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Abstract

Global trading of plant materials, in combination with agricultural practices, may facilitate the spreading of cyst nematodes to so far non-infected areas. Recently Potato Cyst Nematode (PCN) was recognized to be present in Indonesia and both diversity and distribution require further study. Assessment of PCN populations was done by collecting soil samples, determination of morphological characteristics in combination with ITS rDNA and COI mtDNA sequencing. Thirty-seven soil samples were collected from potato fields in the Indonesia archipelago. The results showed the presence of *Globodera rostochiensis* in 22 out of 37 sampling fields, namely North Sumatra (6 fields), Central Java (12 fields), East Java (3 fields), and -for the first time- in Sulawesi (North Sulawesi) (1 field). The highest observed density was found in Banjarnegara (Central Java), *i.e*., 872 cysts 100 ml soil^{-1}. *Globodera pallida* was not recovered. Both ITS and COI characterisation of Indonesian PCN (*G. rostochiensis*) revealed the virtual absence of sequence variation as compared to most PCN from the rest of the world; the COI sequences were identical to the most common and mostly distributed haplotype around the world. Microsatellite genotyping indicated a higher genetic diversity for populations from East Java than for populations from North Sumatra, suggesting that cysts at the origin of populations in North Sumatra were coming from populations in East Java. These data on species identification, population density, genetic diversity, and distribution of potato cyst nematode over the Indonesian archipelago constitute the very basis for the design of environmentally-sound and effective PCN control strategies.

Keywords: *Globodera*, ITS, COI, microsatellite, phylogenetic tree.

Introduction

Cyst nematodes (*Heterodera* and *Globodera* spp.) are together with *Meloidogyne* spp. and *Pratylenchus* spp. included in the top three plant-parasitic nematodes based on economic and scientific importance (Jones et al. 2013). The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are quarantine pests that are associated with potatoes and some other Solanaceous species. *G. ellingtonae* Handoo, Carta, Skantar & Chitwood, 2012 (the Ellington potato cyst nematode) should be seen as a PCN. So far, its distribution seems to be restricted to the USA (Handoo et al. 2012) and in Argentina (Lax et al. 2014).

Globodera rostochiensis and *G. pallida* are known to cause losses that are estimated at about 9% of total potato production worldwide (Turner and Subbotin 2013). The nematodes can reduce tuber size, and infected roots are extensively branched (OEPP/EPPO 2017). To illustrate the economic damage, potatoes tuber yield loss was estimated to be more than 50% if 32 to 64 eggs g^{-1} soil of an Iranian population *G. rostochiensis* were inoculated without nematicide application under greenhouse conditions (Hajihassani et al. 2013). An inoculation pot experiment of an Indonesian population of *G. rostochiensis* estimated yield decrease of 17 to 45% after inoculation with 2 up to 256 cysts per pot (Mulyadi et al. 2005).

It is well known that PCN originates from the Andean region of South America and have accidentally been introduced into Europe and subsequently to the Americas, Africa, Asia, Australia, and New Zealand with infested tubers (Phillips 1989; Mugniéry and Phillips 2007). Despite the substantial crop losses due to PCN in Indonesia, which are estimated to be 30-90% based on limited farmer interviews in Batu-East Java (Hadisoeganda 2006), their actual distribution and diversity are not yet well known. PCN were for the first time detected in Indonesia on potato plants in Batu, East Java-Indonesia, and identified morphologically as *G. rostochiensis* (Mulyadi et al. 2003; Indarti et al. 2004). Their densities ranged from 1-2 cyst g^{-1} of soil for plants with moderate damage to 6-7 cysts g^{-1} of soil for severely affected plants (Indarti et al. 2004). Later, Lisnawita et al. (2012) identified *G. rostochiensis* in Bandung (West Java), Banjarnegara and Wonosobo (Central Java), and Batu (East Java) using ITS rDNA-based species-specific primers. In the same study, *G. pallida* was for the first and so far only time recorded in Banjarnegara and Wonosobo (Central Java). The presence of *G. rostochiensis* in Bandung (West Java) and Probolinggo (East Java) was confirmed by Nurjanah et al. (2016) using species-specific primers based on the internal transcribed spacer (ITS1 and ITS5) regions. Furthermore, Nugrahana et al. (2017) also reported *G. rostochiensis* in Magetan and Pasuruan (East Java), using species-specific primers targeting the ITS region.

Whereas species-specific primers are based on specific DNA motifs, and can be used for direct identification, molecular barcoding includes a sequencing step that would facilitate both species identification and an assessment of the intraspecific variation (Floyd et al. 2002; Castagnone-Sereno et al. 2011). ITS rRNA is a molecular barcode region for the diagnosis of *Heterodera* and *Globodera* spp. (Subbotin et al. 2001; Jones et al. 2011).

Recently, the mitochondrial COI gene has been used for the characterisation of several Cereal Cyst Nematodes (CCN) species, and the sequences proved to be a powerful tool for assessing intraspecific genetic patterns and phylogeography in cyst nematodes of the *Avenae* group (Subbotin et al. 2018). However, hitherto, molecular barcodes were found to contain only limited intraspecific variation to asses phylogeographical patterns of cyst nematodes. For example, the internal transcribed spacer region (ITS1-5.8S-ITS2) of the rRNA gene and D2-D3 expansion segments of the 28S rRNA appeared to be not informative in clarifying the origin of Canadian *G. rostochiensis* populations (Madani et al. 2010). The use of mitochondrial barcodes in the phylogeography of potato cyst nematodes was only recently explored by Subbotin et al. (2020). This study revealed a high haplotype diversity of *G. rostochiensis* in Bolivia, while *G. rostochiensis* from Europe and North America and other globally distributed populations of this species appeared to belong to a single COI or cytb haplotype.

Microsatellites, a tract of repetitive DNA in which certain DNA motifs are repeated, being highly polymorphic, co-dominant and generally neutral, are genetic markers widely used in population genetics (Selkoe and Toonen 2006; Schlötterer 2000; Jarne and Lagorda 1996). Microsatellites may be valuable for population genetic structure and progeny analyses in *Globodera* species (Thiéry and Mugniéry 2000). In *Globodera pallida*, microsatellites have previously been used successfully to investigate the origin of the European populations of this pest (Plantard et al. 2008), and to reveal the phylogeographical history and reduction of the allelic richness of Peruvian *G. pallida* populations (Picard et al. 2007). Microsatellite markers have also been developed for *G. rostochiensis* to reveal the phylogeographical history of *G. rostochiensis* populations in Canada (Boucher et al. 2013).

Given the large economic importance of cyst nematodes in Indonesia, identifying their current distribution, diversity, intraspecific variation, and population density are very important to define appropriate control methods and would help to restrict the spread of the pest. The aims of this study were: i) to give an overview of the distribution of PCN in Indonesia; ii) to provide a morphological and molecular, using sequences of ITS-rDNA and COI-mtDNA, characterisation of Indonesian PCN populations; iii) to characterise the intra-population genetic diversity and, investigate the genetic structure of Indonesian *G. rostochiensis* populations using microsatellite genotyping.

