Specific Diagnosis of Two Root-Knot Nematodes, *Meloidogyne chitwoodi* and *M. fallax*, with Satellite DNA Probes

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ABSTRACT

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Meloidogyne chitwoodi and *M. fallax* are serious pests of potato, and both species have been recently designated as quarantine organisms in the European Community and in Canada. The sympatric and less damaging species *M. hapla* is often found associated with both of them under temperate climates. Here, we describe the use of satellite DNA (satDNA) sequences previously isolated from these three root-knot nematode species for the development of specific diagnostic procedures. In dot-blot experiments, it was unambiguously possible to separate *M. chitwoodi* and

M. fallax from *M. hapla* using satDNA monomers as probes. In squash-blot experiments, satDNAs allowed discrimination between single individuals of *M. chitwoodi* or *M. fallax* from *M. hapla*, even within root tissues, without the need for DNA purification. The same results were obtained with radioactive or digoxigenin-labeled probes with no loss of sensitivity in detection. *M. fallax* and *M. chitwoodi* could not be distinguished. From this study, it is concluded that such cloned satDNA sequences may constitute a powerful tool for the identification and management of *Meloidogyne* spp. populations in the field and for the implementation of quarantine regulations against these pests.

Additional keywords: species-specific probe, squash-blot procedure, tandem repeats.

The root-knot nematode species *Meloidogyne chitwoodi* Golden, O'Bannon, Santo, & Finley and *M. fallax* Karssen are important pathogens of potato and other crops in the western part of Europe, where a significant increase of the populations has been observed recently (12). *M. chitwoodi* is also a major pest of potato in the northwestern states of the United States, where *M. fallax* has not yet been reported (13). These nematodes cause severe galling on the surface and necrotic spotting below the skin of the tubers, which become unmarketable for either fresh consumption or processing. Because of their current emergence as serious pests, *M. chitwoodi* and *M. fallax* have recently been designated as quarantine organisms in the European Community and in Canada (1,14). *M. hapla* Chitwood, which causes less economic damage on potato than does *M. chitwoodi* or *M. fallax* (13), is very often found associated with both species under temperate climates. Because the three species resemble each other morphologically, simple and reliable identification tools for these *Meloidogyne* spp. are urgently needed for both the identification and management of populations in the field and for the implementation of quarantine regulations by official plant protection services.

Recently, different methodologies based on the polymerase chain reaction (PCR) have been developed to identify these root-knot nematode species. Restriction analysis of PCR-amplified ribosomal DNA (rDNA) sequences containing the internal transcribed spacer (ITS) was successfully used to distinguish between *M. hapla* and *M. chitwoodi* isolates (21). In the same way, amplification of the intergenic spacer (IGS) region of the genomic rDNA produced polymorphic fragments between *M. hapla*, *M. chitwoodi*, and *M. fallax*, although the size difference of the amplified bands was very low between *M. chitwoodi* and *M. fallax* (13). Design of specific primers derived from the sequence of the amplified ITS re-

gion resulted in fragment-size polymorphisms that distinguish the three species, without the need for subsequent restriction analysis (20). The same results were also obtained with primers specific for the IGS region of nematode rDNA (15).

Satellite DNA (satDNA) is present in the genome of almost all eukaryotic organisms and is composed of highly repetitive sequences organized as long arrays of tandemly repeated elements, present from 10^3 to 10^5 copies per haploid genome (2). Recently, satDNA sequences have been characterized from a number of plant-parasitic and entomopathogenic nematodes and have proven to be species specific, thus constituting useful diagnostic tools for the identification of species of agronomic interest (9). Therefore, an alternative strategy for specific identification that does not involve PCR amplification of nematode DNA could be considered in the case of *M. chitwoodi* and *M. fallax*, based on such sequences. A *StyI* satDNA family was isolated from *M. hapla* (16), and its use as a probe demonstrated that it does not hybridize with the genomic DNA from *M. chitwoodi* isolates (17). Very recently, we cloned and characterized two *AluI* satDNA families from the genome of *M. chitwoodi* and *M. fallax*, respectively (4,5). The objectives of the study reported here were to evaluate the species specificity of both sequences, compared with *M. hapla*, and then to design a diagnostic procedure that could be easily manipulated on a routine basis.

