Differences Between ITS Regions of Isolates of Root-knot Nematodes *Meloidogyne hapla* and *M. chitwoodi*

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ABSTRACT

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Restriction enzyme analysis of ribosomal DNA (rDNA) sequences was used to distinguish species and isolates of root-knot nematodes. DNA fragments containing the internal transcribed spacer (ITS) rDNA were amplified from total DNA of 27 geographic isolates each of *Meloidogyne hapla* and *M. chitwoodi* and from one isolate each of *M. incognita* and *M. javanica* by polymerase chain reaction (PCR). The amount of DNA present in a single juvenile was sufficient to amplify these PCR products. The amplified ITS fragments were relatively short compared to those that have been found for the genera *Aphelenchoides*, *Caenorhabditis*, *Ditylenchus*, *Heterodera*, and *Xiphinema*. Digestion of the ITS regions with *Alu*I, *Dra*I, and *Hin*fI distinguished *M. hapla* and *M. chitwoodi* from each other as well as from *M. incognita* and *M. javanica*. Results indicated that different ITS sequences are present within a single individual of *M. hapla*. Three isolates of *M. chitwoodi* that produce isozyme patterns distinct from other *M. chitwoodi* isolates also could be distinguished by ITS restriction fragment length polymorphisms. The possibility that they do not belong to *M. chitwoodi* proper is discussed.

Additional keywords: diagnostics, identification, RFLP.

Species of *Meloidogyne* pose a significant threat to agriculture. *M. hapla* Chitwood, which is found worldwide, and *M. chitwoodi* Golden, O'Bannon, Santo, & Finley, known to be present in Europe and the northwestern region of the United States, cause damage to a variety of crops grown in temperate climates. *M. incognita* and *M. javanica* occur in temperate and tropical regions and in glasshouses.

In Dutch agriculture, an increase of the root-knot nematodes *M. hapla* and *M. chitwoodi* has been observed recently. *M. hapla* causes problems on all light soils in the Netherlands when susceptible plants are grown, whereas *M. chitwoodi* mainly has been reported in the southeastern region of the country. Root-knot nematodes have a very broad host range. Crops susceptible to *M. hapla* and *M. chitwoodi* include potato, beet, pea, bean, and carrot, whereas *M. chitwoodi* also is able to reproduce on monocotyledonous crops such as wheat, barley, oat, and maize.

In the past, nematodes generally could be controlled by soil pesticides. However, increased environmental awareness makes the use of nonchemical control strategies, such as crop rotation and resistant cultivars, necessary. Accurate, timely, and reliable identification of nematode populations at the species and subspecies levels (races, biotypes, pathotypes, etc.) is important, since crop rotation and resistant cultivars can be species-specific. To identify species and populations of *Meloidogyne*, several methods are being applied that all have limitations.

Most methods of nematode diagnostics have some limitations. Species identification based on differences in morphological characters (18) requires a lot of skill and is often inconclusive for individuals. Isozyme analysis is a relatively fast way to identify species of the genus *Meloidogyne* (11,12,13). Differences between species-specific esterase and malate dehydrogenase patterns show great consistency and are useful for separating species. However, for clear, reliable results, the isozyme analysis can only be done with females of a specific developmental stage. DNA-based diagnostics provide attractive solutions to the problems associated with these identification methods, because they do not rely on the expressed products of the genome and are independent of environmental influence or developmental stage.

The majority of DNA analyses conducted on root-knot nematodes have used total DNA (genomic and mitochondrial DNA [mtDNA]) or mtDNA and have predominantly assayed restriction fragment length polymorphisms (RFLPs) between reference populations, detecting some interspecies or even intraspecies variation (2,3,6,7,8,9,16,19,23,24,26,33). However, as sensitive as this technique may be, it requires micrograms of DNA from several thousand nematodes. Polymerase chain reaction (PCR)-based techniques, such as random amplified polymorphic DNA analysis (1,4,5,27), are able to identify single juveniles. However, although only small amounts of template DNA are needed, equal amounts of template DNA among samples are required to obtain reproducible results (22). This problem can be overcome by amplifying specific sequences in *Meloidogyne*, such as the mtDNA (17,19,25,34) and the heat shock gene 70A (34). RFLPs from such sequences permit discrimination of several *Meloidogyne* species (5,17,25,34).

Comparative analysis of coding and noncoding regions of ribosomal DNA (rDNA) is becoming a popular tool for species and subspecies identification of many organisms. 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. The more conserved sequences are most useful for classification

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at higher taxonomic levels (genus to phylum), whereas the ITS sequences are useful at species and subspecies levels (21,32).

Several populations of cyst-forming nematodes can be discriminated by comparing partial ITS sequences obtained by PCR with the published sequence for *Caenorhabditis elegans* (10) and with each other (14,15). Species within the *Xiphinema americanum* group (29,30) and *Ditylenchus* (31) have been identified by examining RFLPs in amplified ITS and the 5.8S gene. Xue et al. (34) amplified rDNA sequences of *M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla* but did not find RFLPs.