Materials and methods

Soil samples and nematode extraction

Soil samples were collected from 37 fields of potato crops in the Indonesian archipelagos (Table 1). In each field, ten plots of 5 x 5 m grid were selected surrounding infected potato plants and in each grid, a 250 ml soil sample was taken of the rhizosphere zone (0 to 20-cm depth). The individual samples of each plot were collected and mixed in a bucket to obtain a single composite sample (Southey 1974). Each composite sample was thoroughly mixed to get a homogenous sample, and a 500 ml subsample of soil was air-dried at 37 °C over two days for PCN cyst extraction (Been and Schomaker 2000; Reid and Pickup 2005; Nurjanah et al. 2016).

PCN was extracted from a subsample of 100 ml of dried soil using the Baunacke method (Baunacke 1922; OEPP/EPPO 2013), *i.e.*, dried cysts that float in water were decanted and collected on a 250 µm sieve. Then, the cysts were air-dried overnight and counted.

Morphological identification

Ten cysts were randomly selected from each field and identified based on the vulval plate and one juvenile of each cyst. Vulval plates were prepared following the method of Subbotin et al. (2010) after soaking dry cysts for a minimum of 15 min. Identification was based on the vulval plate fenestral diameter, the anus-fenestral edge distance, the Granek´s ratio (the anus-fenestral edge distance divided by fenestral diameter), and the number of cuticular ridges between anus and fenestra. Additionally, juveniles (J2) were characterised using body, stylet, tail and hyaline region length (OEPP/EPPO 2017; Subbotin et al. 2010) (Table 2).

DNA extraction, PCR and sequencing

Morphological vouchers were made from a heat-killed second stage juvenile from the cysts. They were examined, photographed, and measured in a temporary slide using an Olympus BX51 DIC Microscope (Olympus Optical), equipped with an Olympus C5060Wz camera. Subsequently, a single juvenile was picked from temporary slides and washed with double-distilled water for ten min in an embryo dish. The nematode was cut into 2-3 pieces using a scalpel and transferred into 20 µl of WLB (50 mM KCl; 10 mM Tris (pH 8.3); 2.5 mM $MgCl_2$; 0.45% NP-40 (Tergitol Sigma); 0.45% Tween-20) thereafter kept in freezer at -20 °C for at least 10 min. Before use, 1 µl Proteinase-K (1.2 mg ml^{-1}) was added and the sample was incubated into PCR thermocycler for 1 h at 65 °C and 10 min at 95 °C followed by centrifugation for 1 min at 20 800 *g* (Nguyen et al. 2019).

The supernatant (2 µl of extracted DNA) was taken as a template for PCR reaction and transferred to an Eppendorf tube containing 25 µl master mix of TopTaq DNA polymerase kit (Qiagen, Hilden, Germany) made of 17 µl ddH_2O, 2 µl $MgCl_2$, 2.5 µl of 10X buffer, 2.5 µl coralLoad, 0.5 µl (10 mM) dNTPs, 0.15 µl (10 µM) of forward primer, 0.15 µl (10 µM) of reverse primer, and 0.05 µl *Taq* Polymerase. The forward primer TW81(5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') were used in PCR for amplification of the ITS1-5.8-ITS2 or ITS1 regions (Tanha Maafi et al. 2003). The PCR amplification profile consisted of 94 °C for 4 min; 94 °C for 1 min in 35 cycles, 55 °C for 1.5 min, and 72 °C for 2 min, and adhered by a final step of 72 °C for 10 min. The primers JB3 (forward: 5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (reverse: 5'-TAAAGAAAGAACATAATGAAAATG-3') (Bowles et al. 1992; Hu et al. 2002) and the forward Het-coxiF (5'-TAGTTGATCGTAATTTTAATGG-3') and the reverse Het-coxiR (5'-CCTAAAACATAATGAAAATGWGC-3') primers (Subbotin et al. 2018) were used for amplification of the partial COI gene of mtDNA. The following thermal profile was taken for COI gene amplification: 94 °C for 4 min, followed 94 °C for 1 min in 40 cycles, 45 °C for 1 min, and 72 °C for 1 min 30 s, with an ultimate extension at 72 °C for 10 min. Five µl of the PCR products were separated on a 1% horizontal agarose electrophoresis at 135 V and stained with Biotium for 23 min. The remaining PCR product was stored at -20 °C until use.

The successful PCR reactions were enzymatically cleaned with 1 µl of EXO-fastAp mix (100 µl Exonuclease I (20 U μl^{-1} Thermo Fisher Scientific); 200 µl FastAp (1 U μl^{-1} Thermosensitive Alkaline Phosphatase); 30 µl buffer; 270 µl H_2O) for 15 min at 37 °C followed by 15 min at 85 °C and sequenced commercially by Macrogen Inc. (Europe) after adding 5µl (10 mM) of the used primers. The contigs were assembled using Geneious 10.1.3 (Biomatters, https: //www.geneious.com). New, unique, sequences were deposited in the GenBank database under accession numbers: MT270180, MT270100, MT270485, MT270444 (ITS) and MT240262 (COI).

PHYLOGENETIC ANALYSIS

Contig sequences were analysed with all available *G. rostochiensis* sequences available in GenBank and EPPO-QBank, and two *G. tabacum* sequences as an outgroup. Multiple alignments of ITS rDNA and COI mtDNA were made using MUSCLE (Edgar 2004) with default parameters and followed by manual trimming to the length of the shortest sequences.

Bayesian phylogenetic analysis (MrBayes 3.2.6; Huelsenbeck and Ronquist 2001) was carried out using the GTR+I+G model; analyses were run for 2×10^6 generations and Markov chains were sampled every 1000

generations and 20% of the converged runs were regarded as burn-in. Posterior probabilities (PP) were plotted on Bayesian 50% majority-rule consensus trees on their corresponding clades. All phylogenetic programs used were implemented in Geneious 10.1.3 (Biomatters, https: //www.geneious.com).

MICROSATELLITE GENOTYPING

Based on the number of available cysts, seven Indonesian *G. rostochiensis* populations were selected for microsatellite genotyping: five from North Sumatra (NRK-1, NRK-3, NRK-4, NRK-5, and NRK-6) and two from East Java (NRM-1 and NRM-2). To explore the genetic diversity at the intra-population level and the genetic structure among those *G. rostochiensis* populations, we used a set of 12 microsatellite markers developed by Boucher et al. (2013) and multiplexed in three panels: Gr50, Gp109, Gp126 and Gp135 (panel 1), Gr85, Gr96, Gp116 and Gp118 (panel 2) and Gr67, Gr75, Gr90 and Gr91 (panel 3).

