

Evaluation of multiple PCR techniques for rapid detection of *Meloidogyne* spp. directly from soil

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ABSTRACT

Meloidogyne spp. (root-knot nematodes; RKNs) are parasitic nematodes that cause significant damage to diverse economically important plants such as various herbaceous species. However, research focusing on direct soil-based detection remains limited research updates, as identifying these pathogens directly from complex soil matrices presents significant technical challenges. This study developed and optimized rapid, specific protocols for end-point polymerase chain reaction (PCR), multiplex PCR, and real-time PCR (qPCR) for the direct detection of *M. incognita* and *M. javanica* in soil. Nematodes from infected roots were identified morphologically, then spiked into soil for DNA extraction. Species-specific primers (Inc-K14-F/Inc-K14-R for *M. incognita* and Fjav/Rjav for *M. javanica*) were used for target detection, with PCR annealing optimized at 60-64 °C (30 s). In end-point PCR, the minimum of detection for *M. incognita* was 6 adult females g⁻¹ soil and 8 second-stage juveniles (J2) g⁻¹ soil. For *M. javanica*, the sensitivity was 4 adult females and 4 J2 g⁻¹ soil. Furthermore, the multiplex PCR protocol developed in this study successfully identified both species in a single reaction, with a minimum of detection of 6 adult females individuals and 20 J2 g⁻¹ soil for each species. Additionally, a qPCR assay was successfully developed using SYBR Green qPCR and conditions adapted from the multiplex PCR for *M. javanica*, achieving a minimum of detection of 4 adult females and 8 J2 g⁻¹ soil. Overall, these molecular protocols provide rapid and accurate tools for nematode identification, helping to reduce crop yield losses.

Key words: Detection sensitivity, plant disease detection, plant protection, root-knot disease, soil-borne pathogen.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are considered the most economically significant group of plant-parasitic nematodes worldwide (Rusique et al., 2023). Over 100 species have been identified and classified within this genus (Mitiku, 2018; Rusique et al., 2023). The most significant *Meloidogyne* spp. are the tropical *M. arenaria*, *M. incognita*, and *M. javanica*, as well as the temperate *M. hapla* (Rusique et al., 2023). Annual global crop losses caused by plant-parasitic nematodes are estimated at over USD 157 billion, accounting for 12.3% yield reduction in susceptible crops (Rehak Biondić et al., 2025). These pathogens parasitize a wide range of over 5500 species (Evlice, 2021). As obligate parasites, they infect an extensive variety of hosts, from woody perennials to various monocotyledonous and dicotyledonous herbaceous plants (Sikandar et al., 2020). The formation of giant galls in the roots is the most characteristic sign of infection by major *Meloidogyne* spp. (Saud et al., 2024). Infected

hosts usually show symptoms such as stunted growth, leaf yellowing, wilting, and plant death (Sikandar et al., 2020). In Thailand, *Meloidogyne* spp. is a major problem for many important crops (Homkajorn et al., 2023; Boonrin et al., 2024; Jojun et al., 2025). To effectively mitigate this continuous threat, rapid and accurate species identification is necessary to plan effective control strategies and select resistant varieties.

Conventional nematode extraction methods, including the sieving technique and the Baermann funnel technique (Zhao and Wang, 2022), are commonly used as initial steps to extract nematodes from soil samples. However, they are constrained, time-consuming and depend on the nematodes being alive and moving. This often leads to inconsistent results and the loss of inactive specimens (Saikai et al., 2021). Following extraction, accurate diagnosis of root-knot disease requires precise species identification, which is critical for developing effective and sustainable management protocols (Rusique et al., 2023). This has traditionally relied on morphological characterization, such as the perineal patterns of adult females (Phanbut et al., 2024; Jojun et al., 2025; Nishantha et al., 2025). However, this method has many limits. It is time-consuming and requires a high level of expertise. Also, diagnosis is often confusing because of overlapping features between species, particularly in the J2 stage (Rehak Biondić et al., 2025).

Species-specific polymerase chain reaction (PCR) primers are highly effective at differentiating major root-knot nematode species (Rashidifard et al., 2019). To date, a diverse array of molecular diagnostic tools utilizing these primers has been developed. In the early stages of nematode diagnostics, sequence-characterized amplified region (SCAR) markers have been successfully implemented to identify various *Meloidogyne* species (Aydinli and Mennan, 2016; Resquín-Romero et al., 2023) and restriction fragment length polymorphism (RFLP) has served as the foundational method for genetic differentiation (Kamran et al., 2019). In recent years, the scientific community has gravitated toward more rapid and sensitive platforms, particularly real-time PCR (qPCR) (Hodson et al., 2023) and loop-mediated isothermal amplification (LAMP), which offer enhanced efficiency in routine diagnostics (Omer et al., 2022). Furthermore, multiplex PCR was developed to enhance diagnostic throughput, enabling the simultaneous detection of multiple species in a single reaction (Devran et al., 2018). The practical value of this approach is further supported by findings that approximately 29% of infested areas contain multiple species at once (Marquez and Hajihassani, 2023).

