Analysis of a Satellite DNA from *Meloidogyne hapla* and Its Use as a Diagnostic Probe

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ABSTRACT

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A *Sty*I satellite DNA previously isolated from *Meloidogyne hapla* gave hybridization signals only with the five *M. hapla* populations tested in Southern blot experiments, indicating that this satellite sequence is species-specific. Because of its high level of reiteration and its variability, this sequence was able to discriminate both between *Meloidogyne* species and among three *M. hapla* populations. The *Sty*I satellite DNA was polymorphic at the intraspecific level, since hybridization patterns of *M. hapla* populations exhibited very different profiles with polymorphisms charac-

terizing each of the three populations tested. In simple squashed-nematode experiments, we were able to unambiguously identify *M. hapla* vs. *M. chitwoodi*, which are sympatric. The procedure was effective even on a single female located in root tissues, with the main advantage that it avoided time-consuming DNA extraction procedures. Therefore, in a case where confusion may occur among some particularly damaging nematodes, this very sensitive technique may prove to be reliable. Such satellite DNA sequences should provide rapid, inexpensive, and user-friendly field tools for *Meloidogyne* species identification.

Additional keywords: diagnostic marker, fingerprinting.

Species of the genus *Meloidogyne* are polyphagous and among the most destructive plant-parasitic nematodes. Crop loss due to *Meloidogyne* spp. is estimated to be 5% worldwide (23). Four species represent 95% of the root-knot nematodes observed in soils: *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; *M. javanica* (Treub, 1885) Chitwood, 1949; and *M. hapla* (Chitwood, 1949). *M. incognita*, *M. arenaria*, and *M. javanica* are from temperate to tropical regions, whereas *M. hapla* is largely encountered in cooler climates (15). In 1980, a new species was described, *Meloidogyne chitwoodi* (12), which damages potatoes grown in the temperate climates of the northwestern United States (11) and of Europe. *M. chitwoodi* resembles *M. hapla* and these two species are often sympatric.

The unambiguous identification of both species and populations of *Meloidogyne* is essential for successful management practices. Until now, characterization of *Meloidogyne* was based on analysis of morphological features, e.g., perineal pattern (8) and enzyme phenotyping, e.g., isoesterases (7). *M. chitwoodi* can be distinguished from *M. hapla* morphologically (12,19), by host range (19,22), and by isozyme analysis (9). These methods are more or less difficult to apply on a routine basis and do not allow discrimination at the intraspecific level among geographic populations. Recently, the value of recombinant DNA techniques in identifying *Meloidogyne* spp. has been demonstrated (13). The first studies were based on analysis of restriction fragment length polymorphism (RFLP) observed in agarose gel stained with ethidium bromide after digestion of genomic DNA with endonucleases (6). In a second step, labeled probes from nuclear and mitochondrial genomes were used. They allowed clear discrimination at both inter- and intraspecific levels but could not be used on a routine basis, since they were efficient only in time-consuming methods and required high amounts of DNA (5,14,21).

In a previous work, we isolated two highly reiterated *Sty*I tandemly arranged sequences from two root-knot nematode species: one from *M. incognita*, population Taiwan, and another

from *M. hapla*, population La Môle (20). They are represented by monomeric units of 295 bp and 169 bp at a reiteration frequency of approximately 4,300 and 15,000 copies, respectively, and comprise 5 and 2.5% of *M. hapla* and *M. incognita* genomes, respectively. These sequences classified as satellite DNA are generally localized in heterochromatin, and in spite of many hypotheses, no precise function has been demonstrated for them (18). We suppose that they play roles in chromosome structure, especially during chromosome pairing at the time of meiosis. As this part of the genome is usually nontranscribed, it escapes selective pressure and therefore contains an important polymorphism due to the accumulation of mutations (4). The heterogeneity between monomers is estimated at 3 and 3.5% for *M. hapla* and *M. incognita* tandemly arranged sequences, respectively.

In this study, we examined the species specificity of a tandemly arranged satellite sequence (20) from *M. hapla*, and we showed the usefulness of this sequence as a probe to identify *M. hapla* vs. *M. chitwoodi* in a simple procedure, avoiding time-consuming DNA extractions.

MATERIALS AND METHODS

Nematode isolates. *Meloidogyne* populations were maintained on potted tomatoes (*Lycopersicon esculentum* Mill. cv. St. Pierre) in a greenhouse at 20–25 C (Table 1). Eggs were extracted from infested roots (17), resuspended in 0.3 M NaCl and 0.7% streptomycin sulfate solution, and placed on a 10-μm-pore sieve at 20 C for 1 wk. The first solution was then replaced by a 0.7% streptomycin sulfate solution, and egg hatch started 3 days later and continued for about 2 wk. Every 3 days, juveniles were harvested by repeated washing on a 0.5-μm-pore sieve, concentrated by centrifugation at 2,000 g for 2 min in 30% sucrose solution, washed in distilled water, pelleted in a microcentrifuge, and stored at −80 C until use.