MATERIALS AND METHODS

Nematode isolates. Root-knot nematode isolates originating from Europe, the United States, and Canada (Table 1) were maintained on tomatoes (*Lycopersicon esculentum* L. cv. Moneymaker) grown in a sand-peat mixture in 1,000-cm³ clay pots at 20°C in a greenhouse. They were specifically identified morphologically, according to their isoelectric electrophoretic pattern, or both (7).

DNA isolation. Eggs were extracted from *Meloidogyne* spp.-infested roots, collected (10), and placed on a 10-µm-pore sieve at 20°C for 3 weeks to allow the eggs to hatch. Second-stage juveniles were collected every week and concentrated by centrifugation

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at $2,000 \times g$ 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. (6), resuspended in Tris-EDTA (TE) buffer (0.01 M Tris, pH 8.0, and 0.001 M EDTA), and stored at -80°C until use.

DNA probes. Three different DNA probes were used in the experiments: pMcCo14 (GenBank accession number U96668), pMfFd1.9 (EMBL accession number AJ223457), and pMhM6 (GenBank accession number L07109). They consisted of cloned monomers of satDNAs previously identified in *M. chitwoodi*, *M. fallax*, and *M. hapla*, respectively (4,5,16).

TABLE 1. *Meloidogyne* spp. and isolates used in this study

Isolates	Origin ^a	Source ^b	Last host
<i>M. chitwoodi</i>			
Ca	The Netherlands (SE)	PD	Maize
Ci	The Netherlands (SE)	PD	Tomato
Cj	The Netherlands (SE)	PD	Unknown
Cl	The Netherlands (NE)	PD	Wheat
Cp	The Netherlands (SE)	PAV	Black salsify
Cu	The Netherlands (SE)	PD	Potato
Cx	The Netherlands (S)	PD	Potato
Cy	The Netherlands (SE)	PD	Potato
Cz	The Netherlands (SE)	PD	Potato
Caj	The Netherlands (SE)	PD	Potato
Caq	The Netherlands	PD	Potato
Cav	Washington	WSU	Potato
Cba (race 1)	Oregon	WSU	Potato
Cbd (race 2)	Washington	WSU	Potato
Cbh (race 3)	Tulelake, California	WSU	Potato
Cbj	The Netherlands (SE)	BLGG	Unknown
Cbk	The Netherlands (SE)	BLGG	Unknown
Cbn	The Netherlands (SE)	BLGG	Unknown
Cbo	The Netherlands (SE)	BLGG	Unknown
Cbs	The Netherlands (NE)	PAV	Unknown
Cbu	The Netherlands (NE)	PAV	Unknown
Cbv	The Netherlands (NE)	PAV	Unknown
Cbz	The Netherlands (NE)	PAV	Unknown
Ccc	The Netherlands (SW)	PD	Onion
<i>M. fallax</i>			
Fa	The Netherlands (SE)	PAV	Primrose
Fb	The Netherlands (SE)	PD	Beet
Fc	The Netherlands (SE)	PD	Potato
Fd	The Netherlands (SE)	PD	Potato
Fe	The Netherlands (SW)	BLGG	Unknown
Ff	Belgium	ARC	Unknown
Fg	The Netherlands (S)	CPRO	Unknown
<i>M. hapla</i>			
Ha	The Netherlands (W)	PD	Peony
Hc	The Netherlands (S)	PD	<i>Aconitum</i> sp.
He	The Netherlands (NE)	PAV	Potato
Hp	The Netherlands	HLB	Potato
Hau	Utah	WSU	Potato
AN2	La Môle, France	INRA	Grape
AN3	England	INRA	Unknown
AN4	Frontignan, France	INRA	Unknown
Hbg	Wyoming	UW	Unknown
Hbj	Canada	UW	Unknown
Hbq	Hungary	PD	Rose
Hbr	The Netherlands (NW)	PD	<i>Veronica</i> sp.
Hbs	The Netherlands (SE)	PD	Fennel
Hbv	The Netherlands	BLGG	Rose
Hcb	The Netherlands	CPRO	Hemp

^a Parts of the Netherlands: SE = southeastern, S = southern, NE = northeastern, SW = southwestern, and NW = northwestern.