Each population consisted of 40 second-stage juveniles (J2) from 40 distinct cysts, randomly chosen, were genotyped. DNA extractions were performed as described in Boucher et al. (2013). DNAs were diluted with a 1:2 dilution ratio, and 2 µl was used for the microsatellite genotyping. PCR multiplex was performed in 5 µl of working volume, containing 1X of Type-it Microsatellite PCR kit (Qiagen, Hilden, Germany) and 0.4 µM of each primer. Cycling settings are the same as those described by Boucher et al. (2013), *i.e*. an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 90 s and extension at 72 °C for 30 s, followed by a final extension at 60 °C for 30 min. PCR products were then diluted to 1:40 in sterile water, and 3 µl of this dilution was mixed with 0.05 µl of GeneScan 500 LIZ Size Standard (Applied Biosystems) and 5 µl of formamide (Applied Biosystems). Analyses of PCR products were conducted on an ABI Prism®3130xl sequencer (Applied Biosystems). Allele sizes were identified using the automatic calling and binning procedure of GeneMapper v4.1 (Applied Biosystems) and completed by a manual examination of peaks. To minimize the rate of genotyping errors, a second round of PCR and electrophoresis was performed.

MICROSATELLITE DATA ANALYSIS

Genetic diversity in each *G. rostochiensis* population was explored by the unbiased estimate of gene diversity (H_{nb}) according to Nei (1978) and the allelic richness for a reduced sample size (Ar). H_{nb} was computed using GENETIX 4.05.2 (Belkhir et al. 2004) and Ar using the rarefaction method implemented in POPULATIONS 1.2.32 (Langella 1999). For each population, deviation from random mating (F_{IS}) was computed using GENETIX and the statistical significances of F_{IS} were estimated using the allelic permutation method (10,000 permutations).

The differentiation coefficients between each pair of populations (F_{ST}) were computed using GENEPOP 4.5.1, according to Weir and Cockerham (1984), and their statistical significances were estimated by 5,000 random permutations of individuals among populations. A Bonferroni adjustment was applied to take into account multiple testing ($\alpha = 0.05$ was lowered to $\alpha = 0.0024$ for 21 comparisons).

The Bayesian clustering algorithms implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003) were run to explore the genetic structure of the seven Indonesian *G. rostochiensis* populations, on a reduced dataset free of any missing data (*i.e*., 250 individuals). According to the recommendations of Wang (2017), the alpha value was set to 0.143 (*i.e.*, 1/p, p is the number of populations) and the uncorrelated allele frequency model was used. The number of Markov Chain Monte Carlo (MCMC) repetitions was 3,000,000 and the initial burn-in period consisted of 1,000,000 iterations. The K value was set from 1 to 8 and twenty independent runs were performed for each K. We applied Structure Harvester Web ver.0.6.94 Earl and vonHoldt (2012) to determine the most likely number of clusters statistically determined using the ad-hoc Evanno statistic ΔK (Evanno et al. 2005).

DISTRIBUTION MAP

All available literature information of PCN incidences in Indonesia (Mulyadi et al. 2003, 2014; Indarti et al. 2004; Lisnawita et al. 2012; Nurjanah et al. 2016; Nugrahana et al. 2017 and Syafi'i et al. 2018) were integrated with data of the current study to create a distribution map of the PCN in the Indonesian archipelagos. The locations of the fields were estimated based on the description in the respective papers, *i.e.*, without GPS coordinates.

Results

THE DISTRIBUTION OF POTATO CYST NEMATODE IN INDONESIA

Globodera rostochiensis (Wollenweber, 1923) Skarbilovich, 1959 was detected in 22 of 37 sampled fields (60%) namely North Sumatra (6 fields), Central Java (12 fields), East Java (3 fields), and North Sulawesi (1 field) (Table. 1). Our results revealed the presence of *G. rostochiensis* in potato fields on 10 new locations in North Sumatra, Central and East Java, and North Sulawesi. The latter reveals, for the first time, the presence of *G. rostochiensis* in Sulawesi. The highest density of cyst (872 cysts per 100 ml soil^{-1}) (~2,616 eggs ml soil^{-1})[1] was observed in Dieng Kulon (Banjarnegara, Central Java) on potato cv. Tedjo MZ (Granola). PCN were apparently absent in West Nusa Tenggara (6 fields) and South Sulawesi (2 fields) and in seven of the 37 investigated fields (North Sumatera,

[1] Based on estimation of presence of eggs in the Dieng Kulon population. The average of eggs *Globodera* from Dieng Kulon = 300 eggs/cyst; therefore 872 cyst 100 ml^{-1} = 872 x ~300 eggs 100 ml soil^{-1} = ~2,616 eggs ml soil^{-1}

1 field; West Sumatra, 4 fields; West Java, 1 field, and North Sulawesi, 1 field) (Table 1). Most of the cyst nematode infestations were located at a relatively high elevation; all eight fields of above an altitude of 1,630 m were found to be positive except for Pattapang (South Sulawesi) on 1,759. Fields at a lower altitude, below 1,257, did not contain cyst nematodes except for Rurukan in Tomohon district (North Sulawesi) at 1,158 m. The age of the potato did not affect PCN recovery: cysts were found at all investigated sampling moments. The presence of cyst appeared to be related to the potato variety; PCN were detected, with a density of 8 to 872 (average 214) cysts 100 ml soil^{-1}, in 22 out of the 27 fields with potato cv. Granola (not in Kuta Rakyat (North Sumatera), West Sumatra and South Sulawesi), while not detected in the fields with the varieties Atlantic (7 fields), Cingkariang (2 fields) and Supejohn (1 field) (Table. 1). However, these varieties are also generally cultured on a lower altitude.

Current data of PCN distribution were combined with the previous distribution data (Mulyadi et al. 2003, 2014; Indarti et al. 2004; Lisnawita et al. 2012; Nurjanah et al. 2016; Nugrahana et al. 2017, and Syafi'i et al. 2018) into Figure 1.

SPECIES IDENTIFICATION

All obtained PCN nematodes (second-stage juveniles and cysts) were morphologically and morphometrically identified as *Globodera rostochiensis* (Fig. 2). Infective second-stage juveniles (J2s) were 385-508 µm long (measurements across all 17 populations) with a slightly C-shaped body after fixation, tapering at the posterior end (Fig. 2A). Anterior part with labial region slightly offset, anteriorly rounded; stylet well developed, 20-24 µm long with rounded basal knobs (Fig. 2B). Lateral field with four lines extending for the most body of the length (Fig. 2C). The tail of 38-56 µm long with a prominent hyaline tail part of 22-33 µm long, with a finely rounded to pointed terminus (Fig. 2D).

Eggs of *G. rostochiensis* were retained within the cyst (Fig. 2E), and the average number of eggs in each cyst was 300 based on Dieng Kulon-Banjarnegara populations. Cysts were tanned dark in colour, spherically shaped with protruded neck, and circumfenestrate without bullae (Fig. 2G). The number of ridges between the vulva and anus ranged from 16 to 28, and the Granek's ratio ranged from 3 to 7 (Fig. 2F; Table 2).