TABLE 1. Isolates and sources of species of *Meloidogyne* used in this study

Code	Location	Isolate	Source	Enzymes tested[a]
M. chitwoodi				
Ca	Rips, NL[b]	C3022	PD[c]	All
Cc	Baexem, NL	Baexem	PAGV[d]	All
Cg	Vredepeel, NL	C4571	PD	All
Cj	Hapert, NL	C5273f	PD	B, RI, RV, Ha, H, R, X
Ck	Spijkenisse, NL	C5273g	PD	B, RI, RV, Ha, H
Cl	Smilde, NL	C2960	PD	All
Cp	Sterksel, NL	Sterksel	PAGV	RI, H, M, R
Co	Horst, NL	Horst	PAGV	All
Cx	Kessel, NL	C6503	PD	A, RI, M, R
Cy	Herkenbosch, NL	C6496	PD	RI, H, M, R
Cae	Roggel, NL	C4666	PD	A, RI, H, M, R
Caq	Wieringermeer, NL	C6289	PD	All
Cat	Wash., USA	WAMC17, race 1	Mojtahedi/Santo	All
Cau	Wash., USA	WAMC18, race 1	Mojtahedi/Santo	
Cav	Wash., USA	WAMC20, race 1	Mojtahedi/Santo	
Caw	Wash., USA	WAMC21, race 1	Mojtahedi/Santo	
Cax	Wash., USA	WAMC22, race 1	Mojtahedi/Santo	M
Cay	Wash., USA	WAMC23, race 1	Mojtahedi/Santo	
Caz	Wash., USA	WAMC25, race 1	Mojtahedi/Santo	
Cba	Oreg., USA	ORMC12, race 1	Mojtahedi/Santo	
Cbb	Wash., USA	WAMC19, race 2	Mojtahedi/Santo	
Cbc	Wash., USA	WAMC24, race 2	Mojtahedi/Santo	
Cbd	Wash., USA	WAMC16, race 2	Mojtahedi/Santo	All
Cbe	Wash., USA	WAMC27, race 2	Mojtahedi/Santo	
Cbf	Oreg., USA	ORMC8, race 2	Mojtahedi/Santo	A
Cbg	Oreg., USA	ORMC11, race 2	Mojtahedi/Santo	B, RI, H, M
Cbh	Calif., USA	CAMC2, race 3	Mojtahedi/Santo	B, RI, H, M
M. hapla				
Hb	Zwaanshoek, NL	C3093	PD	All
Hc	Bavel, NL	C3064	PD	All
He	Drouwenerveen, NL	Drouwenerveen	PAGV	H
Hh	Wachtum, NL	Wachtum	PAGV	A, RI, H, M, R
Hi	Smilde, NL	Smilde	PAGV	All
Hk	Slochteren, NL	Slochteren	PAGV	A, RI, H, M, R
Ham	Queensland, Australia	Q114	Fargette	All
Han	South Korea	C2346	PD	RI
Hak	Mussel, NL	Mussel	HLB[e]	RI, H, M, R
Has	Wash., USA	WAMH2	Mojtahedi/Santo	All
Hat	Wash., USA	WAMH3	Mojtahedi/Santo	RI
Hau	Utah, USA	UTMH1	Mojtahedi/Santo	All
Hav	Ariz., USA	AZMH1	Mojtahedi/Santo	
Haw	France	AN2	Castagnone-Sereno	All
Hax	England	AN3	Castagnone-Sereno	
Haz	Fla., USA	FL	MacGuidwin	
Hba	Calif., USA	CA	MacGuidwin	
Hbb	N.C., USA	NC	MacGuidwin	
Hbc	Tenn., USA	TN	MacGuidwin	
Hbd	Ohio, USA	OH	MacGuidwin	
Hbe	Conn., USA	CT	MacGuidwin	
Hbf	Md., USA	MB	MacGuidwin	
Hbi	Wis., USA	WI	MacGuidwin	
Hbg	Wyo., USA	WY	MacGuidwin	
Hbh	Mich., USA	MI	MacGuidwin	
Hbk	Mo., USA	MO	MacGuidwin	
Hbq	Hungary	C6611	PD	All
M. incognita				
Ia	Western NL	IPO-DLO[f]		All
M. javanica				
Ja	Taiwan		PD	All

[a] All populations were tested with *Dra*I and *Hin*fI. Other enzymes tested are indicated for the relevant populations: A: *Alu*I; B: *Bam*HI; RI: *Eco*RI; RV: *Eco*RV; Ha: *Hae*III; H: *Hin*dIII; M: *Msp*I; R: *Rsa*I; S: *Sst*I; X: *Xho*I; and All: all of the restriction enzymes presented in Table 2.

[b] NL: the Netherlands.

[c] PD: Plant Protection Service, Wageningen, the Netherlands.

[d] PAGV: Research Station for Arable Farming and Field Production of Vegetables, Lelystad, the Netherlands.

