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MOLECULAR IDENTIFICATION OF ROOT-KNOT NEMATODES MELOIDOGYNE SPP. ISOLATED FROM CUCUMBER FARMS IN DIFFERENT IRAQI REGIONS

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Abstract

Meloidogyne spp. is one of the most common crop diseases globally. Due to the similarity in morphological features between various *Meloidogyne spp.*, molecular techniques were used to identify their species. In this study, conducted in October 2022 and January 2023, genetic identification was undertaken on 61 samples collected from nine distinct regions of Iraq. PCR was used to amplify the part of the *Meloidogyne spp*. genome between the COI and 16S mtDNA genes. Then, *M. incognita*, *M. hapla*, *M. javanica*, *M. enterolobii*, and *M. arenaria* were genotyped using sequence characterized amplified region (SCAR-PCR), with decreasing prevalence rates, respectively. The data revealed that the 50 isolates of *M. incognita* were in the Shuan Kara region, which means they exhibited the highest percentage. Interestingly, *M. arenaria* was the least common species in all areas and was isolated from Ghanaia. Additionally, *M. enterolobii's* representation of other species led to the discovery of a new one in Iraq. The coexistence of more than two species within a single region has never been documented. The study employed molecular detection to determine how different *Meloidogyne* species infect cucumber plants and the importance of being able to spot infected regions.

Keywords: COI Barcode, Molecular Markers, mtDNA, Nematodes.

التوصيف الجزيئي لنيماتودا تعقد الجذور Meloidogyne spp. المعزولة من مزارع الخيار في مناطق عراقية مختلفة

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الخلاصة

Meloidogyne spp. هي واحدة من أسوء الأمراض الزراعية على مستوى العالم. بسبب تشابه الصفات المظهرية بين أنواع ال *Meloidogyne spp* تم استخدام التقنية الجزيئية لتحديد أنواعها. تضمنت هذه الدراسة التمييز الوراثي لـ 61 عينة من *Meloidogyne spp.* التي جمعت من تسعة مناطق في العراق في شهر تشرين الأول لعام 2022 وشهر كانون الثاني لعام 2023. تم تضخيم المنطقة الواقعة بين جيني COI وال S16 mtDNA لـ *Meloidogyne spp.* باستخدام تفاعل البلمرة المتسلسل PCR. باستخدام مؤشرات *SCAR-PCR* تم تحديد *M.javanica ,. M.incognita, M. hapla ,M. enterolobii* و *M. arenaria* بالترتيب التنازلي وفقاً لأعلى نوع انتشاراً .أظهرت النتائج أن البادئ المصمم *Inc* ارتبط بشكل متخصص ب 50 عزلة من النوع *M.incognita*، حيث سجلت أعلى نسبة ظهور من هذا النوع في منطقة شوان كارا. في منطقة غنايا سجل النوع *M.arenaria* أقل ظهوراً بواقع عينة واحدة فقط. كذلك، أدى إيجاد *M. enterology* بالنسبة للأنواع الأخرى الى اكتشاف نوع جديد في العراق. لم يوثق أي ظهور لأكثر من نوعين في نفس المنطقة مطلقاً. الهدف من هذا البحث هو استخدام الطرق الجزيئية لتحديد عدد الأنواع المختلفة من جنس *Meloidogyne spp.* التي تصيب نبات الخيار ومدى أهمية تحديد المناطق المصابة.

كلمات مفتاحية: الخيار، الباركود سايتوكروم اوكسيديس، مؤشرات جزيئية، الدنا المايتوكونديري.

Introduction

Meloidogyne spp. ranks first among the ten most significant genera of plant parasitic nematodes globally and is among the top five most dangerous plant infections (7). It induces root-knot formation, affecting *Cucurbitaceae* family members. Symptoms include stunted root growth, a shift in leaf coloration from light green to yellow, and wilting, notably exacerbated during the hottest hours of the day (22).

The distribution of the *Meloidogyne* species within a region is contingent upon their ability as obligatory root parasites to persist throughout winter. Among the

documented 90 *Meloidogyne spp.* the most detrimental include *M. incognita*, *M. javanica*, and *M. arenaria* (27). The species has been identified based on female and juvenile morphology, host response, isozyme analyses, and molecular techniques (21). Accurate identification of *Meloidogyne spp.* can be achieved through the application of various molecular methods (17). Currently, the predominant molecular marker employed in *Meloidogyne* molecular systematics is nuclear ribosomal DNA (2). Varying genes and spacers within a ribosomal rDNA transcription unit exhibit varying mutation rates, resulting in sections of neighboring DNA segments in the cistron that are valuable across several taxonomic levels (15). This category encompasses both the conserved and variable sections of the 18S and 28S subunits, along with the more variable section of the Internal Transcribed Spacer (ITS) (24). Mitochondrial Cytochrome c Oxidase subunit I (COI) is a key barcode sequence for animals in the Barcode of Life Data System (BOLD) identification system (3). DNA barcoding is a major asset in identifying species and defining overlap and divergence regions (18). The optimal DNA-based technique for identifying quarantine nematode species and their near relatives would ideally utilize two distinct genes, such as COI and Small Subunit SSU rDNA (19). This study aimed to detect the species of root-knot nematodes (*Meloidogyne spp.*) infecting Iraqi cucumber plants through the application of molecular markers.

Materials and Methods

Nematodes collection: Sixty-one samples of *Meloidogyne spp.* were isolated from roots and rhizosphere of cucumber cultivars across various Iraqi regions. They included Isolate numbers 1 to 31 from Sayff, Seegreen, and Kamaal cucumber varieties, respectively in Sulaymaniyah/Banjan, Bazian, and Shuan Kara regions; Isolate numbers 32 to 46 from Joud, Mohannad, and Super Faris cucumber varieties respectively in Wasit/Rahmaniyah, Ghanaia, and Saouira regions; Isolate numbers 47 to 51 from the Jana cucumber variety in Tikrit/Khizamiah; Isolate numbers 52 to 56 from the Valkon cucumber variety in Samarra/Haouish; and isolate numbers 57 to 61 from the Super Faris cucumber variety in Baghdad/Yusufiya, as per (6). Soil samples were collected approximately 10 cm from the roots, extending to a depth of 30 cm, following the methodology outlined in (28). Female and egg samples were extracted from the root tissues of cucumber, as shown in Figure 1. Moreover, second-stage juveniles (J2s) were collected from hatching eggs.

