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Orijinal araştırma (Original article)

Molecular identification of root-knot nematode *Meloidogyne incognita* from kiwi fruit orchards in Ordu province, Turkey[1]

Ordu ili kivi bahçelerinde görülen kök-ur nematodu *Meloidogyne incognita*'nın moleküler teşhisi

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Summary

Root-knot nematodes (*Meloidogyne* spp.) are one of the major groups of nematodes identified on kiwi fruit (*Actinidia deliciosa* Chev.). This investigation was conducted to identify root-knot nematodes encountered the in kiwi fruit orchards in the Ordu province of Turkey by means of Polymerase Chain Reaction (PCR). For this purpose, infected plant root samples were collected from 17 kiwi fruit orchards located in Center, Perşembe, Ünye, Gülyalı, İkizce and Ulubey districts of the province during the years 2011-2012. Females directly obtained from the infected roots of orchard populations were used for the molecular identifications. It was carried out with an identification key using ribosomal DNA (rDNA) region, Intergenic Spacer (IGS-2) region between 5S-18S genes, and the species-specific Sequence Characterized Amplified Region (SCAR) markers. All samples from the districts of Ordu province were identified as *Meloidogyne incognita* by the exact size of 502 bp amplicons by SEC1F/SEC1R scar primers. This study was the first detailed investigation both in the province and country on kiwi fruit.

Key words: *Actinidia deliciosa,* Kiwi, *Meloidogyne incognita*, rDNA , Root-knot nematodes, SCAR

Özet

Kök-ur nematodları, kivi bitkisi (*Actinidia deliciosa*)'nde zararlı olan önemli nematod gruplarından birisidir. Bu çalışma, Ordu ili kivi bahçelerinde görülen kök-ur nematodlarının, PCR (Polymerase Chain Reaction) yöntemi ile teşhisini gerçekleştirmek için yapılmıştır. Bu amaçla 2011-2012 yıllarında Ordu ili Merkez, Perşembe, Ünye, Gülyalı, İkizce, Ulubey ilçelerini kapsayacak şekilde 17 farklı kivi bahçesinden bulaşık kök örnekleri toplanmıştır. Kök-ur nematodu ile enfekteli kivi köklerinden elde edilen dişiler doğrudan teşhis için kullanılmıştır. Moleküler teşhisler, teşhis anahtarı kapsamında sunulan 5S-18S genleri arasındaki rDNA bölgesi IGS-2 için kullanılan primerler ve türe özgü SCAR primerlerinin kullanılması suretiyle gerçekleştirilmiştir. Ordu ili kivi bahçelerinden elde edilen bütün örneklerin teşhisi sonucunda tespit edilen tür *Meloidogyne incognita* olmuş ve SEC1F/SEC1R türe özgü primerleri 502 bp değerinde amplicon vermiştir. Bu çalışma, kivi bitkisi üzerinde ilk detaylı çalışma niteliğindedir.

Anahtar sözcükler: *Actinidia deliciosa,* Kivi, *Meloidogyne incognita*, rDNA, Root-knot nematodes, SCAR

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Introduction

Kiwi fruit (*Actinidia deliciosa* A. Chev.) has become one of the popular perennial horticultural plant species in Turkey where it is a relatively new commercial fruit crop. Recently, It has also become very popular in the Ordu area of the Black Sea Region of Turkey. The attempts to introduce it as an alternative crop to hazelnut have been supported goverment's subsidization programme in the region.On the other hand, kiwi fruit production is an important and profitable element of pomology in other countries such as: China, Italy, New Zealand, Chile, Greece, France, USA, Iran, Japan and Portugal (ZhengWhang *et al*., 2009). The Turkish kiwi fruit production in 2011 was 29,321 tons, out of which the Ordu province produced 5,951 tons, i.e. 20.4% of the total production (Anonymous, 2013).

