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Molecular Biological Diagnosis of *Meloidogyne* Species Occurring in Korea

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Root-knot nematode species, such as *Meloidogyne hapla*, *M. incognita*, *M. arenaria*, and *M. javanica* are the most economically notorious nematode pests, causing serious damage to a variety of crops throughout the world. In this study, DNA sequence analyses were performed on the D3 expansion segment of the 28S gene in the ribosomal DNA in an effort to characterize genetic variations in the three *Meloidogyne* species obtained from Korea and four species from the United States. Further, PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism), SCAR (Sequence Characterized Amplified Region) PCR and RAPD (Randomly Amplified Polymorphic DNA) were also utilized to develop methods for the accurate and rapid species identification of the root-knot nematode species. In the sequence analysis of the D3 expansion segment, only a few nucleotide sequence variations were detected among *M. incognita*, *M. arenaria*, and *M, javanica*, but not *M. hapla*. As a result of our haplotype analysis, haplotype 5 was shown to be common in *M. arenaria*, *M. incognita*, *M. javanica*, but not in the facultatively parthenogenetic species, *M. hapla*. PCR-RFLP analysis involving the amplification of the mitochondrial COII and large ribosomal RNA (lrRNA) regions yielded one distinct amplicon for *M. hapla* at 500 bp, thereby enabling us to distinguish *M. hapla* from *M. incognita*, *M. arenaria*, and *M. javanica* reproduced via obligate mitotic parthenogenesis. SCAR markers were used to successfully identify the four tested root-knot nematode species. Furthermore, newly attempted RAPD primers for some available root-knot nematodes also provided some species-specific amplification patterns that could also be used to distinguish among root-knot nematode species for quarantine purposes.

Keywords : PCR-RFLP, RAPD, Root-knot nematodes, SCAR PCR, 28S rDNA sequence

The genus *Meloidogyne* Goeldi, 1887 encompasses more than 80 described species (Karssen and Van Hoenselaar, 1998), among which *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* inflict severe damage to economically important agricultural crops. These species are reproduced via parthenogenesis and are the most broadly distributed root-knot nematodes (RKN), and are found from tropical to temperate regions.

Currently, in Korea, *M. arenaria*, *M. hapla*, and *M. incognita* are distributed broadly among a wide range of hosts, including melons, Korean leeks, ginseng, medical herbs, yams and sweet potatoes, but *M. javanica* is quite scarce (Cho et al., 2006; Park et al., 1994). In a previous study, Cho et al. (2006) surveyed 11 major potato-growing areas and identified root-knot nematodes as a dominant species. Repeated cultivation is a principal component in the economic damage inflicted by root-knot nematodes. Application of resistant varieties, crop rotation, and enemy plants are the most effective control strategies in crop growing areas containing contaminated *Meloidogyne* species, because they result in less negative environmental effects and do not require chemical control.

In Korea, several *Meloidogyne* species, including *M. mayaguensis*, *M. minor*, and *M. fallax* have been designated as quarantine nematodes. They are endoparasites in the root, and thus are not readily detectable even under close examination. It is therefore essential that an accurate and reliable diagnostic method for the detection of root-knot nematodes for quarantine purposes and subsequent control strategies be developed. The traditional identification of root-knot nematodes generally relies not only on close observation of three important taxonomic criteria, including the perineal patterns of females, male head shape, and stylet morphology, but also relies on different host tests (Hartman and Sasser, 1985) and enzyme phenotypes (Cho et al., 2000). However, these diagnostic methods suffer from some technical limitations, including the necessity of experienced taxonomists, applicability of limited life stage, and time requirements.

Recently, a PCR technique involving the sequence analysis of ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA) has become increasingly popular, as this method is applicable to all life stages. The comparative sequence

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analysis of rRNA, however, is also somewhat limited as the result of sequence homology among *Meloidogyne* species (Powers et al., 1997; Zijlstra et al., 1995). Powers and Harris (1993) established a method for the diagnosis of four *Meloidogyne* species via PCR-RFLP analysis using the cytochrome oxidase subunit II (COII) gene and the 16S ribosomal RNA (lrRNA) gene in the mtDNA. According to the results of a previous study, Han et al. (2004) applied this method to the differentiation of three *Meloidogyne* species occurring in Korea and determined that variations in the size of the COII/LrRNA region can be used to readily distinguish between two obligatory mitotic parthenogenesis species, such as *M. arenaria* and *M. incognita* from *M. hapla* which reproduce via facultative meiotic parthenogenesis. The application of genetic markers such as nuclear DNA or mtDNA to distinguish root-knot nematodes requires a consideration of the unique evolutionary history of the mitotic root-knot nematode lineages; as a result, no high clonal diversity is expected within individuals of the species (Castagnone-Sereno, 2006). However, Hugall et al. (1999) demonstrated a high level of sequence diversity among populations via detailed sequence analyses of the ITS region among root-knot nematode species. He ascribed these findings to reticulate evolution from closely related amphimictic species. Chen et al. (2003) evaluated nucleotide substitution patterning within the D3 region of the 28S rDNA of *Meloidogynes,* and determined that the three obligatory mitotic parthenogenesis species evidence the same D3 haplotype (haploid genotype), suggesting recent divergence among them.

