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Molecular Characterization and Phylogeny of *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley 1980 Obtained from Potato Production Areas in Turkey

Ayşenur ASLAN[1]✉, Dilek DİNÇER[2], Tohid BEHMAND[3], Adem ÖZARSLANDAN[4], Lerzan ÖZTÜRK[5]
İbrahim Halil ELEKCİOĞLU[6]

[1]Atatürk University, Faculty of Agriculture, Department of Plant Protection, 25040, Yakutiye, Erzurum, Türkiye, [2]Biological Control Central Research Institute, 01360, Adana, Türkiye, [3]Çukurova University, Faculty of Agriculture, Department of Plant Protection, 01360, Balcalı, Adana, Türkiye, [4]Applied Technology and Management School of Silifke 33940 Silifke, Mersin, Türkiye, [5]Viticulture Research Institute, 59100, Süleymanpaşa, Tekirdag, Türkiye, [6]Çukurova University, Faculty of Agriculture, Department of Plant Protection, 01360, Balcalı, Adana, Türkiye

[1]https://orcid.org/0000-0002-2752-5769, [2]https://orcid.org/0000-0002-1671-5647, [3]https://orcid.org/0000-0001-7227-2484
[4]https://orcid.org/0000-0001-7129-8543, [5]https://orcid.org/0000-0003-2199-6807, [6]https://orcid.org/0000-0002-1707-7392
✉: aysenur.aslan@atauni.edu.tr

ABSTRACT

Meloidogyne chitwoodi is an invasive nematode that can cause economic damage to agricultural areas in many parts of the world. The identification of plant parasitic nematodes is basically carried out by observation of morphologic characteristics and morphometric parameters. However, it is not always possible to obtain certain results with the use of published original descriptions and diagnostic keys. Therefore, in this study, nematode samples isolated from potato tubers collected from the Central Anatolia region during the production season of 2018 and 2019 were determined molecularly by PCR-based diagnosis method using JMVhapla, JMV1 and JMV2 primer sets; and morphologically using morphometric measurements and perineal pattern. PCR reactions yielded 540 bp bands. As a result of both methods, the nematode species was determined as *Meloidogyne chitwoodi*. Phylogenetic analysis and pairwise distance were performed to evaluate the relationships of local populations with other *Meloidogyne* species. After phylogeny studies, it was determined that the populations were 99% similar to both the Turkish population and other populations. Compared to other sequences of published local isolates, the Niğde isolate in this study showed quiet similarity with Nevşehir (KF557791.1) isolate. As a result of this study, the data on *M. chitwoodi*, which causes damage in the potato growing areas of the Central Anatolia Region, has been updated.

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Türkiye'deki Patates Ekiliş Alanlarından Elde Edilen *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley 1980'nin Moleküler Karakterizasyonu ve Filogenisi

ÖZET

Meloidogyne chitwoodi, dünyanın birçok yerinde tarım alanlarında ekonomik zarara neden olabilen istilacı bir nematoddur. Bitki paraziti nematodların tanımlanması temelde morfolojik özelliklerin ve morfometrik parametrelerin gözlenmesiyle yapılmaktadır. Ancak yayınlanmış orijinal betimlemeler ve tanılama anahtarlarının kullanılmasıyla her zaman doğru sonuç elde edilememektedir. Bu nedenle, bu çalışmada 2018 ve 2019 yılı üretim sezonu boyunca İç Anadolu Bölgesi'nden toplanan patates yumrularından izole edilen nematod örnekleri JMVhapla, JMV1 ve JMV2 primer setleri kullanılarak PCR tabanlı tanı yöntemi ile moleküler tanılama ile morfometrik ölçümler ve perineal pattern kullanılarak morfolojik olarak tanılanmıştır. İki yöntem sonucunda da nematod türü *Meloidogyne chitwoodi* olarak tespit edilmiştir. Ayrıca çalışmada yerel popülasyonların diğer *Meloidogyne* türleri ile ilişkilerini araştırmak için filogenetik analiz ve ikili uzaklık değerlendirilmesi yapılmıştır. Filogeni çalışmalarından sonra popülasyonların hem Türkiye popülasyonuna

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hem de diğer popülasyonlara %99 benzer olduğu tespit edilmiştir. Yayımlanmış yerel izolatların diğer sekansları ile karşılaştırıldığında bu çalışmadaki Niğde izolatı, Nevşehir (KF557791.1) izolatı ile oldukça benzerlik göstermiştir. Bu çalışma sonucunda İç Anadolu Bölgesi patates yetiştirme alanlarında zarar yapan *M. chitwoodi* ile ilgili veriler güncellenmiştir.

