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IDENTIFICATION AND DISTRIBUTION OF ROOT-KNOT NEMATODE SPECIES IN POMEGRANATE ORCHARDS OF EASTERN IRAN

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A B S T R A C T

Root-knot nematodes are the most damaging plant-parasitic nematodes in pomegranate and are widely distributed in Iran. During the 2015/16 growth season, 195 root and soil samples were collected from major areas of pomegranate orchards in the eastern of Iran (Khorasan Razavi, Northern and Southern provinces). Species identification was carried out according to the molecular method by sequences of D2a-D3b expansion domains of 28S rDNA, sequence characterized amplified regions (SCAR). Morphological diagnostic was conducted on perineal pattern of females. Pomegranate infection to *Meloidogyne* spp. was observed in almost all regions, but infection severity was very variable. Several species of *Meloidogyne* were identified and showed band399 bp in *M. incognita* by Inc-14 primer, 670 bp in *M. javanica* by Jav primer, and 420 bp in *M. arenaria* by ar primer. *M. cruciani* showed 820 bp band by D2-D3 primer but the sequence was not distinct from other species and use perineal pattern morphology. Subcuticular punctuation around the anus on lateral posterior sides has characterized the perineal pattern in *M. cruciani*. *M. incognita* is the predominant species with 60/8% and showed the highest distribution in the city of Bardaskan orchards in Khorasan Razavi province.

Keywords: Root-knot nematode, Pomegranate, D2a-D3b, SCAR, Eastern of Iran.

INTRODUCTION

Pomegranate (*Punica granatum* L., Punicaceae) is an ancient, popular tree in Iran. This tree has a broad geographical dispersion spread of Iran to the Himalayas and has been planting since old times throughout the Mediterranean regions of Asia, Africa, and Europe (Levin 1994; Holand *et at*. 2009). These fruits have achieved great attention for their antioxidant and nutritional values that are important for human health (Tehranifar *et at*. 2010). In 2019, Iran was the world's largest producers and exporters country contributing 1,009,885 tons of pomegranates to the world's annual production (Anonymous, 2019). Root-knot nematodes-members of the genus *Meloidogyne*-can be said to illustrate the definitive success between plant-parasitic nematodes.

The numbers of this genus especially for species (*Meloidogyne arenaria*, *Meloidogyne javanica* , *Meloidogyne incognita* and *Meloidogyne hapla*) have host ranges encompassing more than a thousand plant species and are spread all over the temperate and (sub)tropical regions of the world. (Jones *et at*., 2013). *Meloidogyne* species are the foremost damaging plant-parasitic nematodes in pomegranate as the firstly create galls of the root system that eventually are seriously prevented in their functions of uptake and transport of water and nutrients. Infected pomegranate show yellowing of foliage, leaf abscission, stunted growth and as a result produced less or undersized fruits (Sudheer *et at*. 2007; El-Deen *et at*. 2016). The species of *Meloidogyne javanica*, *M. incognita*, *M. arenaria*, and *M. hapla* recognized from infected pomegranate orchards in regions of Yazd, Gillan, Tehran, Saveh, Ghom, Fars provinces of Iran (Akhyani 1987; Kargarbideh 1989; Hatamabadi Farahani *et at.* 2018). *M.incognita,* and *M.*

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javanica are serious plant parasite nematode of pomegranate in several other parts of the world (El-Deen *et at*. 2016). Recognition of *Meloidogyne* species are more important for the plan of nematode management such as crop rotation and plant resistance but due to highly conserved morphology across species is more difficult. Morphology and morphometry on the second stage juvenile, male, female and perineal patterns, host races and isozymes are used for the identification of *Meloidogyne* species (Ye *et at*. 2015). Nowadays, employed of molecular methods is usual for the identification of *Meloidogyne* species. The most important of molecular markers are available as restriction fragment length polymorphisms, random amplified polymorphic DNA, sequence characterized amplified region and molecular techniques including real-time identify PCR assays based on sequences of rDNA, mt DNA, ITS, and IGS (Zijlstra *et at*. 2000; Blok & Powers, 2009; Ye *et at.* 2015; Zeng *et at*. 2015). Recently, SCAR primers are extracted of sequences of species-specific RAPD markers and enable to detection of the species *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. chitwoodi* and *M. fallax* present in mixed populations in proportions of less than 1 %.(Fourie *et at*. 2001).

Due to Iran's position in the pomegranate export market, identification of pests and diseases is an important way to safe control and enhance productivity. This study reports the identification, incidence, and diversity of various *Meloidogyne* species in the pomegranate orchards in the Razavi, Northern, and Southern Khorasan provinces Eastern of Iran.