Despite these advancements, molecular-based soil testing remains relatively underutilized. This represents a significant knowledge gap, considering that J2 in the soil serves as the primary infectious agents (Wendimu, 2021). However, the robustness of molecular tools for soil analysis has been validated in recent literature. For instance, qPCR has demonstrated superior sensitivity compared to traditional PCR methods (He et al., 2021). Furthermore, contemporary qPCR protocols have been optimized to quantify nematode populations accurately, even within complex environmental matrices (Hodson et al., 2023). However, the application of PCR for direct soil-based nematode detection still faces practical limitations and requires further methodological updates. To get accurate results, molecular tools must be optimized for local nematode populations, as genetic traits can change from one region to another (Lopes et al., 2019; Zhou et al., 2024). Consequently, this study aims to optimize conditions and evaluate the sensitivity of end-point PCR, multiplex PCR, and qPCR protocols for the rapid detection and quantification of both *M. incognita* and *M. javanica* directly from soil samples, using easily dissected adult females to confirm that DNA extraction and PCR work effectively despite soil inhibitors, and infective juveniles (J2) as field-representative targets.

MATERIALS AND METHODS

Isolation of *Meloidogyne* spp.

Root-knot disease samples were collected from celery (*Apium graveolens* L.) from Phayao Province, Thailand, and lettuce (*Lactuca sativa* L.) from Chiang Mai Province, Thailand. After microscopic examination, tissues were softened by soaking in 4 °C water overnight to facilitate isolation. Adult females were isolated for morphological and molecular identification. To ensure accurate species identity, J2 were obtained by incubating eggs retrieved from individual adult females previously identified based on perineal patterns of *Meloidogyne incognita* and *M. javanica*.

Sample preparation of *Meloidogyne* spp. for morphological identification

Sample preparation of *Meloidogyne* spp. for morphological identification was adapted from the method of Hartman and Sasser (1985). The preparation of perineal patterns was performed under a stereo microscope (Stemi 305, ZEISS, Oberkochen, Germany). An incision was made in the female cuticle near the midbody using a

fine needle under a stereomicroscope, and the body contents were gently expelled into a drop of distilled water on a clean microscope slide. The cuticle section adjacent to the anus was transferred to a new drop of distilled water on a new microscope slide to clean the cuticle of any remaining internal tissues. The tissue surrounding the perineal pattern was cut and removed. The specimen was then transferred to a drop of glycerin, trimmed into a square, aligned in a straight line and covered gently with a coverslip. Finally, the slide was examined using a compound microscope (CX31, Olympus Corporation, Tokyo, Japan) for morphological identification.

DNA extraction

Genomic DNA was extracted from 1-10 *Meloidogyne* spp. individuals (adult females and second-stage juveniles, J2) using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and used as positive controls in end-point PCR assays. For environmental simulation, nematode-free soil samples (0.25 g) were artificially spiked with defined quantities of nematodes, ranging from 1 to 8 adult females and 2 to 20 J2s for *M. incognita* and *M. javanica*, respectively. Specifically, adult females were dissected from infected root tissues, their perineal sections excised for morphological verification, and the remaining anterior body portions collected individually using a micropipette. For juvenile preparation, egg masses were handpicked and maintained in sterile distilled water at room temperature until hatching, after which freshly hatched J2s were collected. The DNA was extracted immediately after spiking using the Quick-DNA Fecal/Soil Microbe MiniPrep Kit (Zymo Research, California, USA). Nematode-free soil served as a negative control (Ns) in multiplex PCR. All DNA templates were diluted to a final concentration of 10-20 ng μL^{-1} prior to amplification.