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INTRODUCTION

Potato, *Solanum tuberosum* L. (Tubiflorales: Solanaceae) is an annual plant with more than 4.000 varieties grown in the world for its nutritionally rich content (Grzebisz et al., 2020). Potato contains higher amount of carbohydrates, proteins, fiber, many vitamins such as vitamin C, potassium, magnesium, phosphorus and iron. Also, potato is good industrial plant which is processed to chips, flour and alcohol (Çalışkan et al, 2010). Potato plant is adapted to grow well in many different climates and is the fourth most cultivated plant in the world with the product of 400 million tons yearly. China is leading producer and consumer while Türkiye ranked 14th with 4.800 tones (Anonymous, 2019).

Although many plant parasitic nematodes have been identified in potato growing areas in the world economically, most harmful species include in genera of *Meloidogyne* spp. Despite *M. hapla* Chitwood, 1949 and *M. chitwoodi* Golden, O'Bannon, Santo & Finley are considered as most damaging root-knot nematodes, *M. chitwoodi* is the most destructive species and is difficult to control by nematicides. (Tiilikkala et al., 1995). *M. chitwoodi* (Golden, O'Bannon, Santo & Finley) is one of the most destructive nematode in potato fields in the world. This species causes economic problems in potato growing areas in the northern, western and eastern regions of Türkiye (Devran et al., 2009; Özarslandan & Elekcioğlu, 2010; Evlice & Bayram, 2013; Özarslandan et al., 2013). Symptom caused by this nematode include small tuber galls, necrosis and above ground plant stunting (Anonymous, 2005). Related yield reduction and gall symptoms of tubers lead to significant reduction of market value of potato plants (Santo, 1994). The damage on plant roots and tubers leads to infection of several pathogens as well. The interactions between *Meloidogyne* spp. and *Fusarium* wilt and *Rhizoctonia solani* have been previously reported (Golden & Van Gundy, 1975; Siddiqui et al., 1999).

M. chitwoodi has two races and these have a wide host range from several families such as Brassicaceae, Cucurbitaceae, Lamiaceae, Liliaceae, Fabaceae, Umbelliferae and Vitaceae (Santo et al., 1980; O'Bannon et al., 1982; Evlice & Bayram, 2016). Despite the widespread distribution all over the world, the species was identified for the first time in Central Anatolia, Türkiye by Özarslandan et al. (2009) and later by Evlice and Bayram (2012).

Morphological methods are widely used in the identification of nematodes, but in some cases (dauer larvae) and due to the morphological proximity of the species, the accuracy of this method needs to be supported by other methods (Gerič Stare et al. 2018; Aslan & Elekcioğlu, 2022). Species identification using molecular technique give reliable and accurate results in a short time (Devran & Söğüt, 2009). Morphological and molecular diagnostic techniques are studies that support each other (Özarslandan & Elekcioğlu, 2010).

During the nematode surveys and inspections in 2018-2019, potato tubers with symptoms resembling *Meloidogyne* spp. damage were detected from samples collected from Central Anatolia. The species were identified by morphologic characteristics and perineal patterns of vulval cuttings. In order to prove morphologic identification, a study that aims development PCR based method with specific primers were carried out, the amplification product was sequenced and compared in universal gene bank to reveal phylogenetic relationships with other nematode species.

MATERIAL and METHODS

Sample collection, nematode isolation and morphologic characterization

Galled potato tubers were obtained from the infested potato fields located in Niğde and Konya province in 2018-2019 (Table 1). A total of 23 populations were brought to the laboratory and examined. Potato slides prepared from necrotic areas were examined with a Leica DM1000 microscope to verify the presence of *Meloidogyne* individuals. The female nematodes were hand-picked with pens after

examination at 10X magnification. Juveniles were isolated by modified Baermann Funnel method described (Baermann, 1917). The morphometrics of nematodes was measured using Leica Application Suite software and the images were taken with Leica ICC50 W camera (Figure 1).