[e] HLB: Hillebrands Laboratorium, Assen, the Netherlands.

[f] IPO-DLO: DLO-Research Institute for Plant Protection, Wageningen, the Netherlands.

The objective of this study was to discover whether isolates of root-knot nematodes, especially *M. hapla* and *M. chitwoodi*, could be distinguished on the basis of ITS sequences, using only small amounts of DNA from single nematodes.

MATERIALS AND METHODS

Nematodes. Isolates of *M. hapla* and *M. chitwoodi* originating from Europe, the United States, Australia, and South Korea (Table 1), were maintained on tomatoes (*Lycopersicon esculentum* L. cv. Moneymaker) grown in a sand/peat mixture in 1,000-cm^3 clay pots at 20°C in a greenhouse. The *Meloidogyne* species were identified morphologically as well as by esterase and malate dehydrogenase phenotypes from single females (13). All isolates tested showed the expected species-specific isozyme phenotypes as published by Esbenshade and Triantaphyllou (13), except for isolates Cc, Cg, and Cae of *M. chitwoodi*, which showed the deviating phenotype recently described by Van Meggelen et al. (28) and referred to as the "Baexem type."

DNA extraction. Eggs were extracted from *Meloidogyne*-infested roots, collected (20), and placed on a 10-µm pore sieve at 20°C to allow the eggs to hatch for 3 weeks. Second-stage juveniles were collected every week and concentrated by centrifugation at 2,000 × *g* for 2 min in a 30% sucrose solution, washed in distilled water, and pelleted in a microcentrifuge. The pellet was transferred to a mortar, frozen by liquid nitrogen and ground. From the resulting fine powder, total DNA was extracted as described by Curran et al. (8). DNA was extracted from single juveniles as described by Harris et al. (17).

rDNA amplification. Figure 1 shows the location of the primers used for amplifying segments of the nuclear rDNA. The primer ITS3 was described by White et al. (32). Primers 5367 and 5368 were sequences as described by Vrain (29). Primers F194 and F195 were the forward and backward primers, respectively, as described in Ferris et al. (14).

The DNA fragments containing the ITS regions were amplified by PCR. The reaction mixture contained 10 mM Tris, pH 9.0; 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% (wt/vol) gelatin; 0.1% Triton X-100; 100 µM each of dATP, dCTP, dGTP, and dTTP (Boehringer GmbH, Mannheim, Germany); 0.6 µM each primer; 0.1 to 10 ng of total DNA or the crude DNA extract from a single juvenile; 0.6 units of *Taq* DNA polymerase (Sphaero Q, Leiden, the Netherlands); and deionized water to a volume of 50 µl. The amplification was carried out in a Perkin-Elmer Cetus (Norwalk, CT) DNA thermal cycler. Initially PCR amplification conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, repeated for 20 cycles. A 5-min incubation period at 72°C followed the last cycle to complete any partially synthesized strands. The fastest available transition between each temperature was used.

To amplify ITS regions from single juveniles, the same conditions were used, but the number of cycles was 35. When large quantities of ITS amplification products of single juveniles were required, amplification was carried out in two phases. First, the homogenate of a single juvenile was amplified for 20 cycles using the conditions described above, then 1 µl of the PCR mixture was reamplified for another 20 cycles using fresh components. In each PCR experiment, a control reaction without template DNA was included. After DNA amplification, 3 to 10 µl of the products was analyzed on 2.5% agarose regular gels.

RFLP. Amplified DNA fragments were digested with one of the following restriction enzymes: *Alu*I, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Msp*I, *Rsa*I, *Sst*I, and *Xho*I. The digested DNA was loaded on a 2.5% agarose gel, separated by electrophoresis, and detected by ethidium bromide staining.

RESULTS

Amplification. When the primers 5367 and 5368 were used for amplification of the ITS region, every isolate from the four *Meloidogyne* species tested gave one major product of approximately 760 bp (Fig. 2). Homogenate of a single juvenile was sufficient to obtain this PCR product when using 35 cycles (Fig. 2). To obtain large quantities of ITS amplification products from single juveniles, the two-step procedure was applied. From the 760-bp product of the first PCR, 1 µl was used for a second PCR. While using the primers 5367 and 5368 for this second PCR, very often a smear was obtained. A strong signal of a 570-bp amplified region was obtained (Fig. 2) using primers F194 and F195. This 570-bp ITS region was 190 bp shorter than the one obtained with the primers 5367 and 5368, which concurs with the published map differences in *C. elegans* of 150 and 40 bp between these forward and reverse primers, respectively (10; Fig. 1).

Additional confirmation that the amplified fragment was the ITS region came from PCR experiments with primers ITS3 and F195, using the 760-bp ITS fragment as a template. The resulting 290-bp fragment (Fig. 2) confirmed the presence of the 5.8S gene. Assuming that the position of the ITS3 primer (Fig. 1) and the length of the 5.8S gene in root-knot nematodes are similar to those published for *C. elegans* (10), the length of both ITS regions could be determined: 210 bp for the one upstream of the 5.8S gene, and about 110 bp for the one downstream of the 5.8S gene.