^b PD = Plant Protection Service, Wageningen, the Netherlands; PAV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, the Netherlands; WSU = Washington State University, Prosser; BLGG = Laboratory for Soil and Crop Testing, Oosterbeek, the Netherlands; ARC = Agricultural Research Centre, Merelbeke, Belgium; CPRO = Center for Plant Breeding and Reproduction Research, Wageningen, the Netherlands; HLB = Hillbrands Laboratorium, Assen, the Netherlands; INRA = Institut National de la Recherche Agronomique, Antibes, France; and UW = University of Wisconsin, Madison.

Radioactive labeling and hybridization conditions. DNA probes were radioactively labeled according to the random oligonucleotide priming method (8) with alpha- ^{32}P dCTP. Hybridizations were conducted at high stringency ($6\times$ SSC [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], $5\times$ Denhardt's, and 0.5% sodium dodecyl sulfate [SDS] at 65°C). After hybridization, filters were washed at 65°C in $2\times$ SSC and 0.1% SDS, and then in $1\times$ SSC and 0.1% SDS. After posthybridization washes, filters were exposed to X-ray film with an intensifying screen at -80°C .

Digoxigenin (DIG) labeling, hybridization, and detection conditions. PCR was used to produce probes directly labeled with DIG using the PCR DIG Labeling Mix (Boehringer GmbH, Mannheim, Germany) that contains a 19:1 ratio of dTTP:DIG-dUTP, according to the manufacturer's instructions. The primers used are show in Table 2, and the thermal profile was as follows: 3 min at 94°C and then 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. A 10-min incubation period at 72°C followed the last cycle to complete any partially synthesized strand.

Membranes were prehybridized for 1 h at 65°C in hybridization buffer ($2.5\times$ SSC, 0.01% SDS, 0.1% *N*-lauroylsarcosine, and 1% blocking reagent) and then incubated overnight at 65°C in the same buffer in which the appropriate probe had been added. After hybridization, membranes were successively washed at 65°C in $2\times$ SSC and 0.1% SDS (30 min), in $0.2\times$ SSC and 0.1% SDS (30 min), and finally in washing buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.3% [wt/vol] Tween 20, pH 7.5). Membranes were then incubated for 1 h in blocking buffer (10% blocking reagent [wt/vol] in 0.1 M maleic acid and 0.15 M NaCl, pH 7.5). For detection, the anti-DIG-alkaline phosphatase (AP) conjugate (Fab fragments from an anti-DIG antibody from sheep, conjugated with AP) was diluted 1:40,000 in blocking buffer, and the membranes were incubated in this solution for 30 min. For the detection procedure, membranes were rinsed in washing buffer (15 min, twice) and incubated for 5 min in detection buffer (0.1 M Tris-HCl and 0.1 M NaCl, pH 9.5). The CDP-star solution (Boehringer GmbH), an ultra-sensitive chloro-substituted 1,2-dioxetane chemiluminescent substrate for AP, was diluted 1:200 in 2 ml of detection buffer, and the membranes were incubated in this solution for 5 min at room temperature. Membranes were then drained on paper and exposed to X-ray film with intensifying screen at room temperature.

Dot-blot procedure. Dot blots of genomic DNA were prepared by first diluting the DNA to the required concentration (10 ng/ μl), denaturing it with 2 M NaOH, and finally neutralizing it with 4 M ammonium acetate. The samples (25 ng of DNA) were then dotted by vacuum onto nitrocellulose membranes in a MiniFold I blotting apparatus (Schleicher & Schuell, Dassel, Germany). Each dot-blot experiment was repeated at least twice.

Squashed nematode experiment. The experiment was conducted on isolates AN2, Cy, and Fa for *M. hapla*, *M. chitwoodi*, and *M. fallax*, respectively. For each isolate, one female, five females, one egg mass, and one gall were hand-picked and placed on a nylon membrane. The nematodes were then ruptured by gentle pressure with a yellow, flat-tipped micropipette tip. Squashed materials were lysed by layering, successively, the filter on Whatman 3MM paper (Whatman International Ltd., Maidstone, England) soaked with 10% SDS (2 min), 0.5 M NaOH and 2.5 M NaCl (twice, 5 min each), and 3 M sodium acetate, pH 5 (three times, 2 min each). The filter was dried at room temperature (30 min) and then

TABLE 2. Primers used in this study

Species	Code	Sequence (5' to 3')
<i>M. chitwoodi</i>	McS1	CTGTGTCGATTACCTCTTCATCCTC
	McS2	CTATGAAATTCAAAAATC
<i>M. fallax</i>	MfX1	AGAATGAAGATTTTGTGAAG
	MfX2	AGCTTCATTATATTCAGAAT
<i>M. hapla</i>	MhS1	GAGTTCCTTGTAAGCAACTTCT
	MhS2	GGACTTTTTTAGGACACAGC

baked at 80°C for 1 h. Prehybridization and hybridization with the DIG-labeled probes were carried out as described above. The whole procedure was carried out three times to verify reproducibility.