Table 1. Isolates collected from potato production areas of Nigde and Konya province in Türkiye
Çizelge 1. Türkiye'de Niğde ve Konya ili patates üretim alanlarından toplanan izolatlar

Isolate	City	District	Isolate	City	District
MCN-1	Niğde	Edikli	MCN-13	Niğde	Alay
MCN-2	Niğde	Orhanlı	MCN-14	Niğde	Alay
MCN-3	Niğde	Orhanlı	MCN-15	Niğde	Alay
MCN-4	Niğde	Yeşilgölcük	MCN-16	Niğde	Ağcaşar
MCN-5	Niğde	Konaklı	MCN-17	Niğde	Ağcaşar
MCN-6	Niğde	Konaklı	MCK-18	Konya	Ovakavağı
MCN-7	Niğde	Konaklı	MCK-19	Konya	Ovakavağı
MCN-8	Niğde	Konaklı	MCK-20	Konya	Ovakavağı
MCN-9	Niğde	Konaklı	MCK-21	Konya	Ovakavağı
MCN-10	Niğde	Konaklı	MCK-22	Konya	Ovakavağı
MCN-11	Niğde	Konaklı	MCK-23	Konya	Ovakavağı
MCN-12	Niğde	Alay			

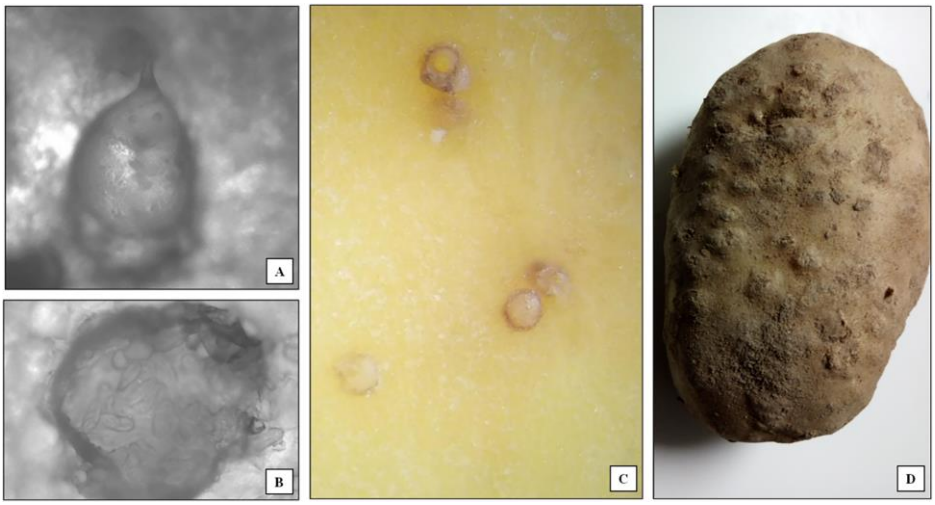

Figure1. *Meloidogyne chitwoodi*; A, C) Female, B) Egg mass of female, D) Galls on potato tuber
Şekil 1. Meloidogyne chitwoodi; A-C) Dişi, B) Dişi yumurta paketi, D) Yumruda bulunan galler

The MC-2 population was selected from the infected tubers. Morphologic identification of this population was carried out with juvenile and female perineal pattern morphology (Figure 2). In order to prepare nematode slides extracted *Meloidogyne* females, were cut from posterior body part and the posterior end of body with perineal patterns and were placed into glycerin. On the other hand, juveniles were collected from egg masses and the isolated individuals were heat killed at 60 ºC for one minute, fixed in double strengthen TAF solution and mounted on slides by wax-ring method (Seinhorst, 1959). Identifications were performed according to previous studies (Jepson, 1987; Karssen, 2002).

Molecular identification and phylogeny

A PCR based diagnostic method with primers set JMVhapla, JMV1 and JMV2 were performed to verify *M. chitwoodi*. Primer sequences used in PCR studies are given in Table 2 and used as stated in the literature (Wishart et al., 2002).