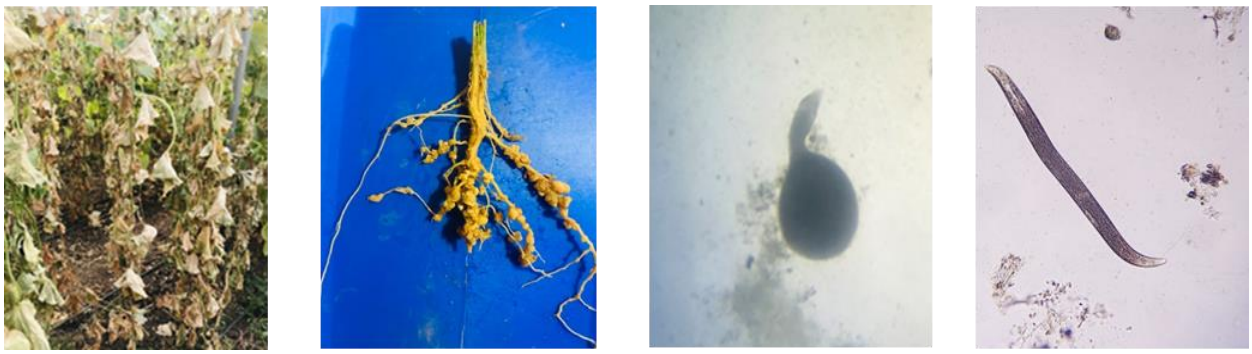


Figure 1: Infected plants and roots and the isolates of nematodes.

Isolation of nematodes from plant material: Cucumber roots were finely chopped and processed in a blender. The primary objective was to minimize blending time, to ensure the sufficient release of nematodes from the tissue (9). The tissue was exposed to 0.5% NaOCl for 2 minutes to increase insulation efficiency (25). Soil samples were thoroughly stirred in four buckets with 1 L of water each and filtered through a 2 mm sieve, recovering each soil suspension in a tube. The tubes were then centrifuged at 1000 rpm for 40 seconds according to (5), after which the supernatant was discarded. Samples produced were cleaned using the Baermann technique, following the procedures outlined by (20).

DNA extraction and primers: The Monarch® HMW DNA Extraction Kit, following the Tissue Protocol outlined by (26), was employed to extract DNA from *Meloidogyne spp*. females and J2. A newly designed Inc primer was designed to bind selectively with *Meloidogyne incognita*. The precise selection of the primer binding region was performed after downloading the *M. incognita* sequence of the most common *Meloidogyne*. The COI primer and other primer sequences were used to bind to specific genes (23). These identified primer sequences of *M. incognita, M. hapla, M. arenaria, M. enterolobii*, and *M. javanica* are listed in Table 1.

Reactions and PCR program: All PCR reactions were performed on a SimpliAmp Thermal Cycler PCR (Applied BioSystems, Singapore) in a 24 µl reaction composed of 12.5 µl of OneTaq 2X Master Mix (Biolabs), 5 µl of template DNA, 1 µl (20 µM) Forward Primer, 1 µl (20 µM) Reverse Primer, and 4.5 µl nuclease-free water.

The PCR components were transported into a thermal cycler to amplify the targeted genes. The PCR procedure incorporated the utilization of the following programs, as outlined by (10). It involved an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds. The annealing temperatures varied: 41°C for the FCOI/RCOI primers for 45 seconds, 47°C for the Finc/Rinc primers for 45 seconds, 41°C for the FHap/RHap primers for 45 seconds, 54°C for the Fare/Rare primers for 45 seconds, 61°C for the FMe/RMe primers for 45 seconds, and 54°C for the FJav/RJav primers for 45 seconds. The extension step occurred at 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Subsequently, the PCR product was analyzed by 1.2% agarose gel electrophoresis.

Table 1: Primer pairs used for polymerase chain reaction.

Primer Pairs	Primer Sequence	Fragment Size	Species	References
F-COI	TTTTTTGGGCATCCTGAGGTTTAT	400 bp	*Meloidogyne spp.*	19
R-COI	TAAAGAAAGAACATAATGAAAATG			
F- Inc	TGTGATGTTCAAATTTAAATTCGCA	447 bp	*Meloidogyne incognita*	This study
R- Inc	ACAACTAGGATAAACAACAATCC			
F- Hap	GCGATGTTCAAATATGAATTTTCG	470 bp	*Meloidogyne hapla*	This study
R- Hap	TTGGTAAAAGTTATGTAAACAAT			
F-are	TCG GCG ATA GAG GTA AAT GAC	419 bp	*Meloidogyne arenaria*	23
R-are	TCG GCG ATA GAC ACT ACA ACT			
F- Me	AAC TTT TGT GAA AGT GCC GCT G	236 bp	*Meloidogyne enterolobii*	23
R- Me	TCA GTT CAG GCA GGATCA ACC			
F- jav	GGT GCG CGATTG AAC TGA GC	660 bp	*Meloidogyne javanica*	23
R- jav	CAG GCC CTT CAG TGG AAC TAT AC			

Statistical analysis: R version 3.0.1 was used to analyze the data on *Meloidogyne spp.* distribution in the Iraqi area by utilizing Pearson's Chi-square test and Fisher's exact test. The statistical degree was considered significant at P> 0.01.

Results and Discussion

Disease symptoms: For this study, 66 cucumber root samples exhibiting symptoms of infection were gathered from nine regions spanning five provinces in Iraq. Among these, only 61 root samples were identified as nematodes responsible for root-knot formation. As described by (22), indications of severe nematode infestation were evident, including stunted growth, reduced yield, yellowing, leaf drop, and an overall dwarfing of the plants. The roots exhibited pronounced twisting, atrophy, and prominent knots, affecting axial and branch roots. Numerous galls, containing white or milky white eggs, were visibly present on the surface of the affected roots, with potential locations either inside the root galls or externally. These symptoms appeared at the same level in all infected cucumber cultivars in multiple regions (Figure 2). Notably, the older roots showed signs of decay and necrosis.

Figure 2: Infected cucumber in Sulaymaniyah showing the yellowing leaves, fruit hanging down and knots on root.