End-point PCR, multiplex PCR and qPCR techniques

Specific detection of *M. incognita* was performed using primers Inc-K14-F (5'-GGGATGTGTAAATGCTCCTG-3') and Inc-K14-R (5'-CCCGCTACACCCTCAACTTC-3'), producing PCR products of 399 bp. Similarly, detection of *M. javanica* was conducted with primers Fjav (5'-GGTGCGCGATTGAACTGAGC-3') and Rjav (5'-CAGGCCCTTCAGTGGAAGTATAC-3'), resulting in a 670 bp (Resquín-Romero et al., 2023). The end-point and multiplex PCR reactions were performed in a 25 μL reaction mixture consisted of 12.5 μL QuickTaq HS DyeMix (Toyobo, Osaka, Japan), 0.25 μL of each 10 μM primer (final concentration of 0.1 μM per primer), 2 μL DNA template and molecular-grade water (10 μL for end-point PCR or 9.5 μL for multiplex PCR). Amplification was performed using a PCR machine (GeneAmp PCR System 9700, Applied Biosystems, Waltham, Massachusetts, USA). For qPCR, a total reaction volume of 10 μL was used, containing 5 μL SYBR Green qPCR Master Mix (MedChemExpress, Monmouth Junction, New Jersey, USA), 0.50 μL of each 10 μM primer, 2 μL DNA template, and 2 μL molecular-grade water. Amplification was performed using a DNA thermal cycler (QIAquant 96 2plex, QIAGEN), and the results were analyzed using QIAquant 96 Software version 1.0.4 (QIAGEN). Thermal cycling conditions for end-point PCR and multiplex PCR included an initial denaturation at 94 °C for 3 min, followed by 45 cycles of denaturation at 94 °C for 30 s. The annealing temperature was optimized based on the target and sample type, and 60 °C was used for *M. incognita* (J2), multiplex PCR (adult females and J2), and qPCR for *M. javanica*. Conversely, 64 °C was utilized for *M. incognita* (adult females) and *M. javanica* (adult females and J2), for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min for all sample types. The PCR products were subsequently analyzed by gel electrophoresis on a 1.5% agarose gel in 0.5X TBE buffer, run at 100 V for 35 min with the DNA marker. Finally, the gel was stained with nucleic acid stain and visualized under a gel documentation system with a CMOS camera (SmartView Pro 1100, Major Science, Saratoga, California, USA). The experimental design for the minimum detection of *M. incognita* (subscripts *in*) and *M. javanica* (subscripts *ja*) in adult females (F), J2 (J) and soil samples (S) using multiplex PCR (Table 1). Plots and charts were visualized in R program using ggplot2 package (R Core Team, Vienna, Austria).

Table 1. Experimental setup for determining the sensitivity of multiplex PCR in soil-based *Meloidogyne* spp. detection. ^aF: Adult females; J: second-stage juveniles (J2); S: soil samples. Subscripts *in* and *ja* represent *Meloidogyne incognita* and *M. javanica*, respectively.

Sample ID ^a	Nematodes g ⁻¹ soil	Nematode species	Nematode stage
FS2 _{in}	2	<i>M. incognita</i>	Adult female
FS4 _{in}	4		
FS2 _{ja}	2	<i>M. javanica</i>	
FS4 _{ja}	4		
FS2 _{in} +2 _{ja}	2 and 2	<i>M. incognita</i> and <i>M.</i>	J2
FS4 _{in} +4 _{ja}	4 and 4	<i>javanica</i>	
FS6 _{in} +6 _{ja}	6 and 6		
JS16 _{in}	16	<i>M. incognita</i>	
JS20 _{in}	20		
JS12 _{ja}	12	<i>M. javanica</i>	
JS16 _{ja}	16		
JS16 _{in} +16 _{ja}	16 and 16	<i>M. incognita</i> and <i>M.</i>	
JS20 _{in} +20 _{ja}	20 and 20	<i>javanica</i>	

Sequence analysis

To definitively confirm the species identity of *M. incognita* due to morphological similarity to *M. enterolobii*, representative amplicons (399 bp) were purified and subjected to bidirectional Sanger sequencing (1st BASE, Apical Scientific, Malaysia). The chromatograms obtained were inspected and trimmed using BioEdit software (version 7.7.1). The cleaned consensus sequences were analyzed against the NCBI GenBank database utilizing BLASTn for homology verification.

RESULTS AND DISCUSSION

Nematode morphology identification

Specimens identified as *Meloidogyne incognita* (Figure 1a) showed a high dorsal arch. The striae were irregular, rough and wavy. Lateral lines were indistinct, characterized by breaks, irregularities and the forking of striae, which occasionally formed the appearance of lateral wings. The vulval-anal region was relatively wide and smooth. Observations remain consistent with high, squarish dorsal arches and indistinct lateral lines reported in Turkey (Aydinli and Mennan, 2016), Sri Lanka (Nishantha et al., 2025), and Thailand (Phanbut et al., 2024). In contrast, specimens identified as *M. javanica* (Figure 1b) showed a lower and rounded dorsal arch. The striae were regularly patterned and smooth. The dorsal and ventral regions were clearly divided by distinct lateral lines. The vulval-anal region was narrower than in *M. incognita* (Nishantha et al., 2025). Findings correspond with *M. javanica* populations in Portugal (Rusique et al., 2023) and Thailand (Jojun et al., 2025), where distinctive double lateral lines remain consistent despite minor variations in dorsal arch height.

