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Identification of Root-Knot Nematodes in the Mediterranean Region of Turkey by Using rDNA and mtDNA Markers

Zübeyir DEVRAN
Citrus and Greenhouse Crops Research Institute, Antalya - TURKEY
Uğur GÖZEL, M. Ali SÖĞÜT, Şenol YILDIZ, İ. Halil ELEKÇİOĞLU
Çukurova University, Agriculture Faculty, Plant Protection Department, Adana - TURKEY

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Abstract: Fragments containing the internal transcribed spacer (ITS) ribosomal rDNA were amplified from *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla* by polymerase chain reaction (PCR). Only digestion of the ITS region with the enzyme RsaI significantly distinguished *M. hapla* from other species, while other enzymes, EcoRI and BamHI, were not effective in distinguishing these nematode species. PCR was also done with mitocondrial DNA (mtDNA) markers for species discrimination. The obtained amplification product of 600 bp was digested with HinfI restriction endonuclease allowing discrimination among the four root-knot species. mtDNA was found to be a useful tool for discriminating between the important *Meloidogyne* species in this study.

Key Words: Root-nematodes, rDNA, mtDNA, diagnostics

Türkiye'nin Akdeniz Bölgesindeki Kök Ur Nematodlarının rDNA ve mtDNA Markerleri ile Tanımlanması

Özet: *Meloidogyne incognita*, *M. javanica*, *M. arenaria* ve *M. hapla*'nın rDNA'sının ITS bölgesini içeren fragmentler PCR ile çoğaltılmıştır. ITS bölgesinin RsaI enzimi ile kesimi sonucu *M. hapla* diğer türlerden ayrılmıştır. EcoRI ve BamHI enzimlerinin bu türleri ayırmada etkisiz olduğu belirlenmiştir. Bu nedenle diğer türleri ayırmada mtDNA markerleri kullanılmıştır. PCR sonucu elde edilen 600 bp'lık fragment HinfI restriction enzimi ile kesilerek türler birbirlerinden ayrılmıştır. Bu çalışmada mtDNA'nın önemli *Meloidogyne* türlerini ayırmada faydalı bir araç olabileceği bulunmuştur.

Anahtar Sözcükler: Kök ur nematodları, rDNA, mtDNA, teşhis

Introduction

Turkey has a total of 21 million tons of vegetable production from 783,000 ha of land, and 28% of this amount is produced in the Mediterranean region (DİE, 1998). Root-knot nematodes belonging to the *Meloidogyne* genera are one of the most economically important plant pathogens (Siddiqi, 2000) especially in high value intensive crops. So far, over 80 species have been described, but more than 90% of the estimated damage worldwide is considered to be caused by four root-knot nematodes: *M. incognita* (Chitwood), *M. javanica* (Chitwood), *M. arenaria* (Chitwood), and *M. hapla* (Chitwood), (Siddiqi, 2000; Netscher and Sikora, 1990).

Due to the economic importance of root-knot nematodes in this region several studies have been conducted (Elekçioğlu and Uygun, 1994; Elekçioğlu et al., 1994). Söğüt and Elekçioğlu (2000 a,b) conducted comprehensive work on the morphological identification and host preferences of various populations of *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*.

Since several species of *Meloidogyne* are commonly found together, unambiguous identification to species level is essential for the successful management of these nematodes. Identification of species within this genus was initially based on morphological characteristics and host preferences (Eisenbeck et al., 1981); however, these features vary and are time consuming and difficult to observe (Cenis, 1993; Stanton et al., 1997). Thus, using molecular methods including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and amplified fragment length

polymorphism (AFLP) (with one or a combination of methods) have been reported in other locations in the world to provide clear discrimination at both inter and intraspecific variations of root-knot nematodes (Powers and Sandall, 1988; Peloquin et al., 1993; Hyman and Whipple, 1996; Powers et al., 1997; Stanton et al., 1997; Semblat et al., 1998; Whipple et al., 1998).