MOLECULAR CHARACTERISATION AND PHYLOGENETIC RELATIONSHIPS

Morphological identification was confirmed by ITS rDNA and COI mtDNA sequence data. Fifty-two ITS sequences of 783 to 1021 bp long were generated; the most different sequence with respect to the other Indonesian sequences was obtained from Lingga Julu in Karo-North Sumatra, *i.e.*, 0.73% (7 bp) difference. The resulting

alignment was 951 bp nucleotides long and contained 52 sequences of Indonesian populations, 74 sequences of GenBank, and two outgroup sequences. The resulting tree topology revealed that Indonesian sequences formed a maximally supported clade with other *G. rostochiensis* (Fig. 3). However, the position of Indonesian populations in respect to *G. rostochiensis* from several regions in the world, *i.e*., Asia, Middle East, North and South America, and Europe, was not resolved (Fig. 3). Indeed, the Indonesian sequences were identical or very similar to most other sequences in the world, with max 1.2% (11 bp) nucleotides differences with a population of Poland, EU855120.

Ninety-four COI sequences of 393 to 548 bp long were generated without any sequence differences. The resulting alignment of 442 bp nucleotide long contained 94 sequences of Indonesian populations, 40 sequences of GenBank and EPPO-Q-Bank, and two outgroups. The Indonesian sequences are also 100% identical to the European ones (The Netherlands, Germany, and Italy), and the rest of the world (Fig. 4). According to the resulting tree topology, the Indonesian sequences were in a maximally supported clade together with the common and globally distributed GrCOIA1 haplotype (Subbotin et al. 2020) (Fig. 4).

GENETIC FEATURES OF *G. ROSTOCHIENSIS* POPULATIONS

Using the set of 12 microsatellite markers developed by Boucher et al. (2013), we identified 25 alleles among the seven Indonesian *G. rostochiensis* populations. Two markers were monomorphic, Gr50 and Gr75, and for the ten other polymorphic loci, the number of alleles ranged from 2 to 4.

The genetic diversity was a little bit higher for populations from East Java than for populations from North Sumatra. H_{nb} ranged from 0.09 to 0.12, and Ar, estimated on a reduced sample of 27 individuals, ranging from 1.33 to 1.49 alleles per locus for populations from North Sumatra, whereas H_{nb} ranged from 0.12 to 0.18 and Ar from 1.39 to 1.64 for populations from East Java (Table 3). Among the seven populations, six were at the Hardy-Weinberg equilibrium, with F_{IS} not significantly different to zero, and the last one (NRK3) showed a significant heterozygote excess (Table 3).

GENETIC STRUCTURE OF INDONESIAN *G. ROSTOCHIENSIS* POPULATIONS

The matrix of pairwise F_{ST} between the seven Indonesian *G. rostochiensis* populations showed significant differences between populations from North Sumatra and East Java, F_{ST} values ranging from 0.052 to 0.270 (Table 4). Among populations from North Sumatra, F_{ST} was low and not significant (the highest value being 0.033-Table 4), and the genetic differentiation between both populations from East Java was significant (F_{ST} = 0.065-Table 4).

Accordingly, the Bayesian clustering analysis identified two genetic clusters, with individuals from North Sumatra mainly assigned to one cluster, and individuals from East Java mainly assigned to the other cluster (Fig. 5). Nevertheless, each population included individuals from both genetic clusters.

Discussion

Potato cyst nematodes have been shown to be present in the Indonesian archipelago since 2003 (Mulyadi et al. 2003). In various subsequent surveys, both *G. rostochiensis* and *G. pallida* have been found (see for instance Indarti et al. (2004), Nugrahana et al. (2017) and Syafi'i et al. (2018), and Lisnawita et al. (2012) for the two PCN species respectively. In the current study, we detected *G. rostochiensis* in most but not all relevant potato growing areas in Indonesia. The presence of *G. pallida* was not confirmed in the current study, even after sampling the fields where *G. pallida* had been found before. Our findings are in agreement with other following up studies in the same districts (Mulyadi et al. 2014; Nurjanah et al. 2016). As a result of these more recent surveys, the Indonesian quarantine status of *G. pallida* was changed from A2 to the A1.

Most likely, the absence of *G. pallida* in the Indonesia Archipelago its related to its preference for a lower soil temperature compared to *G. rostochiensis* (Jones et al. 2017). The optimal temperature for reproduction of *G. pallida* is lower than for *G. rostochiensis* (Kaczmarek et al. 2014; Jones et al. 2017), and unlike *G. pallida*, fluctuating diurnal heat stress from 17.5 to 32.5°C had no significant effect on the development of growing females of *G. rostochiensis* (Jones et al. 2017).

The potato cv. Tedjo MZ (Granola) resulted in the highest cyst infestation, up to 872 cysts in 100 ml soil^{-1}(~680 cysts 100 g^{-1})[2] in Banjarnegara, similar to Syafi'i et al. (2018) who also reported the highest population density, 131 cysts 100 g^{-1} soil, in Banjarnegara. Literature data and the results in the current study together, list PCN in 117 potato fields in the following districts, in highland areas with altitudes between 1,158-2,081 m: Pengalengan and Sindangkerta in West Java; Banjarnegara and Wonosobo in Central Java; Batu, Malang, Probolinggo, Magetan, and Pasuruan in East Java; Karo and Simalungun in North Sumatra; and Tomohon in North Sulawesi.

Although PCN is distributed in the most important Indonesian islands, *i.e.*, Java, Sumatra, and Sulawesi. West Sumatra, Nusa Tenggara, and South Sulawesi are still potentially free of PCN and it is crucial to prevent PCN from spreading to these potato production areas. In line with this, West Nusa Tenggara and South Sulawesi were suggested as seed potato production fields in eastern Indonesia (Dawson et al. 2011).

[2] Conversion dried soil in Dieng Kulon-Banjarnegara from ml to gram: 100 ml = 78 gram

For all analysed samples, morphological and molecular results, based on both ITS rDNA and COI mtDNA sequences, always agreed. The COI sequence of *G. rostochiensis* from Indonesia populations was identical to the *G. rostochiensis* haplotype GrCOIA1 (Subbotin et al. 2020), being the most common haplotype and globally mostly distributed haplotype. Hence, COI sequences are not informative to explore the intra-population genetic diversity and to speculate about gene flow among *G. rostochiensis* populations in Indonesia. Therefore, other markers, such as microsatellites, need to be used.

While heterozygote deficits have been previously highlighted, using microsatellite markers, for several cyst nematode species, *i.e*., for *Globodera pallida* (Picard et al. 2004), *Heterodera schachtii* (Plantard and Porte 2004), *G. tabacum* (Alenda et al. 2014), *H. glycines* (Wang et al. 2015), *H. avenae* (Wang et al. 2018) and *H. carotae* (Gautier et al. 2019), with one exception showing a heterozygote excess all-Indonesian *G. rostochiensis* populations showed no deviation from the Hardy-Weinberg equilibrium. It is consistent with results from Boucher et al. (2013), indicating that only three out of 15 populations showed a significant heterozygote deficit. The heterozygote deficit in cyst nematodes being attributed to the low active dispersal ability of juveniles, we have currently no hypothesis to explain this particular feature of *G. rostochiensis* populations. Investigations are needed to determine whether the mode of reproduction of *G. rostochiensis* may differ from the one of its sister species *G. pallida* and penalize mattings between siblings.

