Identification of Japanese species of the genus *Meloidogyne* (Nematoda: Meloidogynidae) by PCR-RFLP analysis

Yukio Orui

Leaf Tobacco Research Laboratory, Japan Tobacco Inc., Oyama, Tochigi 323-0808, Japan

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Abstract

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis for identification of ten *Meloidogyne* species from Japan was attempted on an individual second-stage juvenile. The PCR was performed with the primer set to amplify the region between the cytochrome oxidase subunit II gene and the large subunit of the 16S ribosomal RNA gene in mitochondrial DNA. A single fragment of about 1.7 kb was produced in *M. incognita*, *M. javanica* and *M. arenaria*. The sizes of amplified products differed in *M. hapla*, *M. suginamiensis*, *M. marylandi*, *M. mali*, *M. camelliae*, *M. sp. near mali* and *M. sp. near arenaria*. The fragment size of each product ranged from 0.5 kb to 0.6 kb. *Dra*I, *Mse*I, *Ssp*I and *Vsp*I produced digested fragments in all ten *Meloidogyne* species. Though a *Ssp*I or *Vsp*I+*Hinf*I-digested pattern separated the ten *Meloidogyne* species into nine groups, we were unable to distinguish *M. arenaria* from *M. javanica* using these patterns. *Mse*I-digested pattern alone distinguished *M. arenaria* from *M. javanica*. *Mse*I and either *Ssp*I or *Vsp*I+*Hinf*I-digested patterns of the PCR product are most suitable for discrimination of these ten *Meloidogyne* species from Japan.

Key words: Identification, PCR-RFLP, mitochondrial DNA, *Meloidogyne*, root-knot nematode

INTRODUCTION

Root-knot nematodes, *Meloidogyne* species, are agriculturally important plant parasitic nematode pests. At present, 13 *Meloidogyne* species: *M. incognita* (Kofoid and White), *M. arenaria* (Neal), *M. javanica* (Treub), *M. hapla* Chitwood, *M. suginamiensis* Toida and Yae-gashi, *M. marylandi* Jepson and Golden, *M. mali* Itoh, Ohshima and Ichinohe, *M. camelliae* Golden, *M. ichinohei* Araki and *M. graminis* (Sledge and Golden), are reported in Japan. In addition 3 undescribed species, *M. sp. near arenaria* on mulberry, *M. sp. near mali* on mulberry and *Meloidogyne sp.* on vine have been described (Araki, 1992a, b, c).

It is important to determine *Meloidogyne* species composition in field populations in order to introduce non-chemical control strategies such as crop-rotation, resistant cultivars and enemy plants. As only second-stage juveniles (J2s) and eggs exist in fields before planting, the identification method for J2s is practical. Identification methods based on morphological characteristics and on host range test are difficult for non-spe-

cialists and time-consuming, respectively. Isozyme analysis is a reliable method of identification for adult females of *Meloidogyne* species, however, this method does not allow direct identification of individual J2.

A polymerase chain reaction (PCR) technique has recently been developed for identification of a single *Meloidogyne* J2. Powers and Harris (1993) developed an identification method by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis between the cytochrome oxidase subunit II (COII) gene and the 16S ribosomal RNA (LrRNA) gene in the mitochondrial DNA (mtDNA) of a single *Meloidogyne* J2. Zijlstra et al. (1995) were able to distinguish *M. hapla* from *M. chitwoodi* by the PCR-RFLP analysis of the internal transcribed spacer (ITS) in the ribosomal DNA (rDNA), however, this method using the rDNA could not discriminate *M. incognita* and *M. javanica*. The random amplified polymorphic DNA-PCR (RAPD-PCR) method successfully identified *Meloidogyne* species (Cenis, 1993; Orui, unpublished). In the present study, the PCR-RFLP analysis between the COII gene and the LrRNA gene of the

mtDNA was attempted on a single J2 of the ten *Meloidogyne* species from Japan.

MATERIALS AND METHODS

Nematodes. The 31 nematode populations used are shown in Table 1. Egg masses of *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, *M. suginamiensis* and *M. sp. near arenaria* were obtained from cultures maintained on tomatoes (Kyoryoku-beiju) in a greenhouse. Those of *M. marylandi* were obtained from a culture maintained on lawn in a greenhouse. Egg masses of *M. mali* on cherry, *M. sp. near mali* on

mulberry, and of *M. camelliae* on camellia were directly removed from roots of each host plant. Each population was identified by esterase (EST) and malate dehydrogenase (MDH) phenotypes (Orui et al., 1996) or perineal patterns.