The rDNA gene family is a multigene family. In most eukaryotes, the 5' to 3' organization of the gene family is an external transcribed spacer (ETS), the gene 18 S, an internal transcribed spacer (ITS 1), the 5.8 S gene, ITS 2, 28 S gene and intergenic spacer (IGS). ITS, located between the repeating array of nuclear 18 S and 28 S ribosomal DNA genes, is a versatile genetic marker. The ITS region does not encode any product, permitting it to evolve at a faster rate than the ribosomal coding regions. The level of variation in this region makes it suitable for detecting genetic variation among genera, species and within species (Ziljstra et al., 1995). ITS have been used in construsting phylogenetic trees, estimating genetic population structures, evaluating population level evolutionary processes and determining taxonomic identity (Powers et al., 1997).

mtDNA polymorphism has been successfully employed as a marker for population and phylogenetic studies, in part due to its rapid evolution, maternal inheritance patterns, apparent absence of recombination and small genome (Powers and Sandall, 1988, Peloquin et al., 1993, Whipple et al., 1998, Hyman and Whipple, 1996, Stanton et al., 1997).

The objective of this study was to show the usefulness, sensitivity and reliability of using rDNA-RFLP and mtDNA-RFLP to distinguish between root-knot nematode species *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* collected from different locations in the İçel and Antalya provinces of Turkey. The potential of these techniques was also discussed.

Materials and Methods

Nematodes and DNA Extraction

Isolates of *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* were collected from vegetable growing areas in the Mediterranean region in 2001. Egg masses were collected from infected vegetable roots. DNA was extracted from egg masses as described by Cenis (1993).

rDNA amplification and digestion of the amplified product

The DNA fragments containing the ITS regions were amplified by PCR using the ITS-specific primers described by Vrain et al. (1992). The reaction mixture contained 20 ng DNA, 2 mM $MgCl_2$, 200 µM dNTP (Bioline, UK), reaction buffer, 0.4 µM of each primers, 1 unit of Taq DNA polymerase (Bioline) and deionized water to a volume of 25 µl. The amplification was carried out in a DNA Engine Dyad Peltier Thermal Cycler (MJ. Research, U.S.A). PCR amplification conditions were as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, repeated for 35 cycles. Seven minutes of incubation 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 on 1% agarose gels and the products visualized with UV illumination after ethidium bromide (0.5 µg/ml) binding to the DNA fragments (Fig. 1).

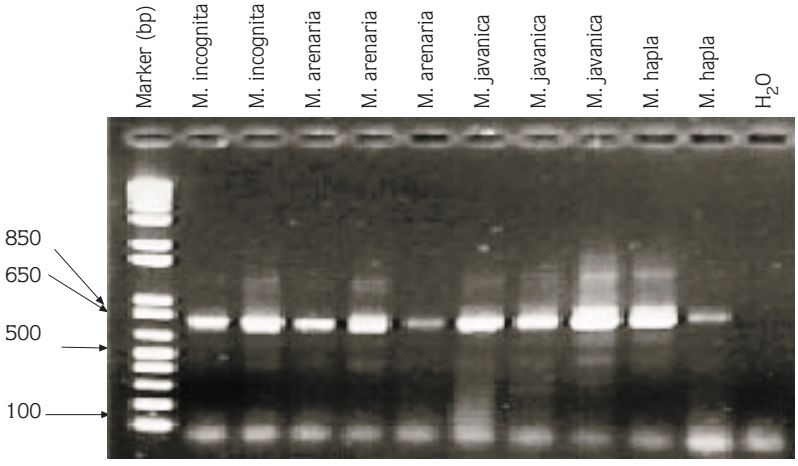

Figure 1. Amplified ITS region of *Meloidogyne* spp 18 S and 28 S primers.

The amplified ITS regions of all species were digested with one of the following restriction enzymes: EcoRI, BamHI and RsaI. The digested DNA was loaded on 2.5% agarose gel, separated by electrophoresis, and detected by ethidium bromide staining (Fig. 2).

mtDNA amplification and digestion of the amplified product

The primers used are described by Stanton et al. (1997). PCR was performed in 25 µl of reaction mix containing about 20 ng of template DNA, 2 mM $MgCl_2$, 200 µM dNTP (Bioline, U.K.), 1 unit of Taq DNA polymerase (Bioline, U.K.), 0.4 µM of each primers, and Reaction Buffer. Amplifications were preceded by a 3 min denaturation at 94 °C followed by 30 cycles of 30 min at 94 °C, 30 min at 50 °C and 1 min at 72 °C. Agarose gel electrophoresis was carried out in TAE. DNA was visualized with ethidium bromide and UV transillumination (Fig. 3).

