

IDENTIFICATION AND GENETIC DIVERSITY OF *MELOIDOGYNE CHITWOODI* IN POTATO PRODUCTION AREAS OF TURKEY

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ABSTRACT

Devran, Z., M. Nedim, Ö. Adem, and E. İ. Halil. 2009. Identification and genetic diversity of *Meloidogyne chitwoodi* in potato production areas of Turkey. *Nematropica* 39:75-83.

Root-knot nematodes (*Meloidogyne* spp.) were first detected in Turkey during regional nematode surveys of potato fields of Niğde province. Twelve populations of *Meloidogyne* were collected and identified based on morphological characteristics as well as molecular methods including species-specific primers, confirming that all twelve populations were *M. chitwoodi*. To estimate genetic relationships among these twelve populations, random amplified polymorphic DNA (RAPD) analysis was done and the results were subjected to UPGM analysis. Analysis of 394 RAPD markers resulted in a similarity coefficient, ranging between 0.64 and 0.81 with a mean similarity of 0.72. The populations Mc3 and Mc4 displayed the greatest genetic similarity (81%). The lowest level of similarity (65%) was observed between Mc9 and Mc7. The large genetic variation among the *M. chitwoodi* populations indicates that multiple sources of inoculum might have been introduced to the region or that the populations formerly existed in these potato growing areas of Turkey.

Key words: Columbia root-knot nematode, genetic similarity, identification, *Meloidogyne chitwoodi*, PCR, RAPD.

RESUMEN

Devran, Z., M. Nedim, Ö. Adem, and E. İ. Halil. 2009. Identificación y diversidad genética de *Meloidogyne chitwoodi* en áreas productoras de papa en Turquía. *Nematropica* 39:75-83.

Los nematodos del nudo radical (*Meloidogyne* spp.) se detectaron por primera vez en Turquía durante censos en cultivos de papa en la provincia de Niğde. Se colectaron 12 poblaciones de *Meloidogyne* se identificaron con base en características morfológicas y utilizando cebadores específicos para especie, confirmando que todas las poblaciones eran *M. chitwoodi*. Para estimar las relaciones genéticas entre estas 12 poblaciones, se utilizó análisis por RAPD (Random Amplified Polymorphic DNA) y se analizaron los resultados por UPGM. El análisis de 394 marcadores de RAPD resultó en un coeficiente de similitud entre 0.64 y 0.81, con una similitud promedio de 0.72. Las poblaciones Mc3 y Mc4 fueron las más similares genéticamente (81%). El menor nivel de similitud (65%) se observó entre Mc9 y Mc7. La amplia variación genética de estas poblaciones de *M. chitwoodi* indica que pudieron existir múltiples fuentes de introducción del nematodo a la región, o que estas poblaciones no fueron introducidas y que ya existían en esta zonas productoras de papa en Turquía.

Palabras claves: identificación, *Meloidogyne chitwoodi*, nematodo del nudo radical, PCR, RAPD, similitud genética.

INTRODUCTION

Potato is one of the most important field crops in Turkey, cultivated in 1593 ha with an annual yield of 4.397.305 tonnes (Anonymous, 2006). The most important areas of production are in Niğde and Nevşehir provinces of Turkey (Onaran *et al.*, 2000).

Although over 80 different species of root-knot nematodes have been described, the most economically important pathogens are *M. incognita*, *M. javanica*, and *M. arenaria*, which are globally distributed in subtropical and tropical regions in the world. *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax* occur in the cooler regions (Netscher and Sikora, 1990; Eisenback and Triantaphyllou, 1991; Siddiqi, 2000; Karssen and Moens, 2006). *Meloidogyne incognita*, *M. javanica*, and *M. arenaria* are the most common and economically important root-knot nematodes in vegetable growing areas in Turkey (Elekçioğlu and Uygun, 1994; Elekçioğlu *et al.*, 1994). The first regional nematode survey carried out in potato fields was in the Niğde province of Turkey. In these studies, *Meloidogyne* species, including *M. chitwoodi*, were identified using morphological and molecular analyses (Özarslandan *et al.*, 2009).

