MITOCHONDRIAL HAPLOTYPE-BASED IDENTIFICATION OF ROOT-KNOT NEMATODES, *MELOIDOGYNE ARENARIA* AND *MELOIDOGYNE HAPLA*, INFECTING KIWIFRUIT IN TURKEY

Faruk Akyazi[1,2]*, Soumi Joseph[2], Anil Firat Felek[1], and Tesfamariam Mekete[2]

[1]Ordu University, Agricultural Faculty, Plant Protection Department, Ordu 52200, Turkey; [2]University of Florida, Entomology and Nematology Department, Gainesville, FL 32611, United States. *Corresponding author:farukakyazi@hotmail.com.

ABSTRACT

Akyazi, F., S. Joseph, A. F. Felek, and T. Mekete. 2017. Mitochondrial haplotype-based identification of root-knot nematodes, *Meloidogyne arenaria* and *Meloidogyne hapla*, infecting kiwifruit in Turkey. Nematropica 47:34-48.

Kiwifruit (*Actinidia deliciosa* [A. Chev.] C.F. Liang et A.R. Ferguson, Actinidiaceae) is an important commercial temperate fruit crop grown in different parts of the world. Root-knot nematodes (*Meloidogyne* spp.), are one of the most damaging plant-parasitic nematodes in kiwifruit growing regions. Among them, *M. arenaria* and *M., hapla* are both important root-knot nematode species of kiwifruit. Northern root-knot nematode, *M. hapla*, is one of the most damaging plant-parasitic nematodes in temperate regions. During the 2016 kiwi growing season, root-knot nematode-infested kiwi roots were obtained from one kiwi orchard located in the district of Altınordu, Ordu, Turkey. The identification of *Meloidogyne* species was confirmed by morphological, morphometric, and molecular characters based on amplification of two mitochondrial DNA regions that span the spacer and part of the adjacent large subunit rRNA (lrDNA) gene. The size of the intergenic spacer and sequence polymorphisms in the lrDNA that were revealed following digestion with the restriction enzymes HinfI and MnlI were used to assign haplotypes. The causal pests were identified as *M. hapla* and *M. arenaria*. This is the first record of *M. hapla* and *M. arenaria* infecting kiwi plants in Turkey.

Key words: Actinidia deliciosa, kiwifruit, *Meloidogyne* spp., *Meloidogyne arenaria*, *Meloidogyne hapla*, rRNA, mtDNA, root-knot nematodes.

RESUMO

Akyazi, F., S. Joseph, A. F. Felek, and T. Mekete. 2017. Identificacion de haplotipos mitocondriales de nematodos agalladores *Meloidogyne arenaria* y *Meloidogyne hapla* infectando kiwi en Turquia. Nematropica 47:34-48.

El kiwi (*Actinidia deliciosa* [A. Chev.] C. F. Liang et A. R. Ferguson, Actinidiaceae) es un importante cultivo comercial frutaL de clima templado que se cultiva en diferentes partes del mundo. Los nematodos agalladores de la raíz (*Meloidogyne* spp.), son uno de los más dañinos nematodos parásitos de las plantas en las regiones de cultivo de kiwi. Entre ellos, *Meloidogyne arenaria* y *Meloidogyne hapla* son dos importantes especies de nematodos de nudo de raíz de kiwi. Nematodo agallador del norte, *Meloidogyne hapla* es uno de los más dañinos nematodos parásitos de plantas en regiones templadas. Durante la temporada de crecimiento 2016, raíces de kiwi infestadas por nematodos de nudo de raíz fueron obtenidos de los huertos de cultivo de kiwi situados en el distrito de Altinordu, Ordu, Turquía. La identificación de especies de *Meloidogyne* fueron confirmados por morfológia, morfométria y caracteres moleculares basados en la amplificación de dos regiones de DNA mitocondrial que abarcan el espaciador y parte del gen rRNA (lrDNA) subunidad grande adyacente. El tamaño del espaciador intergénico y la secuencia de polimorfismos en el lrDNA se reveló después de la digestión con las enzimas de restricción HinfI y MnlI para asignar haplotipos. La plagas fueron identificados como *M. hapla* y *M. arenaria*. Este es el primer registro de ambas especies infectando plantas de kiwi en Turquía.

Palavras chave: *Actinidia deliciosa*, El kiwi, *Meloidogyne* spp., *Meloidogyne arenaria*, *Meloidogyne hapla*, rRNA, mtDNA, Los nematodos agalladores.

INTRODUCTION

Kiwifruit (*Actinidia deliciosa* A. Chev.) is an important commercial crop in the world. It is cultivated extensively in different parts of the world including China, New Zealand, Italy, Chile, Greece, Turkey, and other temperate to sub-tropical areas (Cruzat, 2014). Turkey has been ranked as the world's 6th biggest producer of kiwifruit after France (Anonymous, 2012). The climatic conditions in the Black Sea region of Turkey are suitable for kiwifruit production. In 2015, Turkey produced 41,640 tons of kiwi, and this production is increasing each year (Anonymous, 2015).

Root-knot nematodes (*Meloidogyne* spp.), are one of the most commonly found nematode groups in kiwi-growing areas in the world. Five species of root-knot nematodes (RKN) including *M. incognita*, *M. arenaria*, *M. hapla*, *M. javanica*, and *M. ethiopica* have been reported from different kiwifruit-produing regions of the world (Vovlas and Roca, 1976; Haygood *et al.*, 1990; Philippi *et al.*, 1996; Nicotra *et al.*, 2003; Carneiro *et al.*, 2004; Carneiro *et al.*, 2007; Ma *et al.*, 2007; Ploetz, 2016). Among them, northern root-knot nematode (NRKN), *M. hapla*, is one of the most damaging species in temperate regions, and kiwifruit is susceptible to this species (Grandison, 1983). *Meloidogyne hapla* is also known to have a wide host range affecting more than 550 crop and weed species (Jepson, 1987). *Meloidogyne hapla* is found to be a common parasite of kiwifruit in different countries in the world (Sale, 1985). Watson *et al.* (1992) found *M. hapla* to be the only plant-parasitic nematode consistently associated with kiwifruit roots in New Zealand. This species has great importance as it displayed virulence against several sources of root-knot nematode resistance genes. It has also been reported in Chile, New Zeland, United States, Iran, China, and Korea (Haygood *et al.*, 1990; Philippi *et al.*, 1996; Ma *et al.*, 2007).

