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Detection and molecular characteristics of *Meloidogyne graminicola* on rice in Java Indonesia based on ribosomal DNA gene

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ABSTRACT

Rice root-knot nematode, *Meloidogyne graminicola*, is widely distributed globally, including in Indonesia. However, information on molecular detection of *M. graminicola* was limited, especially in Java, as one of the rice-producing areas in Indonesia. This study aimed to detect and investigate the molecular characteristics of *M. graminicola* in Java, Indonesia. Population samples were collected purposively from three locations in West Java, Central Java, and East Java provinces. Species identification was performed using specific primers Mg-F3/Mg-R2. All samples were sequenced and subjected to phylogenetic analyses, genetic distances, and nucleotide sequence analysis across populations. The results confirmed that all samples from Java provinces were *M. graminicola* and several isolates were closely related to the isolate from the Philippines. The multiple sequence alignment displayed high single-nucleotide polymorphisms on the first 100 bp, then tended to be identical on the next 200 bp, and varied on the last 78 bp. This research highlighted that the *M. graminicola* population was detected on samples from the west-to-east region of Java, Indonesia. A rigorous study on the molecular characteristics of *M. graminicola* in rice grown in Java is crucial because this nematode poses a serious threat to the production of rice, a staple food in Indonesia.

1. INTRODUCTION

Rice root-knot nematodes (RKN), specifically *Meloidogyne graminicola,* are reportedly among the most devastating plant parasitic nematodes affecting rice production worldwide. The first trace of *M. graminicola* in Indonesia was found on rice in Yogyakarta Province in 1993 [1]. Previous research reported that *M. graminicola* infected over 100 plant species from cereals to grasses and dicotyledonous plants, but the most severe was on rice, causing up to 70% loss of harvest [2]. *Meloidogyne graminicola* is claimed to be responsible for 16% to 73% yield loss across various crop cultivation systems [3].

The symptom characteristics of *M. graminicola* appear below the ground in the form of hook-like galls on the roots and above the ground, such as stunted growth and chlorotic leaves [4,5]. Diagnosis based on the nematode symptom characteristics is currently challenging, so it is crucial to identify nematode species to obtain a more precise diagnosis and to manage the *M. graminicola* population. Traditional identification methods based on morphological and morphometric characteristics need specialized expertise, are time-consuming, and potentially subjective, especially for limited samples. In contrast, molecular identification is empirically more efficient, effective for larger samples, and feasible for studying phylogenetic relationships and nucleotide variation among populations [6,7]. Recent studies have increasingly focused on ribosomal DNA (rDNA) genes to identify species and phylogenetic analysis [8], assess phylogenetic relationships, analyze genetic variation, and study genome evolution [9]. The rDNA genes consist of conserved coding regions (e.g., 28S, 18S, and 5.8S subunit genes) and noncoding regions. The 18S gene, universally present in eukaryotes, is an effective marker for nematode barcoding and biodiversity surveys using the Sanger sequencing technique [10]. The 5.8S rDNA gene is more highly conserved than the 18S and 28S rDNA genes, making it ideal for phylogenetic studies at the species and population levels [11].

In Indonesia, morphological and morphometric detections of *M. graminicola* are reported in West Java [12] and South Sulawesi [13], but molecular detection and characteristic methods are understudied in Java Island. This study aimed to fill this gap by investigating the molecular characteristics of the *M. graminicola* population in Java, Indonesia, to obtain an accurate diagnosis of the disease for better preventive measures.

2. MATERIALS AND METHODS

2.1. Nematode Collection

Rice RKN samples were collected purposively from three rice fields in three Java provinces: Sarwadadi Village in Cirebon District, West

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Java (6°47'17.7"S+108°30'13.0"E; 6°47'16.1"S+108°30'11.6"E; 6°47'18.3"S 108°30'11.9"E), Tambaksari Village in Banyumas District, Central Java (7°24'04.1"S 109°15'34.3"E; 7°23'49.5"S 109°15'38.1"E; 7°23'51.6"S 109°15'37.3"E), and Wonorejo Village in Tulungagung District, East Java (8°05'33.8"S 111°56'15.7"E; 8°05'34.0"S 111°56'14.1"E; 8°05'33.9"S 111°56'16.4"E). The total sampling site was nine fields as three fields of each province. Symptomatic plants (stunted growth and clumped distribution) were sampled for each field, three plants per field. To obtain the DNA sequence, three individuals (one J2 per plant) were pooled of each field for analysis. The nematode population was obtained by dissecting the root for further DNA extraction.