Kiwi fruit is susceptible to several pests including insects, mites and nematodes (Tomkins 1996; Steven *et al*., 1997; El-Borai & Duncan 2005; Hill *et al*., 2008; McKenna *et al*., 2009). As for the plant-parasitic nematodes, the root-knot nematodes (*Meloidogyne* spp.) are the one major groups found on kiwi fruit. The important root-knot nematode species of kiwi fruit are *Meloidogyne incognita*, *M. javanica*, *M. arenaria, M. hapla* and *M. ethiopica* (Carneiro *et al*., 2004; Ma *et al*., 2007; Ploetz, 2011). They are considered as an economically important, polyphagous group of highly adapted obligate plant parasites. They are distributed worldwide and, reportedly, parasitize nearly every species of higher plant (Moens *et al*., 2009). Four species of them: *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, according to the literature, account for 95% of all root-knot nematode infestations in all agricultural lands. These important pathogens cause an estimated average crop loss of 5% worldwide (Hussey & Janssen, 2002). Moreover, *M. incognita* has been reported to be the most economically important species (Haygood *et al*., 1990; Carneiro *et al*., 2004; Ma *et al*., 2007; Ploetz, 2011). *Meloidogyne hapla* and *M. incognita* are both widespread in kiwi orchards in Chile (Philippi *et al*., 1996). This species was also reported as widespread on kiwi fruit in the eastern US (Haygood *et al*.,1990).

Recently, a number of studies have been conducted on root-knot nematodes in Turkey. However, so far, a comprehensive study has not been done on the identification of the root-knot nematodes on kiwi fruit in the country. There were few literature records about plant parasitic nematodes on kiwi fruit in the Turkey. *Pratylenchoides bacilisemenus* Sher, 1970 and *P. camachoi* Barcina, Castillo and Pais, 1990 were reported by Kepenekci & Öztürk (1999) and Kepenekci & Öztürk (2000) on *Actinidia deliciosa* cv. Hayward in the Black Sea region. Despite all this information, relatively little attention regardinghorticultural practices has been paid to this problem.

Therefore, the main goal of this study was to identify root-knot nematodes (*Meloidogyne* spp.) collected from different kiwi fruit orchards in the Ordu province of Turkey by using molecular methods, including species-specific primers.

Material and Methods

Nematode sampling

Kiwi fruit root samples were collected from 17 kiwi fruit orchards located (Table 1) in the Center, Perşembe, Ünye, Gülyalı, İkizce and Ulubey districts of Ordu city in the years 2011-2012. To carry out the root sampling for identification of females, a total of 5-10 kiwi fruit vines from each orchard were selected and sampled by cutting roots from four sides of the trunk of each vine. Then, the infected roots and soil were put into polythene bags, labeled and brought to the laboratory of the Plant Protection Department, Agricultural Faculty, Ordu University, for identification of root-knot nematode species.

Table 1. The sampled districts of Ordu province and the coordinates of the locations

District	Code	Location	Coordinates (latitude - longitude)
Merkez	M1	Emen	40^0 92'191 N - 38^0 00'119 E
Merkez	M2	Emen	40^0 92'730 N - 38^0 00'305 E
Merkez	M3	Şenocak	40^0 87'615 N - 37^0 94'831 E
Merkez	M4	Kayabaşı	40^0 57'422 N - 37^0 56'252 E
Merkez	M5	Kayabaşı	40^0 56'580 N - 37^0 56'122 E
Perşembe	P-1	Merkez	41^0 04'363 N - 37^0 45'988 E
Gülyalı	G-1	Turnasuyu	40^0 95'474 N - 38^0 00'221 E
Gülyalı	G-2	Turnasuyu	40^0 95'496 N - 38^0 00'298 E
Gülyalı	G-3	Turnasuyu	40^0 95'877 N - 38^0 00'037 E
İkizce	İ-1	Merkez	41^0 05'835 N - 37^0 07'982 E
İkizce	İ-2	Merkez	41^0 06'337 N - 37^0 08'913 E
İkizce	İ-3	Düzpelit Mah.	41^0 05'907 N - 37^0 08'841 E
Ulubey	U-1	Kömürocağı Mah.	40^0 81'732 N - 37^0 78'897 E
Ulubey	U-2	Sokak Mah.	40^0 82'212 N - 37^0 80'307 E
Ulubey	U-3	Dölbentli Mah.	40^0 83'427 N - 37^0 81'189 E
Ünye	Ü-1	T. İlçe deneme bahçesi	41^0 10'429 N - 37^0 38'427 E
Ünye	Ü-2	Ataköy	