Thus, molecular diagnostics techniques using additional molecular markers are required in order to overcome the microheterogenity existing within the genomes of three apomictic species. Random amplified polymorphic DNA (RAPD) PCR successfully revealed genetic diversity between closely related species, even though this technique has drawbacks, most notably in reproducibility. Some of the sequence information acquired from amplified RAPD fragments is specific to *Meloidogyne* species, and was thus employed in the development of species-specific primers for SCAR that provide reliable and rapid diagnostic tools for the identification of target *Meloidogyne* species (Randing et al., 2002; Zijlstra et al., 2000). In the present study, four different molecular approaches, namely comparative sequence analysis, SCAR, PCR-RFLP, and RAPD were applied to identify the four *Meloidogyne* species.

Materials and Methods

Samples. Adult females of *M. arenaria*, *M. hapla*, and *M. incognita* were removed from contaminated roots using a dissecting microscope. Also, second juveniles (J2) or DNA of *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* were acquired from Professor Agudelo Paula, USA in 2007 and 2009 with the permission of the National Plant Quarantine Office. The species name and geographical origin of the nematode populations utilized in this study are shown in Table 1.

DNA extraction. Total genomic DNA was extracted from adult female or individual J2. Each individual was hand-picked and moved to a 0.5 ml microcentrifuge tube with 0.5 μl of RNA-free water. DNA was extracted with a RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich Co., St. Louis, MO). Remaining individuals of the hand-picked samples were placed in 0.5 ml microcentrifuge tubes with 1 M NaCl and stored at −80°C until use.

28S rDNA PCR, cloning and sequencing. The primer utilized for the amplification of the 28S rDNA region was LSUD-1f, 5'-ACCCGCTGAACTTAAGCATAT-3' for forward primer and LSUD-2r, 5'-TTTCGCCCCTATACCCA-

Table 1. Populations and haplotypes of *Meloidogyne* isolates used in this study

Species	Origin	Haplotype[a] (Chen et al)	GenBank Accession number	Collector
M. arenaria	Clemson, SC, USA	5, MaUs5-1	GQ227797-GQ227801 GQ227804-GQ227806	P. Agudelo
M. arenaria	Seongju, Korea	5	GQ227803, GQ258784 GQ258786-GQ258789	C. H. Bae
M. incognita	Clemson, SC, USA	5, 6	GQ229425-GQ229430 GQ229423-GQ229424	P. Agudelo
M. incognita	Suwon, Korea	5, MiKo5-1	GQ229418-GQ229420, GQ229422, GQ229421	C. H. Bae
M. hapla	Clemson, SC, USA	1, MhUs1-2	GQ227807, GQ227810, GQ227812	P. Agudelo
M. hapla	Canada	1, MhCa1-1, MhCa1-3	GQ227808, GQ227809, GQ227811, GQ227813	P. Agudelo
M. hapla	Naju, Korea	1	GQ258790, GQ258791	K. C. Ma
M. javanica	Clemson, SC, USA	5	GQ229431-GQ229433	P. Agudelo

[a]Haplotype analysis quote from Chen et al. (2003). Haplotype 1; AY335815, Haplotype 5; AY355413, Haplotype 6; AY355414.

AGTC-3' for reverse primer (Bae et al., 2008). The PCR reaction was conducted in a total volume of 20 μl, each containing 1 μl of primer, 0.2 μl of 25 mM $MgCl_2$, 17.8 μl of distilled water, and 1 μl of template DNA. After an initial 3-min denaturation step at 95°C, a 35-cycle amplification (95°C for 45 s, 51-54°C for 1 min 30 s, and 72°C for 2 min) was conducted. The final extension step was continued for 10 min at 72°C. In order to confirm the successful amplification of DNA by PCR, electrophoresis was performed using 1× TAE buffer on 1% agarose gel. The PCR product was subsequently purified with a PCR Purification Kit (Bioneer, Korea). The amplicons were cloned in pGEM-T Easy vector (Promega), and the resultant plasmid DNA was isolated with a Wizard Plus SV Minipreps DNA Purification System (Promega). Both strands of the PCR amplicons were cycle-sequenced with an ABI PRISM® BigDye® Terminator version 1.1 Cycle Sequencing Kit and electrophoresed in each direction on an ABI Prism ABI 377 Genetic Analyzer (PE Applied Biosystems, USA). Sequence alignment was performed using CLUSTAL X software (ver. 1.8; Thompson et al., 1997).