Meloidogyne chitwoodi is well known as a nematode of potato and other crops in western Europe, Netherlands, Portugal, Belgium, Germany, and in the United States (Nyczepir *et al.*, 1982; Molendijk and Mulder, 1996; Karssen and Moens, 2006). The nematode causes severe galling on the surface and necrotic spotting below the skin of the potato tubers (Castagnone-Sereno *et al.*, 1999), leading to reduction of tuber quality of potatoes to be consumed fresh or for processing. *Meloidogyne chitwoodi* has been designated as an EPPO A2 quarantine pest in Europe and Canada due to its serious damage potential (Petersen and Vrain, 1996; Anonymous, 2004).

Meloidogyne chitwoodi and *M. fallax* are commonly found together. Although *M. chitwoodi* was first detected in the USA, *M. fallax* has not been reported first in the USA. Identification of these two species is quite difficult because of their close morphological resemblance to each other. Molecular methods based on PCR (polymerase chain reaction) have recently been developed to identify root-knot nematodes (Hyman and Powers, 1991; Powers and Harris, 1993; Zijlstra *et al.*, 2000). Ribosomal DNA as genetic markers is commonly used to identify them. Variations in the ITS region renders it as a convenient region for detecting genetic variation among genera, species and within species (Zijlstra *et al.*, 1995). Molecular methods are now seen as relatively simple and reliable identification tools for successful management and quarantine services. Another method, RAPD analysis (random amplified polymorphic DNA) can be used to estimate genetic variation. RAPD has been used to investigate genetic variation of *Meloidogyne* species (Blok *et al.*, 1997; Guirao *et al.*, 1995; Carneiro *et al.*, 1998).

The objectives of this study were to identify root-knot nematodes collected from different potato growing locations of Niğde province in Turkey by morphological and molecular methods and to evaluate genetic variation among the nematode populations present.

MATERIALS AND METHODS

Root-knot nematode populations

Isolates of *Meloidogyne* species were collected from twelve multiple locations in the Alay, Tirhan, Gölcük, and Kiledere potato growing areas in Niğde province of Turkey (Table 1). Egg masses were collected from infected symptomatic potato tubers. The isolates were maintained on a susceptible tomato (*Lycopersicon esculentum* L. cv. Tuez).

Table 1. Isolates of *Meloidogyne* were collected from potato production locations of Niğde province in Turkey.

Isolates	Location
Mc1	Alay 1
Mc2	Alay 2
Mc3	Alay 3
Mc4	Tırhan 1
Mc5	Tırhan 2
Mc7	Gölcük 1
Mc8	Gölcük 2
Mc9	Gölcük 3
Mc10	Kiledere 1
Mc11	Kiledere2
Mc12	Kiledere 3
Mc13	Kiledere 4

Morphological Analysis

The perineal region of mature females were cut off in a large drop of 45% lactic acid and transferred to a drop of glycerin on a clean glass slide. From each isolate, ten to fifteen perineal patterns were arranged as two rows on a slide and a cover glass applied and sealed (Hooper, 1986). Perineal patterns were examined by light microscopy (Leica, DM2500, Meyer Instruments, Houston, TX, USA).

DNA Extraction

Eggs were collected from infected potatoes. DNA was extracted from egg masses with DNAeasy Tissue and Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Specific PCR Amplifications for Species

The three different species-specific primers used for DNA analysis of the root-knot nematode samples are listed in Table 2. The reaction mixture contained 20 ng DNA, 2 mM MgCl₂, 200 µM dNTP, reaction buffer, 0.4 µM of each primer, 1 unit of Taq DNA polymerase, plus deionized water to a volume of 25 µl. The amplification was carried out in a DNA Engine PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA).

The thermocycler was programmed for 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 48°C (all primers), and 2 min at 72°C. A seven min incubation period at 72°C followed the last cycle in order to complete any partially synthesized strands. The products from these PCR reactions were separated by electrophoresis in TAE buffer 2% agarose gels and the products were visualized with UV illumination after ethidium bromide (0.5 µg/ml) staining.

Table 2. The primers used for molecular identification of root-knot nematode samples.

Name of Primer	Sequences of Primers (5'-3')	References
JMV1	GGATGGCGTGCTTTCAAC	Wishart <i>et al.</i> , 2002
JMV2	TTTCCCTTATGATGTTTACCC	
JMVhapla	AAAAATCCCCTCGAAAAATCCACC	
18s1.2	GGCGATCAGATACCGCCCTAGTT	Powers <i>et al.</i> 2005
18sr2b	TACAAAGGGCAGGGACGTAAT	
C2F3	GGTCAATGTTTCAGAAATTTGTGG	Powers and Harris,1993
1108	TACCTTTGACCAATCACGCT	

Amplification products obtained by 18s1.2/18sr2b primers (Powers *et al.* 2005) and C2F3/1108 primers (Powers and Harris, 1993) were respectively digested with AluI and DraI restriction enzymes, according to manufacturer's recommendations. The fragments generated were separated in a 4% SFR agarose gel (Amresco, Solon, OH) in TAE Buffer.