Digestion of the 760-bp amplification product. ITS regions of all isolates were digested with restriction enzymes (Table 1). All *M. hapla* isolates showed identical restriction patterns. All *M. chitwoodi* isolates showed identical restriction patterns, except for isolates Cc, Cg and Cae, which belong to the Baexem type. Some of the typical restriction patterns of the 760-bp amplification product from isolates of *M. hapla*, *M. chitwoodi*, *M. incognita*, and *M.*

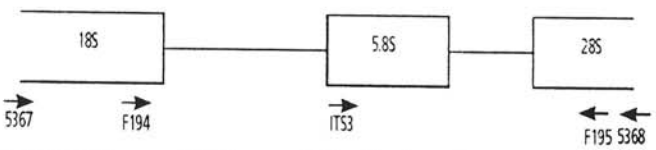

Fig. 1. Locations on *Meloidogyne* rDNA of the internal transcribed spacer (ITS) primers used in this study. The regions in between the rDNA genes (18S, 5.8S and 28S) are the ITS. The 18S and 28S genes are truncated.

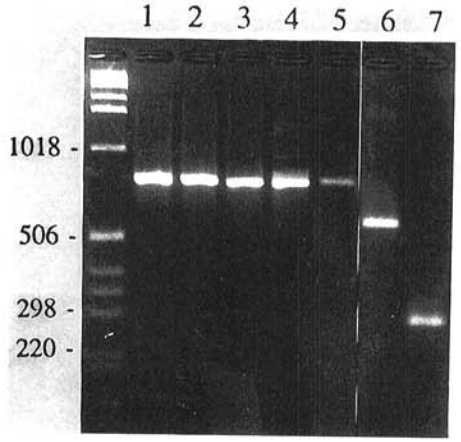

Fig. 2. Typical amplification of 760-bp polymerase chain reaction (PCR) product from template of total DNA extracted from batches of *Meloidogyne* juveniles from isolates of lane 1, *M. hapla*; lane 2, *M. chitwoodi*; lane 3, *M. chitwoodi* Baexem type; and lane 4, *M. incognita*; and from a single juvenile of lane 5, *M. hapla*, with internal transcribed spacer (ITS) primers 5367 and 5368. Lane 6, amplification of a 570-bp PCR product from the 760-bp template of a single juvenile with primers F194 and F195, and lane 7, amplification of a 290-bp PCR product from the 760-bp template with ITS primers ITS3 and F195. The sizes (in base pairs) of some of the fragments of the 1-kb marker are indicated on the left.

javanica are shown in Figure 3. Figure 4 shows some of the differences between *M. chitwoodi* isolates. The sizes of the DNA fragments obtained after digestion are summarized in Table 2. *Hin*fI restriction patterns originating from 1 µg or more of 760-bp PCR product of *M. hapla* showed additional minor bands of 320 and 440 bp, identical to the *Hin*fI restriction pattern of *M. incognita* (Fig. 3D and E; Table 2). The same phenomenon occurred when *M. hapla* ITS fragments were digested with *Hin*dIII (appearance of minor bands identical to the restriction pattern of *M. chitwoodi* and *M. incognita* [Fig. 3C]) and *Msp*I (appearance of minor bands identical to the restriction pattern of *M. chitwoodi* [Fig. 3D]). No such minor bands appeared when the same batches of 760-bp PCR product were digested with the other restriction enzymes used in this study.

Restriction with 10 of the 12 enzymes tested gave RFLPs for some or all of the species tested. There were no restriction sites found for *Xho*I and *Eco*RV. No restriction polymorphisms were found between *M. incognita* and *M. javanica*. Digestion with *Alu*I,