PCR-RFLP. In order to identify the root-knot nematodes, we conducted PCR-RFLP and subsequent digestion of the amplicon with restriction enzyme. The primers used for PCR-RFLP analysis were designed according to the method described by Powers and Harris (1993), which amplifies the region between the COII gene and the lrRNA gene of the mtDNA. 20 μl of the PCR reaction contained 1 μl of primer, 0.5 μl of 25 mM $MgCl_2$, 17.5 μl of distilled water, and 1 μl of template DNA. After an initial 5-minute denaturation step at 95°C, a 35-cycle amplification (95°C for 1 min, 46-52°C for 2 min, and 72°C for 3 min) was performed. The final extension step was continued for 10 min at 72°C. In order to confirm the successful amplification of DNA by PCR, electrophoresis was conducted using 1× TAE buffer on 1% agarose gel. The amplified products were digested separately with the *Hinf*I restriction enzyme. Digestion was performed in a mixture of 10 μl of the PCR product, 1 μl of the enzyme, 2 μl of the 10X buffer, and 7 μl of the distilled water for 2 hrs at 37°C. In order to confirm the successful digestion of the PCR products, electrophoresis was conducted using 1× TAE buffer on 1.4% agarose gel.

SCAR PCR. In order to acquire species-specific amplicons from *M. hapla*, *M. incognita*, *M, arenaria*, and *M. javanica*, SCAR PCR was employed, in accordance with the methods described by Zijlstra et al. (2000). The four primer sets used for species-specific amplification were: Fh, 5'-TGACGGCGGTGAGTGCGA-3' and Rh, 5'-TGACGGCGGTACCTCATAG-3' for *M. hapla*; Finc, 5'-CTCTGCCCAATGAGCTGTCC-3' and Rinc, 5'-CTCTGCCCTCACATTAAG-3' for *M. incognita*; Far, 5'-TCGGCGATAGAGGTAAATGAC-3' and Rar, 5'-TCGGCGATAGACACTACAACT-3' for *M. arenaria*; and Fjav, 5'-GGTGCGCGATTGAACTGAGC-3' and Rjav, 5'-CAGGCCCTTCAGTGGAACTATAC-3' for *M. javanica*. 20 μl of the PCR reaction contained the

Table 2. Operon primer lists used for RAPD

Primer	Sequence	Primer	Sequence	Primer	Sequence
OPA-01	5'-CAGGCCCTTC-3'	OPB-01	5'-GTTTCGCTCC-3'	OPC-01	5'-TTCGAGCCAG-3'
OPA-02	5'-TGCCGAGCTG-3'	OPB-02	5'-TGATCCCTGG-3'	OPC-02	5'-GTGAGGCGTC-3'
OPA-03	5'-AGTCAGCCAC-3'	OPB-03	5'-CATCCCCCTG-3'	OPC-03	5'-GGGGGTCTTT-3'
OPA-04	5'-AATCGGGCTG-3'	OPB-04	5'-GGACTGGAGT-3'	OPC-04	5'-CCGCATCTAC-3'
OPA-05	5'-AGGGGTCTTG-3'	OPB-05	5'-TGCGCCCTTC-3'	OPC-05	5'-GATGACCGCC-3'
OPA-06	5'-GGTCCCTGAC-3'	OPB-06	5'-TGCTCTGCCC-3'	OPC-06	5'-GAACGGACTC-3'
OPA-07	5'-GAAACGGGTG-3'	OPB-07	5'-GGTGACGCAG-3'	OPC-07	5'-GTCCCGACGA-3'
OPA-08	5'-GTGACGTAGG-3'	OPB-08	5'-GTCCACACGG-3'	OPC-08	5'-TGGACCGGTG-3'
OPA-09	5'-GGGTAACGCC-3'	OPB-09	5'-TGGGGGACTC-3'	OPC-09	5'-CTCACCGTCC-3'
OPA-10	5'-GTGATCGCAG-3'	OPB-10	5'-CTGCTGGGAC-3'	OPC-10	5'-TGTCTGGGTG-3'
OPA-11	5'-CAATCGCCGT-3'	OPB-11	5'-GTAGACCCGT-3'	OPC-11	5'-AAAGCTGCGG-3'
OPA-12	5'-TCGGCGATAG-3'	OPB-12	5'-CCTTGACGCA-3'	OPC-12	5'-TGTCATCCCC-3'
OPA-13	5'-CAGCACCCAC-3'	OPB-13	5'-TTCCCCCGCT-3'	OPC-13	5'-AAGCCTCGTC-3'
OPA-14	5'-TCTGTGCTGG-3'	OPB-14	5'-TCCGCTCTGG-3'	OPC-14	5'-TGCGTGCTTG-3'
OPA-15	5'-TTCCGAACCC-3'	OPB-15	5'-GGAGGGTGTT-3'	OPC-15	5'-GACGGATCAG-3'
OPA-16	5'-AGCCAGCGAA-3'	OPB-16	5'-TTTGCCCGGA-3'	OPC-16	5'-CACACTCCAG-3'
OPA-17	5'-GACCGCTTGT-3'	OPB-17	5'-AGGGAACGAG-3'	OPC-17	5'-TTCCCCCCAG-3'
OPA-18	5'-AGGTGACCGT-3'	OPB-18	5'-CCACAGCAGT-3'	OPC-18	5'-TGAGTGGGTG-3'
OPA-19	5'-CAAACGTCGG-3'	OPB-19	5'-ACCCCCGAAG-3'	OPC-19	5'-GTTGCCAGCC-3'
OPA-20	5'-GTTGCGATCC-3'	OPB-20	5'-GGACCCTTAC-3'	OPC-20	5'-ACTTCGCCAC-3'

following: 1 μl of primer, 0.5 μl of 25 mM $MgCl_2$, 17.5 μl of distilled water, and 1 μl of template DNA. After an initial 5-minute denaturation step at 94°C, a 45-cycle amplification (94°C for 30 s, 54-64°C for 30 s, and 72°C for 1 min) was conducted. The final extension step was continued for 7 min at 72°C. In order to confirm the successful PCR amplification of DNA, electrophoresis was conducted using 1× TAE buffer on 1% agarose gel.

