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Morphological and molecular identification of root-knot nematodes infecting pomegranate in Assiut Governorate, Egypt

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Abstract

Root-knot nematodes are the most plant parasitic nematodes causing serious damage in pomegranate. Accurate identification of *Meloidogyne* species is of great importance for effective management of root-knot nematodes. Morphological identification of *Meloidogyne* spp. has been long and extensively used. However, molecular diagnostics was reported as a replacement or a complement method. Examination of the perineal patterns of the female was typically identified the nematode isolates as *M. javanica* which was dominant in all five pomegranate samples. For further confirmation of identification, a polymerase chain reaction (PCR)-based assay with two species-specific sequence characterized amplified regions (SCAR) primer sets was performed. The Fjav/Rjav and MJ-F/MJ-R primers efficiently amplified SCAR markers of 670 and 517 bp, respectively, which were previously reported for *M. javanica*. These results support that SCAR markers are a powerful tool for rapid and effective detection and could be used as a complementary tool together with the morphological identification of root-knot nematodes.

Key words: Root-knot nematodes, *Meloidogyne javanica*, pomegranate, perineal patterns, polymerase chain reaction, SCAR markers.

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Introduction

The pomegranate (*Punica granatum* L.) is an ancient fruit belongs to the family *Punicaceae* which includes one genus and two species. It is a widely grown as horticultural crop in many tropical and subtropical countries. In Egypt, pomegranate is considered one of the most important fruit trees cultivated in warm regions such as Assiut Governorate where the climate is characterized by long hot summer and low air humidity. The total area devoted for pomegranate was 26851 ikres and fruiting area was 9746 acres producing about 89035 tons with the average of 9.136 tons/acre. Assiut Governorate is considered the first in pomegranate cultivation that′s where the biggest area cultivated in Egypt (Ministry of Agriculture and Land Reclamation, 2012). Most of the pomegranate orchards found infested with root-knot nematodes (Khan and Shaukat, 2010; Khan et al., 2005). Root-knot nematodes are sedentary obligate endoparasitic nematodes, which common in Egypt and worldwide and cause severe damage especially in light soils that cause major economic damage to crops (Khan et al., 2008). Accurate and careful identification of *Meloidogyne* species infecting crops is a core for efficient use of plant resistance and successful management of root-knot nematodes (Mwesige et al., 2016). Several methods were used to identify root-knot nematode species such as morphological characters (Eisenback & Triantaphyllou, 1991), host plant response (Hartman & Sasser, 1985), isozyme analyses (Esbenshade & Triantaphyllou, 1990) and molecular techniques (Daramola et al., 2015; Hassan et al., 2013; Powers et al., 2005; Powers et al., 1997; Powers & Harris, 1993). The perineal pattern is often an unreliable character when used alone for making diagnostic conclusions but, when used as a complementary tool together with enzyme characterization or molecular analysis, is essential for checking the morphological consistency of the identification (Carneiro et al., 2004). Therefore, molecular diagnostics of *Meloidogyne* species has been sought as a replacement or complement for these procedures (Powers, 1992; Hyman, 1990; Burrows, 1990). Over the past years, different molecular analyses and PCR-based detection methods have been developed and widely used for nematodes identification, including amplified fragment length polymorphism (AFLP) (Hyman & Whipple, 1996; Hyman, 1990), random amplified polymorphic DNA (RAPD) (Naz et al., 2013; Carneiro et al. 2004), restriction fragment length polymorphism (RFLP) (Tomaszewski et al., 1994) and species-specific or sequence characterized amplification region (SCAR) primers (Daramola et al., 2015; Akyazi & felek, 2013; Naz et al., 2012;Devran & Sogut, 2009). RAPD technique is a simple molecular marker and easy to develop, but lack of reproducibility makes it less reliable for authentication. Therefore, to improve the reliability of RAPDs, (Paran & Michelmore, 1993) developed SCAR technique. SCARs are based on sequencing the polymorphic fragment derived from RAPD primers and designing longer primers that will specifically bind to this fragment. SCAR markers are more advantageous than RAPD markers because they usually detect only a single locus and it is more

specific. Moreover, their PCR amplification is less sensitive to reaction conditions and therefore they are reproducible (Idrees & Irshad, 2014). The present study was carried out in order to identify five root-knot nematode isolates collected from five localities cultivated with pomegranate in Assiut Governorate based on morphological identification and molecular analysis.