Molecular characterization: Molecular characterization relies on the occurrence of polymorphisms in DNA sequences among groups of nematodes, especially in rDNA and mitochondrial DNA (mtDNA). The COI gene has been the primary target of mtDNA. The electrophoresis results revealed precise PCR amplification, as shown in Figure 3 a-f. A total of 61 PCR reactions were performed to identify root-knot nematodes obtained during this study. Subsequently, PCR amplification was obtained from 61 DNA templates available from female nematodes. Among the total samples analyzed, *M. incognita* accounted for 50 identifications, comprising 23 isolates from Sulaymaniyah province, 12 from Wasit province, 5 from Tikrit province, 5 from Samarra province, and 5 from Baghdad. Other *Meloidogyne spp*. were distributed as follows: *M. javanica* with 3 isolates from Banjan district in Sulaymaniyah province, *M. enterolobii* with 3 isolates from Bazian district in the same province, and *M. hapla* with 4 isolates, divided between 2 from the Shuan Kara district in Sulaymaniyah province and 2 from the Rahmaniyah district in Wasit province. Additionally, a single isolate of *M. arenaria* was identified in the Ghanaia region of Wasit province. The use of PCR with species-specific (SCAR) primers successfully confirmed the presence of five distinct *Meloidogyne spp*: 447 bp for *M. incognita*, 470 bp for *M. hapla*, 419 bp for *M. arenaria*, 236 bp for *M. enterolobii*, and 660 bp for *M. javanica*. The presence of *M. incognita*, *M. hapla*, *M. enterolobii*, *M. javanica*, and *M. arenaria* further validates the findings. The amplified DNA segments corresponded to various species, aligning with the findings of (13).

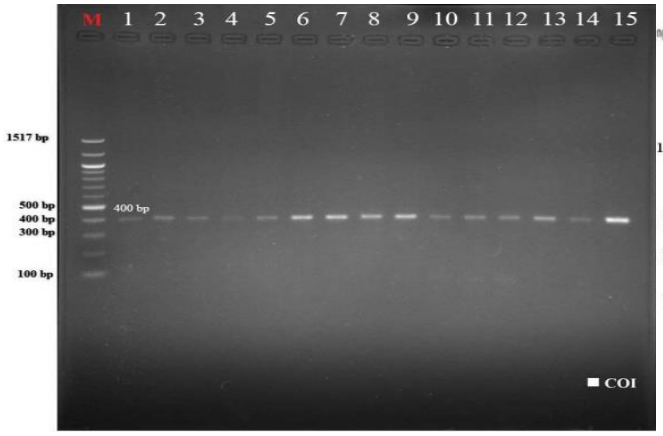


Figure 3 (a)

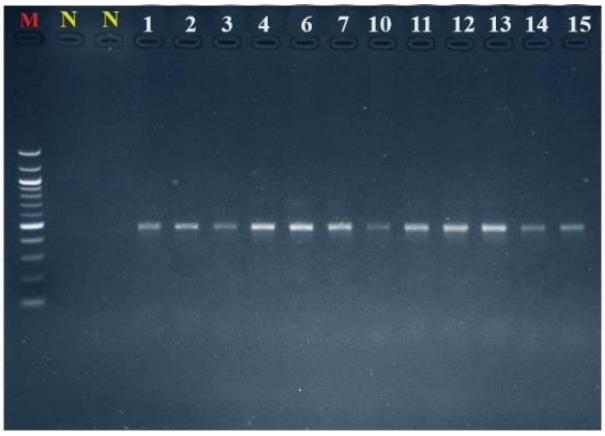


Figure 3 (b)

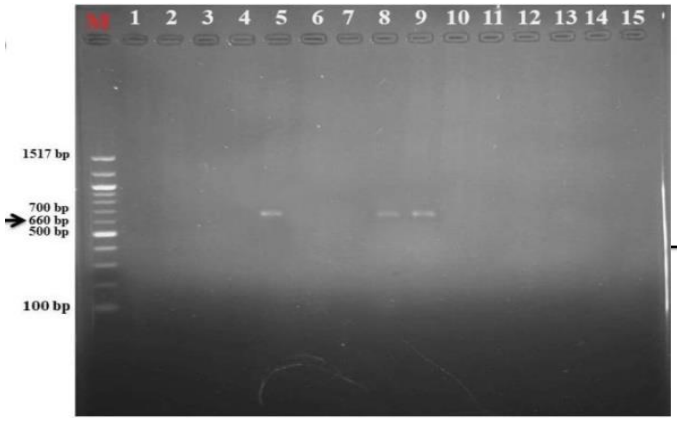


Figure 3 (c)

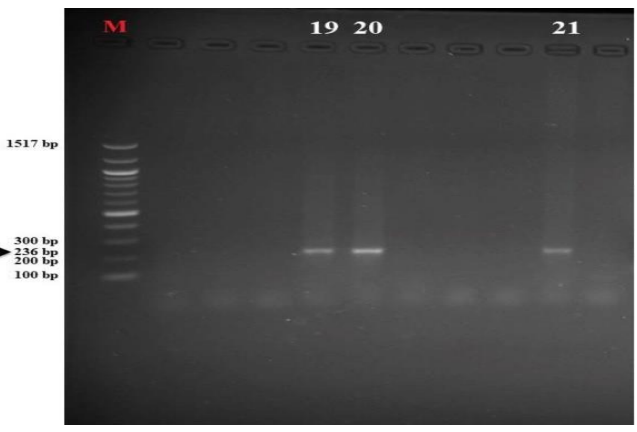


Figure 3 (d)

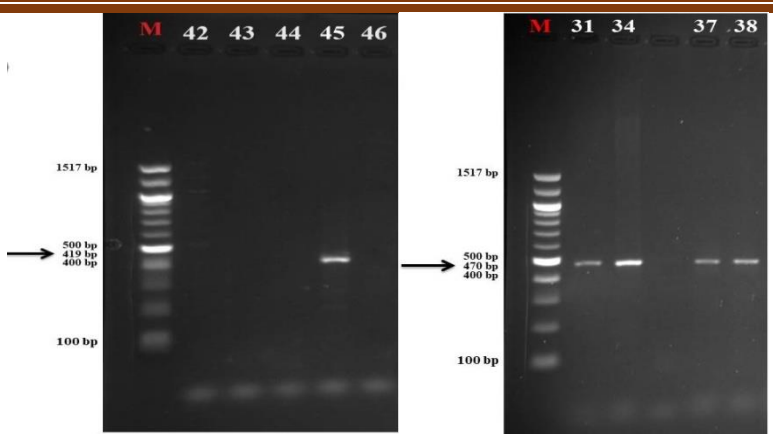


Figure 3 (e) **Figure 3 (f)**

Figure 3: PCR product for five common Meloidogyne spp. from Iraq using species-specific primers. (A) M: is a Marker ladder 100-1517bp, Lane 1, 2, 3; L15 are various Meloidogyne spp. (B) M: is a Marker ladder 100-1517bp, Lane 1, 2, 3; L15 are a Meloidogyne incognita (c) M: is a Marker ladder 100-1517bp, Lane 5, 8 and L9 are a Meloidogyne javanica (d) M: is a Marker ladder 100-1517bp, Lane 19, 20 and L21 are a Meloidogyne enterolobii (e) M: is a Marker ladder 100-1517bp, Lane 45 is a Meloidogyne arenaria (f) M: is a Marker ladder 100-1517bp, Lane 31, 34, 37 and L 37 are a Meloidogyne hapla.