Extraction of nematode DNA. DNA extractions from mass adult females or individual J2 were compared for better template DNA. A miniprep method (miniprep), slightly modified from Cenis (1993), was used for mass DNA extraction. Adult females were removed from roots of each host plant, and were trans-

Table 1. Populations of *Meloidogyne* species used in this study

Species	Population	Cultural host plant	Locality in Japan (City, Prefecture)	Original host plant	Source
<i>M. arenaria</i>	MzKt (A-1)*	Tomato	Kunitomi, Miyazaki	Tobacco	Y. Orui
	KsKd (A-1)	Tomato	Kaseda, Kagoshima	Tobacco	Y. Orui
	OySt (A-2)	Tomato	Sakuto, Okayama	Tobacco	Y. Orui
	MgKd (A-2)	Tomato	Kashimadai, Miyagi	Tobacco	Y. Orui
	FsAb (A-2)	Tomato	Aizubange, Fukushima	Tobacco	Y. Orui
	TgKi (A-2)	Tomato	Kuroiso, Tochigi	Tobacco	Y. Orui
	TsMm (A-2)	Tomato	Mima, Tokushima	Tobacco	Y. Orui
	SgKt (A-2)	Tomato	Karatsu, Saga	Tobacco	Y. Orui
<i>M. incognita</i>	KsKp	Tomato	Kinpo, Kagoshima	Tobacco	Y. Orui
	IkMh	Tomato	Miho, Ibaraki	Tobacco	Y. Orui
	FsIs	Tomato	Iwashiro, Fukushima	Tobacco	Y. Orui
	OyKn	Tomato	Kumenan, Okayama	Tobacco	Y. Orui
	OnHrI	Tomato	Hirara, Okinawa	Tobacco	Y. Orui
	TtKt	Tomato	Ketaka, Tottori	Tobacco	Y. Orui
	AtSw	Tomato	Showa, Akita	Tobacco	Y. Orui
<i>M. hapla</i>	AmKg	Tomato	Kanagi, Aomori	Tobacco	Y. Orui
	ItKh	Tomato	Kunohe, Iwate	Tobacco	Y. Orui
	ItKm	Tomato	Kuzumaki, Iwate	Tobacco	Y. Orui
	YnKz	Tomato	Kobuchizawa, Yamanashi	Carrot	Y. Orui
	CbTu	Tomato	Tomiura, Chiba	Carnation	S. Uematsu ¹
<i>M. javanica</i>	OnHrJ	Tomato	Hirara, Okinawa	Tobacco	Y. Orui
	OnUn	Tomato	Ueno, Okinawa	Tobacco	Y. Orui
	TgUm	—	Utsunomiya, Tochigi	Camellia	Y. Orui
<i>M. camelliae</i>	IkYk	—	Yuki, Ibaraki	Camellia	Y. Orui
	TgMkC	—	Minamikawauchi, Tochigi	Cherry	Y. Orui
<i>M. mali</i>	TgOyC	—	Oyama, Tochigi	Cherry	Y. Orui
	IkUk	Lawn	Ushiku, Ibaraki	Lawn	T. Nishizawa ²
<i>M. suginamiensis</i>	IkTb	Tomato	Tsukuba, Ibaraki	Mulberry	T. Narabu ³
<i>M. sp. nr. arenaria</i>	LMA	Tomato	Maebashi, Gunma	Mulberry	T. Narabu
<i>M. sp. nr. mali</i>	TgMkM	Mulberry	Minamikawauchi, Tochigi	Mulberry	Y. Orui
	IkYkM	—	Yuki, Ibaraki	Mulberry	Y. Orui

*Esterase pattern in parentheses, A-1 and A-2: *M. arenaria* with one and two bands, respectively.