Materials and methods

Identification of nematode isolates: Five isolates of root-knot nematodes were collected from five localities of Assiut Governorate, Egypt (El-Badary, Sedfa, Sahel-Selim, El-Fath and Manfalout Counties) cultivated with pomegranate orchards. Adult females were extracted to prepare perineal patterns.

Perineal patterns: Infected roots prepared from pure culture collected and washed. Perineal patterns were prepared according to a method described by Taylor and Netscher (1974), Nono-Womdim et al. (2002), Eisenback (2010) and Khan (2014), the root tissues were teased apart with forceps and half spear to remove adult females. Females were transferred to a drop of tap water on a glass microscope slide. The cuticle of the female ruptured near the neck and gently pushed the body tissue out. After that the cuticle was placed in a drop of 45% lactic acid on a glass microscope slide for 30 minutes. The cuticle was cutting in half with blade (razor) and the perineal patterns trimmed to a square. The perineal patterns were transferred to a drop of glycerin on a clean glass microscope slide. The interior surface of the cuticle was placed on the glycerin drop against the glass slide. Cover slip was sealed and the slide has been labeled and examined under research microscope.

DNA Extraction and PCR assay: DNA extraction from nematode isolates was performed following cetyl trimethyl ammonium bromide (CTAB) method (Mondino et al., 2015; Sambrook et al., 1989) with slight modifications. In order to confirm morphological identification of nematode isolates, two species-specific SCAR primer sets selected from previous studies as markers specific for *M. javanica*, namely Fjav/Rjav (Zijlstra et al., 2000) and MJ-F/MJ-R (Meng et al., 2004) were used in the PCR (Table 1). PCR amplifications were performed in 25µl reaction mixtures, each containing 5-10 ng of genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.8 µM of each primer, and 1U Taq DNA-polymerase. Amplifications were performed in a SensoQuest Lab Cycler (SensoQuest GmbH, Göttingen, Germany) using the following PCR profile: initial denaturation at 94°C for 4 min, followed by 45 cycles each consisting of 30 sec at 94°C, 30 sec at 60°C for Fjav/Rjav and 55°C for MJ-F/MJ-R primers, followed by 90 sec at 72°C, with a final extension at 72°C for 10 min.

Electrophoresis: PCR products were separated using horizontal gel electrophoresis unit on 1.5% agarose gel stained with ethidium bromide in 0.5 X TBE buffer. A 100 bp DNA ladder was used to estimate the size of each amplified DNA fragment. The gel was run for approximately 2-3 hours using

constant voltage of around 80 V and then visualized and photographed under UV light using a gel documentation system. The specific band with the expected size was then detected for each SCAR marker separately.

Table 1: SCAR primers used for molecular identification of M. javanica.

Primer name	Fragment size (bp)	Sequence (5'-3')
Fjav/Rjav	670	F: GGTGCGCGATTGAACTGAGC R: CAGGCCCTTCAGTGGAACTATAC
MJ-F/MJ-R	517	F: ACGCTAGAATTCGACCCTGG R: GGTACCAGAAGCAGCCATGC

Results and Discussion

Examination of the perineal patterns of the females, hand picked up from infected pomegranate roots exhibited features typical to *M. javanica*. This species was dominant in all five pomegranate samples collected from Sedfa, El-Fath, Sahel-Selim, El-Badary and Manfalout counties Fig. (1). The important diagnostic characters of perineal patterns of the *M. javanica*, were summarized as low and rounded dorsal arch, contain lateral ridges that divide the pattern into dorsal and ventral regions or striae. Striae were coarse and smooth to slightly wavy and tail terminus often with distinct whorl. This species *M. javanica* was differed from the three other common species (*M. arenaria, M. hapla* and *M. incognita*) by containing its pattern on lateral field as described by (Eisenback et al., 1981). A combination of identification methods was used to separate root-knot nematode species. Results from the sampled areas indicated that, the presence of *M. javanica* which is not surprise as it is mentioned as the most common *Meloidogyne* species in tropical and subtropical regions (Moens *et al.,* 2009;Taylor & Sasser, 1978) like Egypt where, annual temperatures are between 17-32˚C. *M. javanica* was dominated at the present studied samples. A similar finding was reported by Nono-Womdin et al. (2002) in Tanzania on tomato plants. This result is in disagreement with Eisenback et al. (1981) where, *M. incognita* was the most prevalent among all *Meloidogyne* species in the studied areas in the international *Meloidogyne* project.