Meloidogyne spp. frequency: The observed distribution of *Meloidogyne spp* across the sampled regions demonstrated statistical significance (p = 0.01) according to Fisher's exact test, while Pearson's Chi-square test showed high statistical significance (P = 0.001), as outlined in Table 2. Notably, the Shuan Kara region in Sulaymaniyah province recorded the highest percentage (22%) of *M. incognita*, while the Rahmaniyah region in Wasit province exhibited the lowest percentage (6%) of the same species, as illustrated in Figure 4.

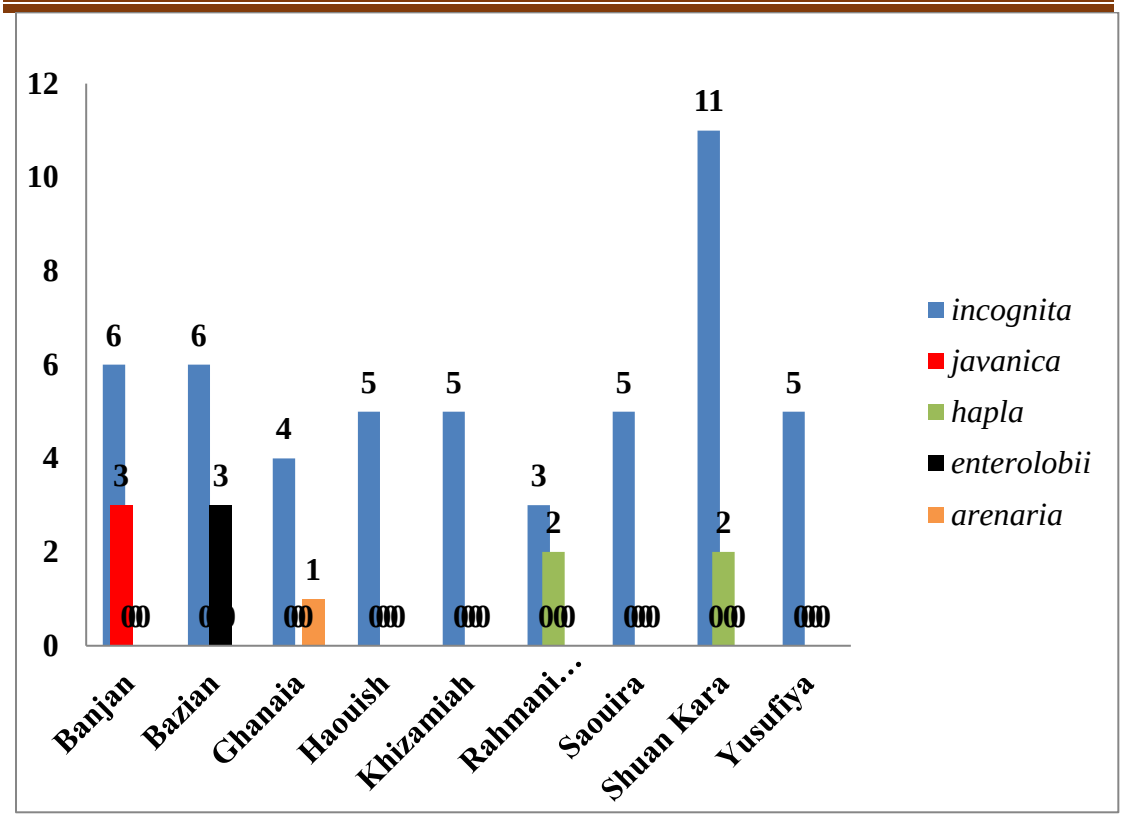


Figure 4: Distribution of Meloidogyne species identified in collection regions.

These findings align with the observations by 12 in noting the prevalence of *M. incognita* in Baghdad, Babylon, and Karbala. Additionally, *M. javanica* was exclusively identified in 33% of the samples collected from the Banjan region in Sulaymaniyah province, with no occurrences in other regions. In Sulaymaniyah, (11) reported the occurrence of *M. javanica* in cucumbers in 30% of greenhouses surveyed and in all districts with RKNs. However, (14) found *M. javanica* the most common species of *meloidogyne* in Iran's greenhouses and vegetable fields. In Paraguay, *M. incognita* and *M. javanica* were found in 39.13% and 26.08% of samples, respectively. The *M. hapla* species was found in two samples from Shuan Kara and two samples from Rahmaniyah, representing 15.4% and 40% of the samples in each respective region. This aligns with (1) findings, indicating that the *M. hapla* is among the four major species prevalent in Iraqi soils. In contrast, (23) observed that only 3% of South African samples were attributed to the *M. hapla* species. A prior study in Brazil by (8), encompassing 36 soil samples and vegetable roots with galls, revealed the least common species were *M. arenaria* (11.1%) and *M. hapla* (8.3%).

M. arenaria was identified at its lowest proportion in the Ghanaia region of Wasit province, accounting for only 1.06% of all species collected, corroborating the findings of (4) who surveyed eggplant farms in Mosul province, indicating the presence of a low percentage of *Meloidogyne arenaria*. In the Bazian region, *M. enterolobii* was moderately present in 33% of all collected samples, in alignment with (23) who found that 62% of samples from South Africa were attributed to it. Also in South China, (16) identified *M. incognita*, *M. enterolobii*, and *M. javanica* as the three most significant species of *Meloidogyne spp*. This study also confirmed that

species present in moderate proportions of the total infections are *M. hapla*, *M. javanica*, and *M. enterolobii*.