2.2. Molecular Identification

DNA was extracted from three juveniles per rice field site with a method described by Morindya *et al.* [14] using a commercial kit (GeneAidTM Tissued/Blood DNA Mini Kit). The final DNA template was subjected to polymerase chain reaction (PCR) amplification as described by Htay *et al.* [15], with specific primers of *M. graminicola* Mg-F3 (5`-TTATCGCATCATTTTATTTG-3`) / Mg-R2 (5`-CGCTTTGTTAGAAAATGACCCT-3`) and an amplified target fragment of 378 bp of rDNA sequence on the 5.8S region. Exactly 10 µl of the PCR mixture (5 µl of MyTaq HS Red Mix Bioline, 1 µl of nuclease-free water, 1 µl of each forward and reverse primer at 0.2 µM concentration, and 2 µl of DNA template) was incubated on the PCR

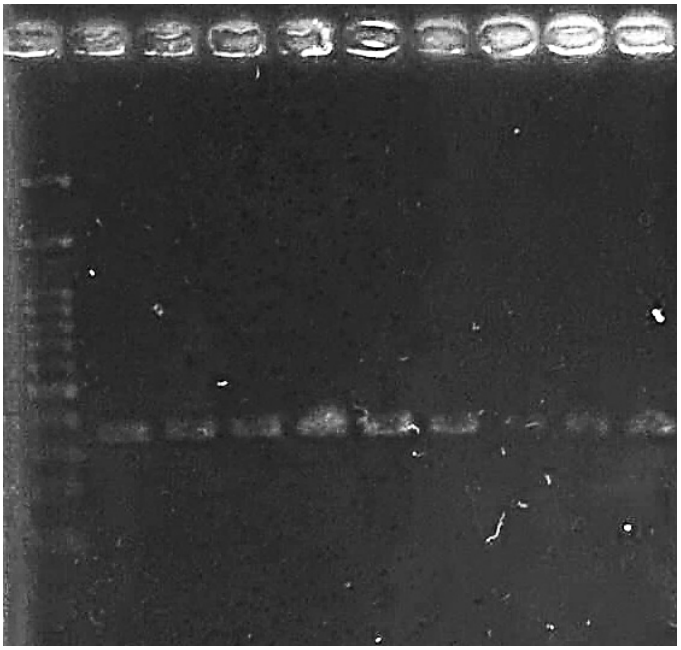

Figure 1. Electrophoresis of PCR products amplified with specific primer of *M. graminicola* Mg-F3/Mg-R2. M: 1 Kb DNA marker. Lanes 1–3: West Java samples, 4–6: Central Java samples, 7–9: East Java samples.