MATERIALS AND METHODS

Collection and reproduction: 195 roots and soil samples were collected of pomegranate orchards in three provinces (Razavi, Southern, and Northern Khorasan) of Iran. Three or four sub-samples were taken from different parts of the orchard and mixed to make a composite sample of about 1 kg containing feeder roots with rhizosphere and bulk soil. Samples were transferred to the laboratory and placed in the cooler box at 7–12°C. Egg masses separated of pomegranate roots and inoculate a susceptible tomato (*Lycopersicum esculantum* Mill. cv. Rutgers) seedling in 4 leaf stage to prepare pure populations of nematodes in the greenhouse. After 60 days, tomato plants were harvested, soil washed from the roots, and several egg masses and female nematodes were collected for species identification. (Madani *et at*. 2012). The incidences of root-knot nematodes of individual pomegranate orchards were determined as follows by (Kayani, Mukhtar *et at*. 2012)

$$\text{Incidence (\%)} = \frac{\text{Total number of infected plants}}{\text{Total number of observed plants}} \times 100$$

Molecular identification: For DNA extraction used two methods. CTAB method used for DNA extraction of eggs and J2 described by Sam-brook *et at* (1989), and AE buffer for DNA extraction of mature female explain by Ye *et at* (2015). Four SCAR primers such as Inc-K14-F GGGATGTGTAAATGCTCCTG and Inc-K14-R CCCGCTACACCCTCAACTTC for *M. incognita*, Far TCGGCGATAGAGGTAAATGAC and Rar TCGGCGATAGACACTACAAACT for *M.arenaria*, Fjav GGTGCGCGATTGAACTGAGC and Rjav AGGCCCTTCAGTGGAACTATAC for *M. javanica* (Zijlstra *et at*. 2000), D2a ACAAGTACCGTGAGGGAAAGTTG and D3b TCGGAAGGAACCAGCTACTA for unknown *Meloidogyne* spp. (Schmitz *et at*., 1998) used for molecular identification. Primers were sourced from the Macrogen Company. Amplifications in PCR assay were performed in the final volume of 25µL including master 12.5 µl, forward and reverse primers 1µl, DNA 2µl, and 8.5 µl water. In PCR reaction to reduce the error rate was made stock solution than to put in micro tubes. Identify PCR reaction was set up at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing temperatures were different such as 62°C (Fjav/Rjav), 61°C (Far/Rar) and (Inc-K14-F/Inc-K14-R) for 30 s, 55°C (D2D3) for 40s extensions were 72°C for 90 s, with a final extension at 72°C for 10 minute.

Morphology of perineal pattern: The tomato roots after removing of soil, were washed and cut to small pieces, then the mature female was removed carefully from galls by using a thin needle. Under a stereomicroscope, cutting the posterior end of the mature female section of perineal were placed in 45% lactic acid for 30sec then perennially get in glycerin (Hooper 1986). Species identification was made according to Jepson (1987) and Karssen(2002).

RESULT

Distribution of pathogen: Investigation of all samples showed 41 % to be infected with Root-knot nematodes but was the difference in the severity of distribution rate. The distribution rate of Meloidogyne species in pomegranate orchards showed 28 populations as *M. incognita,* eight as *M. arenaria,* seven as *M. javanica*, one as *M. cruciani,*

and two unknown of *Meloidogyne* spp. *M. incognita* was observed in almost all orchards in the sampled regions and was recorded as the most common root-knot nematode. The distribution of root-knot nematode in all populations recorded *M. incognita* 60/86%, *M. arenari* 17/4%, *M.javanica* 15/23%, *M.cruciani* 2/17% and 4/34% unknown Meloidogyne (Figure1). Distribution percentage of the *Meloidogyne* spp. species were in Razavi Khorasan with17 %, Northern Khorasan with 4 %, and Southern Khorasan with 2.4 %. The infection rate of root-knot nematodes recorded 30/5 % Bardaskan, 17/03% Bajestan, 9/4% Khalil Abad, 6/3% Kashmar, 4/02% Torbat Heydarieh in Razavi, Ferduws 5.05%, and Nehbandan 1/9% in Southern, Mane 2.7%, and Jajrm 1.8% in Northern provinces (Figure 2). The unidentified *Meloidogyne* sp. was extracted in Bardaskan and Ferdows. Anabad in Bardaskan city showed the highest incidence of root-knot nematodes 57/5% in Razavi Khorasan, of course, this province had most pomegranate orchards are cultivated. Anabad-mohammadkhan in Bardaskan city showed more variety in species of *Meloidogyne* and to have *M. incognita*, *M. javanica*, *M. arenari,* and Meloidogyne spp. *Meloidogyne incognita* is the most abundant species that were recognized in all of the samples. *Meloidogyne cruciani* is the first report in the pomegranate orchards of Nehbndan.