DNA was extracted from single female by using Sigma Aldrich Extract N Tissue PCR kit containing 2.5 µl tissue preparation and 10 µl extraction solution. Tubes were incubated at 55 °C for 10 minutes followed by 94 °C for 3 minutes. PCR assay was performed in a volume of 25 µl mixture containing 8 µl of water, 12 µl of master mix, 1 µl of each primer (JVM1, JVM2 and JVM hapla), 2 µl of DNA. The PCR reaction was performed at 94 °C, 3 min; (94 °C, 50 sec; 59 °C, 50 sec; 72 °C, 1 min) × 35; 72 °C, 7 min. Amplicons were controlled by agarose gel 2 % staining with ethidium bromide. The gel was run 50 minutes at 50 V and visualized UV Transilluminator. After UV visualization, the PCR product was sequenced for approval of identification. For DNA sequence, one population representing the area were chosen. The PCR products were sequences with specific forward primer of *M. chitwoodi.*

Table 2. The primers used for molecular identification for *M. chitwoodi*
Çizelge 2. M. cihwoodi'nin moleküler tanılamasında kullanılan primerler

Primer *Primer*	Primer Sequences (5'-3') *Primer Dizilimleri (5'-3')*	Species *Türler*	Fragment (bp) *Uzunluk (bp)*	Reference *Literatür*
JMV1	GGATGGCGTGCTTTCAAC	***M. chitwoodi***	540	**Wishart et al., 2002**
JMV2	TTTCCCCTTATGATGTTTACCC	***M. fallax***	670	
JMVhapla	AAAAATCCCCTCGAAAAATCCACC	***M. hapla***	440	

Phylogenetic analysis was conducted to evaluate relationships of *M. chitwoodi* populations with other local species and other published foreign species. On this purpose, the sequence data were subjected to GenBank sequence comparison with the BLAST records of NCBI. Neighbour joining with Kimura 2-parameter model was performed on Mega X software comparing *M. chitwoodi* with other sequences. Pairwise distance was calculated after aligning the sequences in ClustalW.

RESULTS and DISCUSSION

Meloidogyne chitwoodi Golden, O'Bannon, Santo & Finley, 1980 (Figure 2)

The measurements of second stage juveniles was; Female body is pyriform and white colour. Head region is offset and cephalic framework is distinct. Stylet is small with rounded knobs that slope posteriorly. Dorsal esophageal gland opens into esophagus lumen. Perineal pattern of female was round to oval with striae curved around anal area. Punctuation not present (Golden et al., 1980).

Male body is vermiform slender, tapering slightly at both extremities. Head is slightly offset, with large labial disc and post labial annule. Lateral field with four incisures. Testis one or two. Spicules are arcuate. Phasmids is located at or anterior to cloaca. Tail is short and rounded (Golden et al., 1980).

Juvenile body small and vermiform. Head not offset, cephalic framework weak, labial disc without striations. Lateral field with four incisures. Phasmids small and located in anterior part of tail. Tail hyaline short and tail terminus rounded (Golden et al., 1980).

The description and morphometrics of *M. chitwoodi* population in this study are compatible with original description of Chitwood (1949) and other reports from Türkiye (Devran et al., 2009; Özarslandan et al., 2009; Özarslandan & Elekcioğlu, 2010; Evlice & Bayram, 2016). Larval morphometric measurements were found similar to Karssen (2002) (Table 3).

Table 3. Second instar larvae measurements of *M. chitwoodi*
Çizelge 3. M. chitwoodi'nin ikinci dönem larva ölçümleri

	Niğde Population	Karssen (2002)
N	30	-
L (µm)	377.12±3.2 (365.8-389.2)	380±11.5 (362-394)
Greatest body diameter (µm)	12.7±0.62 (11.7-13.5)	13.1±0.5 (12.6-13.9)
Body diam. at stylet knobs (µm)	9.15±0.01 (8.9-9.3)	-
Body diam. at S-E pore (µm)	11.9±0.3 (11.6-12.0)	11.8±0.3 (11.4-12.0)
Stylet length (µm)	9.23±0.27 (8.9-9.8)	9.7±0.3 (9.5-10.1)
DGO (µm)	3.14±0.21 (2.89-3.5)	3.4±0.4 (2.5-3.83)
Anus uzunluğu (µm)	9.32±0.3 (8.2-10.2)	9.4±0.4 (8.9 -10.1)
Tail length (µm)	41.63±0.8 (38.8-44.6)	43.2±1.6 (39.8-44.8)
Tail terminus length (µm)	10.03±0.25 (9.2-11.5)	10.9±0.8 (8.9-12.0)
a	28.42±0.5 (26.65-30.52)	29.1±1.3 (26.0-31.0)
b'	6.9±0.1 (6.6-7.35)	7.6±0.9 (5.7-8.8)
c	8.69±0.12 (8.52-8.90)	-
c'	4.02±0.1 (4.89-5.1)	4.6±0.2 (4.2-5.0)

Molecular characterisation of *Meloidogyne chitwoodi*

PCR product of *M. chitwoodi* DNA with JMVhapla, JMV1 and JMV2 primers formed 540 bp bands at 2 % agarose gel electrophoresis. The results were similar with previous studies of *M. chitwoodi* (Wishart et al., 2002; Adam et al., 2007; Devran et al., 2009; Evlice & Bayram, 2016)(Figure 3).