In addition to the NRKN, *Meloidogyne arenaria* is also reported as the most common species associated with kiwi in many countries. This species was identified for the first time in kiwifruit by Maafi and Mahdavian (1997) in Iran. In Brazil, *M. arenaria* is reported in 66.65% of the samples tested (Somavilla *et al.*, 2011).

In Turkey, many root-knot nematodes have been reported from different crops including *M. arenaria*, *M. artiellia*, *M. chitwoodi*, *M. ethiopica*, *M. exigua*, *M. hapla*, *M. incognita*, *M. javanica*, and *M. thamesi* (Ertürk and Özkut, 1973; Yüksel, 1974; Di Vito *et al.*, 1994; Elekçioğlu *et al.*, 1994; Mennan and Ecevit, 1996; Kaşkavalcı and Öncüer, 1999; Söğüt and Elekçioğlu, 2000; Devran *et al.*, 2009; Devran and Söğüt, 2009; Özarslandan *et al.*, 2009; Özarslandan and Elekçioğlu, 2010; Akyazı and Ecevit, 2011; Aydınlı *et al.*, 2013; Kepenekçi *et al.*, 2014; İmren *et al.*, 2014; Aydınlı and Mennan, 2016). Few studies have been conducted on root-knot nematodes on kiwi. *Meloidogyne incognita* is the only root-knot nematode reported so far on kiwi in Turkey (Akyazı and Felek, 2013).

The objective of this study was to identify root-knot nematode infecting kiwi in order to add some new *Meloidogyne* species based on mitochondrial haplotype using specific primers that direct amplification of a mitochondrial DNA (mtDNA) fragment from parthenogenetic RKN. DNA-based identification using the primers that amplify a specific genomic fragment from a target RKN species has limited application because a negative result could indicate either a failed reaction or that the nematode is a different species or variant (Adam *et al.*, 2007; Blok and Powers 2009). Although the DNA sequence of ribosomal RNA (rDNA) genes has been used as a diagnostic tool for many organisms, this approach is limited to resolve the RKN species because sequence differences of rDNA copies within an individual RKN shows greater diversity than between species (Pagan *et al.*, 2015). In contrast to this, the mitochondrial genome of apomictic RKN species, due to its uniparental inheritance, has revealed a useful source of diagnostic markers. The identification based on the length polymorphisms of non-coding intergenic spacer between cytochrome oxidase II (COII) and large subunit of rDNA of mtDNA and restriction fragment length polymorphisms (RFLPs) of this region has become a reliable diagnostic marker to distinguish major RKN species (Powers *et al.*, 1986; Powers and Harris, 1993). Pagan *et al.* (2015) adopted a diagnostic strategy based on amplification of two mtDNA regions that, together, span the spacer and part of the adjacent large subunit (16S) rRNA (lrDNA) gene to characterize different RKN species by assigning the mitochondrial haplotype. In this study, we adopted this strategy to identify the RKN species asscociated with kiwi in Turkey.

MATERIALS AND METHODS

Nematode sampling and extraction

During the 2016 kiwi growing season, root-knot nematode symptoms, such as stunting and extensive root galling (Fig. 1) were observed on roots of kiwi plants in a commercial kiwi orchard located in Altınordu district of Ordu, Turkey. Root samples were collected randomly in a zigzag pattern between rows and root samples carrying galls and egg masses were taken from both sides of the

Fig. 1. Kiwi roots infected with *Meloidoygne* spp. Typical root-galling symptom, known as a nodule-like galls, on the roots.

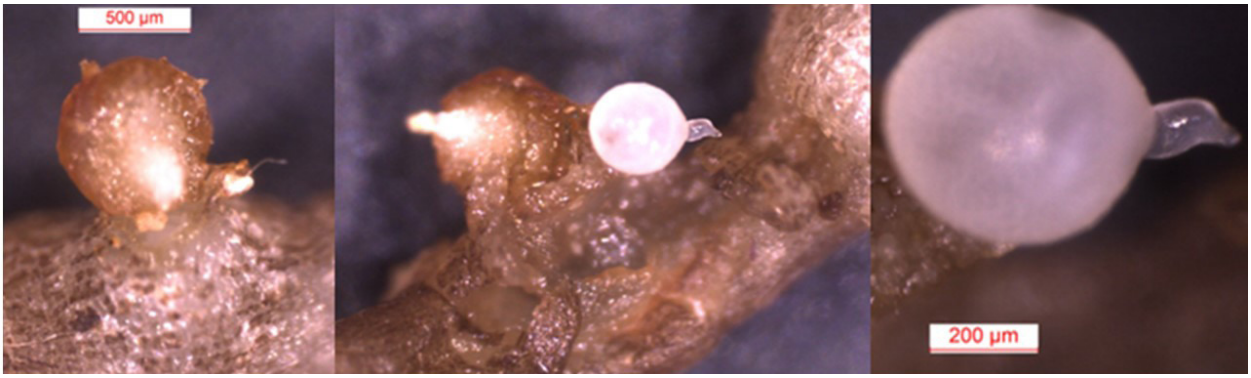

Fig. 2. Egg mass and female of *Meloidogyne* spp. on infected kiwi roots.

vines. A total of 20 root samples were collected and placed in a plastic bag, sealed, and brought back to the laboratory and stored at 4°C until processed. For the extraction of egg laying *Meloidogyne* spp. females and egg masses (Fig. 2), infected roots were dissected using a needle under a stereomicroscope (Leica, S8APO) at 10× magnification. Females and egg masses were collected seperately. The females collected were used for morphological and molecular characterization. To obtain second-stage juveniles (J2), a single egg mass (SEM) was placed in a 35-mm petri dish with 2 ml of sterile tap water and incubated for 48 h in an incubator at 28°C (Esser *et al.*, 1976). The hatched juveniles were hand picked and used for morphological and morphometric characterization.