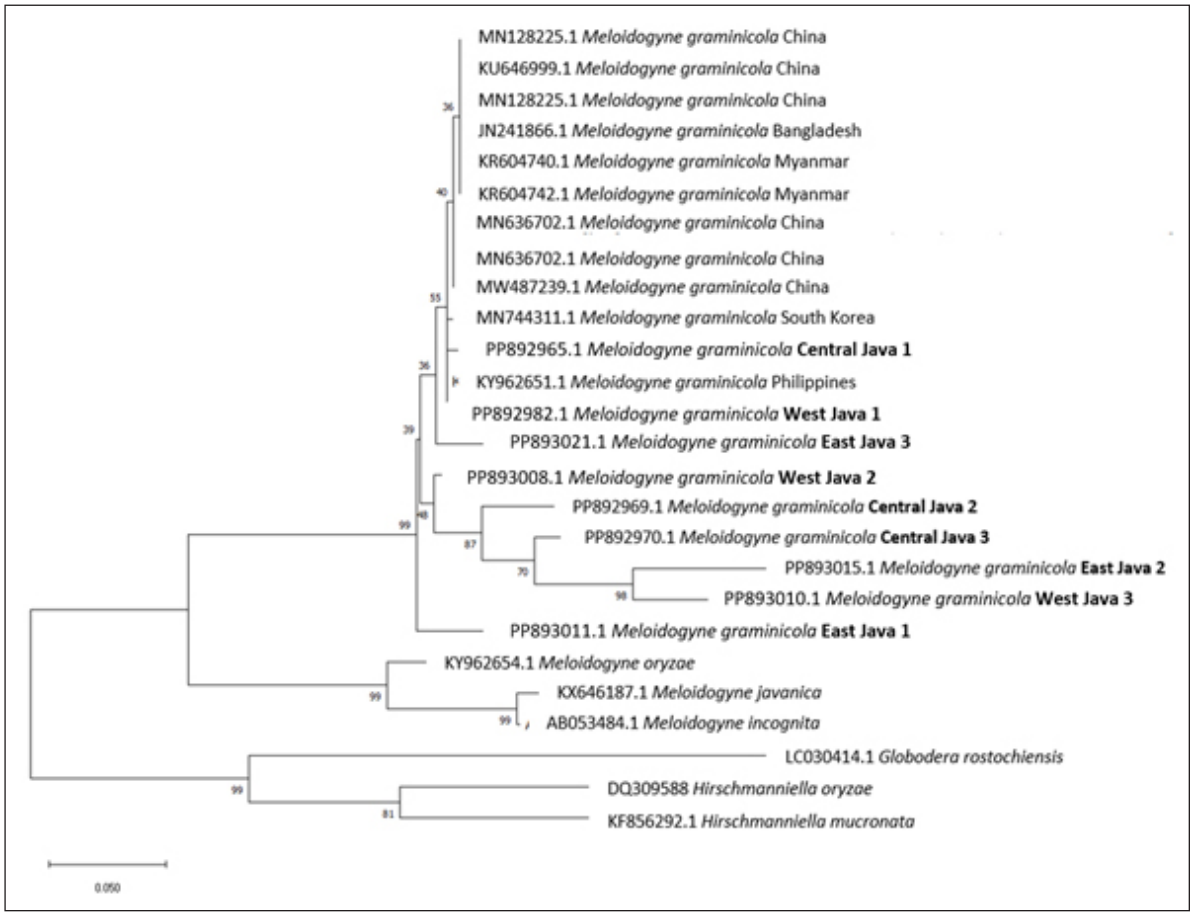

Figure 2. Phylogenetic tree of *M. graminicola* population in Java. The evolutionary distance was measured using maximum likelihood method and Kimura 2-parameter with evolutionarily invariable component (K2+I) of tree model.

machine and run for initial denaturation at 94°C for 2 minutes. Then, 35 cycles were performed: denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 60 seconds. The final synthesis was carried out at 68°C for 5 minutes, then 4°C for the final temperature. All PCR products were then separated by electrophoresis on a 2% agarose gel and 0.1% FloroSafe DNA Stain (1st BASE FloroSafe DNA Stain BIO-5170) at 0.01% v/v at 100 V for 25 minutes and then visualized in the UV transilluminator. The amplified products were sequenced using Sanger sequencing at the Integrated Laboratory for Research and Testing, Universitas Gadjah Mada, and the sequences were submitted to GenBank to obtain the accession numbers.

2.3. Data Analyses

All DNA sequences were aligned and trimmed using ClustalW on BioEdit software (version 7.2). A phylogenetic tree was constructed in the Molecular Evolutionary Genetic Analysis (MEGA) software (version 11).

3. RESULTS

Molecular detection using the specific primer Mg-F3/Mg-R2 amplified a specific band of 378 bp in nine samples of the Java population (Fig. 1). Nucleotide sequence data of this study were identified as *M. graminicola,* deposited on GenBank with the accession numbers for samples from West Java (PP892982.1; PP893008.1; PP893010.1), Central Java (PP892965.1; PP892969.1; PP892970.1), and East Java (PP893011.1; PP893015.1; PP893021.1).