RESULTS

Use of pMcCo and pMfFd repetitive sequences as diagnostic probes. The structural characteristics of the *Alu*I repetitive sequences pMcCo and pMfFd, isolated from the genome of *M. chitwoodi* and *M. fallax*, respectively, have been described previously (4,5). To test their distribution, the genomic DNA of 22 *M. chitwoodi* isolates, 7 *M. fallax* isolates, and 13 *M. hapla* isolates was dot-blotted on a nylon membrane and hybridized first with a radioactively labeled monomer from the pMcCo satDNA. Hybridization occurred with all the *M. chitwoodi* and *M. fallax* populations and never with any of the *M. hapla* populations (Fig. 1A).

After dehybridization, the same membrane was hybridized again using a monomer of the pMfFd satellite family cloned in *M. fallax*, and the results were absolutely similar to those obtained with the probe isolated from *M. chitwoodi* (Fig. 1B). These results demonstrated that both satDNAs are able to separate *M. chitwoodi* and *M. fallax* from the sympatric species *M. hapla*.

DIG labeling of the probes. It is clear that radioactive compounds are not suitable for a routine diagnostic protocol. Therefore, we initiated experiments on a subset of nematode populations in order to test the "cold" labeling of the probes with DIG. In addition to the two previous satDNAs, pMhM, a *S*tyI satDNA cloned in *M. hapla*, was used because its distribution was restricted to this root-knot nematode species only (3,16,17). The three probes were PCR-labeled with DIG and used to successively hybridize a dot blot containing genomic DNAs from populations of *M. hapla*, *M. chitwoodi*, and *M. fallax*. Use of pMcCo and pMfFd gave the

Fig. 1. Dot-blot experiments. A dot-blot of *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla* populations was successfully hybridized with an alpha-³²P-labeled satellite DNA probe from **A**, *M. chitwoodi* and **B**, *M. fallax*. Population codes are *M. chitwoodi* isolates Ca = A1, Ci = A2, Cj = A3, Cl = A4, Cu = A5, Cx = A6, Cy = A7, Cz = A8, Caj = A9, Cag = A10, Cba (race 1) = B1, Cbd (race 2) = B2, Cbh (race 3) = B3, Cbj = B4, Cbk = B5, Cbn = B6, Cbo = B7, Cbs = B8, Cbu = B9, Cbv = B10, Cbz = C1, and Ccc = C2; *M. fallax* isolates Fa = D1, Fb = D2, Fc = D3, Fd = D4, Fe = D5, Ff = D6, and Fg = D7; and *M. hapla* isolates Ha = E1, Hc = F2, He = F1, Hp = E3, AN2 = E10, AN3 = E9, Hbj = E8, Hbq = E7, Hbr = E6, Hbs = E5, Hbv = E4, and Hcb = E2.

same hybridization patterns as previously, thus clearly separating *M. chitwoodi* and *M. fallax* on the one hand, and *M. hapla* on the other hand (Fig. 2A and B). Complementarily, pMhM hybridized specifically with genomic DNAs from the *M. hapla* populations

only (Fig. 2C). Due to the repetitive nature of satDNA, no loss of sensitivity in the detection was observed with the cold probes compared with the radioactive ones. Moreover, the exposure time needed to obtain a readable hybridization signal were considerably reduced with the DIG-labeled probes (from 30 min to 1 h) compared with the radioactively labeled ones (12 to 24 h).