The amplified product was digested with HinfI for 4 h at 37 °C. DNA fragments were separated on a 2.5% agarose gel and stained with ethidium bromide (Fig. 4).

Results

When the primers 18S and 28S were used for amplification of the ITS region, every isolate from the four *Meloidogyne* species tested gave one major product of approximately 700 bp (Fig. 1). RsaI digestion of the approximately 700 bp amplification product produced a characteristic banded pattern in *M. hapla*, versus no band in *M. incognita*, *M. arenaria* and *M. javanica*. After digestion with EcoRI and BamHI, no restriction

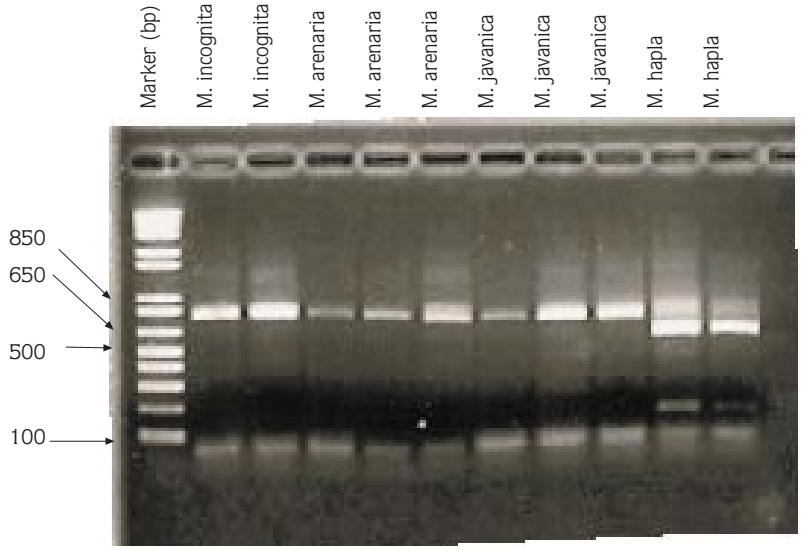

Figure 2. Digestion of amplified ITS region of *Meloidogyne* spp by RsaI.

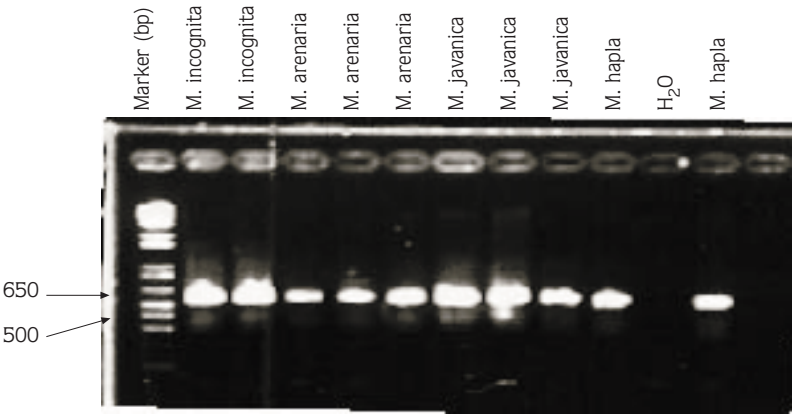

Figure 3. Amplification of mtDNA region of *Meloidogyne* spp by mtDNA primers.