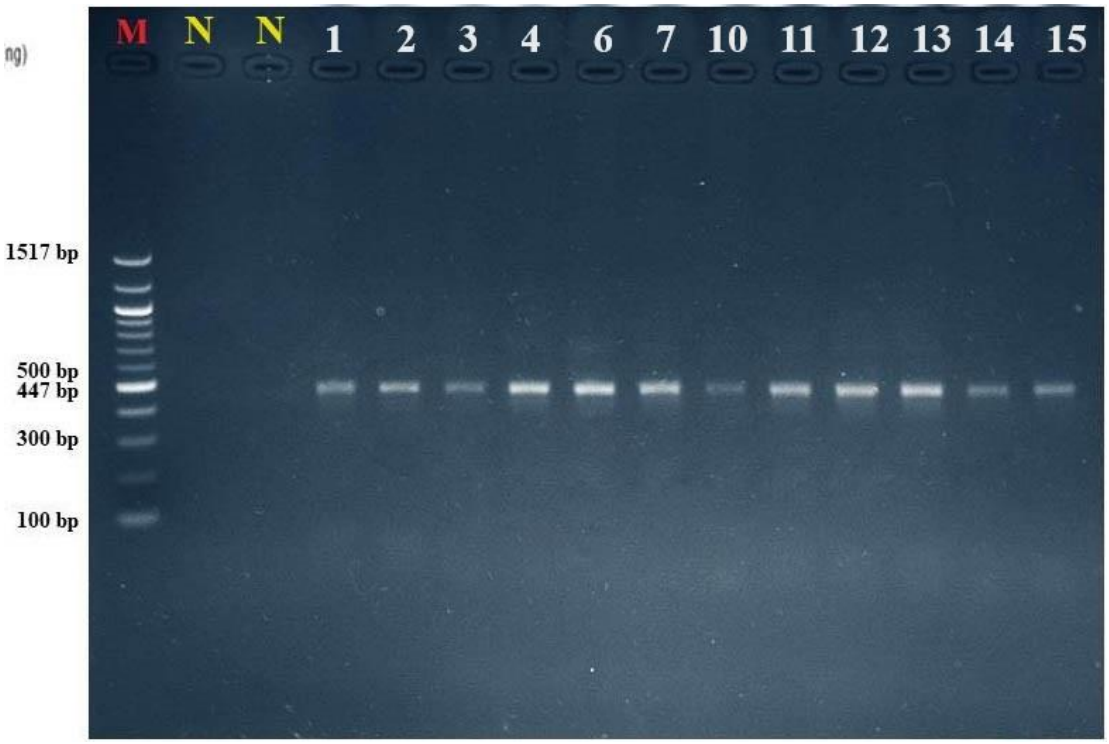
Table 2: Statistical analysis of the chi-squared test for distribution and percentage of Meloidogyne species in collecting regions. The Chi-square includes cell contents showing: *N: Number of samples *N / Row total *N / Col total *N / Table total.

Region	*arenaria*	*enterolobii*	*hapla*	*incognita*	*javanica*	Total
Banjan	0	0	0	6	3	9
	0.148	0.443	0.590	0.257	14.776	
	0.000	0.000	0.000	0.667	0.333	0.148
	0.00	0.00	0.00	0.12	1.00	
	0.000	0.000	0.000	0.098	0.049	
Bazian	0	3	0	6	0	9
	0.148	14.776	0.590	0.257	0.443	
	0.000	0.333	0.000	0.667	0.000	0.148
	0.00	1.00	0.00	0.12	0.00	
	0.000	0.049	0.000	0.098	0.000	
Ghanaia	1	0	0	4	0	5
	10.282	0.246	0.328	0.002	0.246	
	0.200	0.000	0.000	0.800	0.000	0.082
	1.00	0.00	0.00	0.08	0.00	
	0.016	0.000	0.000	0.066	0.000	
Haouish	0	0	0	5	0	5
	0.082	0.246	0.328	0.198	0.246	
	0.000	0.000	0.000	1.000	0.000	0.082
	0.00	0.00	0.00	0.10	0.00	
	0.000	0.000	0.000	0.082	0.000	
Khizamiah	0	0	0	5	0	5
	0.082	0.246	0.328	0.198	0.246	
	0.000	0.000	0.000	1.000	0.000	0.082
	0.00	0.00	0.00	0.10	0.00	
	0.000	0.000	0.000	0.082	0.000	
Rahmaniyah	0	0	2	3	0	5
	0.082	0.246	8.528	0.294	0.246	
	0.000	0.000	0.400	0.600	0.000	0.082
	0.00	0.00	0.50	0.06	0.00	
	0.000	0.000	0.033	0.049	0.000	
Saouira	0	0	0	5	0	5
	0.082	0.246	0.328	0.198	0.246	
	0.000	0.000	0.000	1.000	0.000	0.082
	0.00	0.00	0.00	0.10	0.00	
	0.000	0.000	0.000	0.082	0.000	
Shuan Kara	0	0	2	11	0	13
	0.213	0.639	1.545	0.011	0.639	
	0.000	0.000	0.154	0.846	0.000	0.213
	0.00	0.00	0.50	0.22	0.00	
	0.000	0.000	0.033	0.180	0.000	
Yusufiya	0	0	0	5	0	5
	0.082	0.246	0.328	0.198	0.246	
	0.000	0.000	0.000	1.000	0.000	0.082
	0.00	0.00	0.00	0.10	0.00	
	0.000	0.000	0.000	0.082	0.000	
Total	1	3	4	50	3	61
	0.016	0.049	0.066	0.820	0.049	