RAPD

Thirty-five random 10-mer primers were used in a RAPD (GL-RAPD Decamer Set, Gene Link, USA) experiment (Table 3). Amplification reactions included 20 ng DNA, 2 mM MgCl₂, 200 μM dNTP, Reaction Buffer, 0.4 μM of each RAPD primer, 1 unit of Taq DNA polymerase, and deionized water to a volume of 25 μl. Reaction mixtures were subjected to a preheating at

94°C for 3 min, followed by 40 cycles of 30 s at 94°C, 30 s at 35°C, 60 s at 72°C, and a final incubation at 72°C for 10 min using a DNA Engine PTC-200 Peltier Thermal Cycler (MJ. Research, U.S.A). Amplification products were resolved by electrophoresis in 2% agarose gels in TAE buffer at a constant current of 100 V for approximately 2.5 h, and visualized after staining with ethidium bromide (0.5 μg/ml) under UV light.

Statistical data analysis

Each RAPD band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 software package (Exeter Software, Setauket, N.Y., USA) (Rohlf, 1993). A genetic similarity (GS) matrix was constructed

Table 3. RAPD primers used in this study.

Name of Primer	Sequence of Primer (5-3)	Name of Primer	Sequence of Primer (5-3)
M1	GTTGGTGGCT	N1	CTCACGTTGG
M2	ACAACGCCTC	N2	ACCAGGGGCA
M3	GGGGGATGAG	N3	GGTACTCCCC
M4	GGCGGTTGTC	N4	GACCGACCCA
M5	GGAACGTGT	N5	ACTGAACGCC
M6	CTGGGCAACT	N6	GAGACGCACA
M7	CCGTGACTCA	N7	CAGCCCAGAG
M10	TCTGGCGCAC	N8	ACCTCAGCTC
M11	GTCCACTGTG	N9	TGCCGGCTTG
M12	GGGACGTTGG	N12	CACAGACACC
M13	GGTGGTCAAG	N13	AGCGTCACTC
M14	AGGGTCGTTC	N14	TCGTGCGGGT
M15	GACCTACCAC	N15	CAGCGACTGT
M16	GTAACCAGCC	N16	AAGCGACCTG
M17	TCAGTCCGGG	N19	GTCCGTACTG
M19	CCTTCAGGCA	N20	GGTGCTCCGT
OPG-13	CTCTCCGCCA	SC10-30	CCGAAGCCCT
OPG-19	GTCAGGGCAA		

within the SIMQUAL module based on Dice's coefficient (Dice, 1945), which considers only one to one matches between two taxa for similarity. The GS matrix was used to construct a dendrogram using the un-weighted pair group method arithmetic average (UPGMA) to determine genetic similarity among the root-knot nematode isolates studied.

RESULTS

Morphological analysis

The nematode infected tubers from the survey of potato fields in Turkey showed the same symptoms as those reported in the literature for *M. chitwoodi* infected tubers. The skin of these tubers showed small pimple-like bumps that in some case covered all tuber surfaces.

The perineal patterns of the 12 populations were characterized by quite distinctive striae which were broken, curved, twisted or curled around and above the anal area as reported for the Columbia root-knot nematode (Golden *et al.*, 1980). Punctuation was not observed. Vulva was sunken in an area variable in shape and devoid of striae. These results indicated that the perineal patterns of the twelve root-knot populations matched those of *M. chitwoodi*.

Species-specific molecular markers and PCR-RFLP

PCR with the *M. chitwoodi* specific SCAR primers (JMV1, JMV2 and JMVhapla) produced a 540 bp fragment characteristic of *M. chitwoodi* for all root-knot nematode samples collected from infected potatoes in potato production areas (Fig. 1).

