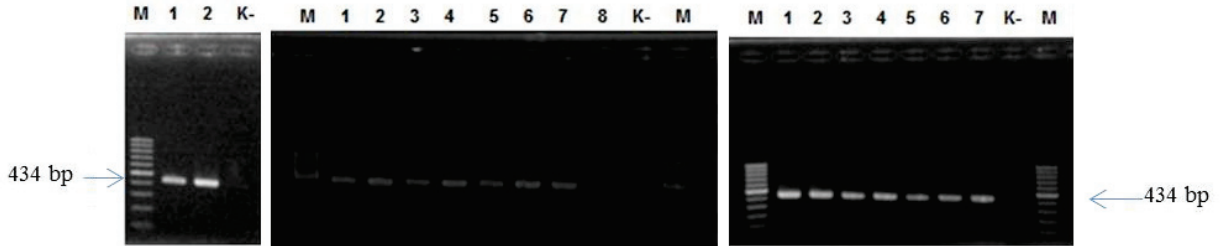

FIGURE 2.PCR product of PCN cyst with the primer specific for *Globodera rostochiensis* and *Globodera pallida*. A) line 1 and 2:Pangalengan West Java; B) line 1, 2, 3 and 4 :Wonosobo Central Java, line 5, 6, 7, and 8 : Banjarnegara Central Java; C) line 1, 2, and 3 : Probolinggo East Java, line 4, 5, 6, and 7 : Malang East Java. M = Marker 100 bp.

Globodera rostochiensis in Java, Indonesia: *G. rostochiensis* has spread in five out of eight districts in Java island (Table 1): Pangalengan (West Java), Wonosobo and Banjarnegara (Central Java), Probolinggo and Malang (East Java) at the altitude of 1,583 – 2,073 m asl. On those five districts, *G. rostochiensis* was found in 17 out of 30 sampling sites. It was the first report of *G. rostochiensis* which was found in soil samples originated from fields used for the production of seed potatoes in Probolinggo (East Java). *Globodera rostochiensis* was absence in Lembang (West Java), Purbalingga and Magelang (Central Java), although the physical and chemical properties of soil were suitable for the development of *G. rostochiensis*.

TABLE 1. Distribution of *Globodera rostochiensis* in potato producing areas in Java

No	District (Province)	Altitude (m asl)	Geographic position	Age of plant (day)	Soil temperature (°C)	*Globodera rostochiensis*
1	Lembang (West Java)	1,185	S : 06°, E : 107°	70	20	_
2	Pangalengan (West Java)	1,339 – 1,619	S : 07° E : 107°	70 - 90	20 - 24	+
3	Purbalingga (Central Java)	1,338 – 1,558	S : 07°, E : 109°	50 - 60	20 - 21	_
4	Banjarnegara (Central Java)	1,331 – 2,073	S : 07°, E : 109°	60 - 90	19 - 21	+
5	Wonosobo (Central Java)	1,330 – 2,034	S : 06° - 07°, E : 109°	50 - 80	18 - 21	+
6	Magelang (Central Java)	1,324 – 1,764	S : 07°, E : 110°	50 - 70	20 - 22	_
7	Malang (East Java)	1,676 – 1,853	S : 07°, E : 111° - 113°	55 - 80	18 - 19	+
8	Probolinggo (East Java)	1,503 – 1,726	S : 07°, E : 112° - 113°	65 - 70	19 - 24	+

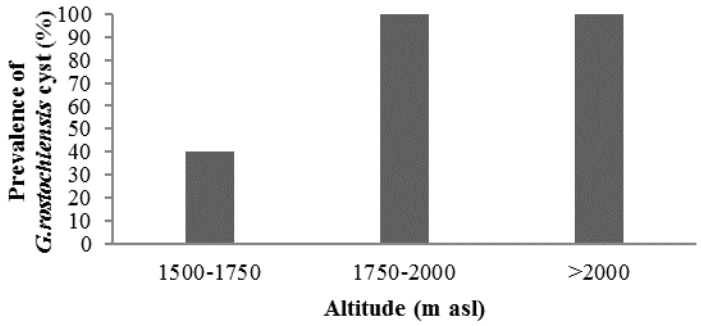

FIGURE 3. The prevalence of *Globodera rostochiensis* cysts in potato producing areas at different altitude in Java, Indonesia.