RAPD PCR. In order to establish new markers for the discrimination of the four root-knot nematodes, 60 oligonucleotide decamer primers were purchased (Table 2; Bioneer, Korea). 20 μl of the PCR reaction mixture contained: 1 μl of primer, 0.5 μl of 25 mM $MgCl_2$, 17.5 μl of distilled water, and 1 μl of template DNA. After an initial 2-minute denaturation step at 94°C, a 45-cycle amplification (94°C for 1 min, 44°C for 1 min, and 72°C for 2 min) was conducted. The final extension step was continued for 10 min at 72°C. In order to confirm the successful PCR amplification of DNA, electrophoresis was conducted using 1× TAE buffer on 1.4% agarose gel for approximately 2 hrs at 100 volts at Agaro-Power™ (Bioneer, Korea) and the products were visualized via UV illumination after ethidium bromide staining.

Results

Sequence analysis of the D3 expansion segment of 28S rDNA. The sizes of the amplified PCR products were all approximately 1.04 kb with a single amplicon, reflecting no variation in size among species (Fig. 1). Using a BLAST sequence similarity search, the 255 bp region of the D3 expansion region was determined and compared with previous haplotype analysis (Chen et al., 2003) (Table 1).

Sequences obtained from six randomly selected clones in the Korean *M. arenaria* isolates consisted of only one haplotype (haplotype 5), whereas 8 sequences obtained from the USA *M. arenaria* isolates were found to be composed of haplotype 5 (5 clones) and a new haplotype (haplotype MaUs5-1; 3 clones), which features three changes from haplotype 5: a T to C change at position 42, a G to A change at position 66, and a C to T change at position 133. Five sequences from the Korean *M. incognita* isolates were comprised of common haplotype 5 (4 clones) and a new haplotype (haplotype MiKo5-1; 1 clone), which differs by one base from haplotype 5: specifically, a T to G change at position 216; 8 sequences from the USA *M. incognita* isolates were found to consist of haplotype 5 (6 clones) and haplotype 6 (2 clones). Three sequences of the USA *M. javanica* isolates were composed of haplotype 5 (3 clones). Two sequences of the Korean *M. hapla* isolates consisted of haplotype 1. Seven sequences of the USA and Canadian *M. hapla* isolates were found to consist of haplotype 1 (4 clones) and 3 new haplotypes (haplotype MhCa1-1, MhUs1-2, MhCa1-3), each of which differs by one base from haplotype 1: specifically, an A to G change at position 52, an A to T change at position 102, and a G to A change at position 99.

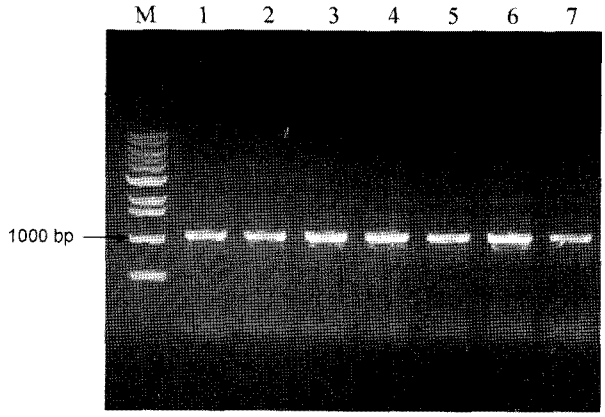

Fig. 1. Amplified PCR products of the combined D1-D3 expansion of the 28S rDNA regions from four *Meloidogyne* species. Lane 1 for *M. arenaria* isolate of Korea, lane 2 for *M. arenaria* isolate of USA, lane 3 for *M. incognita* isolate of Korea, lane 4 for *M. incognita* isolate of USA, lane 5 for *M. hapla* isolate of Korea, lane 6 for *M. hapla* isolate of USA, and lane 7 for *M. javanica* isolate of USA. M, molecular marker (100 bp).