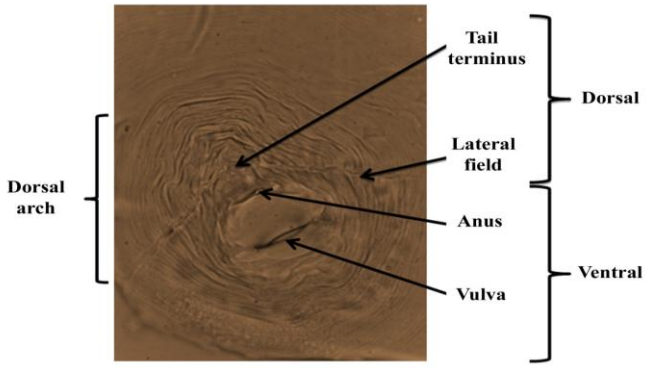

Figure 1: Perineal pattern of *M. javanica*.

The PCR assay for the five nematode isolates with the specific SCAR primer Fjav/Rjav clearly produced a specific DNA fragment of 670 bp (Fig. 2A), which was expected for *M. javanica* as reported by Zijlstra et al. (2000). Similar findings were reported by Devran and Sogut (2009) in Turkey and Naz et al. (2012) in Pakistan. Consistently, the *M. javanica*-specific primers MJ-F/MJ-R generated a SCAR product of 517 bp with also the five nematode isolates (Fig. 2B), which was identical to those previously reported for *M. javanica* (Meng et al., 2004). In accordance Song et al. (2017) have extracted DNA from a single J2 hatched from egg masses and used the MJ-F/MJ-R primers to further confirm species identification. They found that PCR products produced a fragment length of 517 bp (GenBank accession no. KX646189), which was identical to those previously reported for *M. javanica*.

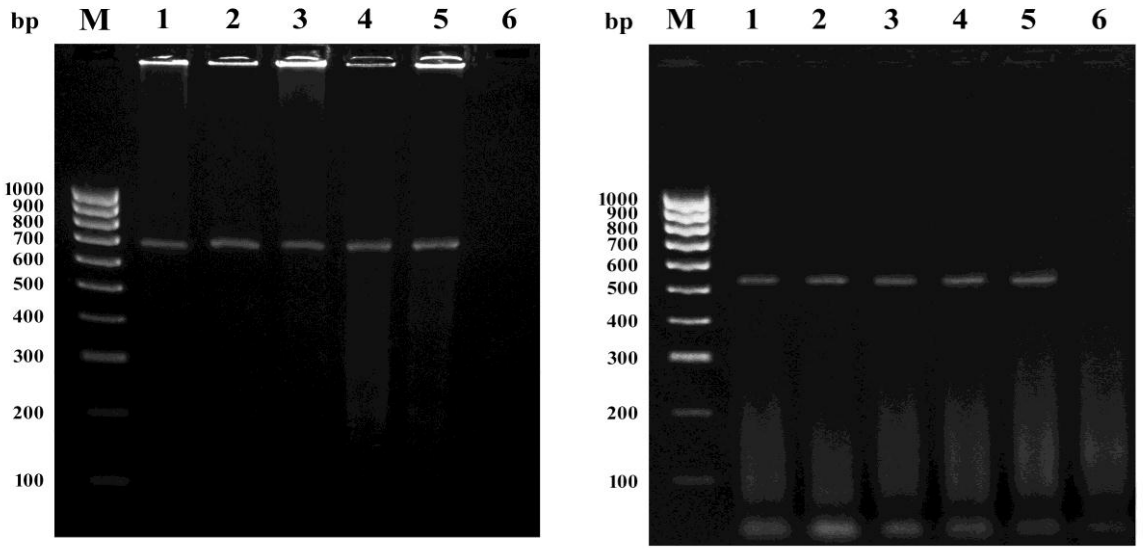

Figure 2: Amplification products (670 and 517 bp) generated with the *M. javanica* species-specific Fjav/Rjav (A) and MJ-F/MJ-R (B) SCAR primers, respectively. M: 100 bp DNA Ladder, 1-5 *M. javanica* isolates 1: El-Badary, 2: Manfalout, 3: Sedfa, 4: Sahel-Selim, 5: El-Fath and 6: water as a negative control (PCR reaction without DNA).