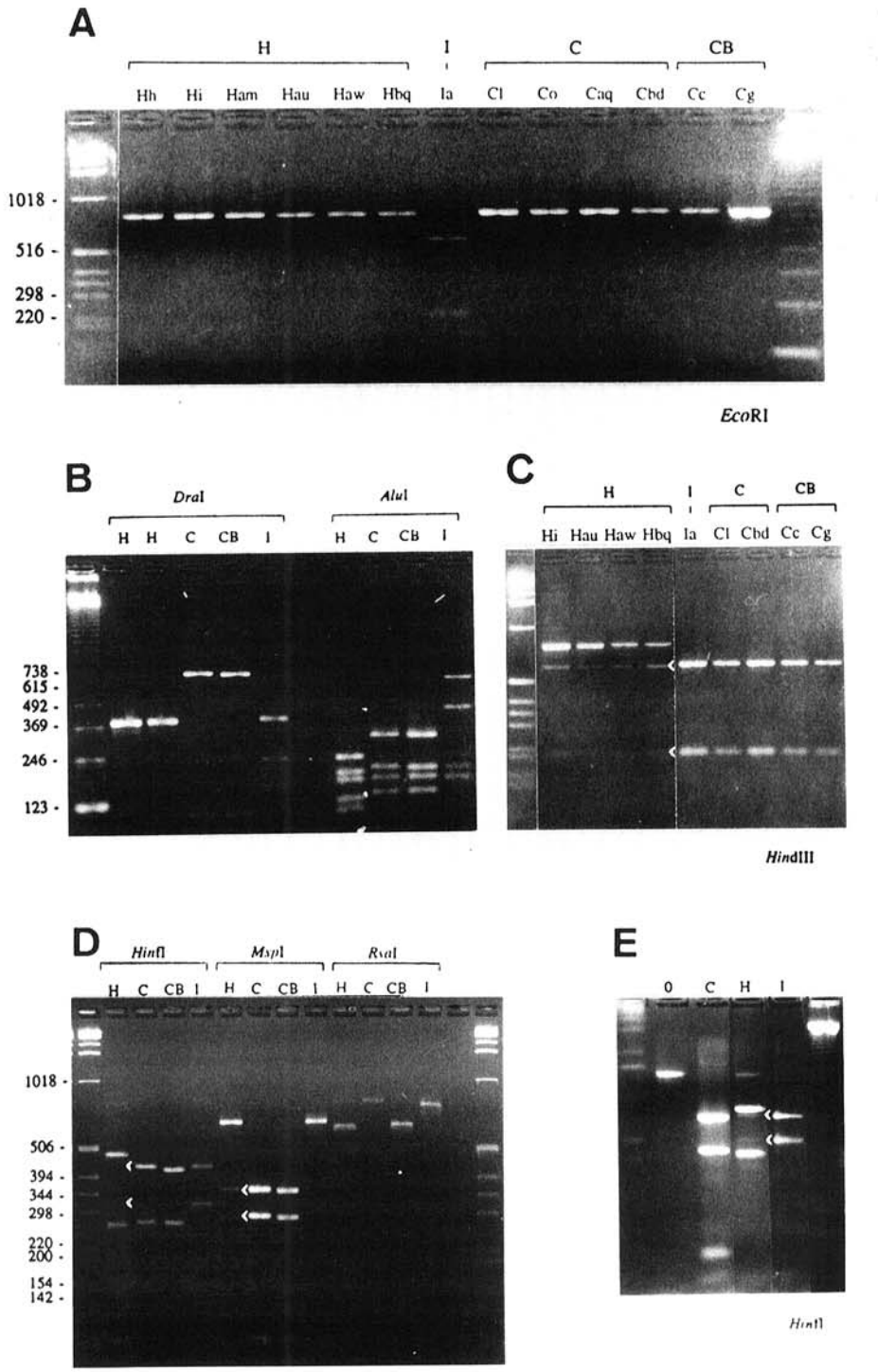

Fig. 3. Typical digestion products of the 760-bp polymerase chain reaction-amplified internal transcribed spacer (ITS) regions upon restriction with **A,** *Eco*RI; **B,** *Dra*I and *Alu*I; **C,** *Hin*dIII; **D,** *Hin*fI, *Msp*I, and *Rsa*I; and **E,** *Hin*fI. The lane designations H, C, CB, I, and 0 correspond to *Meloidogyne hapla*, *M. chitwoodi*, *M. chitwoodi* Baexem type, *M. incognita/M. javanica,* and undigested, respectively. The *Alu*I digestion products always ran slower on the gel than did the other samples loaded. For this reason, the estimated sizes of the *Alu*I restriction products given in Table 2 are not in accordance with the sizes that would be deduced from **B**. The upper band of the *Alu*I restriction pattern shown for *M. incognita/M. javanica* in **B** is partially digested. The sizes (in base pairs) of some of the fragments of the 1-kb markers are indicated. The minor bands listed in parentheses in Table 2 have been marked with <.

*Dra*I, and *Hin*fI distinguished *M. hapla* and *M. chitwoodi* from each other as well as from *M. incognita* and *M. javanica*. To confirm the species specificity of the *Dra*I and *Hin*fI restriction patterns, ITS regions from all isolates presented in Table 1 were digested with *Dra*I and *Hin*fI. *M. hapla* and *M. chitwoodi* products also were differentiated by digestion with the enzymes *Bam*HI, *Hin*dIII, *Msp*I, and *Sst*I.

The *M. chitwoodi* isolates Cc, Cg, and Cae of the Baexem type showed restriction patterns that differed from the other *M. chitwoodi* isolates (Figs. 3 and 4). Digestion with *Rsa*I most clearly differentiated the Baexem type from the other *M. chitwoodi* isolates (Fig. 3D). When digested with *Alu*I, *Dra*I, *Hin*dIII, *Hin*fI, and *Msp*I, the largest restriction fragments of the Baexem type were smaller than those for the other *M. chitwoodi* isolates. After digestion with *Alu*I, *Hin*dIII, and *Msp*I, in the case of the Baexem type, one of the smaller restriction fragments also appeared to be smaller (Fig. 4; Table 2). For this reason, the size of the ITS region of the Baexem type was estimated to be 750 bp (Table 2; Fig. 4, lanes un). The slightly different patterns were consistent and reproducible among DNA preparations from different batches of juveniles from these isolates.