PCR amplification of the COII/LrRNA region. In order to determine whether or not the tested nematodes were accurately identified as the target species, the COII/lrRNA region of mtDNA was amplified (Figs. 2, 3, 4) (Table 3). Different annealing temperatures were applied to the PCR reaction, resulting in differences in band intensities among them. For *M. incognita* and *M. arenaria*, the size of the amplified PCR product was approximately 1.7 kb in the

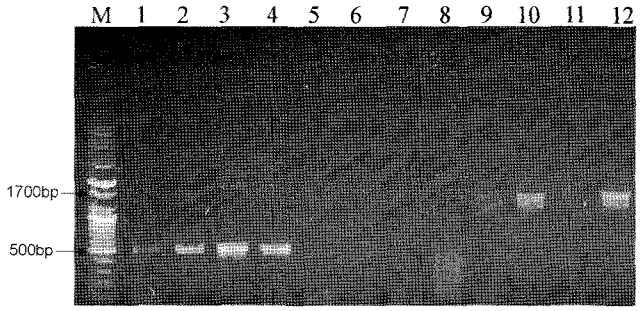

Fig. 2. Amplified PCR products of COII/lrRNA region of the mtDNA from *Meloidogyne hapla* isolates of Korea and USA, and the *M. incognita* isolates of Korea according to different annealing temperatures. Lane 1, 59.6°C; 2, 59.9°C; 3, 60.1°C; and 4, 60.4°C for *M. hapla* isolate of Korea. Lane 5, 59.6°C; 6, 59.9°C; 7, 60.1°C; and 8, 60.4°C for *M. hapla* isolate of USA. Lane 9, 53.6°C; 10, 53.9°C; 11, 54.1°C; and 12, 54.4°C for *M. incognita* isolate of Korea. M, molecular marker (100 bp).

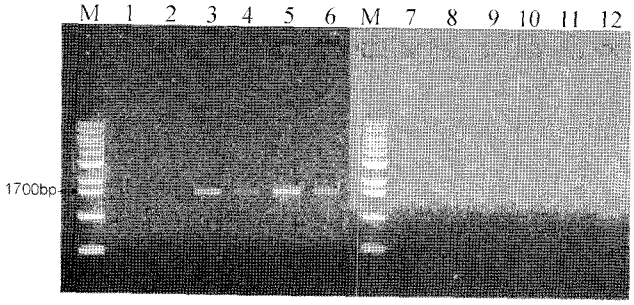

Fig. 3. Amplified PCR products of COII/lrRNA region of mtDNA from *Meloidogyne incognita* isolates of USA and *M. javanica* isolate of USA according to different annealing temperatures. Lane 1, 53.3°C; 2, 53.6°C; 3, 53.9°C; 4, 54.1°C; 5, 54.4°C; and 6, 54.7°C for *M. incognita* isolates of USA. Lane 7, 63.3°C; 8, 63.6°C; 9, 63.9°C; 10, 64.1°C; 11, 64.4°C; and 12, 64.7°C for *M. javanica* isolate of USA. M, molecular marker (100 bp).

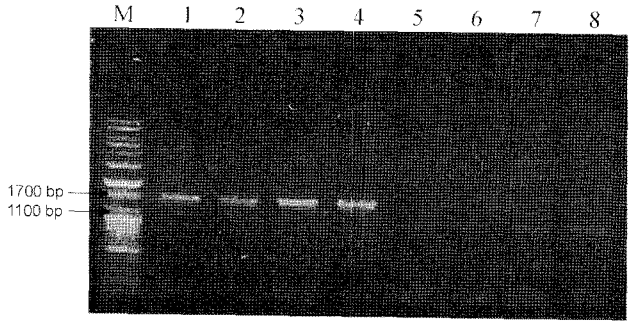

Fig. 4. Amplified PCR products of COII/lrRNA region of mtDNA from *Meloidogyne arenaria* isolates of Korea and *M. arenaria* isolate of USA according to different annealing temperatures. Lane 1, 61.6°C; 2, 61.9°C; 3, 62.1°C; and 4, 62.4°C for *M. arenaria* isolates of Korea. Lane 5, 61.6°C; 6, 61.9°C; 7, 62.1°C; and 8, 62.4°C for *M. arenaria* isolate of USA. M, molecular marker (100 bp).

Korean isolates (lanes 9-12 in Fig. 2 and lanes 1-4 in Fig. 4, respectively), and was 1.7 kb for the USA *M. javanica* isolate (lanes 7-12 in Fig. 3) and 540 bp for the Korean and USA *M. hapla* isolates (lanes 1-8 in Fig. 2). In the case of the USA *M. arenaria* isolate, however, the size of the PCR product differed from that of the Korean isolate, as 1.1 kb (lanes 5-8 in Fig. 4). Thus, a large variation in size was detected from two different *M. arenaria* populations (1.7 kb in Korean isolate vs 1.1 kb USA isolate). Subsequent sequence analysis of the isolates revealed a large deletion in the intergenic region of the COII/LrRNA genes of mtDNA in the USA isolates (GenBank accession numbers, GQ266685 and GQ266686).