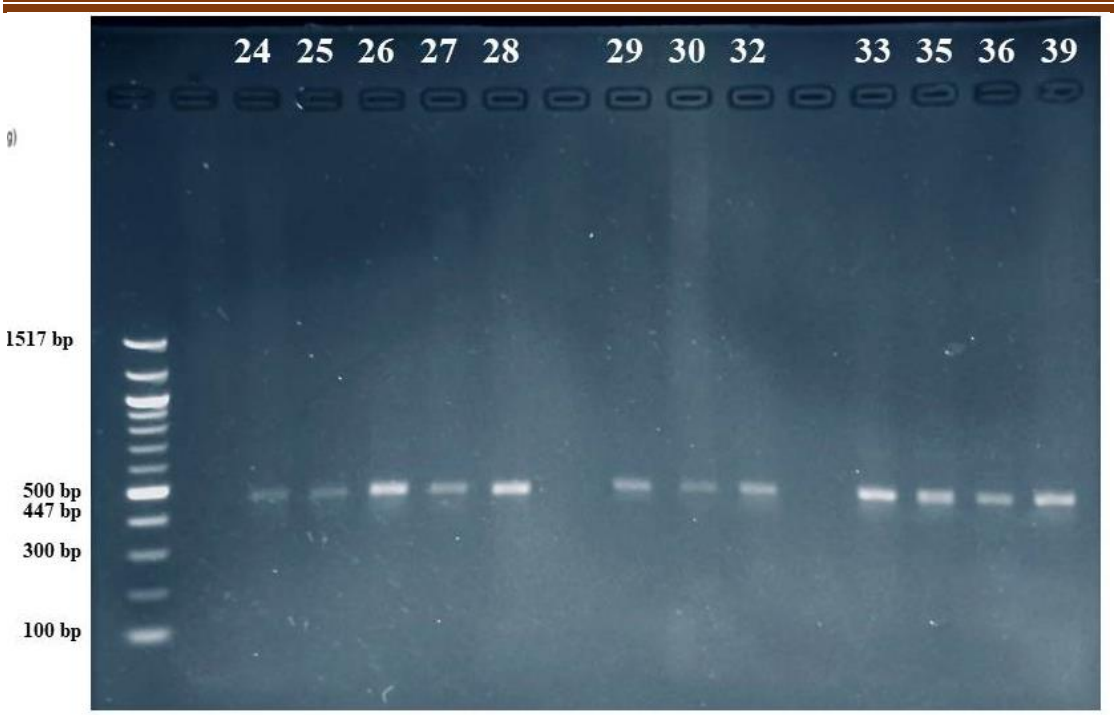
*Chi^2 = 60.37436 *d.f. = 32 * p = 0.00176.

Conclusions

This study concluded that severe cases of infection had an impact on root and vegetative systems, including fruit-dropping, leaf yellowing, and root damage due to inadequate water and nutrients for the plants. It also noted that most infections appear in the northern region due to high humidity and moderate temperatures and that the most dominant species was *M. incognita* representing 81.97% of all *Meloidogyne* species. Moreover, some other species diagnosed by SCAR-PCR regarding the cucumber crop were not very well known locally and had not been diagnosed previously in this area, specifically *M. enterolobii*. The results of this study reveal the absence of two species coexisting in the same location.



The *Meloidogyne incognita* PCR product (447 bp) with marker ladder 100–1517 bp.



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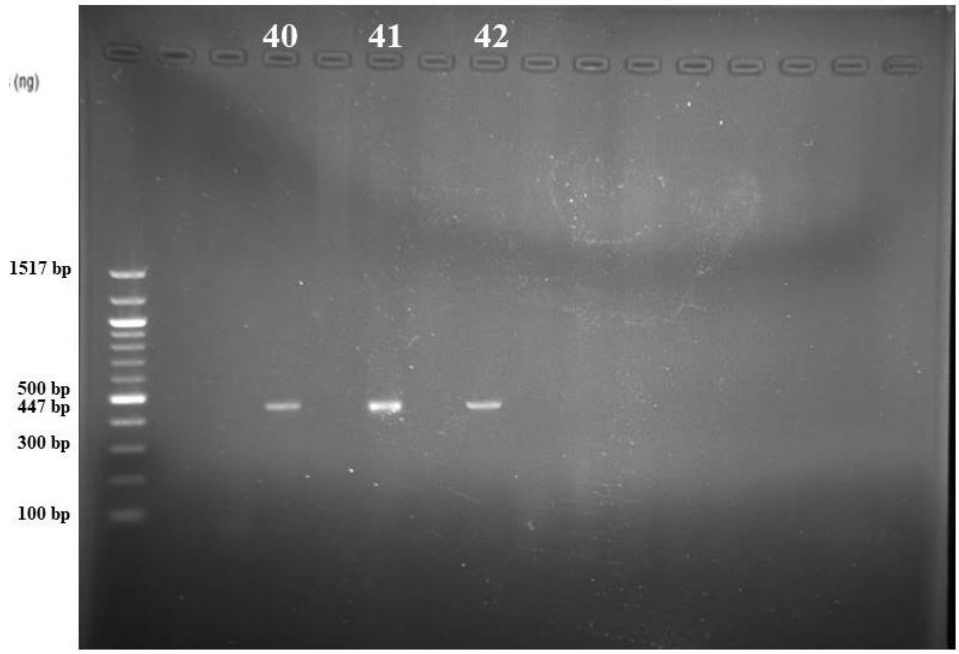

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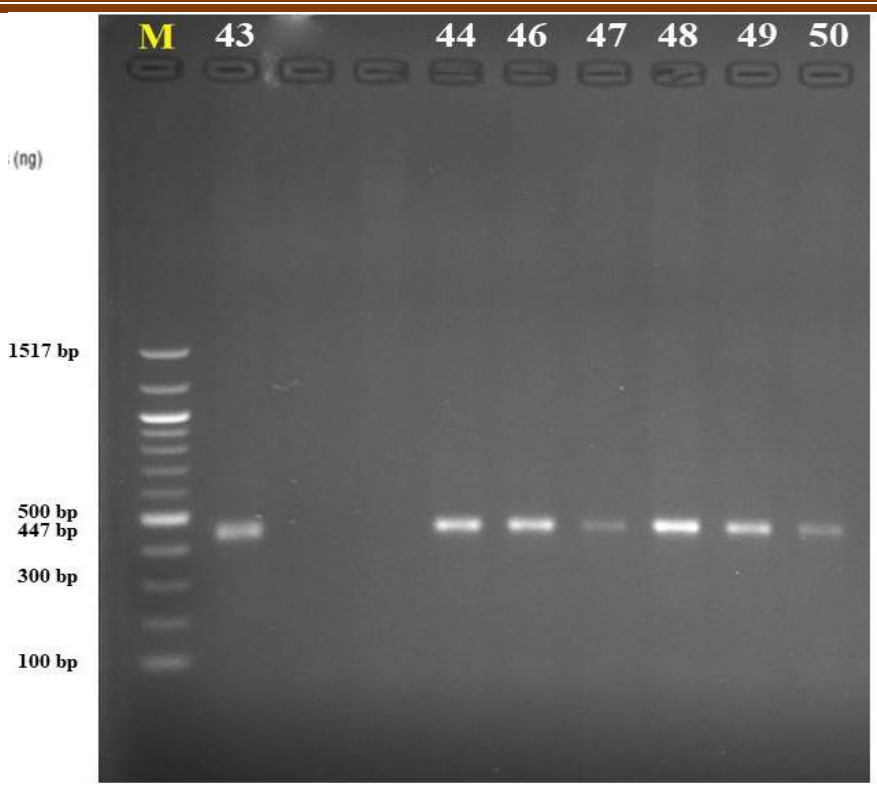