Identification by morphological characters and host plant response is time consuming and needs extensive labor. Isozymes analysis can be carried out only on female individuals and can be affected by environmental factors (Esbenshade & Triantaphyllou, 1990). Unlike, molecular techniques based on DNA can be used in every stage of the nematode's life cycle, and they are rapid, and reliable (Devran & Sogut, 2009). For molecular identification of species, the characteristic sequence of genomic DNA of different species should differ to allow the delineation of species, but at the same time, no/minor variation within the species should exist (Devran & Sogut, 2009; Blok & Powers, 2009). PCR-based markers have been used especially to allow a clear and rapid species diagnosis when external morphological characters are not fully discriminated. SCAR

markers have been commonly used in genomic analysis and widely used for molecular identification of root-knot nematodes to confirm morphological identifications as well as to identify unknown isolates (Akyazi & Felek, 2013; Naz et al., 2012; Jones et al., 2009; Devran & Sogut, 2009; Randig et al., 2002; Zijlstra et al., 2000). Species-specific SCAR primers amplify the DNA fragment(s) belonging to only one species and are desirable to accurately identify nematode species Moreover, SCAR markers can be amplified from DNA from egg masses, second stage juveniles and females and was successfully applied using DNA extracts from infested plant material. Therefore, the method has potential to be optimized for routine practical diagnostic tests facilitating the control of these economically important pest organisms (Zijlstra et al., 2000). In conclusion, it is clear that, results from perineal pattern examination and molecular analysis were consistent with each other, suggesting that molecular identification using SCAR markers could be used as a complementary tool together with the morphological identification of root-knot nematodes.

References

Akyazi F, Felek AF, 2013. Molecular identification of root-knot nematode *Meloidogyne incognita* from kiwi fruit orchards in Ordu province, Turkey. Turkish Journal of Entomology **37**(4): 449–456.

Blok VC, Powers TO, 2009. Biochemical and Molecular identification. In: Perry R. N.; M. Moens; J. L. Starr (eds.). Root-knot Nematodes. Wallingford/UK, CABI Publishing, United Kingdom.

Burrows PR, 1990. The use of DNA to identify plant parasitic nematodes. Nematological Abstracts **59**: 1–8.

Carneiro RMDG, Tigano MS, Randig O, Maria R, Almeida MRA, Sarah JL, 2004. Identification and genetic diversity of *Meloidogyne* spp. (Tylenchida: Meloidogynidae) on coffee from Brazil, Central America and Hawaii. Nematology **6**: 287–298.

Daramola FY, Popoola JO, Eni AO, Sulaiman O, 2015. Characterization of root-knot nematodes (*Meloidogyne* spp.) associated with *Abelmoschus esculentus*, *Celosia argentea* and *Corchorus olitorius.* Asian Journal of Biological Sciences **8**(1): 42–50.

Devran Z, Söğüt MA, 2009. Distribution and identification of root-knot nematodes from Turkey. Journal of Nematology **41**(2): 128–133.

Eisenback DE, Triantaphyllou HH, 1991. Root-knot nematodes: *Meloidogyne* species and races. In W. R. Nickle, (ed.) Manual of Agricultural Nematology, New York: Marcel Dekker Inc., USA, 191–274 pp.

Eisenback JD, 2010. A new technique for photographing perineal patterns of root-knot nematodes. Journal of Nematology **42**(1): 33–34.

Eisenback JD, Hirschman H, Sasser JN, Triantaphyllou AC, 1981. A guide to four most common species of root-knot nematodes (*Meloidogyne* spp.), with a pictorial key. International *Meloidogyne* Project, the Department of Plant Pathology and Genetics North Carolina, United State, Part 3: 22–26 pp.

Esbenshade PR, Triantaphyllou AC, 1990. Isozyme phenotypes for the identification of *Meloidogyne* species. Journal of Nematology **22**: 10–15.

Hartman KM, Sasser JN, 1985. Identification of *Meloidogyne* species on the basis of differential host test and perineal patterns morphology. In Barker KR, Carter CC, Sasser JN, eds. An Advanced Treatise on *Meloidogyne* volume II: Methodology. North Carolina State University Graphics, USA, 69–77 pp.

Hassan MA, Pham TH, Shi H, Zheng J, 2013. Nematodes threats to global food security. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science **63**(5): 420–425.

Hyman BC, 1990. Molecular diagnosis of *Meloidogyne* species. Journal of Nematology **22**: 24–31.

Hyman BC, Whipple LE, 1996. Application of mitochondrial DNA polymorphism to *Meloidogyne* molecular population biology. Journal of Nematology, **28**(3): 268–276.

Idrees M, Irshad M, 2014. Molecular markers in plants for analysis of genetic diversity: A review. European Academic Research **2**(1): 1513–1540.

Khan A, Shaukat SS, 2010. An analysis of phytonematode associated with pomegranate in Khuzdar and Kalat district, Balochistan. Pakistan Journal of Agricultural Sciences **23**: 147–150.