¹Chiba Horticultural Experiment Station; ²Research Laboratory, Japan Protection Association; ³National Agriculture Research Center.

ferred into a sterile plastic dish (9 cm diameter) containing sterile water to wash the nematodes. About 20 females were transferred onto a slide glass, and crushed with a pestle, and 150 μ l of extraction buffer (200 mM Tris-HCl: pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) was added. This extract was transferred to a microcentrifuge tube. After addition of 75 μ l of 3 M potassium acetate, pH 5.2, the lysate was placed at -20°C for 10 min. After centrifugation and transfer of the supernatant to a new microcentrifuge tube, the nucleic acids were precipitated with an equal volume of isopropanol added at room temperature for 30 min and pelleted by centrifugation at 15,000 rpm for 15 min. The pellet was washed with 70% ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl: pH 8.0, 1 mM EDTA). The concentration of the template DNA was adjusted to about 1 ng/ μ l.

A single J2 was transferred with a needle to a well of GenNunc Modules (Nunc) containing 1 μ l of sterile water, and crushed with a micropipet tip whose top was rounded in a flame. Twenty microliters of sterile water was added to the homogenate, which was transferred to a microcentrifuge tube as template DNA. The DNA extraction was also performed with lysis buffer (Orui, 1996) instead of sterile water according to the above procedure. All template DNA was stored at -20°C until use.

PCR primer set. The primer set designed by Powers and Harris (1993) was used in this study. The PCR by this primer set amplified the region between the COII gene and the LrRNA gene of the mtDNA. The primer sequences were 5'-GGTCAATGTTTCAGAAATTTGTGG-3' (C2F3) and 5'-TACCTTTGACCAATCACGCT-3' (1108). These primers were synthesized at Takara Shuzo Co., Ltd.

PCR amplification. Amplifications were performed in 25 μ l of reaction buffer (10 mM Tris-HCl: pH 8.3, 50 mM KCl, 1.5 mM MgCl_2), with 100 μM of each dNTP, 0.1 μM of each primer, 1.0 U *Taq* DNA polymerase (Takara Shuzo Co., Ltd.), and template DNA (5 ng or 5 μ l of crude DNA extract in the case of a single juvenile). The PCR thermal program in a DNA thermal cycler (PTC-100, MJ Research, Inc.) was 35 cycles of 94°C for 1 min, 48°C for 1 min, 70°C for 2 min, and 72°C for 5 min as a

final extension after the last cycle. The amplified products were stored at -20°C until use.

The electrophoresis of the amplified DNA was performed through 7.5% polyacrylamide gels (PAGEL: NPU-7.5L, Atto Corp.) and the fragment sizes were analyzed from the gels. Moreover, 1.4% Agarose I (Wako Pure Chemical Industries, Ltd.) gels were used for confirmation of the size of the large fragment. The electrophoresis condition using the polyacrylamide gel was performed at a constant current of 20 mA/gel for the Atto AE-6450 apparatus at room temperature for about 70 min. The bridge buffer was 25 mM Tris-192 mM glycine. The condition using the agarose gel was performed at a constant voltage of 50 V for the Mupid-2 apparatus (Cosmo Bio Co., Ltd.) at room temperature for about 100 min. The bridge buffer was $0.5 \times \text{TBE}$ buffer (50 mM Tris, 50 mM boric acid and 1 mM EDTA, pH 8.2). The gels were stained with ethidium bromide solution (0.5 $\mu\text{g}/\text{ml}$), and were photographed using Polaroid type 667 films (Polaroid Corp.) on a UV transilluminator.

Restriction endonuclease digestion. Twelve restriction enzymes (BRL Life Technologies, Inc.) were used to digest the PCR product of the mtDNA: *AluI*, *BclI*, *DdeI*, *DraI*, *HindIII*, *HinfI*, *MboI*, *MseI*, *NdeII*, *SspI*, *StyI* and *VspI*. Digestion was conducted in a mixture of 5 μ l of the PCR product, 1 μ l (4–12 U/ μ l) of endonuclease and 14 μ l of buffer for at least 6 h at the appropriate temperature. Digestion with two endonucleases was also conducted in a mixture of 5 μ l of the PCR product, 1 μ l of each endonuclease and 13 μ l of buffer. Digested DNA fragments were separated by electrophoresis through the 7.5% polyacrylamide gels or the 1.4% agarose gels, and the gels were stained and photographed as described above.