Digestion of the 570-bp amplification product of single juveniles. From all isolates listed in Table 1, 570-bp ITS fragments were generated from single juveniles. Each fragment was digested with *Eco*RI, *Hin*dIII, *Hin*fI, and *Msp*I. By comparing the restriction patterns of the 760-bp fragments with those of the 570-bp fragments, restriction endonuclease cleavage maps for ITS regions of *Meloidogyne* were established (Fig. 5). The *Hin*dIII, *Hin*fI, and *Msp*I restriction patterns of the *M. hapla* 570-bp ITS region showed minor bands identical to the restriction patterns of *M. chitwoodi* and *M. incognita* as had been found upon digestion of *M. hapla* 760-bp fragments. To rule out the possibility that observed minor bands were caused by contamination, an experiment was designed to compare different restriction patterns resulting from digestions of the same batch of ITS templates.

DNA from a single juvenile of *M. hapla* was used as a template for amplification of the 760-bp PCR product. The resulting 760-bp reaction product was used as a template for another 10 PCR experiments using the nested primers F194 and F195. The 10 resulting 570-bp PCR products were pooled and digested with *Dra*I, *Eco*RI, *Hin*dIII, *Hin*fI, and *Msp*I. These experiments were done with juveniles of isolates Ham and Haw. Results showed that minor bands appeared in *Hin*dIII, *Hin*fI, and *Msp*I restriction patterns as had been found for the restriction patterns of the 760-bp fragments of *M. hapla*. No minor bands were visible in *Dra*I and *Eco*RI restriction patterns (Fig. 6).

DISCUSSION

Comparison of restriction patterns derived from amplified ITS regions proved to be a useful molecular approach to separate the species *M. hapla* and *M. chitwoodi* from each other, as well as from *M. incognita* and *M. javanica*. The latter two species could not be distinguished from each other, which supports previous conclusions (34) based on a comparison of seven *M. incognita* isolates and one *M. javanica* isolate. Moreover, the method described in our study differentiated the Baexem-type isolates from other isolates.

The 760-bp PCR product we obtained for the amplified ITS region of *Meloidogyne* was considerably shorter than the product reported for *C. elegans* (29), *Xiphinema* (30), *D. dipsaci*, and *D. destructor* (31) (1.4, 1.5, 0.9, and 1.2 kb, respectively). Using the primers F194 and F195, Ferris et al. (14) amplified a 1.1-kb fragment for *Heterodera*, whereas in this study a 570-bp fragment was obtained for *Meloidogyne*.

Our finding that digestion of ITS regions of *M. hapla* isolates with restriction enzymes *Hin*dIII, *Hin*fI, and *Msp*I always resulted in patterns that contained minor bands identical to the restriction patterns of *M. incognita* and *M. chitwoodi* was unexpected. The patterns obtained after digestion with *Hin*dIII and *Msp*I initially were thought to be caused by partial digestion. However, addition of more restriction enzyme did not change the ratio of the weak and strong patterns. A more obvious explanation would be that the amplified *M. hapla* ITS fragments were contaminated with ITS fragments of *M. chitwoodi* and/or *M. incognita*. However, upon

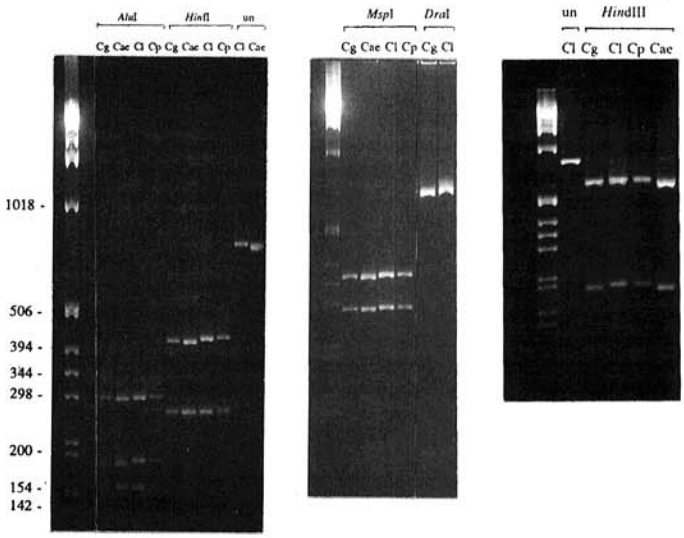

Fig. 4. Typical restriction products of the 760-bp polymerase chain reaction-amplified internal transcribed spacer regions of *Meloidogyne chitwoodi* (represented by populations Cl and Cp) and *M. chitwoodi* Baexem type (represented by populations Cg and Cae) upon restriction with *Alu*I, *Hin*fI, *Msp*I, *Dra*I, and *Hin*dIII. Lanes un, undigested. The sizes (in base pairs) of some of the fragments of the 1-kb marker are indicated on the left.