Khan A, Shaukat SS, Siddiqui IA, 2005. A survey of nematodes of pomegranate orchards in Balochistan province, Pakistan. Nematologia Mediterranea **33**: 25–28.

Khan MR, 2014. A simple technique for preservation of female perineal pattern of *Meloidogyne*s pp. The Journal of Plant Protection Sciences **6**(1): 35–36.

Khan Z, Kim SG, Jeon YH, Khan HU, Son SH, Kim YH, 2008. A plant promoting rhizobacterium, *Paenibacillus polymyxa* strain GBR-1, suppresses root-knot nematode. Bioresource Technology **99**: 3016–3023.

Meng QP, Long H, Xu JH, 2004. PCR assays for rapid and sensitive identification of three major root-knot nematodes, *Meloidogyne incognita, M. javanica*and *M. arenaria.* Acta Phytopathologcia Sinica **34**: 204–210.

Ministry of Agriculture and Land Reclamation, 2012. Bulletin of the Agricultural Statistics, Part (2), 276 pp. (In Arabic)

Moens M, Perry RN, Starr JL, 2009. *Meloidogyne* species a diverse group of novel and important plant parasites. In Perry, R.N.; M. Moens and J.L. Starr (eds). Root-knot nematodes CABI International, Cambrige, MA, USA, 1–17 pp.

Mondino EA, Covacevich F, Studdert GA, Pimentel JP, Berbara RL, 2015. Extracting DNA of nematodes communities from Argentine Pampas agricultural soils. Annals of the Brazilian Academy of Sciences **87**(2): 691–697.

Mwesige R, Seid A, Wesemael W, 2016. Root-knot nematodes on tomatoes in Kyenjojo and Masaka districts in Uganda. African Journal of Agricultural Research **11**: 3598–3606.

Naz I, Palomares-Rius J, Blok V, Saifullah SA, Ahmad M, 2012. Prevalence, incidence and molecular identification of root-knot nematodes of tomato in

Pakistan. African Journal of Biotechnology **11**(100): 16546–16556.

Naz I, Saifullah SA, Palomares-Rius JE, Blok V, Ahmad M, Ali S, 2013. Species identification of root knot nematodes in Pakistan by random amplified polymorphic DNA (RAPD-PCR). Sarhad Journal of Agriculture **29**(1): 71–78.

Nono-Womdim R, Swai I, Mrosso L, Chadha M, Opeňa R, 2002. Identification of root-knot nematode species occurring on tomatoes in Tanzania and resistant lines for their control. Plant Disease **86**: 127–130.

Paran I, Michelmore RW, 1993. Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. Theoretical and Applied Genetics **85**: 985–933.

Powers TO, 1992. Molecular diagnostics for plant nematodes. Parasitology Today **8**: 177–179.

Powers TO, Mullin PG, Harris TS, Sutton LA, Higgins RS, 2005. Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. Journal of Nematology **37**: 226–235.

Powers TO, Todd TC, Burnell AM, Murray PCB, Fleming CC, Szalanski AL, Adams BA, Harris TS, 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. Journal of Nematology **29**: 441–450.

Powers TO, Harris TS, 1993. A polymerase chain reaction method for identification of five major *Meloidogyne* species. Journal of Nematology **25**: 1–6.

Randig O, Bongiovanni M, Carneiro RMDG, Castagnone-Sereno P, 2002. Genetic diversity of root-knot nematodes from Brazil and development of SCAR marker specific for the coffee damaging species. Genome **45**: 862–870.

Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular cloning: A Laboratory Manual (2nded.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Song ZQ, Cheng FX, Zhang DY, Liu Y, 2017. First report of *Meloidogyne javanica* infecting Hemp (*Cannabis sativa*) in China. Plant Disease **101**(5): 842.

Taylor A, Sasser J, 1978. Biology, identification and control of root-knot nematodes. North Carolina State University Graphics.

Taylor DP, Netscher C, 1974. An improved technique for preparing perineal patterns of *Meloidogyne* spp. Nematological **20**(2): 268–269.

Tomaszewski EK, Khalil MAM, El-Deeb AA, Powers TO, Starr JL, 1994. *Meloidogyne javanica* parasitic on peanut. Journal of Nematology **26**(4): 436–441.

Zijlstra C, Donkers-Venne DTHM, Fargette M, 2000. Identification of *Meloidogyne incognita, M. javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based PCR assays. Nematology **2**: 847–853.