End-point PCR for the detection of *M. incognita* in soil

Polymerase chain reaction (PCR) amplification using primers Inc-K14-F/Inc-K14-R successfully detected *M. incognita* in soil samples. To confirm that DNA extraction and PCR amplification work effectively despite soil inhibitors, adult females were initially tested, and target fragments of 399 bp were amplified from soil samples containing 6, 8, and 10 adult females g⁻¹ soil (Figure 2a). For field-representative J2 detection, the target fragment was observed in soil samples containing 8, 12, 16, and 20 J2 g⁻¹ soil (Figure 2b). No amplification was detected in soil samples containing adult females for 2 and 4 individuals g⁻¹ soil (Figure 2a: lane 1 and 2), and J2 for 4 individuals g⁻¹ soil (Figure 2b: lane 1). Specificity was maintained at an annealing temperature of 64 °C for adult females and 60 °C was used for J2, with minimum detection was established at 6 adult females g⁻¹ soil and 8 J2 g⁻¹ soil, respectively. The protocol optimization aligns with established protocols that utilize 60 °C for the successful detection of *M. incognita* across various life stages, including egg masses, adult females, and J2 (Uysal et al., 2017). Consequently, the present study successfully expands the application of specific primers using end-point PCR to direct detection within complex soil matrices.

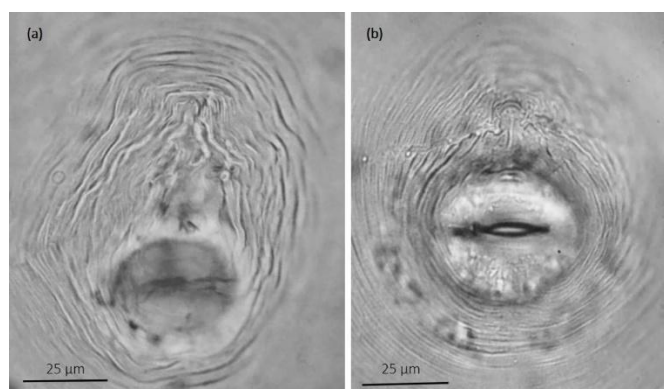


Figure 1. Morphological characteristics of adult female perineal patterns of *Meloidogyne incognita* (a) and *M. javanica* (b).

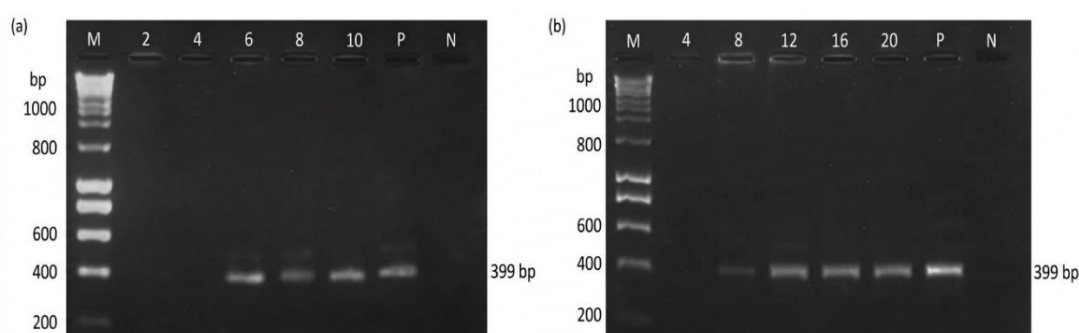


Figure 2. End-point PCR products using species-specific primers Inc-K14-F/Inc-K14-R (target fragment at 399 bp) for the detection of *Meloidogyne incognita* in soil: Detection of adult females in soil at an annealing temperature of 64 °C (a), detection of second-stage juveniles (J2) in soil at an annealing temperature of 60 °C (b). Lanes 2, 4, 6, 8, and 10 are numbers of adult females g⁻¹ soil (a); 4, 8, 12, 16, and 20 are numbers of J2 g⁻¹ soil (b). M: DNA marker; P: positive control; N: negative control.

End-point PCR for the detection of *M. javanica* in soil

The PCR amplification was performed using the species-specific primers Fjav/Rjav for the detection of *M. javanica* in soil samples containing varying numbers of nematodes. A single DNA fragment of 670 bp was successfully amplified in samples containing adult females and J2 at densities of 4, 6, 8, and 10 individuals g⁻¹ soil (Figure 3), and no amplification was observed in samples containing 2 adult females and 2 J2 g⁻¹ soil (Figures 3a and 3b: Lane 2). The successful detection of *M. javanica* 4 adult females and 4 J2 g⁻¹ soil was achieved at an annealing temperature of 64 °C. Previous reports from Paraguay successfully employed the primers at 62 °C for species identification, demonstrating versatile performance and functional stability across various thermal conditions (Resquín-Romero et al., 2023). Consistent amplification suggested that Fjav/Rjav primers possess high efficiency and specificity, effectively overcoming PCR inhibitors within soil matrices (Bott et al., 2023). While earlier studies primarily focused on identifying species from isolated nematodes or infected plant tissues (Phanbut et al., 2024), this research confirms the stability of the system for direct environmental DNA applications.