Morphometric and morphological characters

Ten J2 hatched from a single egg mass were measured for morphometric and morphological characterstics including: juvenile body length, maximum body diameter, DGO, stylet length, stylet knob height, stylet knob width, procorpus length, median bulb length, median bulb diameter, median bulb valve length, median bulb valve width, anterior end to pharyngo-intestinal junction, anterior end to secretory-excretory pore, metacorpus valve from anterior end, nerve ring from anterior end, tail length, anal body diameter, and hyalin. These characters were examined using a Zeiss (Axio Vert. A1) inverted compound microspe equipped with a digital camera (AxioCam MRc 5, Carl Zeiss Meditec, Inc. Dublin, CA). The J2 measurement data were compared with the earlier descriptions of these species (Chitwood, 1949; Whitehead, 1968; Jepson, 1987).

To observe morphological characters of females, perineal patterns were examined. Freshly collected females were placed in a drop of 45% lactic acid on a glass slide, and the perineal patterns were cut and cleaned (Taylor and Netscher, 1974). The perineal patterns were transferred into a drop of glycerin on a glass slide and covered with a round cover slip.

Photomicrographs of female perineal patterns were made using the digital camera attached to the light microscope (Zeiss PrimoVert).

DNA extraction, PCR amplification and sequence analysis

Genomic DNA was extracted from each of 10 individual females. A single female was transferred into 10 µl of extraction buffer (10 mM Tris, 1 mM EDTA, 0.1 % triton X, and 0.1 mg/ml proteinase K) in a 1.5-ml eppendorf tube, and the nematodes were disrupted using a probe (Pagan *et al.*, 2015). Samples were frozen in 0.2 ml PCR tubes at –20°C overnight. For DNA extraction, samples were incubated at 56°C for 1 h followed by 95°C for 10 min, then used immediately for PCR or stored at –20°C.

Mitochondrial DNA fragments were amplified using primer sets TRNAH (TGAATTTTTTATTGTGATTAA) and MRH106 (AATTTCTAAAGACTTTTCTTAGT) or MORF (ATCGGGGTTTAATAATGGG) and MTHIS (AAATTCAATTGAA ATTAATAGC) developed by Stanton *et al.* (1997). Amplification was carried out in 25 µl reaction mix containing 2x Apex Hot Start Taq Master Mix (Genesee Scientific, San Diego, CA), 1.5 µl of DNA, and 0.5 µM of each primer. The thermal cycling was performed in a Mastercycler nexus (Eppendorf AG, Hamburg, Germany). The thermo cycling reactions using the primers TRNAH/MRH106 and MORF/MTHIS (Pagan *et al.*, 2015; Stanton *et al.*, 1997) were as follows: 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 68°C for 1 min, and a final extension step of 68°C for 10 min. DNA fragments were separated by electrophoresis in Tris-acetic acid-EDTA buffer (TAE) (Genesee scientific, San Diego, CA) using 1.5% agarose gels for 30 min at 150 V and visualized under UV light using the ChemiDoc XRS Quantity One 4.5.2 program (Bio-Rad Laboratories, Life Science Group, Hercules, CA) after staining in ethidium bromide (100 ppm) for 20 min. To determine the mitochondrial haplotype, the fragments amplified using the primer set, TRNAH and MRH106 were subjected to restriction digestion using the restriction enzymes HinfI and MnlI (New England Biolabs, Ipswich, MA) as recommended by the manufacturer. To confirm the identity of the sample, an additional PCR using species specific primers was performed. *Meloidogyne hapla* specific SCAR primer set (Wishart *et al.*, 2002) JMV (5' GGATGGCGTGCTTTCAAC 3') JMV-R (5' AAAAATCCCCTCGAAAAATCCACC 3') and *M. arenaria*-specific SCAR primer set (Zijlstra *et al.*, 2000) Far (5' TCGGCGATAGAGGTAAATGAC 3') and Rar (5' TCGGCGATAGACACTACAACT 3') were used for further confirmation of species identification. Additionally, the mitochondrial DNA (a region of variable size in *Meloidogyne* between the mitochondrial cytochrome oxidase subunit II (COII) gene and the large (16S) ribosomal gene) was amplified using the primers C2F3 (5′-GGTCAATGTTCAGAAATTTGTGG-3′) and 1108 (5′-TACCTTTGACCAATCACGCT-3′) designed by Powers and Harris (1993).

The amplified PCR products of the mtDNA regions (COII fragments) were purified using either the QIAquick Gel purification kit (Qiagen, Valencia, CA) or the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified PCR fragments were sequenced in both directions at the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL. To identify the *Meloidogyne* species, raw sequences obtained were checked and edited manually using BioEDIT v. 7.0.9 (Hall, 1999). Consensus sequences obtained were compared to those deposited in the GenBank database using a BLAST engine search tool for sequence homology (Benson *et al.*, 2013). Additionally, the restriction pattern of TRNAH/MRH106 fragments from both nematode species were analysed in silico using restriction mapper version 3 and compared with the restriction pattern observed on the gel.

Phylogenetic analysis

To perform the phylogenetic analysis, the COII sequences obtained from this study and those retrieved from the GenBank databases (Table 1) were aligned using Clustal W for multiple alignments of 30 nucleotide sequences using MEGA 7.0 software (Kumar *et al.*, 2016). The alignment was analysed to get the base substitution model for these sequences using MEGA 7 software. Out of maximum likelihood fits of 24 different nucleotide substitution models, HKY+G+I model with lowest BIS score (Bayesian Information Criterion), where HKY is Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985), G is the gamma distribution and I represents the proportion of invariable sites, was selected and considered to describe the substitution pattern best. The phylogram was generated using base substitution model HKY+G+I and running Maximum Likelihood Model with 1000 bootstrap replicates using MEGA 7.0 (Kumar *et al.*, 2016) to assess the degree of support for each branch on the tree (Landa *et al.*, 2008).Unique data sequences obtained from this study were submitted to GenBank under accession numbers, KX962312, KX962313, KX962314, and KX983450.