RESULTS

Extraction of nematode DNA

The extraction method for template DNA which was obtained with lysis buffer and sterile water on a single J2, was as efficient as the miniprep from mass adult females in preparing DNA for amplification. However, the PCR amplification pattern from the template DNA obtained with sterile water sometimes showed a

faint band. In contrast, the template DNA obtained with lysis buffer always gave a stable band.

PCR amplification

The PCR amplification patterns from the DNA extracted with sterile water and the DNA using miniprep were identical (Fig. 1). The approximate size of each single fragment of amplified products was 1.7 kb for *M. incognita*, *M. javanica* and *M. arenaria*, 580 bp for *M. sp. near mali*, 560 bp for *M. camelliae*, 540 bp for *M. hapla*, *M. suginamiensis* and *M. marylandi*, and 520 bp for *M. mali* and *M. sp. near arenaria* (Table 2).

Restriction endonuclease digestion

AluI, *BclI*, *DdeI*, *DraI*, *EcoRI*, *HinfI*, *HindIII*, *MboI*, *MseI*, *SspI*, *StyI* and *VspI* cut the PCR products into several fragments in at least one *Meloidogyne* species used. The restriction patterns are shown in Figs. 2 and 3, and approximate fragment sizes are shown in Table 2. Some small fragments (<75 bp) and superimposed fragments may have been overlooked in these analyses (Table 2).

DraI, *MseI*, *SspI* and *VspI* cut the PCR products into several fragments in all ten *Meloidogyne* species, and the *MseI*-digested pattern alone distinguished *M. arenaria* from *M. javanica*. *MseI*-digested patterns in all ten

Meloidogyne species had many small fragments of less than 75 bp in length. Though the *SspI*-digested pattern separated the ten *Meloidogyne* species into nine groups, we could not use this pattern, to distinguish *M. arenaria* from *M. javanica*. The *DraI* and *VspI*-digested patterns separated the ten *Meloidogyne* species into eight groups, and the patterns of *M. incognita*, *M. javanica* and *M. arenaria* were identical to each other. *HinfI* cut only the PCR product of *M. incognita* into two fragments (Fig. 2).

When the PCR products of the ten *Meloidogyne* species were digested with *VspI*+*HinfI* or *DraI*+*HinfI*, *M. incognita* was distinguished from *M. arenaria* and *M. javanica*. Therefore, the patterns of the ten *Meloidogyne* species were separated into nine groups. The *VspI*+*HinfI*-digested pattern more clearly distinguished *M. mali* from *M. sp. near mali* than did the *DraI*+*HinfI*-digested one (Fig. 3). The A-1 and A-2 types of *M. arenaria*, which had been characterized by the band number of the EST pattern (Esbenshade and Triantaphyllou, 1985; Orui et al., 1996), were indistinguishable with all the endonucleases used in this study. *MseI* and either *SspI* or *VspI*+*HinfI*-digested patterns of the PCR product were most suitable for discrimination of these ten *Meloidogyne* species from Japan.

When 19 populations of *M. arenaria*, *M. incognita* and *M. hapla* were digested with the

Fig. 1. Effect of the mextraction methods for template DNA on amplification patterns of PCR products of the COII/LrRNA region of the mtDNA in the ten *Meloidogyne* spp. on a 7.5% polyacrylamide gel. The extraction methods for template DNA: A, DNA extracted from mass adult females; B, DNA extracted from a single J2 with sterile water. Nematodes: 1, *M. arenaria* (A-1) (MzKt); 2, *M. arenaria* (A-2) (OySt); 3, *M. javanica* (CbTu); 4, *M. incognita* (KsKp); 5, *M. hapla* (AtSw); 6, *M. mali* (TgMkC); 7, *M. marylandi* (IkUk); 8, *M. sp. near arenaria* (LMA); 9, *M. sp. near mali* (TgMkM); 10, *M. camelliae* (IkYk); 11, *M. suginamiensis* (IkTb). The size marker on both edge lanes is a 1 kb DNA ladder (Gibco BRL).