Table 3. PCR amplification of the COII/lrRNA region of mitochondrial DNA

Species	Origination	Expected size	Size of PCR fragment
Meloidogyne hapla	Korea	500 bp	500 bp
Meloidogyne hapla	USA	500 bp	500 bp
Meloidogyne incognita	Korea	1700 bp	1700 bp
Meloidogyne incognita	USA	1700 bp	1700 bp
Meloidogyne javanica	USA	1700 bp	1700 bp
Meloidogyne arenaria	USA	1100 bp	1100 bp
Meloidogyne arenaria	Korea	1700 bp	1700 bp

PCR-RFLP analysis was conducted in order to differentiate among *M. incognita*, *M. arenaria*, and *M. javanica*, and the expected sizes of the fragments digested with two restriction enzymes are provided in Table 4. The restriction enzyme digestion of *Hinf*I generated three different fragment length patterns. First, the Korean and USA *M. incognita* isolates evidenced fragments of 1,300 bp and 400 bp. Second, *M. arenaria* from Korea evidenced no digestion, resulting in a fragment of 1,700 bp (Fig. 5). We remain uncertain about the *M. arenaria* isolates from the USA, as nothing could be accomplished due to the limited sample. The original PCR-RFLP study of *M. javanica* showed that the species would evidence fragments of 11,000 bp and 700 bp (Powers and Harris, 1993). Nevertheless, it was determined that *M. javanica* from USA evidenced no digestion, exhibiting a fragment of 1,700 bp (Fig. 5). Third, the *M. hapla* PCR products from the Korean and USA isolates digested with *Dra*I were 270 bp and 230 bp in size (Fig. 5).

SCAR primers for species identification of *Meloidogyne* spp. In order to select primer pairs appropriate for the

Table 4. Restriction enzyme digestion patterns of *Meloidogyne* species for COII/lrRNA region

Species	Origination	Enzyme	Expected size	Fragment
Meloidogyne hapla	Korea	*Dra*-I	230 bp, 270 bp	230 bp, 270 bp
Meloidogyne hapla	USA	*Dra*-I	230 bp, 270 bp	230 bp, 270 bp
Meloidogyne incognita	Korea	*Hinf*-I	1300 bp, 400 bp	1300 bp, 400 bp
Meloidogyne incognita	USA	*Hinf*-I	1300 bp, 400 bp	1300 bp, 400 bp
Meloidogyne arenaria	Korea	*Hinf*-I	1,700 bp	1,700 bp
Meloidogyne arenaria	USA	*Hinf*-I	1,100 bp	N/A*
Meloidogyne javanica	USA	*Hinf*-I	1000 bp, 700 bp	1,700 bp

*Not applicable due to limited samples.

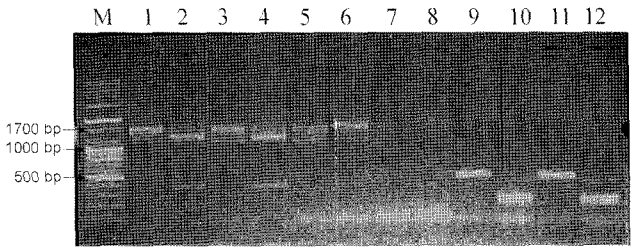

Fig. 5. Restriction enzyme digestion of COII/lrRNA region of *M. incognita*, *M. arenaria*, *M. hapla* using *Hinf*I and *Dra*I. Lanes 1 and 3, PCR product of *M. incognita* isolates of Korea and USA, respectively; lanes 2 and 4, PCR-RFLP (*Hinf*I) of *M. incognita* isolates of Korea and USA, respectively; lane 5, PCR product of *M. arenaria* isolate of Korea; lane 6, PCR-RFLP (*Hinf*I) of *M. arenaria* isolate Korea; lane 7, PCR product of *M. javanica* isolate of USA; lane 8, PCR-RFLP (*Hinf*I) of *M. javanica* isolate of USA; lanes 9 and 11, PCR product of *M. hapla* isolates of Korea and USA; lanes 10 and 12, PCR-RFLP (*Dra*TI) of *M. hapla* isolates of Korea and USA, respectively; M, molecular marker (100 bp).

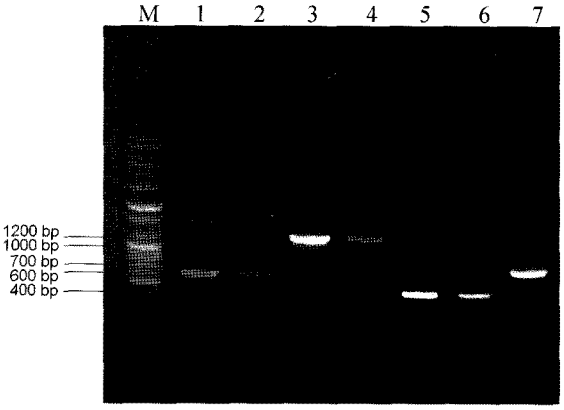

Fig. 6. Amplification products of *Meloidogyne* spp. isolates using the species-specific SCAR primers. Lanes 1 and 2, *M. hapla* isolates from Korea and USA, respectively, amplified using primer sets Fh and Rh; lanes 3 and 4, *M. incognita* isolates from Korea and USA, respectively, amplified using primer sets Finc and Rinc; lanes 5 and 6, *M. arenaria* isolates from Korea and USA, respectively, amplified using primer sets Far and Rar; and *M. javanica* isolate from USA amplified using primer sets Fjav and Rjav; M, molecular marker (100 bp).