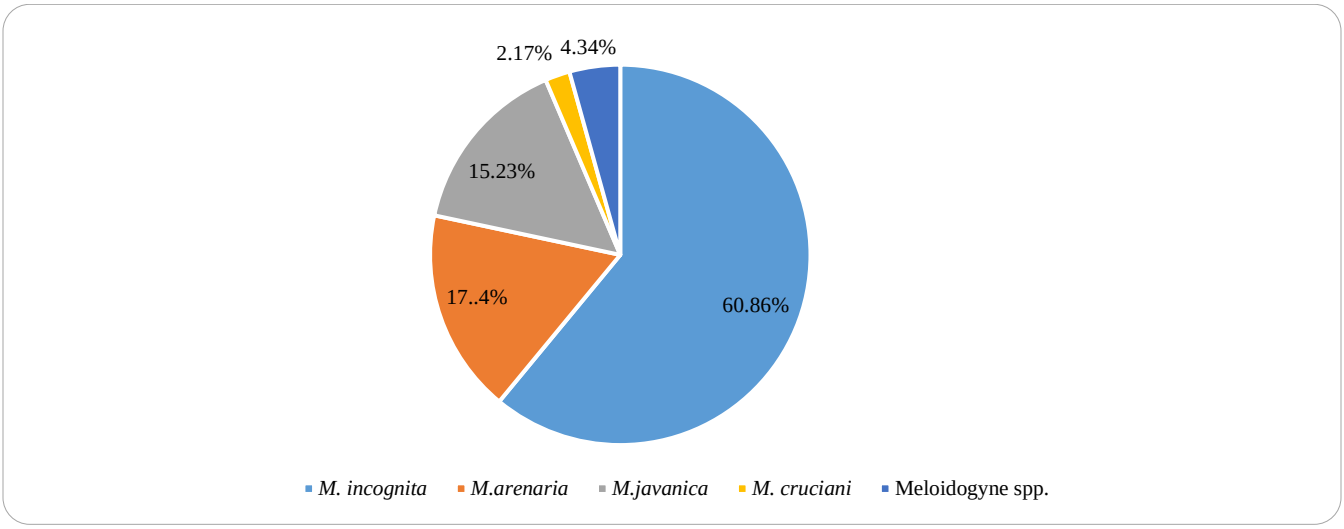
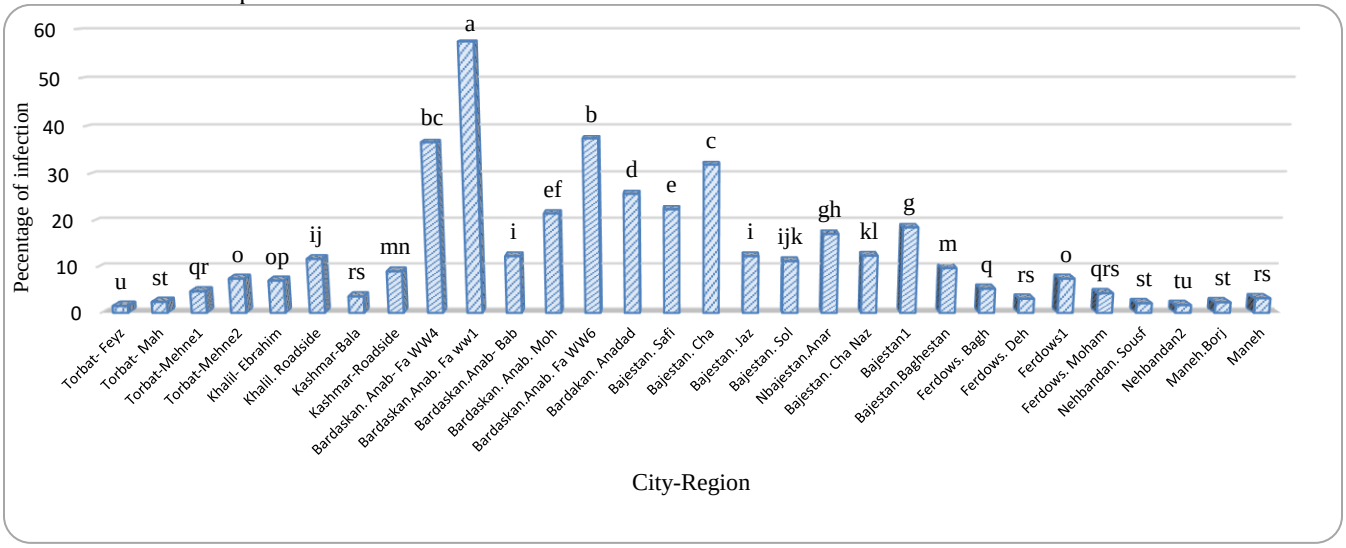