A 520 bp fragment was amplified using the C2F3/1108 primer set in all populations of *M. chitwoodi*. The fragment was then digested with the *Dra*I restriction enzyme which yielded 260 bp, 120 bp and

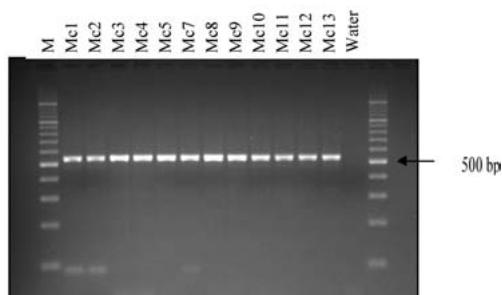


Fig. 1. Amplification product of 540 bp product using JMV multiplex primers on twelve root-knot nematode samples.

85 bp digestion fragments indicative of the genetic fragment properties of *M. chitwoodi* (Fig. 2).

A third PCR of the genomic DNA was conducted using the 18s1.2/18sr2b primer set. This PCR analysis produced a 636 bp amplification fragment consistent with *M. chitwoodi*. Digestion with *Alu*I resulted in 360 bp, 260 bp, 125, 120, 110 bp, 85 bp and 50 bp fragments further indicating that the samples were all *M. chitwoodi* (Fig. 3).

RAPD-PCR

Thirty-six RAPD primers were tested on the twelve populations of *M. chitwoodi*. All primers resulted in amplification and different patterns were tested for population

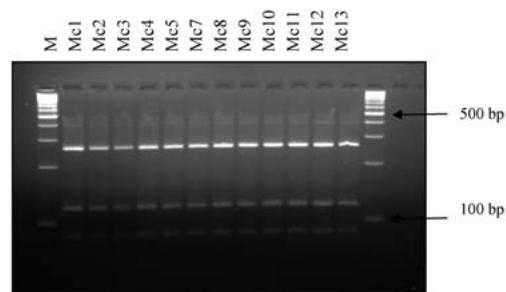


Fig. 2. Digestion of the PCR amplification product of C2F3/1108 primers with the *Dra*I restriction enzyme.

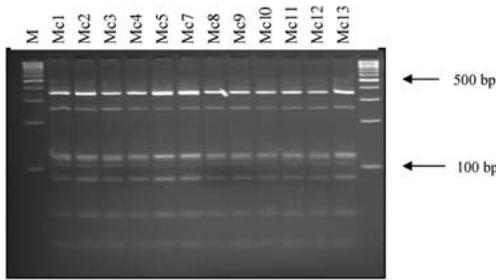


Fig. 3. Digestion of the PCR amplification product of 18s1.2/18sr2b primer with AluI enzyme.

differences. Each RAPD primer generated from five to fourteen bands (Fig. 4).

Thirty-six RAPD primers produced 394 polymorphic markers in populations of *M. chitwoodi*. The mean genetic similarity was 72% among the twelve isolates. The highest genetic similarity was 81% between Mc3 and Mc4 of the *M. chitwoodi* populations. The lowest genetic similarity was 64% between Mc9 and Mc7 (Fig. 5).

DISCUSSION

Niğde is one of the most important potato production regions in Turkey. Infected potatoes were detected in an ongoing regional survey. Identification of twelve *Meloidogyne* spp. populations collected from Niğde province of Turkey was done using morphology and species-specific molecular markers. Morphological

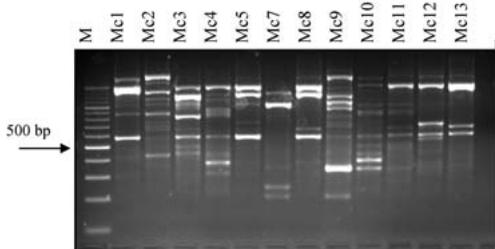


Fig. 4. RAPD amplification patterns obtained using M-12 primers.

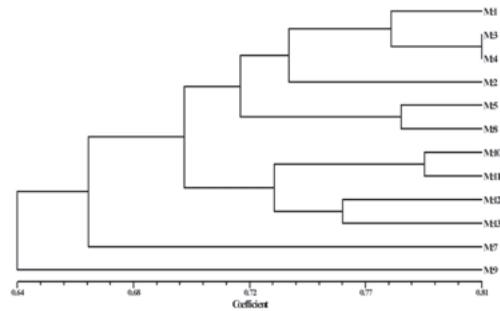


Fig. 5. UPGMA dendrogram based on correlation coefficients among 12 populations of *M. chitwoodi*.

results indicated that all twelve isolates were *M. chitwoodi*. Data obtained by molecular studies fully corroborated the morphological results.