After phylogeny studies, populations in this study showed 98 % Blast identity with Turkish populations from Niğde, Isparta and Nevşehir KF557828 (467/475), KF557817 (467/475), KF557779 (467/475), KF5599 (467/475), KF557793 (467/475), KF557791 (467/475), KF557771 (467/475), of Evlice & Bayram, (2013) (Figure 4). Compared to populations from other countries, the population in this study showed 97 % identity with populations AJ421701 (464/475) and AF013992 (512/541), 96 % identity with GQ395598 (452/460) from Holland, United Kingdom and USA.

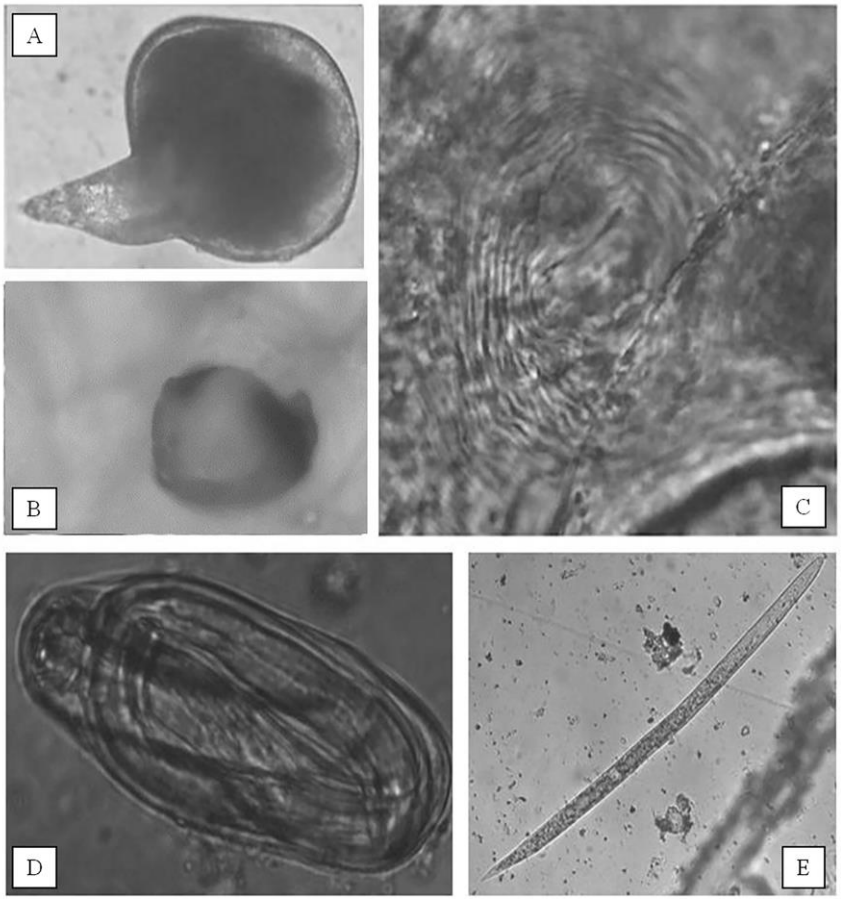

Figure 2. *M. chitwoodi*; A, B) Female body, C) Lateral line of perineal pattern, D) Egg, E) Juvenile
Şekil 2. M. chitwoodi; A-B) Dişi vücudu, C) Anal kesitin lateral hattı, D) Yumurta, E) Larva