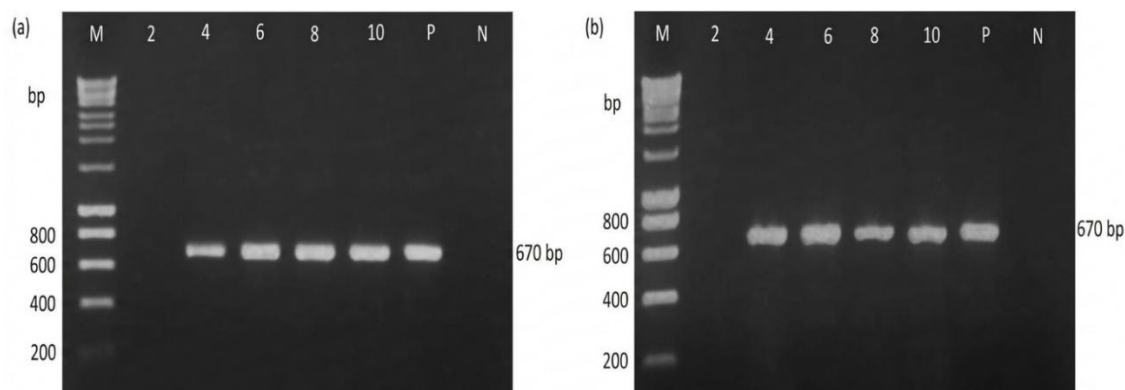


Figure 3. End-point PCR products using species-specific primers Fjav/Rjav (670 bp) for the detection of *Meloidogyne javanica* in soil at an annealing temperature of 64 °C: Adult females (a), second-stage juveniles (J2) (b). Lanes 2, 4, 6, 8, and 10 are number of adult females and J2 g⁻¹ soil. M: DNA marker; P: positive control; N: negative control.

Multiplex PCR for detection *M. incognita* and *M. javanica* in soil

A multiplex PCR assay was developed for the rapid and simultaneous detection of *M. incognita* and *M. javanica* in soil samples. The assay utilized combinations of species-specific primers Inc-K14-F/Inc-K14-R (399 bp for *M. incognita*) and Fjav/Rjav (670 bp for *M. javanica*). The minimum detection for adult females was 4 individuals g⁻¹ soil in single-species samples and 6 individuals g⁻¹ soil in mixed-species samples (Figure 4a). In contrast, no amplification was detected in single-species samples containing 2 individuals g⁻¹ soil (Figure 4b: Lanes 1 and 3). Similarly, in mixed-species samples, no band was observed at a density of 2 individuals g⁻¹ soil (Figure 4b: Lane 5). Mixed-species samples containing 4 individuals g⁻¹ soil showed amplification only for *M. javanica* (Figure 4b: Lane 6). During this competitive process, more efficient primers, such as primers Fjav/Rjav, outcompete others by binding more stably to their target DNA, leading to the selective amplification of *M. javanica* over *M. incognita* (Crego-Vicente et al., 2024). The minimum adult female detection in mixed-species samples was established at 6 individuals g⁻¹ soil (Figure 4b: Lane 7). Testing samples at concentrations below the identified thresholds resulted in no detectable bands, confirming the accuracy of the identified minimum detection. For the J2 stage in single-species samples, the minimum detection was 20 individuals g⁻¹ soil for *M. incognita*, and 16 individuals g⁻¹ soil for *M. javanica* and mixed samples containing 20 individuals g⁻¹ soil of each species (Figure 4c). However, no amplification was observed in single-species samples of *M. incognita* containing 16 individuals g⁻¹ soil (Figure 4d: Lane 1) and *M. javanica* containing 12 individuals g⁻¹ soil (Figure 4d: Lane 3). Similarly, in mixed-species samples, no band was detected at a density of 16 individuals g⁻¹ soil of each species (Figure 4d: Lane 5), but a clear detection of both species at 20 individuals g⁻¹ soil of each species (Figure 4d: Lane 6). The increase in the J2 detection threshold within the multiplex system is attributed to primer competition and varying amplification efficiencies (Crego-Vicente et al., 2024). Consequently, the minimum detection for the multiplex assay was established at 6 adult females g⁻¹ soil and 20 J2 g⁻¹ soil of each species using the original Inc-K14-F/Inc-K14-R and Fjav/Rjav primer sets. While the previous study did not successfully detect nematodes at the juvenile, female, and egg mass stages without DNA smears using the original primer sets, new primer design was required (Devran et al., 2018). This research precisely optimized the conditions for effective direct soil detection, streamlining the diagnostic workflow by eliminating time-consuming nematode extraction and egg hatching. This advancement confirms the reliability of primers for high-throughput soil monitoring without prior nematode isolation.