Table 1. Species of nematodes used in the phylogenetic analysis, including GenBank accession numbers and geographical origin.

Species	GenBank accession no.	Country
Meloidogyne haplanaria	KT783539	USA
Meloidogyne hapla1	L76262	Australia
Meloidogyne hapla2	KX214348	Kenya
Meloidogyne hapla3	KX214349	Kenya
Meloidogyne hapla4	KP306538	South Korea
Meloidogyne hapla	KX962312	Turkey
Meloidogyne partityla	AY672412	USA
Meloidogyne naasi	JN241944	USA
Meloidogyne graminicola	KF751065	Taiwan
Meloidogyne graminis	JN241925	USA
Meloidogyne arabicida	KF993631	USA
Meloidogyne floridensis	AY635609	USA
Meloidogyne fallax	JN241952	USA
Meloidogyne chitwoodi	JQ041627	USA
Meloidogyne incognita	KJ476151	USA
Meloidogyne arenaria1	AY635610	USA
Meloidogyne arenaria2	EU364879	USA
Meloidogyne arenaria3	KP202350	USA
Meloidogyne arenaria	KX962313	Turkey
Meloidogyne hispanica	JN673274	Portugal
Meloidogyne paranaensis	KF993638	Costa Rica
Meloidogyne lopezi	KF993629	Costa Rica
Meloidogyne enterolobii	KP202351	USA
Meloidogyne thailandica	EU364883	USA
Meloidogyne morocciensis	AY942849	Brazil
Meloidogyne javanica	L76261	Australia
Meloidogyne ethiopica	KM042848	Portugal
Globodera pallida	AJ249395	UK
Heterodera glycines	HM640930	Australia
Heterodera cardiolata	HM640929	Australia

RESULTS

Morphometric and morphological characterization

Morphometrics of J2 of the population of *M. arenaria* and *M. hapla* were examined and compared with previous descriptions. Morphometrics of the J2 of the *M. hapla* and *M. arenaria* are listed in Table 2 and 3. Several measurements of J2 of *M. arenaria* and *M. hapla* were overlapping with the respective previous descriptions. Most of the morphological characters of *M. hapla* J2 did not differ significantly from those reported in the original description. However, some of the morphological characters such as body length (383.1 ± 25.9) and stylet length (13.7 ± 0.6) showed higher values than reported in the previous description (Table 2). Tail length was 42.6 ± 4.2 µm and the mean length of the hyaline tail terminus was 15.1 ± 2.1 µm. Additionally, two tail tip shapes were encountered within the populations of *M. hapla*. The tail tip was either uniformly tapered to a bluntly rounded tip (Fig. 3A) or 'toe-shaped' (Fig. 3B).

Meloidogyne arenaria J2 averaged 402.8 ±

Table 2. Measurements of second-stage juveniles of *Meloidogyne hapla* (in µm) (n=10).

			Handoo *et al*., 2005	
Characters	Mean + SD	Range	Mean + SD	Range
Body length	383.1 ± 25.9	[340.1-440.3]	323.1 ± 18.4	[284-355]
a	25.4 ± 3.5	[14.0-30.3]	30.2 ± 2.6	[24.1-35.5]
b	4.4 ± 0.2	[4.2-4.9]	4.7 ± 0.3	[4.3-5.2]
c	7.3 ± 0.5	[6.4-8.3]	7.7 ± 0.6	[6.8-9.4]
c'	4.8 ± 0.3	[4.4-5.3]	5.4 ± 0.6	[4.5-6.0]
Body width	15.5 ± 3.4	[13.4-28.0]	10.8 ± 0.9	[10-12]
DGO	3.1 ± 0.3	[2.7-3.5]	2.5 ± 0.0	[2.0-2.5]
Stylet length	13.7 ± 0.6	[12.7-14.9]	-	-
Stylet knob height	1.6 ± 0.2	[1.5-2.0]	-	-
Stylet knob width	1.9 ± 0.2	[1.6-2.7]	-	-
Procorpus length	31.1 ± 1.9	[28.1-34.6]	-	-
Median bulb length	10.8 ± 0.9	[9.5-12.9]	-	-
Median bulb diameter	7.7 ± 1.2	[6.1-11.2]	-	-
Median bulb valve length	3.5 ± 0.2	[3.0-3.9]	-	-
Median bulb valve width	2.9 ± 0.3	[2.5-3.5]	-	-
Pharyngo-intestinal junction from anterior end	86.2 ± 4.0	[80.7-91.7]	-	-
Excretory pore from anterior end	71.5 ± 5.3	[60.7-82.4]	66.9 ± 7.6	[60-88]
Metacorpus valve from anterior end	50.4 ± 2.9	[45.8-55.0]	46.4 ± 2.3	[40-50]
Nerve ring from anterior end	62.3 ± 3.9	[55.7-71.3]	-	-
Tail length	52.7 ± 1.1	[50.2-54.8]	42.6 ± 4.2	[30-47.5]
Anal body diameter	10.9 ± 0.6	[9.9-11.8]	-	-
Hyaline	15.1 ± 2.1	[12.0-18.5]	10.9 ± 2.1	[5-15]