Table 2. DNA fragment sizes (bp) obtained after endonuclease digestion of the PCR products of the COII/LrRNA region of the mtDNA from 31 populations of the 10 *Meloidogyne* species

Species	PCR product size	Endonuclease							
		<i>AluI</i>	<i>BclI</i>	<i>DdeI</i>	<i>DraI</i>	<i>DraI</i> + <i>HinfI</i>	<i>EcoRI</i>	<i>HindIII</i>	<i>HinfI</i>
<i>M. arenaria</i> (A-1, A-2)	1,700	1,000, 460, 250	—	—	660, 600, 150, 105, 85	660, 600, 150, 105, 85	1,200, 520	950, 700	— (850(S))
<i>M. javanica</i>	1,700	as above	—	—	as above	as above	as above	as above	—
<i>M. incognita</i>	1,700	820, 460, 250, 150	— (860, 800)	—	as above	620, 600, 150, 105, 90, 85	as above	as above	1,300, 400 (850(S))
<i>M. hapla</i>	540	460, 95	—	—	250, 200 (250, 210)	250, 200 (250, 210)	—	—	—
<i>M. mali</i>	520	470, 100	—	—	180(S), 105, 75	180(S), 105, 75	—	—	—
<i>M. marylandi</i>	540	—	—	—	240(S), 75	240(S), 75	—	—	—
<i>M. sp. nr. arenaria</i>	520	460, 95	—	240(S), 80	450, 85	450, 85	—	—	—
<i>M. sp. nr. mali</i>	580	470, 110	—	—	180(S), 105, 75	180(S), 105, 75	—	—	—
<i>M. camelliae</i>	560	480, 100	—	—	270, 150, 85, 75	270, 150, 85, 75	—	—	—
<i>M. suginamiensis</i>	540	450, 100	—	—	150(S), 75	150(S), 75	—	—	—

Species	Endonuclease					
	<i>MboI</i>	<i>MseI</i>	<i>SspI</i>	<i>StyI</i>	<i>VspI</i>	<i>VspI</i> + <i>HinfI</i>
<i>M. arenaria</i> (A-1, A-2)	680, 500, 450	160	370, 310, 290, 210, 145, 135, 110, 95 (370, 310, 290, 210, 145, 135, 95)	1, 100, 540	660, 450, 250, 190, 180	660, 450, 250, 190, 180 (660, 450, 250, 240, 190, 180)
<i>M. javanica</i>	as above	85	370, 310, 290, 210, 145, 135, 110, 95	as above	as above	660, 450, 250, 190, 180
<i>M. incognita</i>	as above (680, 500, 370, 80)	100	315, 300, 295, 210, 145, 135, 110, 95, 70	as above	as above	660, 450, 250, 180, 140
<i>M. hapla</i>	—	110 (130)	230, 220, 90 (225(S), 90)	—	160, 115, 95, 80 (260, 115, 95, 80)	160, 115, 95, 80 (260, 115, 95, 80)
<i>M. mali</i>	—	90	290, 135, 95	—	200, 100, 95, 90, 70	200, 100, 95, 90, 70
<i>M. marylandi</i>	—	*	155, 120, 115, 90	—	260, 180, 105	260, 180, 105
<i>M. sp. nr. arenaria</i>	—	120	450, 95	—	280, 120, 110	280, 120, 110
<i>M. sp. nr. mali</i>	—	110, 100	230(S), 95	—	220, 160, 95, 92	220, 160, 95, 92
<i>M. camelliae</i>	—	*	300, 150, 90	—	300, 105, 75	300, 105, 75
<i>M. suginamiensis</i>	—	100	200, 135, 125, 80	—	500, 90	500, 90

Fragment sizes are approximate; some small fragments (< 75 bp) and superimposed fragments may have been overlooked in these analyses. —: no restriction site. (): an intra-specific variation pattern. (S): superimposed fragment ?. *: fragments under 75 bp in length.

above 12 endonucleases, intraspecific variation in the RFLP patterns was detected in *M. hapla* (YnKz) with *DraI*, *DraI* + *HinfI*, *MseI*, *SspI*, *VspI* and *VspI* + *HinfI*, in *M. arenaria* (KsKd) with *SspI*, in *M. arenaria* (FsAb) with *HinfI* and *VspI* + *HinfI*, and in *M. incognita* (IkMh) with *BclI*, *MboI* and *HinfI*, respectively (Table 2). Some examples of intraspecific variations in the RFLP with *MseI*, *SspI* and *VspI* + *HinfI* are shown in Fig. 4. There was no intraspecific variation in the restriction patterns of three populations of *M. javanica* and each two populations

of *M. mali*, *M. camelliae* and *M. sp. near mali* (Table 2).