TABLE 2. Sizes of the DNA fragments (bp) obtained after restriction enzyme digestion of the 760-bp internal transcribed spacer regions of *Meloidogyne hapla*, *M. chitwoodi*, *M. chitwoodi* Baexem type, *M. incognita*, and *M. javanica*

Restriction enzyme	*M. hapla*[a]	*M. chitwoodi*	*M. chitwoodi* Baexem type	*M. incognita*/*M. javanica*
*Alu*I	230, 180, 160, 100, 90	290, 190, 170, 110	285, 185, 170, 110	400, 190, 170
*Bam*HI	430, 330	760	750	760
*Dra*I	380, 380	660, 100	650, 100	220, 200, 180, 160
*Eco*RI	760	760	750	520, 240
*Eco*RV	760	760	750	760
*Hae*III	760	760	750	360, 250, 150
*Hin*dIII	760 (+560, 200)	560, 200	555, 195	560, 200
*Hin*fI	490, 270 (+440, 320)	440, 270, 50	430, 270, 50	440, 320
*Msp*I	650, 110 (+360, 290)	360, 290, 110	355, 285, 110	650, 110
*Rsa*I	620, 140	760	620, 130	760
*Sst*I	660, 100	760	750	760
*Xho*I	760	760	750	760

[a] Numbers in parentheses indicate minor bands.

digestion of the same batches of *M. hapla* 760-bp PCR product with *Dra*I, *Eco*RI, or *Hae*III, no *M. chitwoodi* or *M. incognita* patterns were observed (Fig. 3A and B; Table 2). Conclusive evidence that the minor bands were not caused by contamination, came from the experiments with digestions of the pooled 570-bp PCR products, originating from one 760-bp PCR product from one single juvenile of *M. hapla*. After digestion with *Hin*dIII, *Hin*fI, and *Msp*I, the minor bands appeared again (Fig. 6, lanes 3, 4, and 5), whereas no minor bands were visible after digestion with *Dra*I and *Eco*RI (Fig. 6, lanes 1 and 2). This indicates that different ITS sequences are present within a single individual of *M. hapla*.

Probably a major part of the *M. hapla* ITS sequences has a restriction map as presented in Figure 5A, no restriction sites for *Hin*dIII, and only one restriction site for *Msp*I and *Hin*fI, whereas a minor part of the ITS regions has a different sequence, with *Hin*dIII and *Hin*fI restriction sites at the same locations as in the ITS region of *M. incognita* or *M. javanica* (Fig. 5C), and *Msp*I restriction sites at the same locations as in the ITS regions of *M. chitwoodi* (Fig. 5B). This could be tested further by sequencing the ITS regions. It also would be interesting to know whether the different homologous types of ITS regions (Fig. 5A, B, and C) are present together in one cluster of repeats or in two or more clusters of repeats, each consisting of identical ITS regions.

In contradiction to our results, Xue et al. (34) found that digestions of ITS fragments of *M. hapla*, *M. incognita*, *M. javanica*, and *M. arenaria* with *Eco*RI and *Hin*dIII did not show differences between species. In our study, *Eco*RI restriction patterns clearly separated *M. hapla* (760 bp) from *M. incognita* and *M. javanica* (520 and 240 bp, respectively [Fig. 3A]). All the *Hin*dIII restriction patterns of ITS regions from *M. incognita*, *M. javanica*, and *M. hapla* contained bands of 560 and 200 bp, whereas the *Hin*dIII pattern of *M. hapla* always showed an additional strong band of 760 bp (Fig. 3C), thereby distinguishing *M. hapla* from *M. incognita* and *M. javanica*.

Our finding that restriction patterns of the ITS region derived from Cc, Cg, and Cae differed from other *M. chitwoodi* isolates provides additional evidence that the Baexem type differs from other *M. chitwoodi* isolates. The most convincing difference is the presence of a *Rsa*I site in the Baexem isolates. The slight size differences shown in Figure 4 indicate there, presumably, are two deletions: one could be located between the *Msp*I site and the second *Hin*fI site (Fig. 5B), and one could be downstream of the *Hin*dIII site.

The isolates Cc, Cg, and Cae were detected in 1992 in the southeastern region of the Netherlands and were initially recognized as *M. chitwoodi*. However, these isolates differ from the other *M. chitwoodi* isolates in morphology, host range, isozyme patterns (28), and, according to our findings, ITS sequence. These findings have caused some confusion concerning the systematic relationship of these isolates to other root-knot nematodes. It may be that the Baexem-type isolates constitute a subspecific group of *M. chitwoodi* or even a new species.