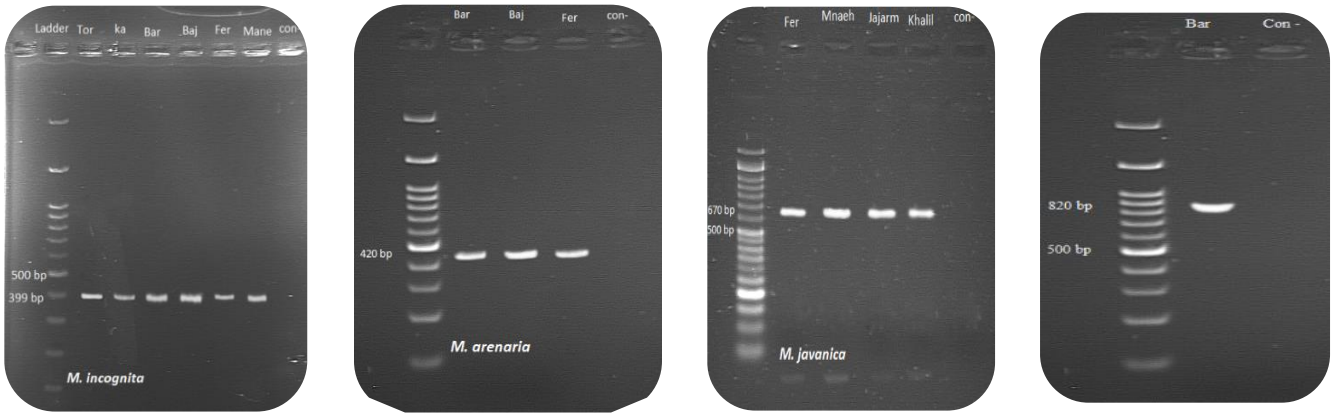
Figure 1. Distribution rate of Meloidogyne species in pomegranate orchards of Razavi, Northern, and Southern Khorasan provinces.



Significant differences according to Duncan's multiple range test $p<0.05$.

Figure 2. Infection rate and region of infected in pomegranate orchards in Razavi, Northern, and Southern Khorasan provinces.

Identification: The nematode species of *M. incognita*, *M.javanica*, *M. arenaria* were identified by analyses of SCAR primers. PCR with the inc-K14-F/inc-K14-R primer produced approximately 399 bp DNA fragments for all *M. incognita* populations. The Far/Rar primer set produced a 420 bp fragment, which was characteristic in all populations of *M. arenaria*. Fjav/Rjav primer sets produced 670 bp for *M. javanica* from all populations. A single specific band of 820 bp showed by D2a-D3b primer in *M. criciani* and unknown *Meloidogyne* but the sequences were not distinguished from other species and used morphology of perineal pattern. Negative control (water) has not produced any band and PCR amplification by other SCAR primer was negative (Figure 3).



(a) (b) (c.) (d)

Figure 3. Produced bands (a) 399 bp by primer Inc-14 for recognition of *M. incognita*, isolated of areas from Torbat Heidaryeh-Feyzabad, kashmar, Bardaskan, Bajestan, Ferdows, Maneh and control -, (b) 420 bp by primer ar for recognition of *M. arenaria* , isolated of areas from Bardaskan, Bajestan, Ferdows and control -, (c) 670 bp by primer Jav for recognition of *M. javanica,* isolated of areas from Ferdows, Maneh, Jajarm, Khalilababd and control-, (d) 820 bp by primer D2a-D3b for identification of *M. cruciani* isolated from Nehbandan, respectively Molecular marker 100 bp.

The result of the perineal pattern appeared present in *M. cruciani*. *M. cruciani* was explain by Garcia-Martinez *et at*. (1982) that characterized in subcuticular punctuation around the anus on lateral and posterior sides. Perineal patterns of *M.cruciani* similar with *M. javanica* in having distinctive lateral lines but differ in tile pronounced lateral lines of *M.cruciani* do not expand as far as of *M. javanica* and this have striae deep, wavy, sometimes broken. Vulva lips jagged, margins with very fine striae, with distinct phasmids. Phasmidial ducts are often visible tail terminus indistinct (Figure 4). Lately, *Meloidogyne cruciani* was reported of cucumber, tomato, pumpkin and pistachio in Iran. In this study the above species was isolated and identified from the roots of pomegranate trees for the first time in Iran.