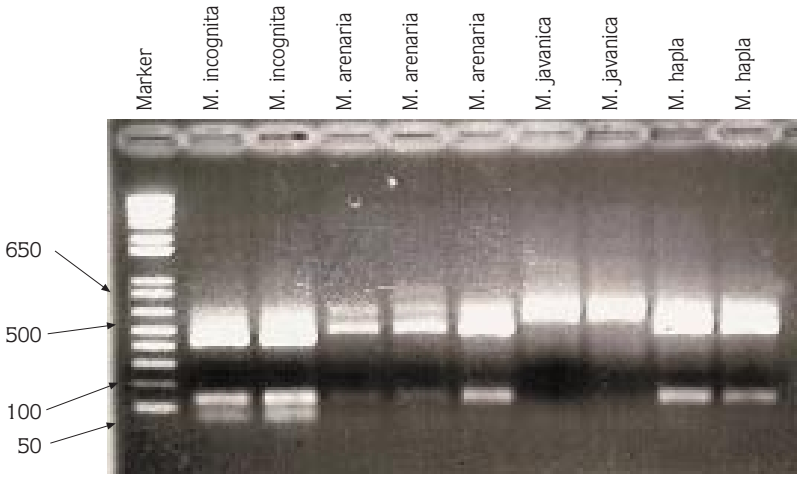

Figure 4. Digestion of amplified mtDNA region of *Meloidogyne* spp by Hinf I.

polymorphisms were found between *M. incognita*, *M. arenaria* and *M. javanica*. The *M. hapla* population was undigested by EcoRI and BamHI.

Amplification with mtDNA species primers produced fragments of approximately 600 bp (Fig. 3). To differentiate between the four *Meloidogyne* species, amplification products were digested with HinfI and produced fragments of approximately 400, 150 and 50 bp for *M. incognita*; 450 bp and 150 bp for *M. arenaria*; 600 bp for *M. javanica* and 450 and 150 bp for *M. hapla* (Fig. 4).

Discussion

Comparative analysis of coding and noncoding regions of ribosomal DNA is becoming a popular tool for the species and subspecies identification of many organisms (Zijlstra et al., 1995; Powers et al., 1997). rDNA has the advantage of being repetitive. The eukaryotic rDNA repeat consists of three genes (18S, 28S and 5.8S), internal and external transcribed spacers and an external nontranscribed spacer region. The sequences of the rDNA genes are highly conserved, whereas there is less conservation within the internal transcribed spacer (ITS) regions and little homology is found in the nontranscribed spacer regions (Zijlstra et al., 1995). The more conserved sequences are most useful for classification of higher taxonomic levels (genus to phylum), whereas the ITS sequences are useful at species and subspecies levels (Hyman and Powers, 1991; Peloquin et al., 1993).

In our study, the *Meloidogyne* species tested with 18S and 28S ITS primers gave one major product of approximately 700 bp (Fig. 1). The RsaI restriction pattern of the ITS region from *M. hapla* contained bands of approximately 550 bp and 150 bp, whereas the same enzyme pattern of three species always showed an additional strong band of 700 bp (Fig. 2). According to Zijlstra et al. (1995), the ITS-RFLP technique can be used to identify *M. hapla*, *M. chitwoodi* and *M. fallax* but *M. incognita* and *M. javanica* were not distinguished from each other by RsaI. In this study, EcoRI and BamHI restriction patterns did not clearly separate the species. Furthermore, Xue et al. (1993) found that digestion of the ITS fragment of *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* with EcoRI and HindIII did not reveal differences between the species.

The mtDNA fragments were amplified by mtDNA primers. Restriction digestion of the 600 bp amplified product with HinfI produced diagnostic patterns for all *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* which were separated, resulted with as the same restriction patterns by HinfI. Powers and Harris (1993) reported a test to differentiate among five mtDNA haplotypes from the species *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. chitwoodi*. In other studies it has been found that mitochondrial DNA may be useful for species identification (Powers and Sandall, 1988; Peloquin et al., 1993; Stanton et al., 1997, Whipple et al., 1998). However, mtDNA is important to consider some theoretical concerns about the relationship of a maternally inherited genome and species boundaries

(Powers and Sandall, 1988, Whipple et al., 1998). In conclusion, ITS and mtDNA markers can be used widely in routine diagnostic tests to identify species. However, the other molecular markers (RAPD, AFLP) allow clear discrimination of intraspecific variation in *Meloidogyne* spp.

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