Though the PCR product in *M. arenaria* (FsAb) appeared to be a single fragment, the PCR product consistently gave a total size of restriction fragments with *HinfI* and *VspI* + *HinfI* over the size of the PCR product (Fig. 4, Table 2).

DISCUSSION

The PCR-RFLP analysis of the COII/LrRNA gene in the mtDNA discriminated the ten

Fig. 2. Restriction fragment patterns of the PCR-amplified COII/LrRNA region of the mtDNA in the ten *Meloidogyne* species on a 7.5% polyacrylamide gel. Template DNA: DNA extracted from mass adult females. Endonucleases: A, *AluI*; B, *DraI*; C, *HinfI*; D, *MseI*; E, *SspI*; F, *VspI*. Nematodes: 1, *M. arenaria* (A-1) (MzKt); 2, *M. arenaria* (A-2) (OySt); 3, *M. javanica* (CbTu); 4, *M. incognita* (KsKp); 5, *M. hapla* (AtSw); 6, *M. mali* (TgMkC); 7, *M. marylandi* (IkUk); 8, *M. sp. near arenaria* (LMA); 9, *M. sp. near mali* (TgMkM); 10, *M. camelliae* (IkYk); 11, *M. sugina-miensis* (IkTb). The size marker on both edge lanes is a 1 kb DNA ladder (Gibco BRL).

Fig. 3. Restriction fragment patterns with a mixture of the two endonucleases, of the PCR-amplified COII/LrRNA region of the mtDNA in the ten *Meloidogyne* species on a 7.5% polyacrylamide gel. Template DNA: DNA extracted from a single J2 with sterile water. Endonucleases: A, *Dra*I + *Hinf*I; B, *Vsp*I + *Hinf*I. Nematodes: 1, *M. arenaria* (A-1) (MzKt); 2, *M. arenaria* (A-2) (OySt); 3, *M. javanica* (CbTu); 4, *M. incognita* (KsKp); 5, *M. hapla* (AtSw); 6, *M. mali* (TgMkC); 7, *M. marylandi* (IkUk); 8, *M. sp. near arenaria* (LMA); 9, *M. sp. near mali* (TgMkM); 10, *M. camelliae* (IkYk); 11, *M. sugina-miensis* (IkTb). The size marker on both edge lanes is a 1 kb DNA ladder (Gibco BRL).

Fig. 4. Intraspecific variation in the restriction fragment patterns of the PCR-amplified COII/LrRNA region of the mtDNA in *Meloidogyne* species on a 7.5% polyacrylamide gel. Template DNA: DNA extracted from a single J2 with sterile water. N: normal restriction pattern, V: intraspecific variation. V₁, *M. arenaria* (FsAb); V₂, *M. arenaria* (KsKd); V₃, *M. hapla* (YnKz). M: 1 kb DNA ladder (Gibco BRL).

Meloidogyne species from each other in this study. This technique can directly identify a single J2 separated with the Baelmann funnel

technique, and will easily classify *Meloidogyne* species in Japan. The DNA was easily extracted with sterile water. When the PCR amplification

Table 3. Comparison of the size (kb) of PCR products and the *Hinf*I-digested fragments by Powers and Harris (1993), Williamson et al. (1994), and the present study

Species	PCR amplification size			<i>Hinf</i> I-digested fragment		
	Powers•Harris	Williamson et al.	Orui	Powers•Harris	Williamson et al.	Orui
<i>M. incognita</i>	1.7	1.7	1.7	1.0, 0.4, 0.3	1.3, 0.4	1.3, 0.4
<i>M. javanica</i>	1.7	1.7	1.7	1.0, 0.7	no digestion	no digestion
<i>M. arenaria</i>	1.1	1.1	1.7	no data	no data	no digestion

pattern from the DNA shows a faint band, DNA extracted with lysis buffer should be used for obtaining a stable pattern.