At the altitude ranging from1,500 to1,750m asl (18-24°C), the prevalence was 40%. The altitude increased the prevalence of *G. rostochiensis* and at the altitude of >1,750 m asl the prevalence was 100%. In that area, temperature ranged 18 – 21°C. Cyst density of *G. rostochiensis* in Java, Indonesia ranging from 0.008–3.428cysts/g soil with 492 eggs within. The highest density of *G. rostochiensis* was in Wonosobo (3.428 cyst/g soil). This condition was out of the tolerance limit of *G. rostochiensis* in some potato producing countries. Brodie *et al.* [22] stated that in Netherlands tolerance limit of *G. rostochiensis* was estimated at 1.5 eggs per g of soil, where as in Italy 1.2 – 2.1 eggs per g of soil. Therefor, *G. rostochiensis* is harmful but it has a limited spread so far in Java, Indonesia.The density of *G. rostochiensis* cysts in West and Central Java were higher than in East Java. Wallace [20] stated that agriculture method and distribution history of *G. rostochiensis* in Indonesia were influenced the current distribution. Potato was planted throughout the year in West and Central Java. Suwardiwijaya *et al.* [23] stated that Banjarnegara Central Java was the first location infected by *G. rostochiensis.* Moreover, the current infestation which established in that location with the highest density of *G. rostochiensis* was observed in Wonosobo (3.428 cyst/g soil).

G. rostochiensis cyst was distributed at temperature ranged from 18-24°C and the altitude ranging from 1,540 to 2,073 m asl. The soil temperature in all location was optimum temperature for *G. rostochiensis* development, so there was no significant response of cyst number to soil temperature. This in accordance to Trifonova [24] explaination, the optimum temperature for *G. rostochiensis* growth is ranging from 15.7 to 23.1°C. Wallace [20] stated that temperature is the key factor to influence nematode activities such as hatching, mobility, invasion, reproduction, development, and moulting.

Genetic Diversity of G. rostochiensis on ITS region: Our sequence analysis of ITS-rDNA revealed intraspecific variation in rDNA of *G. rostochiensis.* The sequence identity matrix of *G. rostochiensis* Java isolates and reference isolates from GenBank demonstrated that five isolates from Java were homologous with nucleotide sequence identity more than 98%. The minor differences observed for nucleotide sequence homology between Java isolates may suggest that all these isolates are closely related.

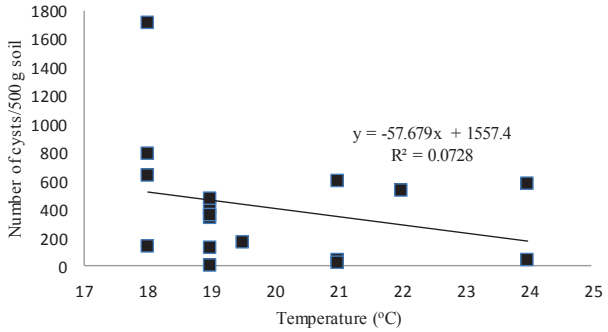

FIGURE 4. The relationship between the soil temperature and the number of *Globodera rostochiensis* cysts in Java, Indonesia

TABLE 2.*Globodera rostochiensis* found in Java and accession number of *G. rostochiensis*in GenBank sequences used for Phylogenetic comparison.