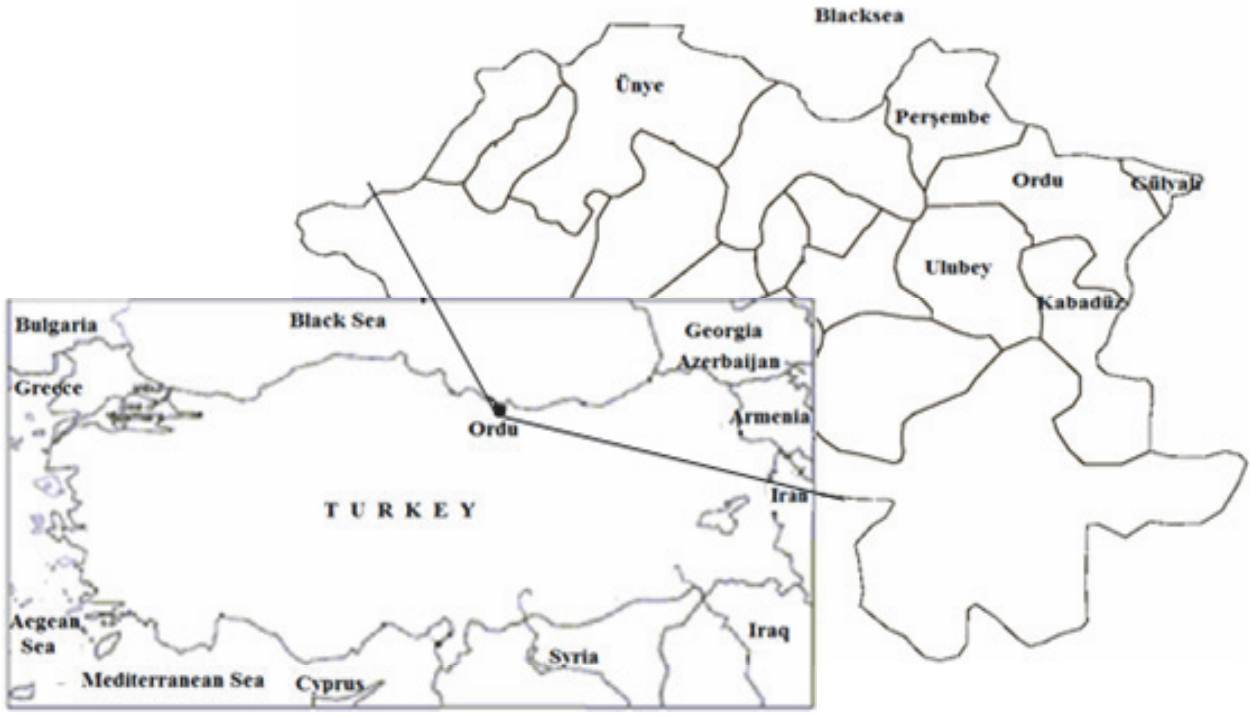

Figure 1. Map of Turkey showing sampled districts of Ordu province.

Extraction of females

Meloidogyne incognita females were dissected from infected kiwi fruit roots using a nematode needle under a stereomicroscope (Leica, S8APO) at 40x magnification.

Extraction of DNA

Nematode DNA was extracted from a single female using a DNeasy Blood & Tissue kit (Qiagen, Maryland, USA). The final volume extracted from each sample was 50 µl in AE buffer. DNA samples in 50 µl in AE buffer were separately kept -20 °C until identification.

Molecular analyses

Molecular analyses were followed by the RKN Molecular Diagnostic Key (Table 2) (Adam *et al*., 2007). All primers used in our investigation were selected from recommended citations of Adam *et al*. (2007) and Devran & Söğüt (2009). Two pairs of primers were used to carry out PCR assays to specifically identify *M. incognita.* Primers used in this study are listed in Table 3. Primers 194 and 195 were used to amplify the IGS-2 (5S-18S) region of the ribosomal DNA (Blok *et al*., 1997). For reactions of the species-specific primers SEC1F and SEC1R were used to obtain species specific primer amplicons (Tesarova *et al*., 2003).