Because the genetic diversity was higher for populations from East Java than for populations from North Sumatra, we hypothesize that cysts at the origin of populations in North Sumatra were coming from populations in East Java. STRUCTURE results support this view, as individuals from both clusters were identified in each population. This hypothesis is congruent with data showing that *G. rostochiensis* was first recorded in East Java (Mulyadi et al. 2003). However, systematic sampling was only done recently and clear historical data regarding the distribution of *G. rostochiensis* are unavailable. Furthermore, seed potato from North Sumatra often originates from Java, and especially from East Java, because farmers prefer cultivars from East Java (Granola Kembang) above cultivars from West Java (Granola Lembang) (Dewantoro 2017).

One can also notice that strong gene flows seem to occur in North Sumatra compared to East Java. It can be explained by either the geographic proximity between the five North Sumatra populations compared to the geographic distance between the two East Java populations or the agronomic practices that allow more soil movements or exchange between the farmers in North Sumatra compared to East Java. However, data of the latter

are not available. Such an impact of the human organization of agricultural practices on the genetic structure of cyst nematode populations was also shown for the tobacco cyst nematode *G. tabacum* (Alenda et al. 2014).

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Human participants and/or animals

The present research did not involve any experimentation on humans or animals.

Informed consent

All the author certify that the work carried out in this research followed the principles of ethical and professional conduct have been followed. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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Table 1 The distribution of Potato Cyst Nematode (PCN) in Indonesia according to current study

Province	District	Fields	Latitude	Longitude	Altitude	*G. rostochiensis*	Cysts/ 100 ml	Plant growth (days)	Cultivars	Sample codes
North Sumatera	Karo	Cinta Rakyat	3.16333	98.49555	1362	+	136	60	Granola	NRK1
		Semangat*	3.17527	98.49444	1373	+	8	55	Granola	NRK2
		Gajah*	3.15333	98.47416	1314	+	78	55	Granola	NRK3
		Lingga Julu*	3.13555	98.47249	1256	+	147	60	Granola	NRK4
		Guru Singa*	3.19416	98.47861	1370	+	42	60	Granola	NRK5
		Suka Ndebi	3.19888	98.47500	1401	+	142	60	Granola	NRK6
		Kuta Rakyat	3.20750	98.41499	1381	-	0	60	Granola	NRK7
West Sumatera	Agam	Nagari Batu Batagak Jorong Simpang	-0.37365	100.38678	1197	-	0	52	Cingkariang	NRP1
		Nagari Kampung Batu Jorong Kampung Batu Utara	-0.37096	100.38059	1455	-	0	55	Granola	NRP2
	Solok	Nagari Jembatan Putih Padang Lambau	-0.98088	100.71755	1508	-	0	60	Granola	NRP3
	Tanah Datar	Nagari Tanung Alam Jorong Koto Laweh	-1.04456	100.78060	1176	-	0	30	Cingkariang	NRP4
West Java	Bandung	Cisondari	-7.13052	107.49628	1384	-	0	40	Atlantic	NRC
Central Java	Banjarnegara	Condong Campur	-7.22733	109.86334	1652	+	117	55	Granola	NRB1
		Bakal Buntu*	-7.28224	109.97797	1950	+	305	60	Granola	NRB2
		Karang Tengah**	-7.31235	109.76660	2037	+	207	60	Granola	NRB3
		Serang*	-7.42105	109.63268	1630	+	160	60	Granola	NRB4
		Legok Sayem*	-7.21977	109.77553	1370	+	35	55	Granola	NRB5
		Dieng Kulon-1	-7.20311	109.90293	2081	+	872	na	Tedjo MZ (Granola)	NRX3
		Dieng Kulon-2	-7.20281	109.90215	2079	+	575	na	Tedjo MZ (Granola)	NRX4
	Wonosobo	Tieng	-7.23916	109.94499	1766	+	232	85	Granola	NRW1
		Parikesit	-7.21583	109.92972	1930	+	110	90	Granola	NRW2
		Patak Banteng-1**	-7.20944	109.92527	1953	+	489	90	Muhzoto (Granola)	NRW3
		Patak Banteng-2**	-7.24371	109.94776	1983	+	342	na	Tedjo MZ (Granola)	NRX2
		Kejajar	-7.21111	109.92476	1507	+	81	na	Granola	NRX1
East Java	Batu	Sumber Brantas	-7.75328	112.53643	1867	+	301	65	Granola	NRM1
		Krajan*	-7.86503	112.55813	1500	+	211	50	Granola	NRM2
		Lemah Putih*	-7.77360	112.53643	1637	+	120	30	Granola	NRM3

West Nusa Tenggara	Lombok Timur	Sembalun Bumbung-Timba Gading 1	-8.35766	116.53305	1092	-	0	50	Atlantic	NRL1
		Sembalun Bumbung-Timba Gading 2	-8.36630	116.53296	1217	-	0	50	Atlantic	NRL2
		Sembalun Lawang	-8.54093	116.48853	1175	-	0	60	Atlantic	NRL3
		Sembalun Bumbung-Jorung 1	-8.38048	116.54224	1178	-	0	55	Atlantic	NRL4
		Sembalun Bumbung-Jorung 2	-8.38075	116.5402	1176	-	0	60	Atlantic	NRL5
		Sembalun Bumbung-Orong Brabas	-8.52840	116.58976	1217	-	0	50	Atlantic	NRL6
South Sulawesi	Gowa	Pattapang	-5.26638	119.92194	1759	-	0	60	Granola	NRG1
		Bulutana	-5.24472	119.8975	1442	-	0	60	Granola	NRG2
North Sulawesi	Tomohon	Rurukan*	1.34573	124.87083	1158	+	3 Juveniles	30	Granola	NRT
	Minahasa Selatan	Kakenturan Barat	0.78574	124.4664	1162	-	0	60	Supejohn	NRO

* New detection of *G. rostochiensis* in this field
** *G. pallida* was found here before (Lisnawita et al. 2012)
na, not available

1 **Table 2** Morphometrics of cysts and J2 of *Globodera rostochiensis* in Indonesia (*all measurements in µm*)