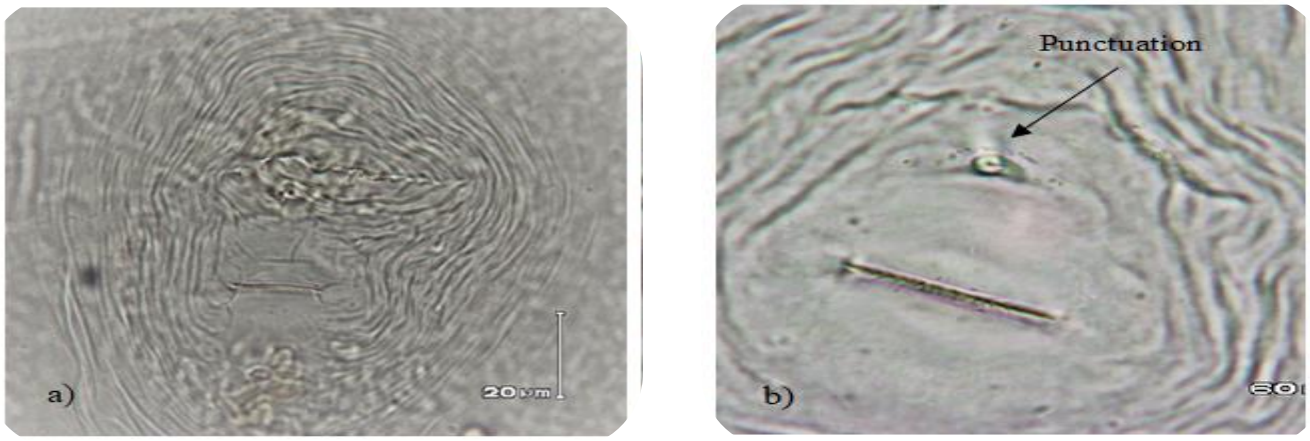


Figure 4. Perineal pattern in the mature female of *M. cruciani* in Nehbandan (a) and (b) subcuticular punctuations around the anus and vulva lips serrated. 100x

DISCUSSION

This study is the first specialized project that screens all *Meloidogyne* species infecting pomegranate orchards in Razavi, Northern, and Southern Khorasan provinces at a molecular level. The results of the present research confirmed that the root-knot nematodes are widely distributed in the pomegranate orchards. Survey results showed the existence of root-knot nematode disease in Razavi province more than in other regions. This study indicated the *M. incognita* has the highest distribution and infection percentage in eastern Iran and conforms to the previous study. Similar results were also reported by Hatam Abadi Farahani (2018), who reported *M. javanica* and *M.incognita* in pomegranate orchards of Saveh in central Iran and showed *M. incognita* is a dominant species. Khan and Shaukat (2010) investigated a survey of phytonematodes associated with pomegranate was 12 regions of Balochistan Pakistan and reported *M. incognita* was the predominant species.

CONCLUSION

In this study, we reports three species of *Meloidogyne*, *M. incognita*, *M. javanica*, and *M*. *arenaria*, among the three provinces were successfully identified by SCAR analysis. Findings indicated that three common tropical species *M. incognita*, *M. javanica*, and *M. arenaria* were the most pomegranate orchards that showed 399 bp, 670 bp, and 420 bp fragments respectively with SCAR primer. Research showed identification based on SCAR primer is fast, accurate, and reliable. Results were also supported by El-Qurashi *et at* (2017). Identified *M. javanica* in pomegranate orchards of Egypt with SCAR primer, they used Fjav/Rjav, and MJ-F/MJ-R primers and showed 670 and 517 bp band. *M. cruciani* was recognized by the perineal pattern morphology and is a new species for pomegranate in Iran. This species was already reported from different crops by (MAHDIKHANI, Kheyri *et at*. 2003) and (RAFIEE, MAHDIKHANI *et at*. 2010) in Iran. This is the first report of *Meloidogyne cruciani* in pomegranate trees of Iran.

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Nafiseh Katooli	:	Conceived idea, conduct research and manuscript write up.
Esmat M. Moghadam	:	Data analysis, help in research conduction and manuscript write up.
Reza Aghnum	:	Critically reviewed and edited manuscript.