IGS and species-specific SCAR amplicons were obtained by using a total of 20 µl of PCR reaction mixture with 10 units of PCR Master Mix kit (APEX), 0.5 µl of 10 pM primers, 4 µl of PCR water (Sigma, St. Louis, MO, USA), and 5 µl of genomic DNA. The PCR amplification conditions of primers are described in Table 4. Amplification products were separated by electrophoresis in 1.8 % Tris borate buffer agarose gel under 120 V for 30 minutes. Products were visualized with UV illumination after ethidium bromide staining (Sambrook *et al*., 1989).

Table 2. Steps to identify the root-knot nematode *Meloidogyne incognita* (modified from Adam *et al*., 2007)

1. Amplify the IGS2 between 5S and 18S ribosomal genes using 194/195 primers
a. 720-bp…… Tropical species…… Go to (2)
2. Tropical RKN specific SCAR Primers
2.1. SEC-1F and SEC-1R primers
502-bp…… *M. incognita*

Table 3. Primers used for molecular identification of root-knot nematode *Meloidogyne incognita*

Locus	Name of Primer	Species	Fragment (bp)	Primer sequences (5'-3')	References
IGS-2	194 195	*M. incognita* *M.arenaria* *M.javanica*	720	TTAACTTGCCAGATCGGACG TCTAATGAGCCGTACGC	Blok *et al*., 1997
	SEC1F SEC1R	*M. incognita*	502	GGGCAAGTAAGGATGCTCTG CGTGGCTATGAAAGAGGTGC	Tesarova *et al*., 2003

Table 4 . PCR amplification conditions of primers used to identify *Meloidogyne incognita* (Adam *et al*., 2007; Devran & Söğüt, 2009)

Name of Primer	**Amplification Conditions**		
194/195	94 °C	3 min.	
	94 °C	30 secs	
	58 °C	30 secs	35 cycles
	72 °C	90 secs	
	72 °C	5 min	
SEC1F/SEC1R	94 °C	3 min.	
	94 °C	30 secs	
	54 °C	30 secs	45 cycles
	72 °C	90 secs	
	72 °C	5 min	

Results and Discussion

In this investigation, gene specific and SCAR primers were used for identification of the root-knot nematode *M. incognita*. Orchard populations were used directly for identification. In total, 17 *Meloidogyne* populations on kiwi fruit were determined. An SEC1F/SEC1R primer set produced a 502 bp amplicon. The species specific SCAR primers Fjav/Rjav for *M. javanica* and Far/Rar for *M. arenaria* did not provide any PCR amplification products. Two different primer sets were used for diagnosis of *M. incognita* (Table 3). Firstly, PCR reaction for the IGS-2 region to provide different size amplicons for different RKN species was performed. For this purpose, amplicons of the IGS-2 region between 5S-18S genes of the rDNA were obtained by using the primers 194/195. The amplification results with 194/195 for all populations were only 720 bp amplicon size,which is expected for the common species *M. javanica, M. incognita* and *M.arenaria,* and consistent with Adam *et al*. (2007) and Wishard *et al*., (2002). After getting amplicons of IGS-2 for the populations, the band size 720 bp of the IGS-2 indicated the second step in the key to use species specific SCAR primers (Figure 2). The results of additional PCR reactions with species specific SCAR primers showed that the unknown *Meloidogyne* species populations from Ordu province were only *M. incognita* by the exact size of 502 bp amplicons by SEC1F/SEC1R primers (Figure 2). In this study, 502 bp amplicon size concurred with the results of Tesarova *et al*., (2003) and Devran & Söğüt (2009).

Identification of *Meloidogyne* species is important to design effective nematode management practices such as crop rotation and plant resistance (Hussey, 1990; Cenis, 1993; Zijlstra et al., 2000; Zijlstra & Van Hoof, 2006). Molecular techniques for identification of *Meloidogyne* species using DNA are being developed using polymerase chain reaction (PCR) technology, which allows analysis on a single nematode (Hussey & Janssen, 2002). In order to identify the most common and important *Meloidogyne* species, *M. arenaria*, *M. incognita, M. hapla* and *M. javanica*, the RKN molecular diagnostic key of Adam et al. (2007) was employed step by step. These results showed that the unknown nematode populations were only *M. incognita*. *Meloidogyne incognita* is the most common root-knot nematode species according to the sampling results of the international *Meloidogyne* project (Taylor *et al*., 1982). Investigations at different times and in different countries also showed that *M. incognita* is the most encountered species (Khan & Ahmad, 2000; Guzman-Plazola *et al*., 2006; Anwar & McKenry, 2010; Kayani *et al*., 2013).