Populations	Cyst				Juvenile-2			
	Fenestral diameter	**Anus-fenestra distance**	**Granek's ratio**	**Number of ridges**	**Body length**	**Stylet**	**Tail**	**Hyaline region**
Karo								
Cinta Rakyat	18.6 ± 2.2(15.1-20.7)	81 ± 13.6(65-98)	4.4 ± 1.0(3.7-6.0)	21 ± 2.6(17-24)	419 ± 14.2(394-436)	22.0 ± 0.5(21.4-22.8)	45.8 ± 3.7(39.4-49.8)	25.8 ± 1.3(24.0-27.6)
	n = 5	n = 5	n = 5	n = 5	n = 10	n = 10	n=10	n = 10
Semangat	17.9 ± 2.2(16.1-21.0)	67 ± 12.7(51-80)	3.7 ± 0.8(3.1-4.8)	18.5 ± 1.9(17-21)	443 ± 29.1(417-491)	21.40 ± 0.9(19.9-22.2)	47.9 ± 2.4(45.9-51.4)	25.2 ± 1.9(23.2-27.9)
	n = 4	n = 4	n = 4	n = 4	n = 5	n = 5	n = 5	n = 5
Gajah	18.4 ± 1.0(17.4-19.4)	87 ± 19.3(71-114)	5.0 ± 1.0(4.1-6.1)	24 ± 4.0(20-28)	427 ± 17.8(400-461)	21.8 ± 0.8(20.6-22.7)	47.4 ± 5.3(37.8-55.9)	26.6 ± 3.5(22.9-32.5)
	n = 5	n = 5	n = 5	n = 3	n = 10	n = 10	n = 10	n = 10
Lingga Julu	18.9 ± 1.5(17.2-21.0)	76 ± 20.1(70-115)	4.0 ± 1.4(3.4-6.5)	18 ± 1.7(17-21)	431 ± 22.8(395-471)	21.2 ± 0.5(20.1-21.9)	48.8 ± 2.2(45.3-51.5)	25.6 ± 2.2(21.6-29.9)
	n = 7	n = 7	n = 7	n = 7	n = 10	n = 10	n = 10	n = 10
Guru Singa	21.0 ± 1.0(19.5-22.0)	73 ± 10.7(64-92)	3.6 ± 0.6(2.9-4.7)	21 ± 0.5(20-21)	465 ± 37.1(405-508)	21.6 ± 0.6(21.6-22.7)	47.9 ± 3.3(44.2-53.5)	25.6 ± 1.3(23.3-27.1)
	n = 6	n = 6	n = 6	n = 6	n = 10	n = 10	n = 10	n = 10
Suka Ndebi	19.7 ± 2.1(17.9-22.0)	73.4 ± 14.5(62-90)	3.7 ± 0.4(3.3-4.1)	20 ± 0.6(20-21)	454 ± 23.1(415-483)	21.6 ± 0.6(20.5-22.7)	48.5 ± 1.4(46.3-50.8)	25.5 ± 2.0(22.1-27.6)
	n = 3	n = 3	n = 3	n = 3	n = 9	n = 9	n = 9	n = 9
Banjarnegara								
Condong Campur	18.9 ± 2.1(16.9-21.6)	71 ± 15.7(55-90)	3.8 ± 1.1(2.7-5.3)	18 ± 2.2(17-22)	415 ± 19.9(392-436)	20.5 ± 0.3(20.3-20.9)	49.3 ± 2.3(46.8-51.7)	27.4 ± 2.8(23.2-30.5)
	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Bakal Buntu	18.4 ± 1.1(17.0-19.8)	67 ± 4.6(63-73)	3.6 ± 0.5(3.0-4.3)	17 ± 0.4(16-17)	428 ± 15.1(417-453)	22.0 ± 0.5(21.9-22.6)	48.7 ± 3.0(47.6-49.5)	27.8 ± 1.4(26.1-28.6)
	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Karang Tengah	18.2 ± 3.7(13.9-21.0)	80 ± 11.9(66-88)	5.0 ± 1.1(4.3-6.3)	19 ± 2.6(17-22)	473 ± 57.8(385-501)	21.7 ± 0.4(21.4-22.1)	49.3 ± 6.7(41.8-54.4)	27.3 ± 2.6(24.3-29.0)
	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3
Serang	17.9 ± 1.4(16.7-20.3)	66 ± 6.7(57-73)	3.7 ± 0.5(3.2-4.4)	18 ± 0.5(17-18)	417 ± 16.7(396-439)	21.9 ± 0.5(21.6-22.5)	48.8 ± 2.4(46.9-52.2)	28.0 ± 1.8(25.7-29.6)
	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Legok Sayem	17.2 ± 2.9(14.5-22.2)	61 ± 13.7(47-79)	3.5 ± 0.4(3.1-4.1)	18 ± 1.6(16-20)	413 ± 16.0(396-430)	22.3 ± 0.8(21.9-23.9)	45.0 ± 1.4(43.8-47.2)	25.5 ± 1.2(23.7-26.3)
	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5	n = 5
Wonosobo								
Tieng	20.5 ± 2.2(16.2-22.6)	77 ± 17.9(47-99)	3.4 ± 0.7(2.9-4.4)	19 ± 1.5(17-21)	419 ± 13.2(396-437)	21.6 ± 0.7(20.5-22.5)	48.4 ± 1.8(45.9-48.4)	27.1 ± 0.9(25.7-28.3)
	n = 8	n = 8	n = 8	n = 9	n = 8	n = 8	n = 8	n = 8

Parikesit	20.1 ± 2.4(16.4-22.5)	76 ± 19.8(52-117)	3.8 ± 0.8(2.7-5.2)	19 ± 1.9(17-22)	4448 ± 25.3(426-499)	21.8 ± 0.7(20.8-23.0)	49.1 ± 2.8(44.0-54.0)	26.7 ± 1.6(24.4-28.8)
	n = 8	n = 8	n = 8	n = 10	n = 9	n = 9	n = 9	n = 9
Patak Banteng-1	18.5 ± 3.5(15.8-22.4)	69 ± 23.2(45-91)	3.8 ± 1.3(2.7-5.2)	21 ± 1.2(17-22)	452 ± 35.6(412-501)	21.8 ± 0.6(20.4-22.7)	49.1 ± 2.7(45.2-53.6)	26.3 ± 2.7(21.7-29.7)
	n = 3	n = 3	n = 3	n = 4	n = 9	n = 9	n = 9	n = 9
Batu								
Sumber Brantas	17.6 ± 1.7(14.2-20.3)	68 ± 17.2(48-100)	3.8 ± 0.8(3.1-5.5)	18 ± 1.2(17-20)	430 ± 36.2(394-491)	21.6 ± 0.4(21.1-22.1)	46.5 ± 2.5(42.4-50.1)	24.9 ± 1.5(22.0-27.2)
	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10
Krajan	20.3 ± 1.6(17.5-21.7)	82 ± 23.9(53-118)	4.0 ± 1.0(2.7-5.4)	19 ± 2.4(16-23)	429 ± 21.3(402-448)	22.2 ± 0.6(21.2-23.0)	48.0 ± 3.8(43.0-53.3)	25.6 ± 2.0(23.1-29.2)
	n = 9	n = 9	n = 9	n = 9	n = 10	n = 10	n = 10	n = 10
Lemah Putih	18.8 ± 2.5(17.1-21.7)	60 ± 16.0(46-77)	3.1 ± 0.5(2.7-3.6)	18 ± 1.0(17-19)	426.9	22.4	47.7	27.2
	n = 3	n = 3	n = 3	n = 3	n = 1	n = 1	n = 1	n = 1

1

Table 3 Indonesian *G. rostochiensis* populations collected in North Sumatra (NRK1, NRK3, NRK4, NRK5 and NRK6) and in East Java (NRM1 and NRM2). For each of the seven populations, the table shows the number of successfully genotyped juveniles (N), the genetic diversity indices (H_{nb} and Ar) and the departure from Hardy-Weinberg equilibrium (F_{IS}).