3.1. Phylogenetic Relationship

We constructed the phylogenetic tree using the maximum likelihood method and the Kimura 2-parameter model with invariant sites (K2 + I) and performed 1,000 bootstrap replicates. The analysis used all sites, treating gaps as missing data. The phylogenetic tree of *M. graminicola* indicated that all isolates from Java were in the same clade as those from China, Myanmar, Bangladesh, South Korea, and the Philippines. Most isolates formed a group with the Philippines and South Korea, while those from China, Bangladesh, and Myanmar were in a separate group. Additionally, several ingroup genera with different species, *Meloidogyne oryzae, Meloidogyne incognita,* and *Meloidogyne javanica,* cluster in a different clade with *M. graminicola.* The other clades, *Globodera rostochiensis, Hirschmanniella oryzae,* and *Hirschmanniella mucronata,* act as an outgroup from different genera (Fig. 2).

The genetic distance analysis revealed a similar trend to that observed in the phylogenetic tree, in which smaller genetic distance values indicate closer relationships among the isolates [16]. Figure 2 shows that several isolates from Java (ID14; ID15; ID19; ID20) were closely related to those from the Philippines (ID5), with a distance ranging from 0.00 to 0.04. The lowest distance among isolates from Java and references was between the isolate from West Java 3 (ID^{20}) and the Philippines (ID^{5}), while the highest distance was from isolates of East Java 2 (ID^{16}) to those of Myanmar, Bangladesh, and China (ID^{6}-ID^{11}) (Fig. 3).

3.2. Multiple Alignment Analysis

The sequences of all populations in Java were aligned using MEGA v.11, and the final trimmed alignment length was 378 bp. Multiple-sequence alignment of *M. graminicola* among Java populations showed a high variation on the first 100 bp, became more identical on the next 200 bp, and varied on the last 69 bp (Fig. S1). Among these samples, there were 69 variable sites and nine distinct haplotypes. Single-nucleotide polymorphisms (SNPs) were counted by comparing