amplification of DNA fragments specific to each of the root-knot nematodes, we initially tested multiple primer sets that had been developed in previous studies. Among them, the following four primer pairs successfully amplified the expected fragments from the respective species: Fh/Rh for *M. hapla*, Finc/Rinc for *M. incognita*, Far/Rar for *M. arenaria*, and Fjav/Rjav for *M. javanica*-all of these were previously developed by Zijlstra et al. (2000) (Fig. 6). For example, the *M. hapla* specific primers-Fh and Rh-yielded 600-bp long fragments when *M. hapla* isolates collected from both Korea and USA were used as temperate DNA. In the same manner, Finc and Rinc for *M. incognita*, Far and Rar for *M. arenaria*, and Fjav and Rjav for *M. javanica*-all amplified the expected fragments (Fig. 6 and Table 5). Excluding *M. javanica*, which lacks a Korean sample, all primer pairs amplified fragments of the same size, regardless of the origin of the root-knot nematodes (Fig. 6). No amplification was noted in the non-primer specific species when the primers were cross-tested (data not shown).

Table 5. Amplification products of PCR reactions using SCAR primers

Species	Origination	Expected size	Size of PCR fragment
Meloidogyne hapla	Korea	600 bp	600 bp
Meloidogyne hapla	USA	600 bp	600 bp
Meloidogyne incognita	Korea	1200 bp	1200 bp
Meloidogyne incognita	USA	1200 bp	1200 bp
Meloidogyne arenaria	Korea	420 bp	420 bp
Meloidogyne arenaria	USA	420 bp	420 bp
Meloidogyne javanica	USA	700 bp	700 bp

RAPD analysis. Owing to the limitations inherent to the acquisition of an *M. javanica* population in Korea and an *M. hapla* population in USA, RAPD analysis was only conducted for *M. javanica* from USA, *M. hapla* from Korea, *M. incognita* from both Korea and USA, and *M. arenaria* from both USA and Korea. Among the 60 RAPD primers we designed, 11 evidenced species-specific amplification. Among them, OPB-05, OPB-06, OPB-10, and OPB-12 yielded RAPD amplification products of 800 bp, 750 bp, 750 bp, and 650 bp, all of which were specific to the Korean *M. hapla* isolate (Table 6). The RAPD primer, OPB-07, yielded a product of 650 bp with the Korean *M. incognita*, and OPB-15 generated 1,100 bp products from both the Korean and USA isolates (Table 6). The RAPD

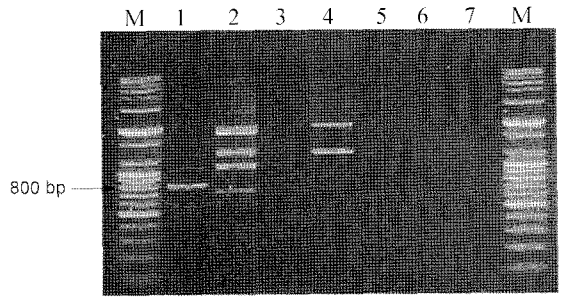

Fig. 7. RAPD patterns for *Meloidogyne* species isolates generated with primer OPB-05. Lane 1, *M. hapla* from Korea; lane 2, *M. incognita* from Korea; lane 3, *M. incognita* from USA; lane 4, *M. arenaria* from Korea; lane 5, *M. arenaria* from USA, lane 6, M. *javanica* from USA; and lane 7, *Aphelenchoides fragariae* from USA. The circle indicates the species-specific target fragment amplified using the RAPD primer.

Table 6. RAPD primers used for amplification of the species-specific products

Primer name	Species	Origination	Size of PCR fragment
OPB-05	*Meloidogyne hapla*	Korea	800 bp
OPB-06	*Meloidogyne hapla*	Korea	750 bp
OPB-10	*Meloidogyne hapla*	Korea	750 bp
OPB-12	*Meloidogyne hapla*	Korea	650 bp
OPB-07	*Meloidogyne incognita*	Korea	650 bp
OPB-15	*Meloidogyne incognita*	Korea, USA	1100 bp
OPA-07	*Meloidogyne arenaria*	Korea, USA	1400 bp
OPB-01	*Meloidogyne arenaria*	Korea, USA	900 bp
OPB-04	*Meloidogyne javanica*	USA	1800 bp
OPB-16	*Meloidogyne javanica*	USA	1300 bp
OPC-02	*Meloidogyne javanica*	USA	1400 bp

primers OPA-07 and OPB-01 amplified 1400 bp and 900 bp fragments, respectively, from the Korean and USA isolates. Finally, three RAPD primers-OPB-04, OPB-16, and OPC-02-generated 1800 bp, 1300 bp, and 1400 bp-long fragments specific to the USA *M. javanica* isolates (Table 6). For the sake of simplicity, the amplification pattern generated from the OPB-05 primer is shown in Fig. 7. Other gel pictures showing the amplification patterns generated by the remaining RAPD primers will be provided on request.