Population	**N**	**H_{nb}**	**Ar**	***F_{IS}***
NRK1	38	0.117	1.486	0.011
NRK3	28	0.108	1.333	-0.242*****
NRK4	40	0.104	1.494	-0.014
NRK5	38	0.085	1.410	0.092
NRK6	39	0.098	1.333	-0.044
NRM1	40	0.124	1.390	-0.056
NRM2	38	0.175	1.641	-0.089

*F_{IS} values significantly different to zero

Table 4 Matrix of pairwise F_{ST} between the seven Indonesian *G. rostochiensis* populations sampled in North Sumatra (NRK1, NRK3, NRK4, NRK5 and NRK6) and in East Java (NRM1 and NRM2).

	NRK1	NRK3	NRK4	NRK5	NRK6	NRM1	NRM2
NRK1	0.0000						
NRK3	0.0199	0.0000					
NRK4	0.0002	0.0041	0.0000				
NRK5	0.0239	0.0328	0.0111	0.0000			
NRK6	0.0138	-0.0018	0.0006	0.0007	0.0000		
NRM1	0.2516*	0.1877*	0.2636*	0.2695*	0.2130*	0.0000	
NRM2	0.0756*	0.0440*	0.0818*	0.0805*	0.0519*	0.0649*	0.0000

*Significant F_{ST}

Fig. 1 The distribution of *Globodera rostochiensis* in Indonesia based on literature data and current study. Sampling locations of current study are enlarged (inserts). Red colors: sample positive for *G. rostochiensis*; green colors: sample negative for *G. rostochiensis*; star and cross symbols: current study; circle and square symbols: literature data).

Fig. 2 *Globodera rostochiensis*. Second-stage juvenile (A: Entire body; B: Lip region & stylet; C: Lateral fields; D: Tail; E: Egg; F: Vulval plates; G: Cysts).

Fig. 3 Bayesian 50% majority-rule consensus tree interfered from ITS rDNA sequences with the GTR+1+G substitution model. Bayesian posterior probabilities are given next to each node. *G. rostochiensis* populations from Indonesia are in bold.

Fig. 4 Bayesian 50% majority-rule consensus tree interfered from COI-*mt*DNA sequences with the GTR+1+G substitution model. Bayesian posterior probabilities are given next to each node. *G. rostochiensis* populations from Indonesia are in bold.

Fig. 5 Structure analysis of the 250 *G. rostochiensis* individuals (*i.e*. the dataset free of any missing data) coming from the seven Indonesian populations (NRK1, NRK3, NRK4, NRK5, NRK6, NRM1, and NRM2). This analysis identified K = 2 genetic clusters. Each vertical line represents an individual for which the genetic assignment is partitioned into two clusters. Vertical white dotted lines separate each of the seven populations.

Figure 1-Distribution map

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Figure 2-Globodera rostochiensis

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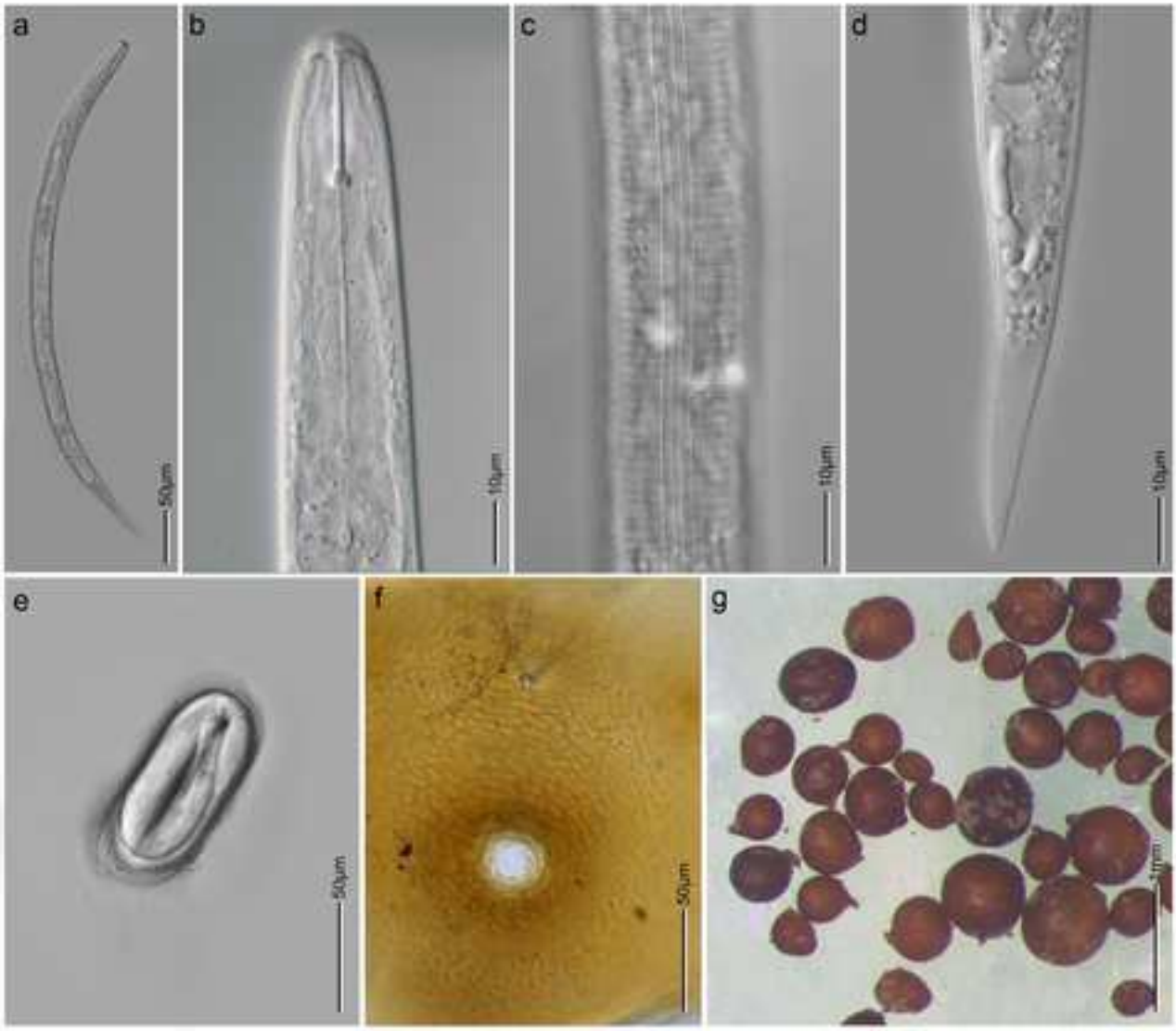