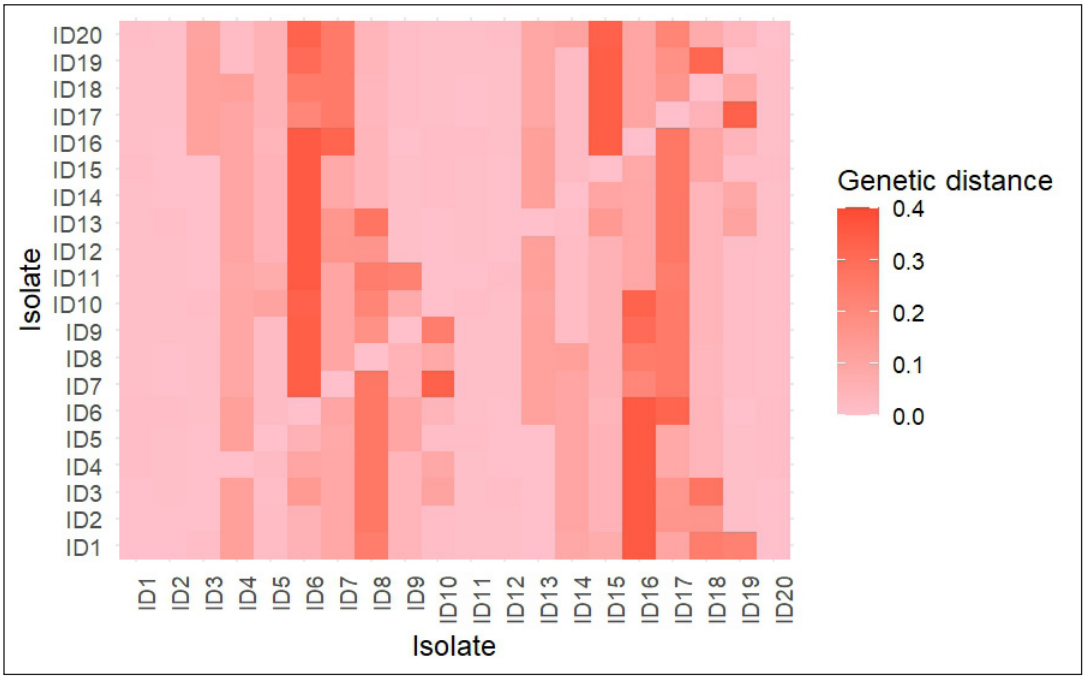

Figure 3. Genetic distance among isolates. ID1 OP941600.1 China; ID2 MW487239.1 China; ID3 MN636702.1 China; ID4 MN744311.1 South Korea; ID5 KY962651.1 Philippines; ID6 KR604742.1 Myanmar; ID7 KR604740.1 Myanmar; ID8 JN241866.1 Bangladesh; ID9 MN521459.1 China; ID10 MN128225.1 China; ID11 KU646999.1 China; ID12 PP892969.1 Central Java 2; ID13 PP892970.1 Central Java 3; ID14 PP892965.1 Central Java 1; ID15 PP893021.1 East Java 3; ID16 PP893015.1 East Java 2; ID17 PP893011.1 East Java 1; ID18 PP893010.1 West Java 3; ID19 PP893008.1 West Java 2; ID20 PP892982.1 West Java 1.

all sequences to the first sequence in the alignment, which was used as the reference sequence (West Java 1). The variable sites of the *M. graminicola* population in West Java, Central Java, and East Java were 31, 25, and 56, respectively, with three distinct haplotypes for each population. SNPs for the population across these locations were determined by comparing all sequences in each location to the first sequence in the alignment of each location, namely West Java 1, Central Java 1, and East Java 1 (Figs. S2–4).

4. DISCUSSION

Meloidogyne graminicola is among the most destructive plant-parasitic nematodes of rice and has been reported in Indonesia, including the sampled Java locations. where the infestation is reported in sampled locations from west to east Java. This study confirmed the rapid detection using species-specific primers for *M. graminicola.* A previous study on RKN detection infesting onion samples using the same primer as this study also showed the same target band for *M. graminicola* detection [17]. Phylogenetic analysis revealed that nine samples of nematodes from rice samples in Java were grouped with *M. graminicola* reference sequences with the highest support branch (99%). It shows a significant difference from *M. oryzae* as the same primary host on rice, as well as other prevalent rice RKNs, such as *M. incognita* and *M. javanica,* on different host species [18–20]. A recent study used a novel method that enables the detection of *M. graminicola* and screening of *M. oryzae* in the same population [21].

The global prevalence of *M. graminicola* on rice-producing countries is evident in Asia, including Bangladesh, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Pakistan, Philippines, Singapore, Sri Lanka, Thailand, and Vietnam [22]. Based on the genetic distance, *M. graminicola* of several Indonesian isolates were closely related to the Philippines, in which *M. graminicola* is the major threat to rice production and has been molecularly detected by ribosomal and mitochondrial DNA genes [23] and on weedy rice roots using the Sequence Characterized Amplified Region marker [24].

This study revealed a nucleotide variation of the DNA sequence within the *M. graminicola* population in Java on the small locus of the ribosomal gene (Figs. S1–4). Although this study found large SNPs in *M. graminicola* population in Java, we cannot claim that this population is diverse because the sample size determined by the small specific rDNA gene was small, namely, less than 1% of the total *M. graminicola* genome, approximately 41.5 Mb [25]. This study reported multi-province molecular confirmation in Java and new sequences deposited. Despite the limitation on the number of samples due to undetected symptoms in the field, this study showed an updated distribution of *M. graminicola* infection, particularly in Java, Indonesia. Future studies can probe into *M. graminicola* populations in Java using whole genome sequencing to evaluate the genetic variation between populations.

5. CONCLUSION

Rice RKN infestation in Java has been detected in sampled locations in West Java, Central Java, and East Java, and molecularly identified as *M. graminicola.* Based on DNA sequences on short loci of ribosomal genes, high nucleotide variations were demonstrated across the population. Further study was required to assess the genetic variation of *M. graminicola* in Java.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. DISCLOSURE

A preprint version of this manuscript is available at Research Square https://www.researchsquare.com/article/rs-5629720/v1.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

14. SUPPLEMENTARY MATERIAL

The supplementary material can be accessed at the journal's website: https://jabonline.in/admin/php/uploadss/1482_pdf.pdf

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