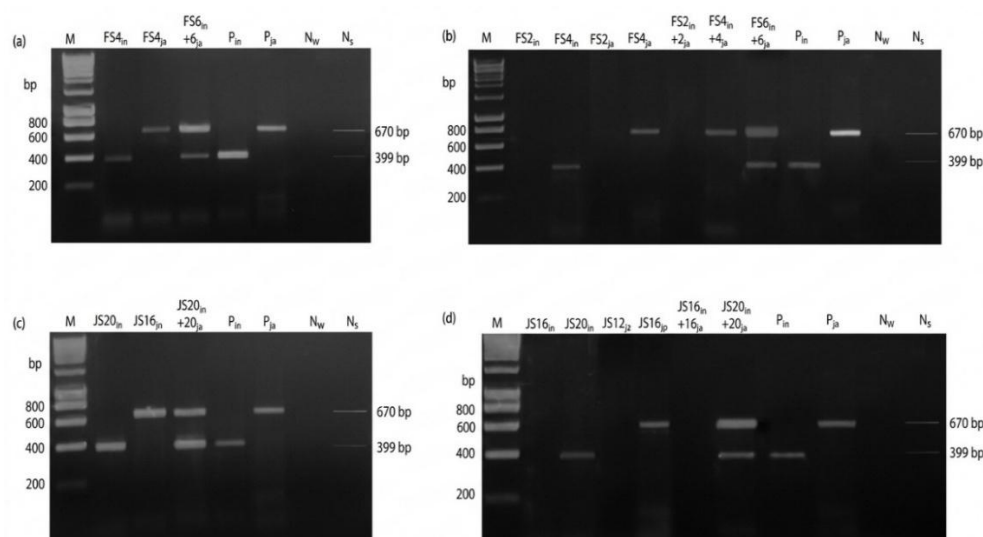


Figure 4. Multiplex PCR products for the detection of *Meloidogyne javanica* (primers Fjav/Rjav, 670 bp) and *M. incognita* (primers Inc-K14-F/Inc-K14-R, 399 bp) in soil at 60 °C: Adult females (a-b); second-stage juveniles (J2) (c-d). Lanes 2, 4, 6, 12, 16, and 20 number of nematode individuals added per sample. M: DNA marker; P: positive control; Nw: negative control using water; Ns: negative control using soil; F: adult females; J: J2; S: soil samples; subscripts *in* and *ja* denote *M. incognita* and *M. javanica*, respectively.

qPCR for detection *M. javanica* in soil

In this study, *M. javanica* was selected as a model nematode to validate the efficiency of the developed real-time PCR (qPCR) detection method in soil samples. The protocol successfully amplified target DNA. The result showed clear amplification curves for 4 adult females g^{-1} soil and 8 J2 g^{-1} soil, which were distinct from the negative control (Figure 5), and no amplification with samples containing 2 adult females and 6 J2 g^{-1} soil. While previous protocols required processing 200 mL soil volumes to achieve detection (Hodson et al., 2023), this assay maintained robust performance within 1 g soil matrix. The optimized protocol overcomes concentrated PCR inhibitors, such as humic acids (Bott et al., 2023), effectively detecting low nematode densities in concentrated samples. Utilizing a smaller soil mass streamlines the diagnostic workflow, reducing sample preparation time and labor. The qPCR system provides a rapid, high-sensitivity tool for direct soil monitoring. Furthermore, adapting standard sequences for qPCR ensures consistency across all methods without requiring primer redesign. The minimum detection for the end-point PCR, multiplex PCR, and qPCR assays was summarized (Figure 6). In summary, our findings offer a more sensitive and rapid alternative to conventional nematode extraction methods. Additionally, the specificity of the primers was cross-validated against other common plant-parasitic nematodes (*Tylenchorhynchus* sp.), with no detectable amplification.

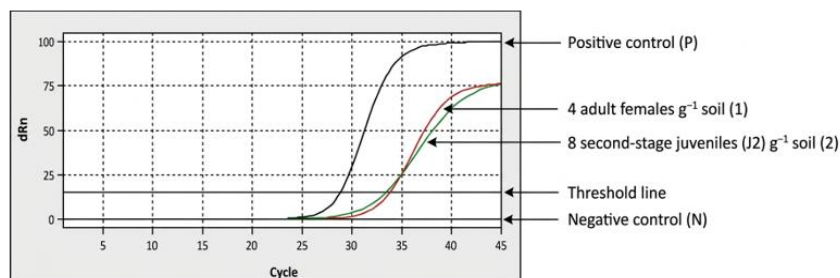


Figure 5. Real-time PCR (qPCR) amplification plot showed the detection of *Meloidogyne javanica* in soil samples containing 4 adult females g^{-1} soil (1) and 8 second-stage juveniles (J2) g^{-1} soil (2), compared to positive control (P), and negative control (N).