Detection of single individuals in squashed nematode experiments. The restricted distribution of the three satDNA families, and also their high level of reiteration in the genome, allowed us to test them as hybridization probes in squashed nematode experiments. The theoretical advantage of such a procedure is that it should work directly on the biological material, without the need for any time-consuming DNA purification step. Once more, probes were PCR-labeled with DIG. Either one single female, one egg mass, or one female in galled root tissues could unambiguously be identified with the corresponding satDNA probe after a 1-h ex-

Fig. 2. Dot-blot experiments. A dot-blot of *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla* populations was successfully hybridized with a digoxigenin-labeled satellite DNA probe from **A**, *M. chitwoodi*; **B**, *M. fallax*; and **C**, *M. hapla*. Population codes are *M. chitwoodi* isolates Cp = B3, Cav = B1, and Cbd (race 2) = B2; *M. fallax* isolates Fa = A1, Fc = A2, Fd = A3, and Fe = A4; and *M. hapla* isolates Hau = C1, AN2 = C3, AN3 = C4, AN4 = C5, and Hbg = C2.

Fig. 3. Squashed nematode experiments. Squashed *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla* materials were successfully hybridized with a digoxigenin-labeled satellite DNA probe from **A**, *M. chitwoodi*; **B**, *M. fallax*; and **C**, *M. hapla*.

posure of the membrane, with the same specificity as displayed in both previous dot-blot experiments (Fig. 3). As expected, hybridization of individuals from other root-knot nematode species and of healthy root material gave no signal (data not shown).

DISCUSSION

Repetitive sequences known as satDNA have been characterized in a number of nematodes of agronomic interest, and because of their species specificity and reiteration in the genome, they have provided very powerful tools to discriminate between closely related species for which specific markers are needed (9). Here, we present evidence that such sequences may be of interest for the development of a routine diagnostic procedure for the identification of the quarantine root-knot nematodes *M. chitwoodi* and *M. fallax* versus the sympatric species *M. hapla*. Using previously cloned satDNAs from these three species (4,5,16) as probes in squash-blot experiments, it was possible to unambiguously discriminate between single individuals of *M. chitwoodi* and *M. fallax* on one side and of *M. hapla* on the other, even within root tissues, without the need for either DNA purification or radioactive compounds for the labeling of the probes.

The monomeric unit of the satDNA from *M. hapla* hybridized only with genomic DNA from populations belonging to this species, without any cross-hybridization observed with populations from either *M. chitwoodi* or *M. fallax*. This specific distribution, inferred from the analysis of a large collection of populations, thus confirmed previous results obtained on a more restricted number of populations (17).

Cross-hybridizations were observed between the satDNA sequences from either *M. chitwoodi* or *M. fallax* and the genomic DNAs of these two species. Because of the usual species restricted distribution of satDNA (9), this result could be considered as unexpected. However, it is easily related to some recent taxonomical, biological, and molecular data. Due to morphological resemblance, *M. fallax* had originally been misidentified as a new race of *M. chitwoodi* (19) and was recognized as a new root-knot nematode species only recently (11). Moreover, successful interspecific hybridization was obtained between the two species, which provided strong evidence supporting their close phylogenetic relationships (18). From a molecular point of view, pairwise sequence comparison of consensus sequences revealed a high sequence similarity (68.39%) between the satDNA from *M. fallax* and satDNA subfamily 2a of *M. chitwoodi* (5). From these data, it was hypothesized that *M. fallax* and *M. chitwoodi* may share a common ancestor and that *M. fallax* could be the result of a recent speciation event. Therefore, the cross-hybridization observed in the current experiment could reflect some ancestral subsequence common to both *M. fallax* and *M. chitwoodi*.

From a practical point of view, the squashed nematode procedure described here presents technical features that should be of interest for the development of a routine diagnostic test for the identification of *M. chitwoodi* and *M. fallax* versus the sympatric species *M. hapla*. First, the use of a nonradioactive labeling procedure is safe compared with the radioactive compounds generally used in hybridization experiments. Second, after hybridization against the blotted target, the hybridization solution still contains large amounts of unannealed DIG-labeled probe, which is stable for at least 1 year when stored at -20°C and can be reused several times. Third, because of the repetitive nature of satDNA, the probe can detect one single nematode, whatever its developmental stage (e.g., eggs and juveniles in egg masses; and females, even in root tissues) and without the need for any time-consuming DNA purification step. Therefore, it appears from this study that cloned satDNAs may constitute a powerful tool for the identification and management of *Meloidogyne* spp. populations in the field and for the implementation of quarantine regulations against these pests.

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