Discussion

The D expansion segments of 28S rDNA are frequently sequenced and compared in order to develop species-specific primers, and also in studies of nematode phylogeny due to the significant genetic diversity existing among closely related species and the existence of phylogenetically informative characteristics. Recently, the D2-D3 expansion segments have been recognized as a promising target region for the DNA barcoding of nematodes (De Ley et al., 2005). Chen et al. (2003) examined the D3 region to evaluate nucleotide variations and evolutionary relationships among the four root-knot nematodes, and determined that the meiotic parthenogenetic species, *M. hapla*, has a unique haplotype featuring a 3-bp insertion at position 204, which differentiates it from the other three mitotic parthenogenetic species. In the current study, *M. hapla* collected from San Francisco in the USA and from Canada also evidenced haplotype 1 and a new haplotype evidencing one base change from haplotype 1. *M. hapla* from Korea was also identified as haplotype 1. Sequence analysis of the Korean *M. arenaria* isolate evidenced only haplotype 5, but haplotypes 3, 4, and 6 were not detected. This is probably because haplotypes 3 and 4, which were located in specified areas in the USA exhibited the presence of new local haplotypes, or possibly because the sequence results we obtained from the Korean isolates did not fully reflect the actual genetic structures of the *M. arenaria* isolates owing to the limited numbers of populations and sequences. Nonetheless, we noted that three apomictic species harbored the common haplotype 5, which is associated with the cytological aspects of the species relationship in which apomictic RKN diverged from a meiotic lineage (Baum et al., 1994; Castagnone-Sereno et al., 1993; Chen et al., 2003). With the common haplotype, multiple haplotypes were noted in the genome of the root-knot nematodes, and these unique features may have stemmed from reticulate evolution, which is characterized by the occasional hybridization and combination of two species (Chen et al., 2003; Hugall et al., 1999). D3 sequence analysis of the *Globodera* species, however, was well conserved, and thus was not ideal for species diagnosis (Subbotin et al., 2000), whereas *Pratylenchus* species are differentiated, with substantial genetic diversity among species (Duncan et al., 1999). In the current study, we detected several new haplotypes that exhibited one base change as compared to the haplotypes reported by Chen et al. (2003). Therefore, additional tests involving PCR-RFLP should be conducted in order to determine whether these new haplotypes exist within each individual genome.

PCR-RFLP analysis of the COII/lrRNA region of mtDNA successfully discriminated important root-knot nematodes (Orui, 1998; Powers and Harris, 1993). Han et al. (2004) applied this diagnostic technique for the discrimination of three *Meloidogyne* species collected from Korea, and found some discrepancies with the USA isolates in the amplified PCR products with C2F3 and 1108 primer pairs and RFLP profiles in the COII/lrRNA region. In the case of *M. arenaria*, the size of the amplified PCR product in the COII/lrRNA region was 1.1 kb in the USA isolates and 1.7 kb in the Korean and Japanese isolates, whereas the Chinese isolates revealed both sizes of the PCR products, thereby indicating that the Chinese isolates have both genotypes, although the authors utilized the C2F3 and MRH106 primers, which produced products 130 bp longer than those in the previous studies (Jianhua et al., 2004; Powers and Harris, 1993). The size variability of PCR products among Chinese, Japanese, Korean, and USA *M. arenaria* isolates was suggestive of intraspecific variation. According to previous studies (Zheng et al., 2000), the cereal cyst nematode, *Heterodera avenae*, has two ITS types, of which genotype A is usually detected in European populations and genotype B in Indian populations. In the case of China, in addition to both types, a new genotype, genotype C, was detected. The amplification of new genotypes within the limited geographical area of the same

species was indicative of the emergence of a new local subtype (Cherry et al., 1997; Szalanski et al., 1997). We used *Meloidogyne arenaria* collected from a small number of areas, so the types obtained from this experiment were not all-inclusive of Korean isolates. Clearly, it will be necessary to evaluate larger populations in order to evaluate the genetic structure of *M. arenaria* in Korea. In our study, PCR-RFLP of *M. javanica* with *Hinf*I revealed results differing from those of a previous study (Power and Harris, 1993), and this might be related to the emergence of a new regional subtype.

The RAPD PCR technique was utilized to successfully differentiate among four important nematodes-*M. hapla*, *M. arenaria*, *M. incognita*, and *M. javanica*-and was recognized as a valuable DNA fingerprinting technique to evaluate genetic variations among different populations of root-knot nematodes (Blok et al., 1997; Randig et al., 2002). However, due to the typical reproducibility drawback of RAPD PCR, a new molecular diagnostic method, such as SCAR PCR, is required for the rapid and reliable identification of root-knot nematodes using species-specific primers derived from polymorphic RAPD sequences (El-Ghore et al., 2004; Randing et al., 2002; Zijlstra et al., 2000). This technique is particularly useful when one or more root-knot nematodes are present in the same soil sample. In this study, the RAPD primer, OPB-07, yielded a 650 bp product with *M. incognita* from the Korean isolates; thus, the SCAR primer designed using the sequence information of OPB-07 will enable us to distinguish Korean *M. incognita* isolates from *M. incognita* isolates found in USA. Further efforts will be required in order to generate favorable results in terms of the identification of root-knot nematodes.

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