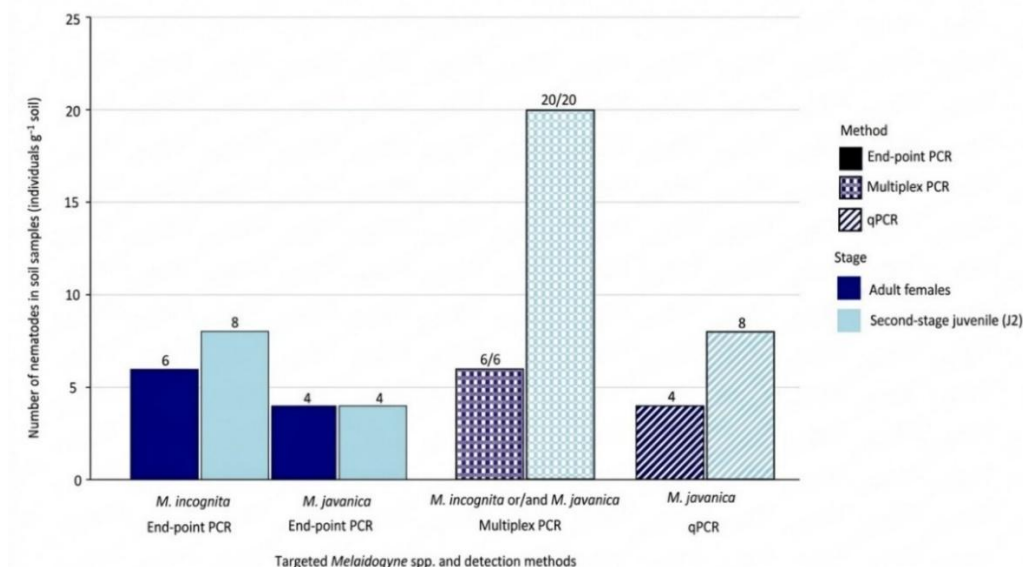


Figure 6. Comparison of the minimum detection for *Meloidogyne incognita* and *M. javanica* g⁻¹ soil using different PCR-based assay. The bar styles indicate the methods used: Solid bars represent end-point PCR, checked patterns represent multiplex PCR, and diagonal hatched patterns represent real-time PCR (qPCR). Bar groups represent adult females (dark blue), second-stage juveniles (J2) (light blue). The values 6/6 and 20/20 indicate detectable number of nematodes in multiplex PCR, representing either 6 individuals from a single species or 6 individuals of each two species, and either 20 individuals from a single species or 20 individuals of each two species, respectively. The qPCR assay was performed specifically for *M. javanica* as a model system for the developed soil detection method.

To conclude the founding of this study, end-point PCR, qPCR, and multiplex PCR were comparatively evaluated based on specificity, sensitivity, cost-effectiveness, and processing time. Each parameter was scored on a three-point scale, where 1 indicated low performance, 2 indicated moderate performance, and 3 indicated the highest performance for the respective aspect. The radar chart was constructed by assigning scores for each parameter based on the experimental findings observed in this study (Figure 7). For specificity, scores were assigned based on the capacity for species-level identification. Methods unable to achieve target detection received the lowest score of 1, whereas methods limited to genus-level identification received a moderate score of 2. Methods capable of accurate species-level detection were assigned the highest score of 3. In this study, all methods successfully demonstrated species-level detection and therefore received the highest specificity score of 3 (Figure 7). For sensitivity, scores reflected the minimum detection limit based on number of nematodes. The highest score of 3 was assigned for the detection of a single individual nematode, the moderate score of 2 was for detection requiring more than one nematode, and the lowest score of 1 was for detection requiring more than 10 nematodes. In this experiment, end-point PCR and qPCR demonstrated moderate sensitivity by detecting a range of 4-8 individual nematodes g⁻¹ soil including both females and J2 stage, thereby receiving a score of 2. Meanwhile, multiplex PCR exhibited lower sensitivity, requiring more than 10 individuals, and therefore received the lowest score of 1. For cost-effectiveness, high costs were assigned with the lowest score of 1, moderate costs a moderate score of 2, and low costs the highest score of 3. Therefore, the qPCR received the lowest score of 1, the end-point PCR received a moderate score of 2, and the multiplex PCR received the highest score of 3 (Figure 7). Lastly, processing time was measured as the total duration of the experiment, where longest experimental times scored the lowest at score of 1, moderate times scored moderately at score of 2, and the fastest times scored the highest at score of 3. This test end-point PCR received the lowest score of 1, multiplex PCR received a moderate score of 2, and qPCR received the highest score of 3 (Figure 7).