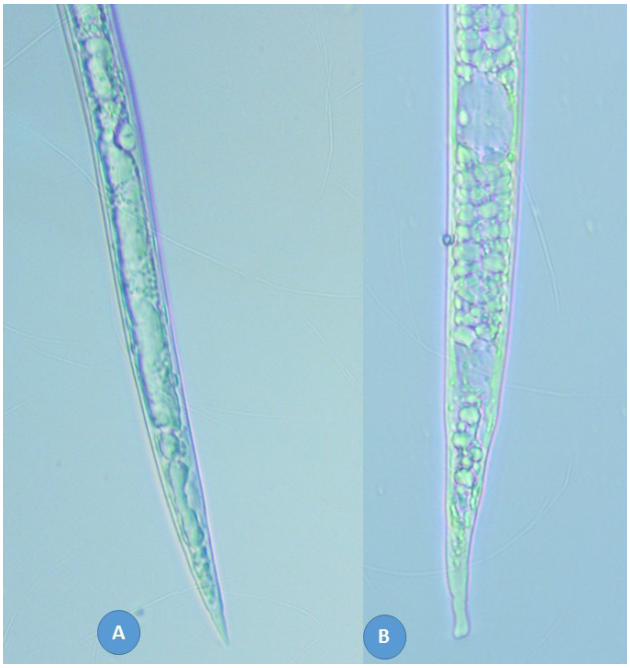

Fig. 3. Photomicrographs of J2 juvenile tails of *Meloidogyne hapla*. A: Tapered tail, B: Toe-shaped tail.

Table 3. Measurements of second-stage juveniles of *Meloidogyne arenaria* (in µm) (n = 10).

			Cliff & Hirschmann, 1985	
Characters	Mean + SD	Range	Mean + SD	Range
Body length	402.8 ± 22.8	[362.2 - 427.4]	503.6 ± 4.26	[391.6 - 605.2]
a	25.5 ± 1.9	[22.5 - 28.5]	-	-
b	4.5 ± 0.5	[3.9 - 5.3]	-	-
c	7.8 ± 0.6	[6.9 - 8.6]	-	-
c'	4.6 ± 0.1	[4.4 - 4.8]	-	-
Body width	15.6 ± 0.7	[14.5 - 16.3]	15.3 ± 0.09	[12.8 - 17.8]
DGO	3.4 ± 0.3	[3.0 - 3.9]	3.7 ± 0.04	[2.7 - 4.7]
Stylet length	14.7 ± 0.7	[13.8 - 15.9]	11.1 ± 0.03	[10.1 - 11.9]
Stylet knob height	1.7 ± 0.2	[1.5 - 2.0]	-	-
Stylet knob width	2.1 ± 0.3	[1.9 - 2.7]	-	-
Procorpus length	29.5 ± 0.9	[28.4 - 30.6]	-	-
Median bulb length	11.8 ± 1.1	[10.4 - 13.6]	-	-
Median bulb diameter	7.7 ± 1.2	[6.5 - 9.7]	-	-
Median bulb valve length	4.3 ± 0.4	[3.8 - 5.0]	-	-
Median bulb valve width	3.3 ± 0.4	[2.5 - 3.8]	-	-
Pharyngo-intestinal junction from anterior end	90.0 ± 4.8	[80.8 - 93.9]	-	-
Excretory pore from anterior end	78.1 ± 1.8	[76.4 - 81.1]	89.8±0.56	[75 - 105.2]
Metacorpus valve from anterior end	50.9 ± 0.8	[50.0 - 51.8]	-	-
Nerve ring from anterior end	68.6 ± 1.2	[66.5 - 70.1]	-	-
Tail length	52.0 ± 1.9	[48.3 - 53.8]	56.0 ± 0.53	[43.6 - 69.4]
Anal body diameter	11.2 ± 0.2	[10.9 - 11.4]	-	-
Hyaline	14.4 ± 4.1	[7.0 - 18.9]	-	-

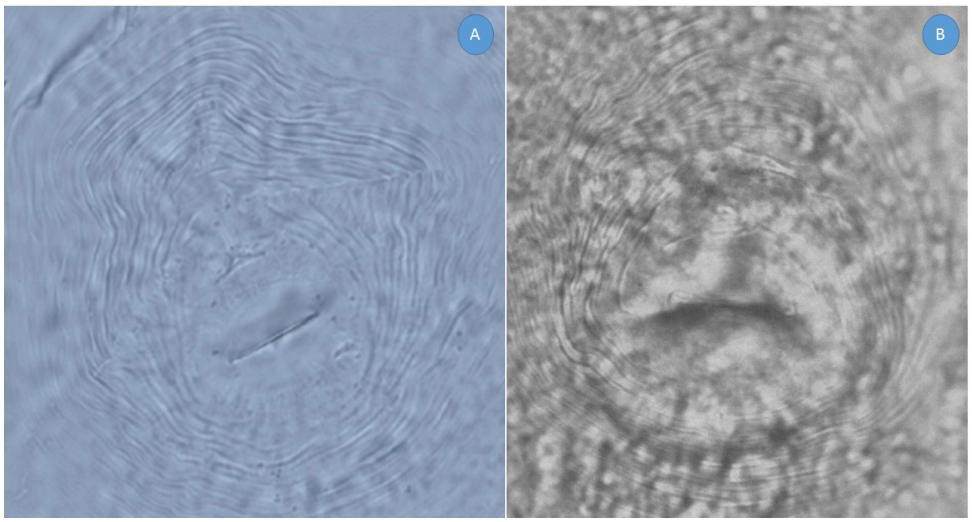

Fig. 4. Photomicrographs of perineal patterns of both species. A: *Meloidogyne hapla*, B: *Meloidogyne arenaria.*