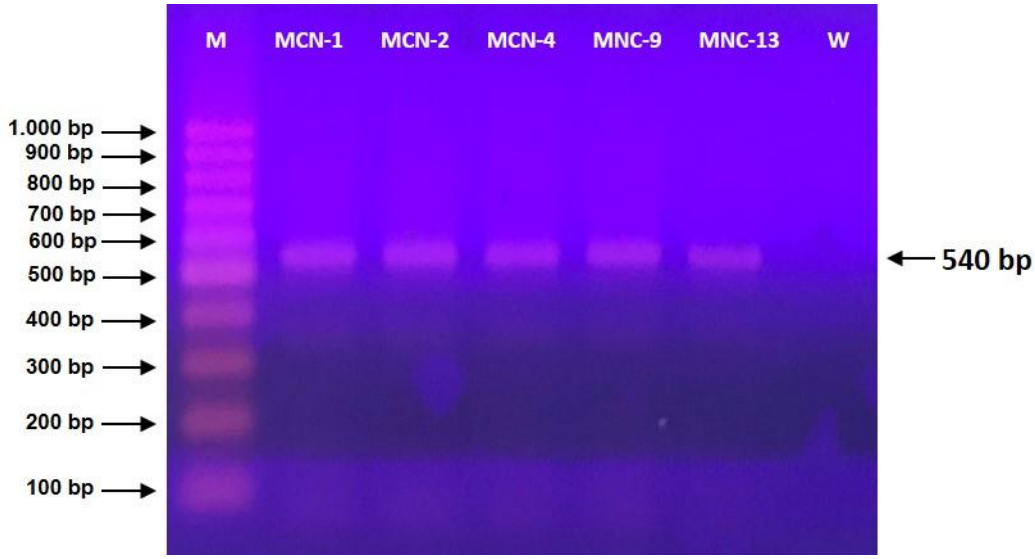

Figure 3. Electrophoresis of PCR products amplified with JMVhapla, JMV1 and JMV2 primers. MC: Sample region, W: water
Şekil 3. JMVhapla, JMV1 ve JMV2 primerleri ile amplifiye edilmiş PCR ürünlerinin elektroforezi. MC: Örnek bölge, W: su

Neighbour joining tree was designed by comparing *M. chitwoodi* isolates with AF013992.1, AJ421701.1, KC262253.1, GQ395598.1, KF557770.1, KF557774.1, KF557772.1, KF557816.1, KF557791.1 accessions of Canada, UK, USA, Switzerland, Türkiye and local isolate in this study. According to tree *M. chitwoodi* higher similarity between Canada, UK, USA, Switzerland in the rate of 99 % was observed and

compared to other Turkish isolates (Aksaray, Konya, Nevşehir, Niğde and Isparta), isolate in this study was found nearest with Nevşehir KF557791.1. The relationship between isolates which belongs to same species in the world was proved with phylogenetic analysis help.

Pairwise distance analysis was conducted on Mega X. differences among 9 *M. chitwoodi* sequences, and the sequences was shown in Figure 5. The distance was varied between 0 to 0,04. The difference was highest in accessions KF557816, AJ421701 and AF013992. P-distance of the samples varied between 0 % to 4 %.