The ITS restriction analysis described here enables the identification of *M. hapla* and *M. chitwoodi*, which makes this approach ideal for identifying the species of field isolates of root-knot nematodes. The *Dra*I and *Hin*fI ITS restriction patterns obtained in this study for *M. hapla* and *M. chitwoodi* can be considered species specific because they appear to be the same for all the isolates tested within one species. The isolates originated from different locations worldwide and represent different races, which means that a large part of the natural diversity of the two species has been covered in the experiments in this study.

The advantages of this rDNA analysis are that the method is fast and reliable; it is useful for identification at any developmen-

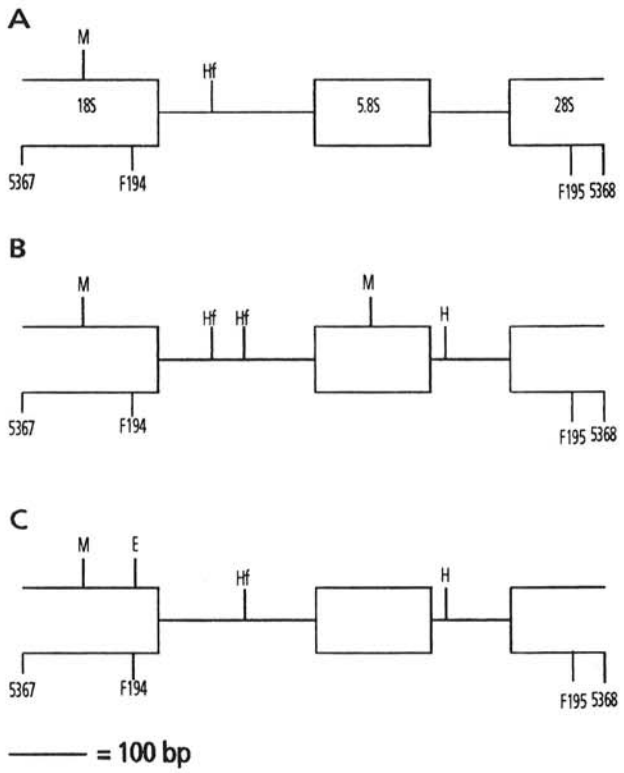

Fig. 5. Restriction endonuclease cleavage maps of internal transcribed spacer (ITS) regions of *Meloidogyne* for four restriction enzymes: E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; and M, *Msp*I. They were established by comparing restriction patterns of the 760-bp fragments with those of the 570-bp fragments. **A,** restriction map of the major part of the ITS regions from *M. hapla*; **B,** restriction map of the ITS region of *M. chitwoodi*; and **C,** restriction map of the ITS regions of *M. incognita* and *M. javanica*. The restriction map of *M. chitwoodi* Baexem type is similar to **B**, although it must contain two small deletions: one between the *Msp*I site and the second *Hin*fI site and one downstream of the *Hin*dIII site. The locations of primers 5367, 5368, F194, and F195 also are indicated.

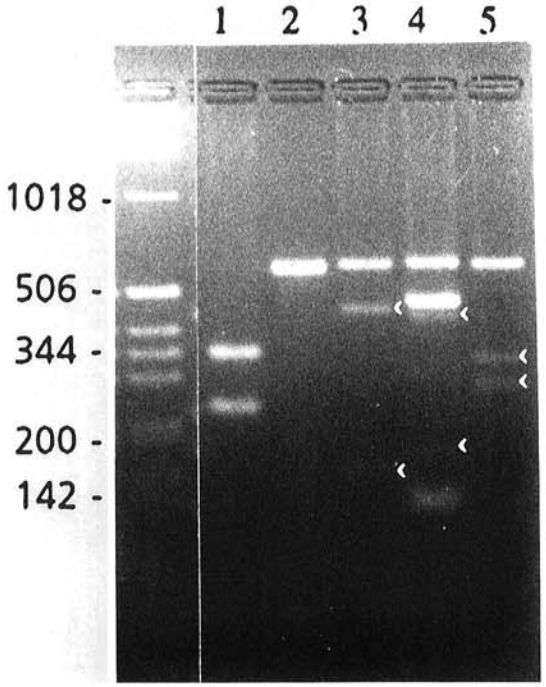

Fig. 6. Restriction patterns obtained after digestion of 10 pooled 570-bp polymerase chain reaction (PCR) products originating from one 760-bp PCR product from a single *Meloidogyne hapla* juvenile of isolate Ham. Restriction enzymes used were lane 1, *Dra*I; lane 2, *Eco*RI; lane 3, *Hin*dIII; lane 4, *Hin*fI; and lane 5, *Msp*I. The sizes (in base pairs) of some of the fragments of the 1-kb marker are indicated on the left. The minor bands that have been listed in parentheses in Table 2, have been marked with <.

tal stage; and because of the abundance of rDNA in the genome, it can even be applied on individual nematodes. Moreover, the method gave additional evidence that the Baexem type of *M. chitwoodi* might not belong to *M. chitwoodi* proper.

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