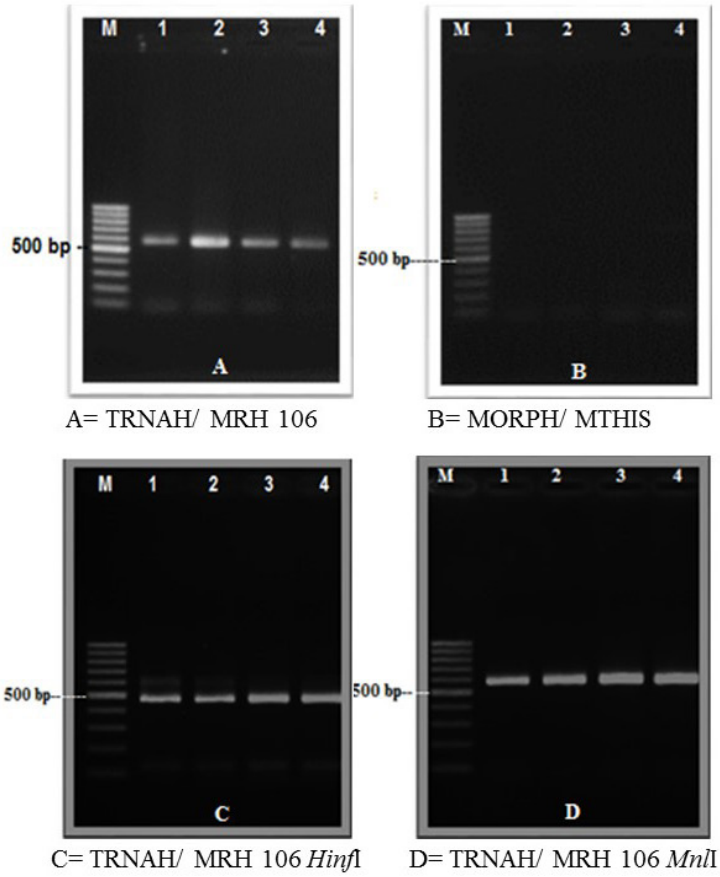

Fig. 5. Diagnostic amplification PCR products from mitochondrial genomes of *Meloidogyne hapla*. A= Agarose gel showing sizes of amplification products from characterized root-knot nematode *M. hapla* obtained using primers TRNAH and MRH106. B= Amplification products obtained with primers MORF/MTHIS. C,D=Fragments obtained after digestion with HinfI or MnlI. Species of DNA source are indicated below each lane: Lanes labeled M contain 100-bp marker ladder, with the position of the 500-bp band indicated by an arrow.

Table 4. Expected fragment sizes following amplification of *Meloidogyne arenaria* and *Meloidogyne hapla* mtDNA with primers MORF, MTHIS, TRNAH, and MRH106 and restriction digestion with Hinfl or MnlI (Pagan *et. al*., 2015)

	MORF	TRNAH	Hinfl	Mnl
Meloidogyne arenaria	214 bp	557 bp	445, 112 bp	340, 140, 77 bp
Meloidogyne hapla	NP[z]	556 bp	446, 110 bp	556 bp

[z]NP: no product, nonspecific bands; The exact size of the restriction fragments were determined in silico using restriction mapper version 3.

22.8 µm in length and 15.6 ± 0.7 µm in width and were consistent with the original description of *M. arenaria* (Table 3). The average stylet length and its range were greater than the measurement in the original description. The tail morphometrics and tail morphology were very similar with the original description. Tail length was 52.7 ± 1.1 µm and the mean length of the hyaline tail terminus was 14.63 ± 2.11µm.

The morphology of the perineal pattern of root-knot nematode females is one of the most important and reliable characters for separation of *Meloidogyne* species. Perineal pattern of *M. hapla* is rounded hexagonal and lateral line on both sides is visible (Fig. 4A). The perineal pattern of *M. arenaria* has a low and rounded dorsal arch (Fig. 4B). The striae in

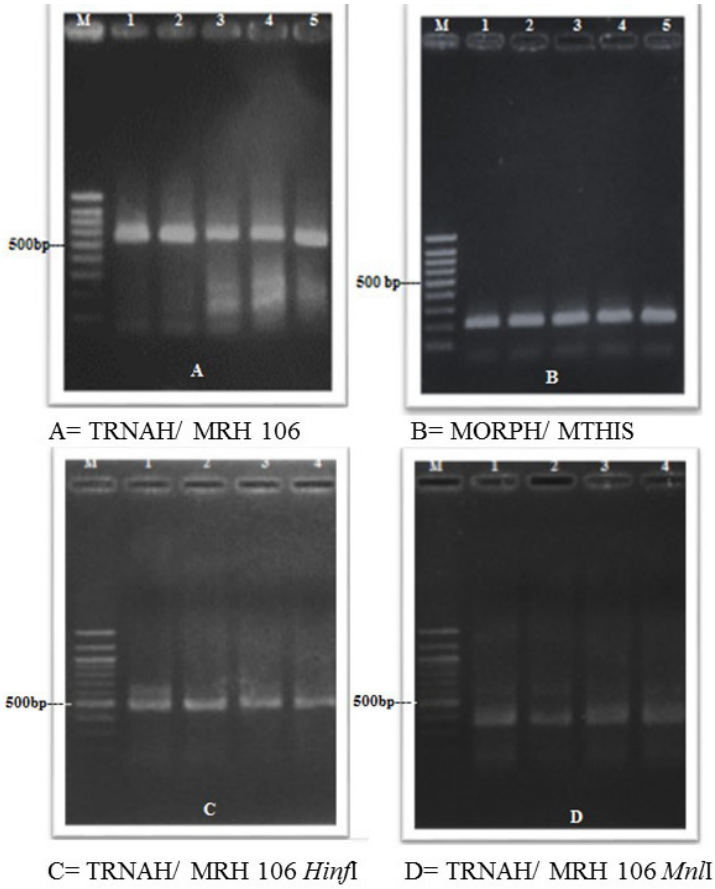

Fig. 6. Diagnostic amplification PCR products from mitochondrial genomes of *Meloidogyne arenaria*. A= Agarose gel showing sizes of amplification products from characterized root-knot nematode *M. arenaria* obtained using primers TRNAH and MRH106. B= Amplification products obtained with primers MORF/MTHIS. C,D=Fragments obtained after digestion with HinfI or MnlI. Species of DNA source are indicated below each lane: Lanes labeled M contain 100-bp marker ladder, with the position of the 500-bp band indicated by an arrow.

the arch are slightly indented at the lateral lines and generally form a shoulder on the arch.