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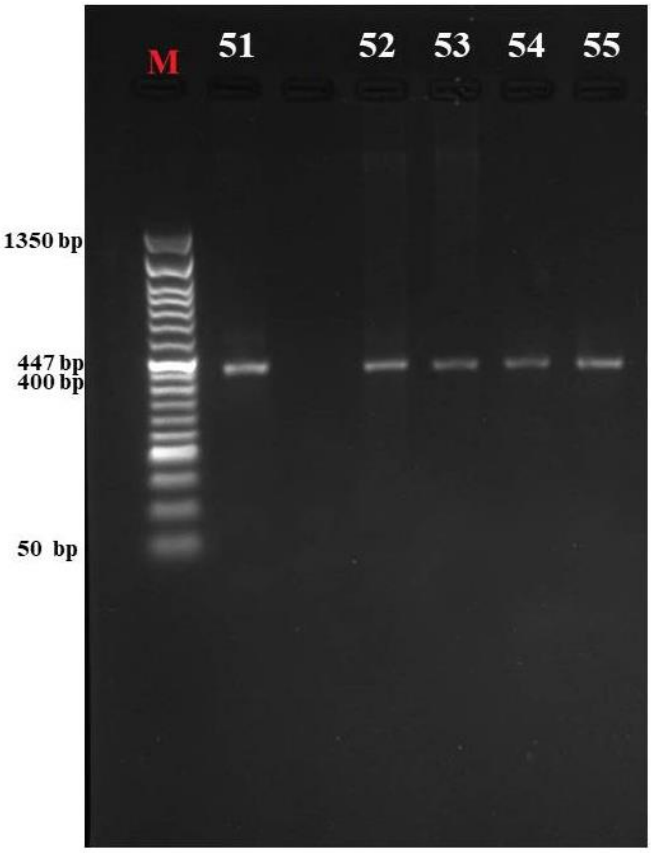
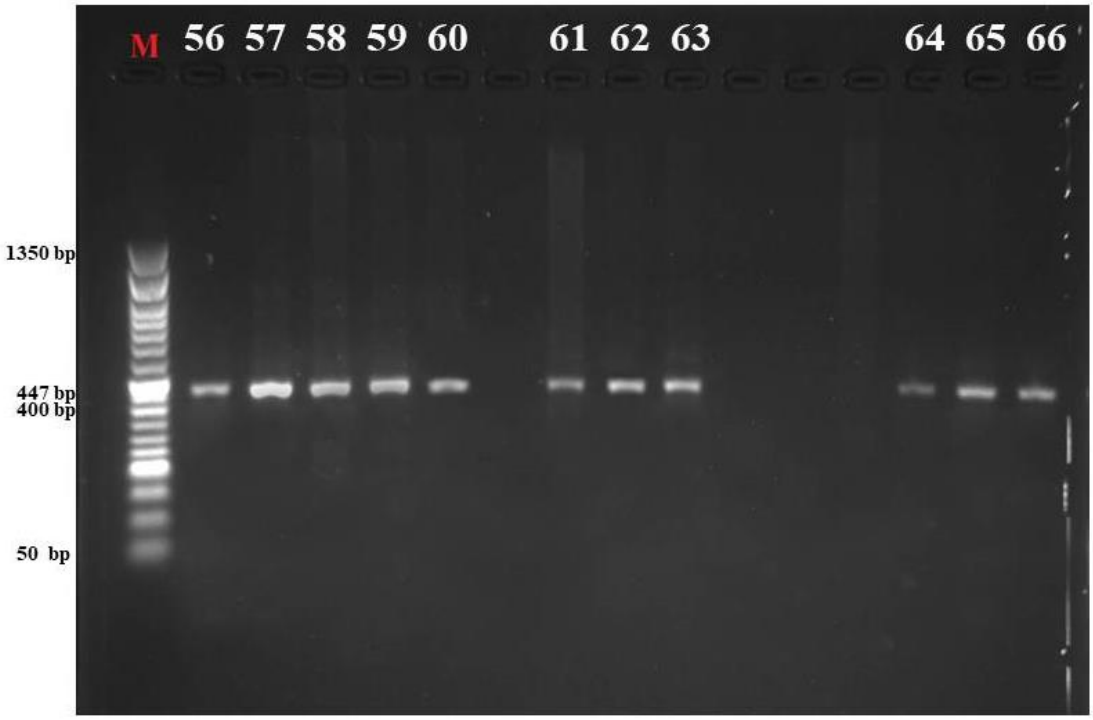
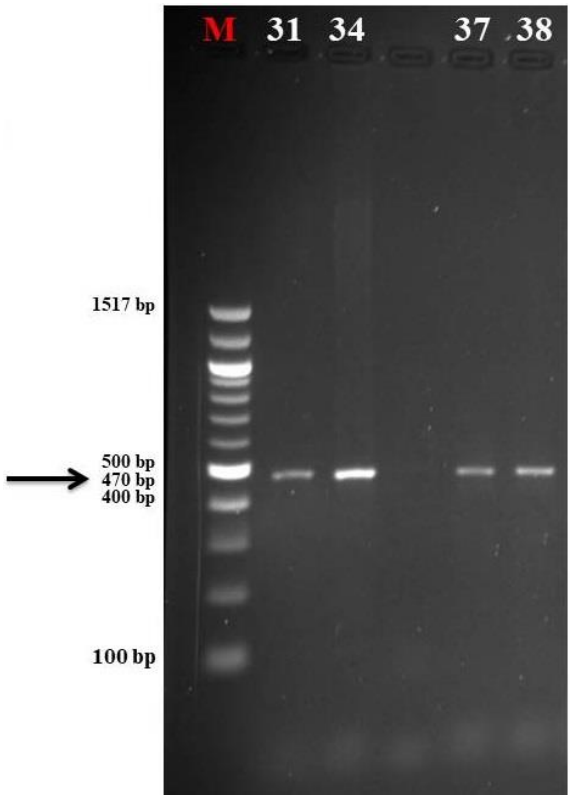