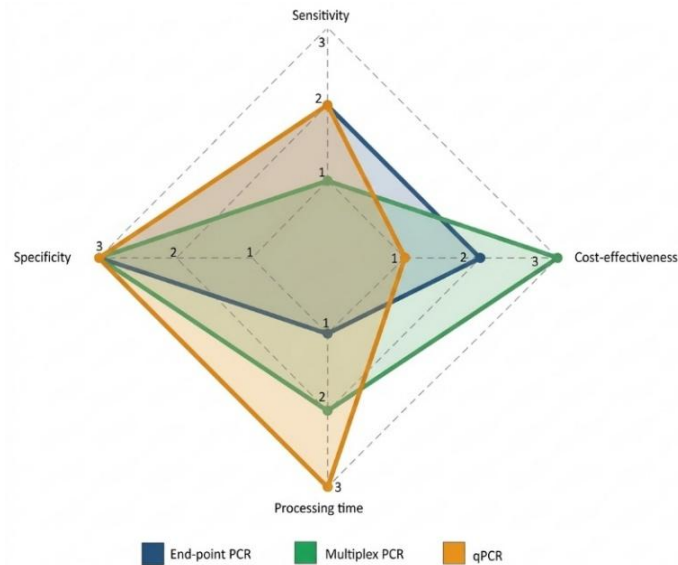


Figure 7. Conceptual radar chart comparing the performance of end-point PCR, multiplex PCR, and real-time PCR (qPCR based on specificity, sensitivity, cost-effectiveness, and processing time. Each parameter was scored on a three-point scale, where 1, 2, and 3 represent low, moderate, and high performance, respectively. For specificity, scores were assigned based on target detection ability, including unsuccessful detection (1), genus-level detection (2), and species-level detection (3). Sensitivity scores were based on the minimum detectable number of nematodes, including detection requiring more than 10 nematodes (1), more than one nematode (2), or a single nematode (3). Cost-effectiveness scores were assigned according to high (1), moderate (2), or low assay cost (3). Processing time scores were determined based on longest (1), moderate (2), or shortest (3) total experimental duration compared to others.

This research highlights a robust system for direct detection from soil samples. Specifically, qPCR demonstrated exceptional performance, matching the high sensitivity of end-point PCR while significantly outperforming it in processing time due to the elimination of post-PCR analysis. Meanwhile, multiplex PCR offers maximum cost-effectiveness for simultaneous species screening. Significantly, the successful application of an annealing temperature range of 60-64 °C across all platforms, end-point, multiplex, and qPCR, demonstrates the robust multi-functionality of the Inc-K14-F/Inc-K14-R and Fjav/Rjav primer sets. This standardized approach not only ensures efficient integration from basic detection to advanced quantification without primer redesign but also establishes a strong foundation for the future development of multiplex qPCR assays for high-throughput soil monitoring.

Molecular confirmation of *Meloidogyne incognita*

Electrophoretic analysis of the SCAR-PCR products yielded a specific, single diagnostic band of approximately 399 bp in the analyzed isolates, consistent with the positive control (*M. incognita*, sequence-verified) with no amplification observed in the negative control (Figures 2-5). Following quality trimming of the chromatograms, a high-quality consensus sequence of 335 bp was obtained from a representative sample. The BLASTn searches against the NCBI GenBank database revealed that the amplicon shared 100.00% nucleotide identity with reference accessions of *M. incognita* (e.g., Accession No. OR453944.1), displaying 100% query coverage and an exceptionally significant E-value (9×10^{-173}). These molecular data conclusively verify the identity of the isolate as *M. incognita*.

Morphological differentiation of root-knot nematodes based solely on female perineal patterns often remains inconclusive, as *M. enterolobii* characteristically exhibits a rounded outline with elevated dorsal arches that closely resemble those of *M. incognita* (Hu et al., 2011). Consequently, molecular validation using species-specific SCAR-PCR coupled with Sanger sequencing was crucial to overcome these phenotypic ambiguities and ensure definitive species verification.

CONCLUSIONS

This study successfully optimized and evaluated three molecular diagnostic tools end-point PCR, multiplex PCR, and real-time PCR (qPCR) for the rapid detection and quantification of *Meloidogyne incognita* and *M. javanica* directly from soil samples. These techniques significantly reduce the time required for traditional nematode identification and offer high specificity at the species level. In terms of sensitivity, end-point PCR established a minimum detection of 6 adult females g⁻¹ soil and 8 second-stage juveniles (J2) g⁻¹ soil for *M. incognita*, while *M. javanica* was detected at 4 individuals g⁻¹ soil for both life stages. The multiplex PCR assay provided a stable method for identifying mixed-species infections, with a detection of 6 adult females g⁻¹ soil and 20 J2 g⁻¹ soil for each species. Furthermore, the qPCR protocol demonstrated superior performance for *M. javanica*, detecting as few as 4 adult females g⁻¹ soil and 8 J2 g⁻¹ soil. While all three methods are highly effective, they offer distinct advantages depending on the application. Overall, the precise detection of nematodes in soil at low densities provides a robust framework for early detection. It facilitates timely prevention and management strategies, to minimize crop yield losses.

Author contribution

Conceptualization: Y.B., P.C., O.R., M.S., N.M. Methodology: Y.B., P.C., O.R., M.S., N.M. Validation: M.S., N.M. Formal analysis: Y.B., M.S., N.M. Investigation: Y.B., M.S., N.M. Resources: N.K., M.S., N.M. Data curation: Y.B., M.S., N.M. Writing-original draft: Y.B., M.S., N.M. Writing-review & editing: Y.B., P.C., O.R., N.K., M.S., N.M. Visualization: Y.B. Supervision: M.S., N.M. Funding acquisition: M.S., N.M. All co-authors reviewed the final version and approved the manuscript before submission.

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