The samples collected from infected potatoes were identified as *M. chitwoodi* using known fragment length profiles for three different specific primer sets. First, amplification using JMV primers resulted in a 540 bp fragment in all twelve samples, consistent with previous studies of *M. chitwoodi* (Wishart *et al.*, 2002; Adam *et al.*, 2007). The second amplification used primer sets (C2F3 and 1108) developed by Powers and Haris (1993). It resulted in a 520 bp fragment, consistent with the identity of *M. chitwoodi*. Afterwards, digestion of the 520 bp amplification products by DraI confirmed to the diagnostic pattern for *M. chitwoodi*. Moreover, our results were consistent with the results reported by Powers *et al.* (2005) after digestion with AluId 18s1.2/18sr2b primers produced a 636 bp amplification fragment in all populations. The identity of *M. chitwoodi* was proved by the use of species-specific primers with resulting products in correspondence with previous reports.

Thirty-six RAPD primers were amplified with genomic DNA isolated from the twelve *M. chitwoodi* populations. A total of 394 polymorphic markers were observed in these Turkish populations of *M. chitwoodi*.

A similarity matrix based on simple matching values was constructed to estimate the level of DNA polymorphism among the twelve *M. chitwoodi* populations. For multi loci marker systems such as RAPD and AFLP, however small percentage, it is possible that some of the DNA bands of the same size may not be the same DNA sequence. This is especially true when different species are compared. But these and similar marker systems have been regularly used for genetic diversity studies because they provide a fast, cheap and random scan of genomes. For instance, the RAPD markers have been solely used to study the genetic variability of burrowing nematode (*Radopholus* sp.) isolates (Marin *et al.*, 1999), potato cyst nematode *Globodera rostochiensis* (Chrisanfova *et al.*, 2008), pine-wood nematode *Bursaphelenchus xylophilus* (Vieira *et al.*, 2007), *Globodera rostochiensis* and *G. pallida* (Da Conceicao *et al.*, 2003), and the soybean cyst nematode *Heterodera glycines* (Lax *et al.*, 2004; Da Silva *et al.*, 2000).

Previous studies have examined genetic variation of *Meloidogyne* spp. with RAPDs. A similarity of >40% was detected in some *M. javanica* populations collected from Brazil (Carneiro *et al.*, 1998). Another study found intraspecific genetic variability of *M. arenaria*, *M. incognita* and *M. javanica* ranged from 40 to 67%, 45 to 56% and 41 to 73%, respectively (Devran *et al.*, 2008). Contrary to these findings, intraspecific genetic variation values were 9.7%, 4.4% and 3.3% in *M. arenaria*, *M. incognita* and *M. javanica*, respectively (Semblat *et al.*, 1998). Similarly, a significant high mean in genetic similarity (99.4%) within each specific cluster in a very homogeneous group of *M. javanica* was also observed (Tzortzakakis *et al.*, 1999). No general correlation was present between genomic similarity and geographical origin of the populations (Semblat *et al.*, 1998; Devran *et al.*, 2008).

In the present study, *M. chitwoodi* populations collected from infected potatoes in Niğde province, showed similarity up to 72% and established five clusters identify the clusters using a number of RAPD markers. Location-specific groupings were also observed. *Meloidogyne chitwoodi* isolates collected in and around Kiledere town (Mc10, Mc11, Mc12, and Mc13) formed a group together. Geographically, Mc1 to Mc5 grouped closely because the towns of Alay and Tırhan are relatively close to each other. Gölcük area isolates showed the most diversity in *M. chitwoodi* populations. This town may have experienced multiple introductions of *M. chitwoodi*. In another report, *M. chitwoodi* and *M. fallax* populations were clustered and separated from the other *Meloidogyne* species and *M. chitwoodi* displayed several clusters with higher genetic diversity than *M. fallax* (Fargette *et al.*, 2005). The large amounts of genetic variability identified in the *M. chitwoodi* population indicates that multiple introductions may have occurred or the existence of numerous populations that have been genetically isolated over a long period of time. A more geographically comprehensive survey encompassing all major potato production areas in Turkey might reveal interesting patterns of *M. chitwoodi* diversity in other regions.

In conclusion, root-knot nematode samples collected from a potato production region in Turkey have been identified as *M. chitwoodi*. Morphology and molecular methods were used to support each other in confirming that *M. chitwoodi* exists in Niğde province and has been distributed throughout the province's potato production areas.

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