Isolate	Number of nucleotide	Geography position	Sources (Accession Number in GenBank)
Pangalengan	409	Ciarileu-Margamukti village, Pangalengan sub-district, Bandung Regency, West Java	This research
Wonosobo	420	Parikesit village, Kejajar sub-district Wonosobo regency, Central Java	This research
Banjarnegara	399	Bakal village Batur sub-district Banjarnegara regency, Central Java	This research
Probolinggo	399	Wonotoro Ngadas village, Sukapura sub-district, Probolinggo regency, East Java	This research
Malang	399	Jurang Kuali village, Bumiaji sub-district, Batu Malang regency, East Java	This research
GRRO 40 UK	1184	United Kingdom	Douda et al.(2014) (KJ409620)
1-Poland	4064	Poland	Nuwaczyk et al. (2008) (EU855120)
KEA049 Canada	434	Central Saanich, Vancouver Island, British Columbia-Canada	Rott et al. (2010) (HM584981)
MAR149 Canada	434	Central Saanich, Vancouver Island, British Columbia-Canada	Rott et al. (2010) (HM584981)
Gr1c Canada	911	Quebec-Canada	Yu et al.(2008) (EU517119)
10 Poland	1003	Poland	Nowaczyk et al.(2011) (JF907549)
Laguna pampa-12 Bolivia	1077	Andes-Bolivia	Grenier et al.(2009) (GU084809)
18 S Jepang	895	Jepang	Uehara et al.(2005) (AB207271)
2 Poland	1003	Poland	Nowaczyk et al.(2011) (JF907541)
010-116-Canada	1191	Quebec, field 2, Canada	Madani et al. (2008) (FJ212166)
Canada	753	Canada	Szalanski et al.(1997) AF016875)

Table 3. Similarity of *Globodera rostochiensis* (%) between Java isolates and reference isolates from GenBank

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Pangalengan	ID															
2	Wonosobo	99.7	ID														
3	Banjarnegara	99.7	100	ID													
4	Probolinggo	99.1	99.4	99.4	ID												
5	Malang	99.7	100	100	99.4												
6	GRRO40 UK	99.7	100	100	99.4	100	ID										
7	1-Poland	99.4	99.7	99.7	99.1	99.7	99.7	ID									
8	KEA049 Canada	99.4	99.7	99.7	99.1	99.7	99.7	100	ID								
9	MAR149 Canada	99.4	99.7	99.7	99.1	99.7	99.7	100	100	ID							
10	Gr1c Canada	99.7	100	100	99.4	100	100	99.7	99.7	99.7	ID						
11	10 Poland	99.4	99.7	99.7	99.1	99.7	99.7	100	100	100	99.7	ID					
12	Laguna Pampa 12Bolivia	98.3	98.6	98.6	99.1	98.6	98.6	98.3	98.3	98.3	98.6	98.3	ID				
13	Japan	99.1	99.4	99.4	100	99.4	99.4	99.1	99.1	99.1	99.4	99.1	99.1	ID			
14	2_Poland	99.1	99.4	99.4	100	99.4	99.4	99.1	99.1	99.1	99.4	99.1	99.1	100	ID		
15	010-116Canada	99.1	99.4	99.4	100	99.4	99.4	99.1	99.1	99.1	99.4	99.1	99.1	100	100	ID	
16	Canada	98.8	99.1	99.1	99.7	99.1	99.1	98.8	98.8	98.8	99.1	98.8	98.8	99.7	99.7	99.7	ID

The West Java isolate (Pangalengan) was 99% in similarity with isolates from Central Java and East Java. Isolates from Central Java (Wonosobo and Banjarnegara) were identic (100% identity). Malang isolate (East Java) is identic with Central Java isolates. Probolinggo isolate (East Java) is homolog with other isolates from Java. This result indicated that Java isolate has high genetic similarity and closely related, reducing the probability of increasing the new pathotype of *G. rostochiensis* in the future.

The West Java isolate (Pangalengan) was 99% similar to isolates from UK, Poland, Canada and Japan. Isolates from Central Java (Wonosobo and Banjarnegara) were identic (100% identity) with an isolate from Canada. Malang isolate (East Java) is identic with isolates from UK and Canada. Probolinggo isolate (East Java) homolog with isolates from Japan, Poland, and Canada. The potato seeds in Indonesia were imported from Dutch, Poland, England, Germany, Scotland, Canada and Australia [25]. These results suggested that there is a strong connection between the origin of potato seeds planted in Indonesia with the genetic similarity of *G. rostochiensis*.

Research to develop novel approaches for control of PCN will significantly enhance by a greater understanding of the molecular basis of the parasitic interaction and the key nematode henes required for this study [26]. The results of this study will facilitate management decisions regarding the presence of *G. rostochiensis*for Java-Indonesia potato farmer. The potato farmers and Agricultural Quarantine Institution should concentrate their management effort to control G. *rostochiensis*. Genetic variability in populations of *G. rostochiensis* is substantial. Therefore preserving the usefulness of host-plant resistance, cultural control measures, and available nematicides is a serious chalenge for practical nematology.

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