Taxonomy of *M. arenaria* and *M. javanica* in Japan has been a topic of recent discussion. Though the perineal pattern of *M. arenaria* from Japan is similar to that of *M. javanica*, the EST phenotype of *M. arenaria* shows one or two bands and that of *M. javanica* shows three bands (Narabu, 1995). These EST phenotypes correspond to typical phenotypes of Esben-shade and Triantaphyllou's report (1985). The Japanese populations which had been regarded to be *M. javanica* by the perineal pattern were considered to be *M. arenaria* by the EST phenotype and the distribution pattern (Narabu, 1995). The PCR-RFLP analysis of these Japanese populations of *M. arenaria* and *M. javanica*, which were identified by EST phenotypes, was attempted in this study for confirming specific status in the world. The present result is different from Powers and Harris (1993) and Williamson et al. (1994) in *M. incognita*, *M. arenaria* and *M. javanica*, as shown in Table 3. The size of the PCR products of *M. arenaria* and *Hinf*I-digested pattern of *M. incognita* and *M. javanica* differed among the authors. However, the result of this study corresponds to Williamson et al. (1994) with the exception of the PCR amplification size in *M. arenaria*. This suggests that *M. incognita* and *M. javanica* in Japan correspond to the respective species in North America. The different result by Powers and Harris (1993) may be attributed to genetically different populations in these nematode species. The size difference of PCR products between Japanese populations and North American populations of *M. arenaria* indicates that Japanese *M. arenaria* may belong to a different species, or that a part of the COII/LrRNA

region of the mtDNA may be lacking in North American populations as an intraspecific variation. The latter will be tested by comparing sequences of the COII/LrRNA region of Japanese populations with North American ones. Though Japanese *M. arenaria* was different from North American *M. arenaria* in the size of the PCR product, this species in the present study was treated as *M. arenaria* according to Narabu (1995). *M. arenaria* is widely distributed in Japan, and is an agriculturally important pest for a wide range of hosts. Its specific status in the world should be established by various techniques including molecular ones in order to disseminate information of this species worldwide.

As *M. arenaria* and *M. javanica* could be considered as one group in dendrograms by RFLP and RAPD-PCR analyses (Fargette et al., 1994; Garate et al., 1991), these two species can be considered to be closely related. In the present study, the 9 endonucleases cut the PCR products of *M. javanica* and *M. arenaria* into some fragments, and the digested patterns by 8 of the 9 endonucleases were identical. This result supports Fargette et al. (1994) and Garate et al. (1991).

M. mali on apple was morphologically similar to *M. sp. near mali* on mulberry, but was different from the latter in the host plant test and some isozyme patterns (Narabu et al., 1991). Five endonucleases cut the PCR products of these 2 species into several fragments, and *Mse*I, *Ssp*I and *Vsp*I-digested patterns were clearly different between the 2 species. This result supports that *M. sp. near mali* is different from *M. mali* at the species level.

Though *M. sp. near arenaria* was morphologically similar to *M. arenaria* or *M. incognita*, these 3 species differed from each other in some

isozyme patterns (Narabu et al., 1991; Orui, unpublished), sizes of the PCR products, and RFLP patterns with *DdeI* in these 3 species: the PCR product of *M. sp.* near *arenaria* alone in these 3 species was cut into fragments with *DdeI*. These results suggest that *M. sp.* near *arenaria* is different from *M. arenaria* and *M. incognita* at the species level.

The total size of the restriction fragments after *HinfI*-digestion was double to the size of the PCR product in *M. arenaria* (FsAb). A similar phenomenon was reported in *M. hapla* and *Pratylenchus vulnus* Allen and Jensen (Zijlstra et al., 1995; Orui, 1996). A slightly different sequence in the COII/LrRNA region of the mtDNA may be present within a single individual of *M. arenaria* (FsAb).

The ten nematode species which represent most *Meloidogyne* species in Japan were distinguished from each other by PCR-RFLP analysis of the COII/LrRNA gene in the mtDNA using DNA extracted from a single J2. However, in the future, when unknown RFLP patterns are seen in *MseI*, *SspI* and *VspI*+*HinfI*-digestion from unknown populations, some of the endonucleases used in this study such as *AhaI*, *DraI* and *DraI*+*HinfI* should also be used for confirmation. Intraspecific variation patterns were demonstrated for some nematode species. The range of the variation should be studied further for each species. This will further improve PCR-RFLP analysis as a useful tool for practical determination of *Meloidogyne* species.

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