Results of this investigation suggest that only *M. incognita* occurs in the kiwi fruit orchards in the Ordu. However, several *Meloidogyne* species such as *M. incognita*, *M. javanica*, *M. arenaria, M. hapla* and *M. ethiopica* (Carneiro *et al*., 2004; Ma *et al*., 2007; Ploetz, 2011) were reported to attack worldwide.The original research in different regions of Turkey showed that *M. incognita* was the most common species on various cultivated plants in the country (Elekçioğlu & Uygun, 1994; Elekçioğlu *et al*., 1994; Mennan & Ecevit, 1996; Kaşkavalcı & Öncüer, 1999; Söğüt & Elekçioğlu, 2000, Örümlü, 2003; Katı, 2006; Özarslandan, 2009). In addition to these results, the detailed investigation by Yüksel (1974) revealed that the Black Sea, Marmara, Aegean and Mediterranean Regions in Turkey have *M. incognita* as the most common species on cultivated plants. Especially, the annual and perennial agricultural plants grown in the eastern part of Samsun Province in Black Sea region were pointed out as the plants damaged by only *M. İncognita,* according to the results of Yüksel (1974). It is therefore clear that *M. incognita* is a widespread species adapted to different ecological conditions worldwide.

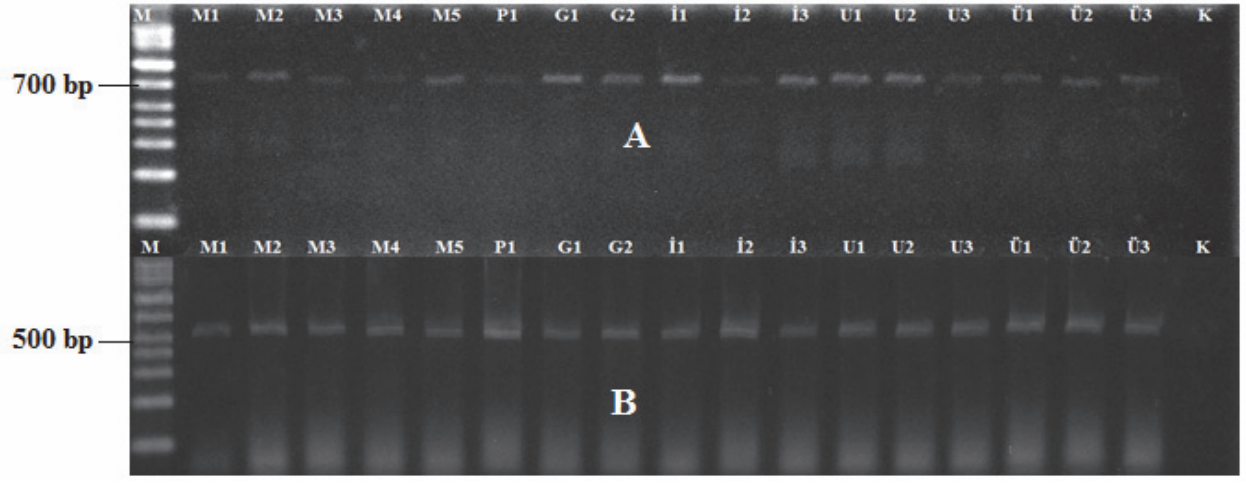

Figure 2. Amplification products of M1-Ü3 orchards. Each well contains only a single female's DNA performed with PCR.
A: Amplicons of IGS-2 (720 bp) region between 5S-18S rDNA with 194/195 primers.
B: Amplicons of *Meloidogyne incognita* species-specific SEC1F/SEC1R primers (502 bp).
M: Marker (Qiagen (1kb+).
K: Water.

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