Molecular characterisation of RKN species

PCR amplification using the primers TRNAH and MRH106 gave fragments of 556 bp and 557 bp for *M. hapla* and *M. arenaria*, respectively (Fig. 5A; 6A). The PCR amplification using MORF and MTHIS primers generated a fragment of 214 bp in *M. arenaria* whereas *M. hapla* did not give any product (Fig. 5B; 6B). The digestion assay of TRNAH/MRH106 using HinfI showed 446 and 110 bp products for *M. hapla*, but the digestion assay of TRNAH/MRH106 using HinfI showed 445 and 112 bp products for *M. arenaria* (Fig. 5C; 6C). The digestion assay with MnlI gave one digestion product of 556 bp in *M. hapla* (Fig. 5D), but *M. arenaria* gave three digestion products of 340, 140, and 77 bp (Fig. 6D). All these fragment size results for *M. hapla* and *M. arenaria* are consistant with results of Pegan *et al.* (2015) (Table 4). In silico restriction analysis of the TRNAH/MRH106 fragments from both species showed 100% similarity with restriction pattern from the known sequence of *M. hapla* and *M. arenaria*.

In this study, the COII fragment of mtDNA and the large ribosomal gene was amplified using the primers C2F3 and 1108. *Meloidogyne hapla* produced a 520 bp fragment (Fig. 7A), which is different from the isolates of *M. arenaria* that produced a 1100 bp fragment products (Fig. 7B). These results agree with those of Powers and Harris (1993), which indicated different size classes of amplification products in reactions with *Meloidogyne* species.

The identification of *M. hapla* and *M. arenaria* species was also confirmed by PCR using species-specific primers. *M. hapla*-specific SCAR primer set

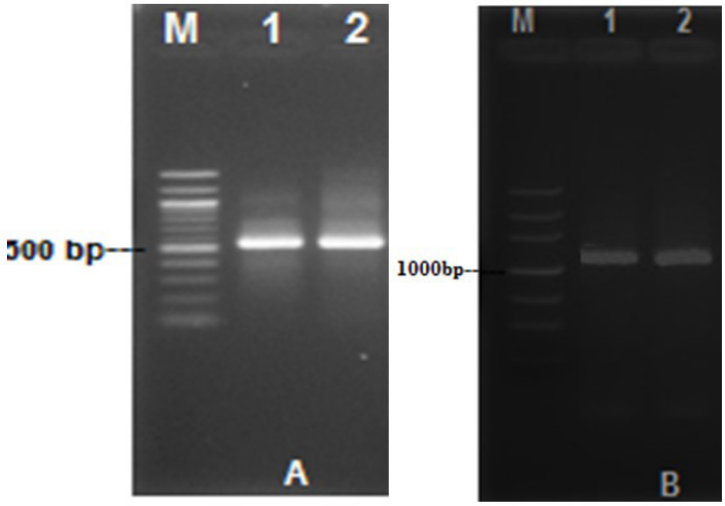

Figure 7. Gel electrophoresis of mitochondrial specific primers C2F3/1108. A: *Meloidogyne hapla*, B: *Meloidogyne arenaria*.

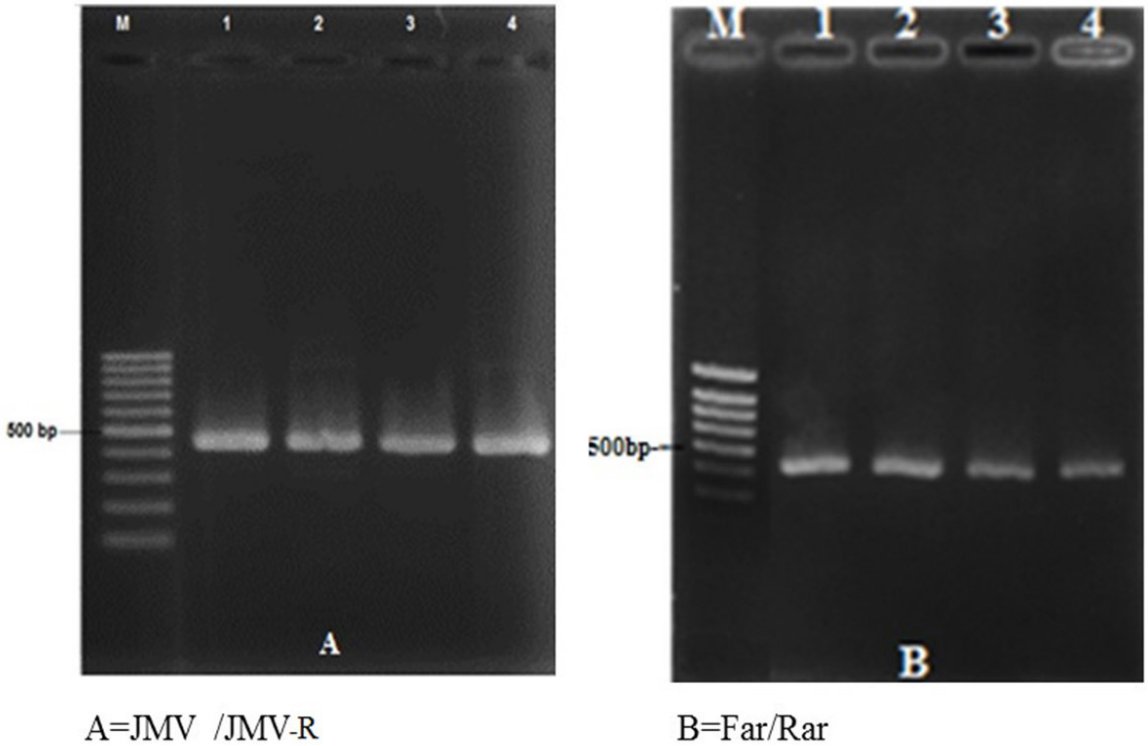

Fig. 8. PCR Amplification on MFT1 and MFT10 using *Meloidogyne hapla* species specific primer set JMV/JMV-R (A) and *Meloidogyne arenaria* species specific primers, Far/Rar (B), respectively.