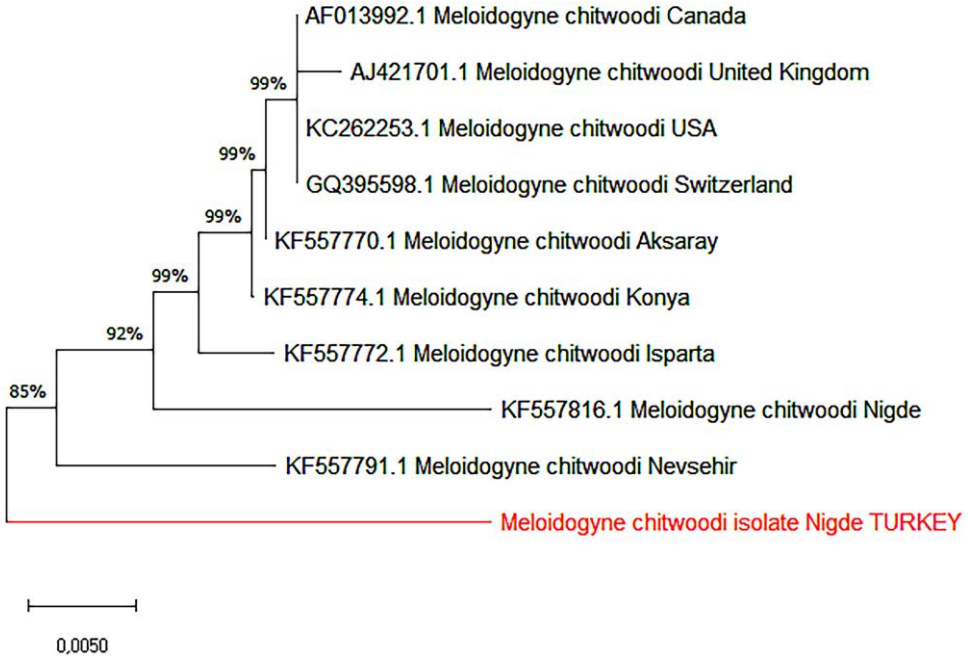

Figure 4. The phylogenetic analysis which belongs to *M. chitwoodi* isolates
Şekil 4. M. chitwoodi izolatlarına ait filogenetik analiz

	1	2	3	4	5	6	7	8	9	10
1. AF013992.1 Meloidogyne chitwoodi Canada										
2. AJ421701.1 Meloidogyne chitwoodi United Kingdom	0.002									
3. KF557791.1 Meloidogyne chitwoodi Nevsehir	0.021	0.023								
4. GQ395598.1 Meloidogyne chitwoodi Switzerland	0.000	0.002	0.021							
5. KC262253.1 Meloidogyne chitwoodi USA	0.000	0.002	0.021	0.000						
6. KF557774.1 Meloidogyne chitwoodi Konya	0.002	0.004	0.021	0.002	0.002					
7. KF557772.1 Meloidogyne chitwoodi Isparta	0.008	0.011	0.021	0.008	0.008	0.006				
8. KF557770.1 Meloidogyne chitwoodi Aksaray	0.000	0.002	0.021	0.000	0.000	0.000	0.004			
9. KF557816.1 Meloidogyne chitwoodi Nigde	0.023	0.026	0.030	0.023	0.023	0.019	0.021	0.017		
10. Meloidogyne chitwoodi isolate Nigde TURKEY	0.035	0.038	0.035	0.035	0.035	0.035	0.035	0.035	0.046	

Figure 5. Pairwise distance of local *M. chitwoodi* and other populations from the same species
Şekil 5. Yerel M. chitwoodi ve aynı türden diğer popülasyonların ikili uzaklıkları

Due to spread by irrigation water and infected seeds, *M. chitwoodi* infected areas increase continuously. Chemical control cannot be effective on individuals which are located and feed in tubers. Hence, toxic chemicals may not reach to nematodes colonized inside cells. Eradication can be achieved only by selection of proper species-specific management methods targeting *M. chitwoodi.*

Meloidogyne spp. are generally identified based on their morphology and morphometrics. Recently esterase and malate dehydrogenase were proven as applicable methods for reliable identification (Karssen et al., 1995). More recently several reports on molecular identification of nematodes were published. Multiplex PCR methods were applied for identification of *M. chitwoodi, M. fallax, M. hapla* and *M. incognita* while classic PCR was found reliable on *M. incognita*, *M. javanica, M. arenaria, M. mayaguensis, M. hapla, M. chitwoodi* and *M. fallax* (Adam et al., 2007; Meng et al., 2004; Devran et al. 2009). Molecular identification is reliable method which can be carried out even by unqualified staff to

any nematode specimen. Furthermore, nematode species at every developmental stage can be used for DNA isolation and diagnostic process.

CONCLUSION

In this study, we were able to identify *M. chitwoodi* from single juvenile sample with PCR using JMVhapla, JMV1 and JMV2 primers. 540 bp expected DNA band was observed on agarose gel electrophoresis. The application of other methods like RFLP analysis of *M. chitwoodi* was previously reported (Devran et al., 2009; Özarslandan et al., 2013). On this study, phylogenetic tree analysis provided to understand better relationship *M. chitwoodi* among other countries. It was determined that the phylogenetic analysis of the isolate showed great similarity with the Türkiye isolates, as expected. In addition, the isolate was highly similar to Canada, UK, USA and Switzerland isolates. These primers and PCR method can be used for identification of local nematode species.

Contribution of the authors as summary

Authors declares the contribution of the authors is equal.

Statement of conflict of interest

Authors have declared no conflict of interest.

Ethics committee decision

Ethics committee approval is not required for this study.

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