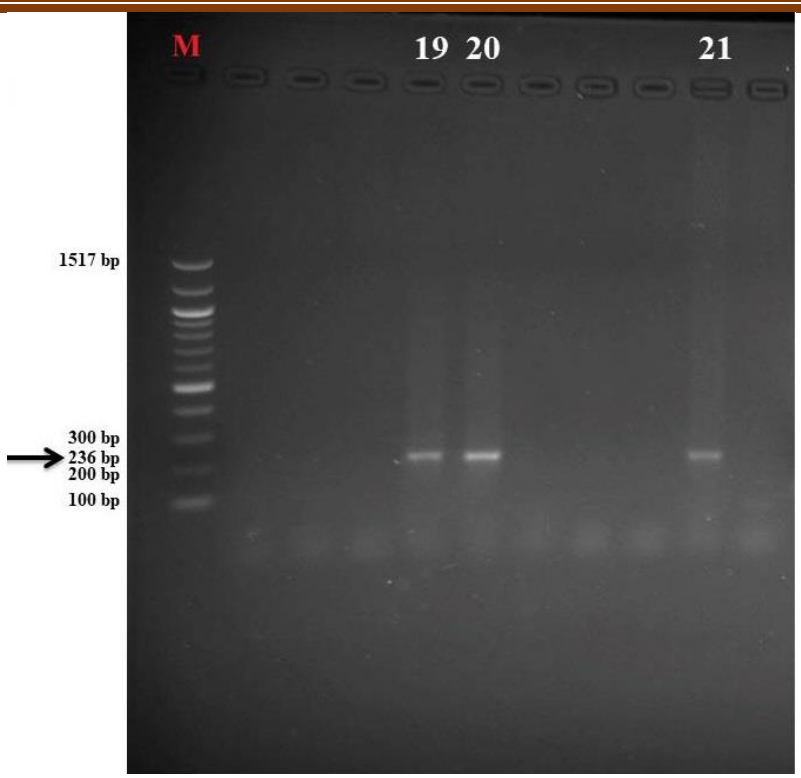
The *Meloidogyne incognita* PCR product (447 bp) with marker ladder 50–1350 bp.



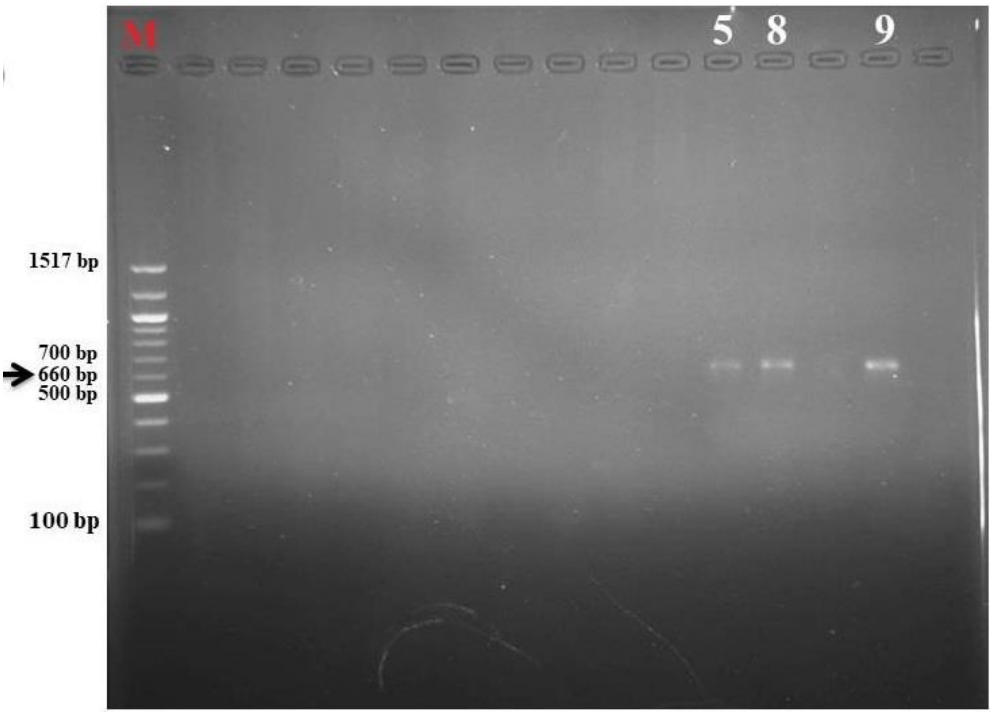
The *Meloidogyne incognita* PCR product (447 bp) with marker ladder 50–1350 bp.



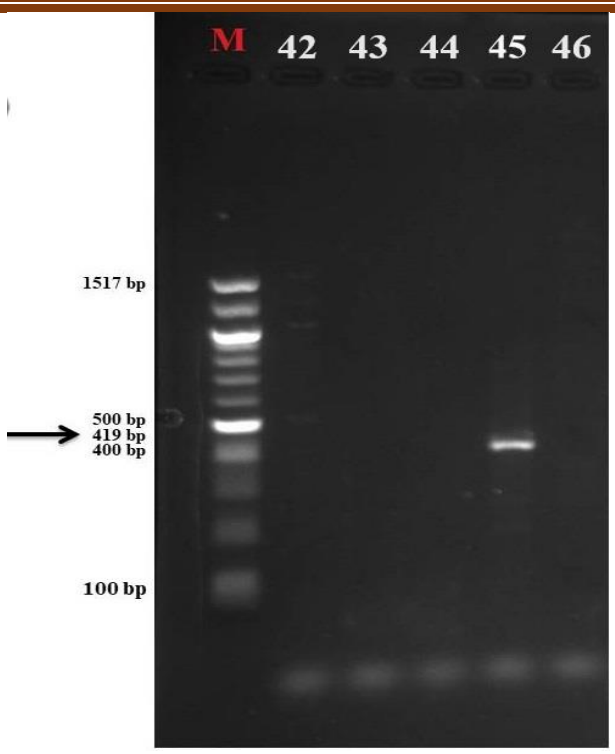
The *Meloidogyne hapla* PCR product (470 bp) with marker ladder 100–1517 bp.



The *Meloidogyne enterolobii* PCR product (236 bp) with marker ladder 100–1517 bp.



The *Meloidogyne javanica* PCR product (660 bp) with marker ladder 100–1517 bp.



The *Meloidogyne arenaria* PCR product (419 bp) with marker ladder 100–1517 bp.

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No Supplementary Materials.

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M. R. A. and A. A. S. wrote the main manuscript text, and S. M. K. conducted statistical analyses. All authors reviewed the manuscript.

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All relevant data are within the manuscript.

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The authors declare no conflict of interest associated with this manuscript.

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