Fig. 9. Maximum likelihood tree inferred from COII- lrDNA region of the mitochondrial genome under HKY+G+I model (BIC= 6500.205; AIC= 6041.328; INL=-2957.291 freqA =0.374; freqT =0.528;. freqC = 0.028; freqG =0.069; R= 0.78). The analysis was done using 1000 bootstrap replicates. The bootstrap value for each clade is indicated on the nodes. GenBank accessions, KX962313, and KX962312 represents the haplotypes of *Meloidogyne arenaria* and *Meloidogyne hapla*, respectively, obtained from this study. Rest of the accession were retrieved from GenBank database as references. Out group sequences from *Globodera pallida*, *Heterodera cardiolata*, and *Heterodera glycines* were used for alignment and for the construction of phylogram.

JMV/JMV-R gave 440 bp fragment (Fig. 8A) and Far/Rar primers for *M. arenaria* successfully gave PCR products of 420 bp (Fig. 8B). Our study results showed agreement with earlier studies of Zijlstra *et al.* (2000) and Adam *et al.* (2007).

Sequence analysis and phylogenetic analysis

The PCR products of 556 bp and 557 bp generated from the samples MFT1 and MFT10, respectively, using the primers TRNAH/MRH106 were deposited in GenBank with the Accession No, KX962312 and KX962313. The BLAST analysis of these sequences from MFT1 and MFT10 revealed 99% and 100% similarity with the GenBank sequences from *M. hapla* (Accession No: L76262.) and *M. arenaria* (Accession No: KP202350.), respectively. Additionally, the sequence fragments between mtDNA COII and 16S ribosomal gene sequences generated from MFT1 (528bp) and MFT10 (1112bp) using the primers C2F3/1108 were deposited in GenBank with Accession No. KX962314 and KX983450, respectively. The BLAST analysis of these fragments from MFT1 and MFT10 also revealed a sequence similarity of 100% with *M. hapla* (GenBank Accession No. AY757887) and 99% with *M. arenaria* (Accession No. AY635610).

Alignment and phylogenetic analysis of COII sequences revealed several clades that were separated by varying bootstrap support (BS) values in the ML analysis (Fig. 9). The optimal phylogenetic tree obtained from the ML analysis revealed that the sequences from Turkey isolate of *M. hapla* and the *M. hapla* sequence retrieved from GenBank formed a monophyletic group with a high boot strap value of 99%. The sequence from the Turkey isolate of *M. arenaria* clustered with Clade I RKN species.

DISCUSSION

Root-knot nematodes are one of the most important pathogens in Turkey causing considerable yield losses in different agricultural crops. Some studies on identification and distribution of root-knot nematodes in Turkey have been conducted by different research groups (Elekçioğlu and Uygun 1994; Elekçioğlu *et al.*, 1994; Mennan and Ecevit, 1996; Kaşkavalcı and Öncüer 1999). The identification of root-knot nematodes to species level based on morphological characters is very difficult due to high morphological similarity among the species. Similarly, different populations of the same RKN species may show significant differences in morphological characters, which may lead to misidentification of the species. For example, in our study, some of the morphometric characteristic values such as body length and stylet length in the Turkey isolate of *M. hapla* showed differences from the original description of *M. hapla* (Cliff and Hirschmann, 1985) even though the molecular characterization of *M. hapla* using a different molecular marker confirmed the identity of the species. Previous reports also showed that the different factors can influence morphogical characters, and these characters can be significantly different not only between species but also among populations within a species (Doucet and Cagnolo, 1998; Carneiro *et al.*, 2008; Skantar *et al.*, 2008). Moreover, multiple species of root-knot nematodes co-exist in the same plant root. Therefore, fast and accurate identification of root-knot nematodes is needed for developing efficient nematode management strategies.

The identification based on molecular approaches gives accurate, fast, and reliable identification of nematodes. Devran *et al.*, (2002) conducted the first molecular identification of root-knot nematodes in Turkey. However, this identification study was conducted mainly using species specific primers available for root-knot nematode species. This approach is highly limited in nematode screening when multiple species of root-knot nematodes are present in the soil. The specific primers are available for only a few species of root-knot nematodes and also some specific primers may give false positive for closely related species. Joseph *et al.* 2016 demonstrated that the species specific primers used for *M. enterolobii* gives positive result for *M. haplanaria*. Therefore, the use of species specific primers in RKN identification should be used with much caution. In this study, we used a diagnosis strategy based on mtDNA analyis. This method has been proven to be an efficient method to identify and address unforeseen plant-parasitic nematodes, which belong to RKN species (Pagan *et al.*, 2015; Joseph *et al.*, 2016). This method can be efficiently used to distinguish morphologically similar but genetically divergent nematode species as well as new and emerging RKN species. Recent studies have shown that the small differences in mtDNA of *Meloidogyne* spp. is in alignment with esterase phenotype of the species (Pagan *et al.*, 2015; Janssen *et al.*, 2016). In our intial screening using mtDNA haplotype, we found that 70% of the RKN species belong to *M. hapla* while 30% belong to *M. arenaria*. Further sequencing of mtDNA fragments from these samples confirmed the species identity. Therefore, the identification approach based on michondrial haplotypes is an easy, accurate, rapid, and cost-effective method that can be efficiently adopted in RKN identtification.

Kiwifruit is becoming a more important fruit

in Turkey. In the northern part of Turkey, climatic conditions are suitable for kiwifruit production. Every year new orchards have been established and production is increasing each year. Before the establishment of a new orchard, it is necessary to know the diversity of root-knot nematodes exist in the orchard. Correct nematode identification can determine the introduction of resistant cultivars and successful rotations with crops or cultivars that are resistant or a non-host for existing RKN population. Here, we report the presence of *M. hapla* and *M. arenaria* on kiwifruit in Turkey for the first time. We also provided a thorough description of a Turkish population of *M. arenaria* and *M. hapla* and employed an efficient molecular approaches to reach an accurate identification. The high incidence of *M. hapla* in kiwi emphasizes the need for further study to develop efficient management strategies to control this aggressive species of RKN in Turkey.

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