Figure 3-ITS Globodera rostochiensis tree

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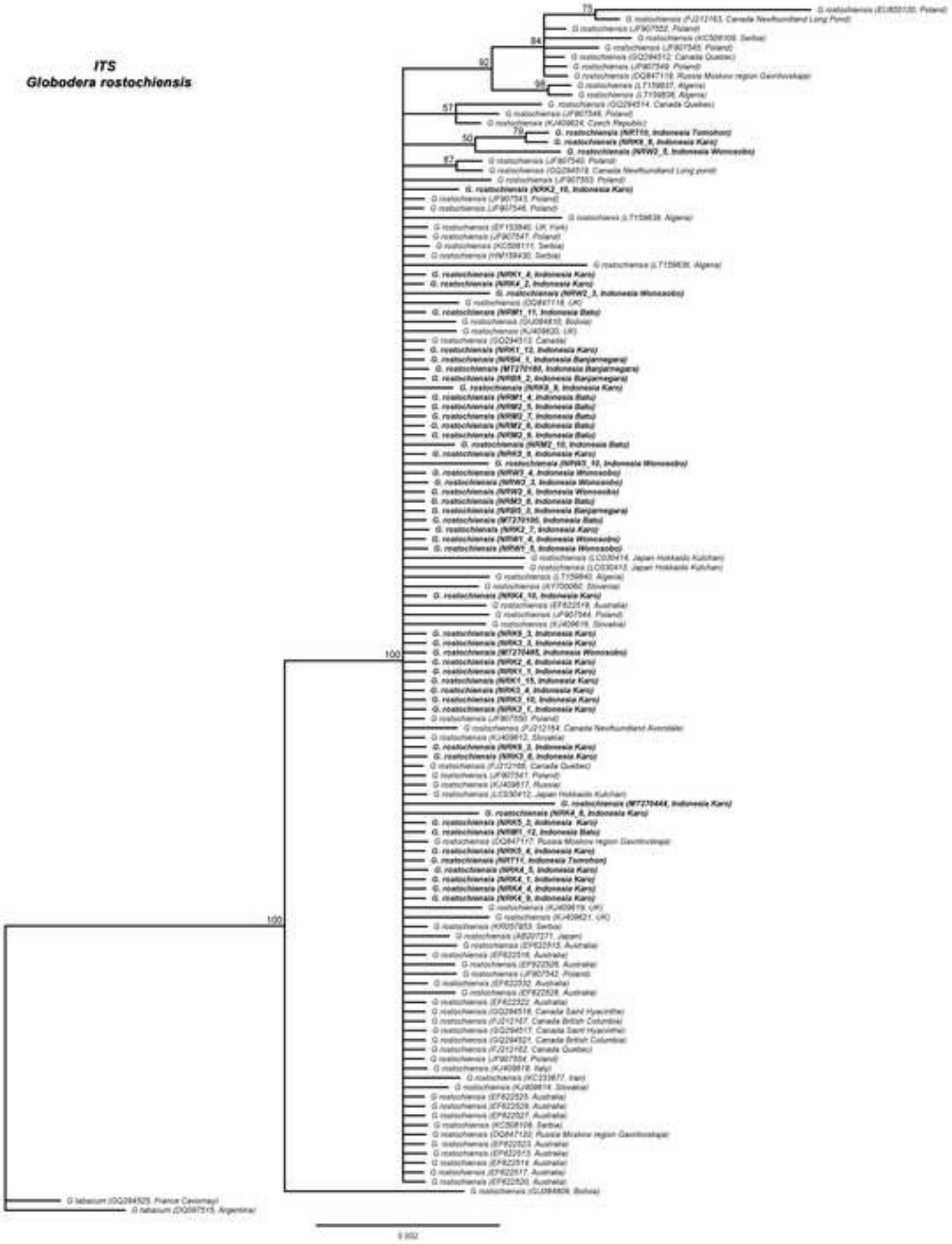

Figure 4-COI Globodera rostochiensis tree

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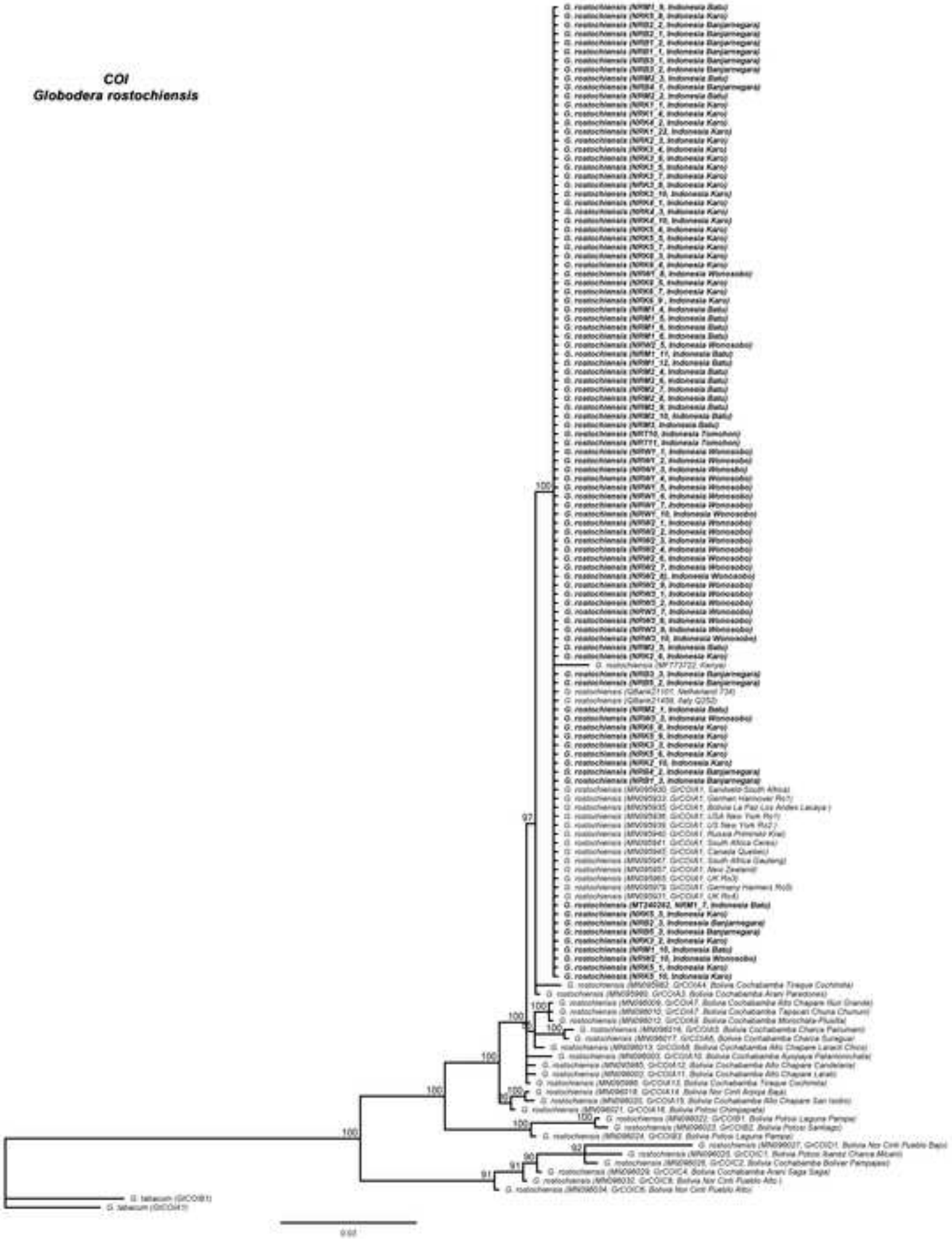

Figure 5-Microsatellite map

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