DNA extraction. An aliquot of each nematode population (100–200 μl) was frozen in liquid nitrogen and ground by mortar and pestle. DNA was extracted from the resulting powder after the lysis of cells by denaturing reagents (10 mM β-mercaptoethanol, 2.3% sodium dodecyl sulfate [SDS], and 0.5% triton)

according to a phenol/chloroform procedure (16). DNA was precipitated by adding 100% ethanol, resuspended in TE (0.01 M Tris, pH 8.0, and 0.001 M EDTA), and stored at −80 C.

Restriction enzyme digestion and gel electrophoresis. *Meloidogyne* DNAs were digested to completion by several enzymes (at least 4 h with 1 unit per microgram of DNA) according to the procedure specified by the manufacturer (Boehringer Mannheim, Meylan, France). Agarose gel electrophoresis was carried out in TBE (0.45 M Tris, 0.01 M EDTA, 0.45 M boric acid). DNA was visualized with ethidium bromide and UV transillumination.

Southern blotting and hybridization. After electrophoresis, DNAs were blotted by capillarity (24) onto nylon membranes (Amersham, Les Ulis, France), and labeled by the random oligonucleotide priming method (10) with [^{32}P] dCTP (Amersham). Hybridizations were conducted at high stringency (6× SSC [0.45 M NaCl and 0.0045 M citrate sodium], 5× Denhardt's [Ficoll 400 at 0.5 g/L, polyvinylpyrrolidine at 0.5 g/L, and bovine serum albumin at 0.5 g/L], and 0.5% SDS, 65 C). After hybridization, filters were washed at 65 C in 2× SSC, 0.1% SDS and then in 1× SSC, 0.1% SDS. After posthybridization washes, filters were exposed to X-ray film with an intensifying screen at −80 C. All hybridizations were repeated twice.

Hybridization of squashed nematodes. Squashed-nematode experiments were conducted on *M. hapla* populations Maasbree and Gilze, with population la Môle as positive control, and *M. chitwoodi* populations Rips and Valks, with *M. incognita* population Taiwan as negative control. For each population, one female, five females, one egg mass, and one gall were hand-picked and placed on a nylon membrane (Amersham). The nematodes were then ruptured by gentle pressure with a yellow, flat-tipped micropipet tip. Squashed materials were lysed by layering, successively, the filter on Whatman 3MM papers (Whatman International Ltd., Maidstone, England) soaked with 10% SDS (2 min), 0.5 M NaOH/2.5 M NaCl (two times, 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 baked (80 C, 1 h) (1). The prehybridization and hybridization were carried out as described for Southern blotting hybridization (65 C). The procedure was carried out three times to verify reproducibility.

RESULTS

***M. hapla* satellite DNA as a species-specific probe.** The characteristics of the two highly reiterated *Sty*I tandemly arranged sequences used in this study have been previously described (20). To test their specificity, the DNA of two populations belonging to each of the following species, *M. incognita*, *M. javanica*, and *M. arenaria*, and three populations of *M. hapla* were hybridized with a cloned monomeric unit representing these two tandemly arranged sequences. After hybridization with *M. hapla* La Môle satellite DNA, only the genomic DNA of the three *M. hapla* populations showed the presence of a ladder of multimers of the 169 bp *Sty*I repeats (Fig. 1), which is typical of the satellite sequences arranged in tandem arrays (25). The hybridization with

the monomeric unit of *M. incognita* Taiwan tandemly arranged sequence resulted in a non-species-specific signal, since all the populations belonging to *M. incognita*, *M. arenaria*, and *M. javanica* hybridized with this sequence (data not shown).

Molecular fingerprinting of *M. hapla* geographic populations with satellite DNA. Genomic DNA of *M. hapla* La Môle was digested by a set of endonucleases, and different types of patterns were obtained according to the enzyme used. The *Sau*3A digestion exhibited a regular ladder pattern where the monomer was absent. In the *Xba*I digestion, hybridization was observed with the monomer and also with the dimer, nanomer, and decamer (Fig. 2). The other restriction enzymes showed profiles with discrete bands corresponding to some multimers of the satellite sequence, but the patterns were all different with no evidence of regular ladder patterns (e.g., *Alu*I and *Msp*I).

When digested by *Sty*I and hybridized with the *M. hapla* satellite sequence, the genomic DNA of the three *M. hapla* populations showed regular ladder patterns (Fig. 1). However, Angleterre population hybridized less than La Môle and Frontignan populations and showed a strong band at 3.8 kb and another that was specific to this population at 3.2 kb (Fig. 1).

Other digestions provided different patterns of numerous discrete bands that varied among populations of *M. hapla*. A pronounced polymorphism was observed in the hybridization patterns of the same genomic DNA from *M. hapla* populations digested by *Alu*I and *Msp*I (Fig. 3). These digestions revealed patterns different from each other, and population-specific bands could be seen. In *Alu*I digestion patterns, a 3.6-kb band identified the La Môle population and a 4.2-kb band characterized the Frontignan population. The absence of hybridization from 1.2 to 4.7 kb separated the Angleterre population from the two others. In *Msp*I digestion patterns, a 2.3 kb and a 3 kb were characteristic for the La Môle population. The absence of hybridization from 1.6 to 4.7 kb for Frontignan and under 4.7 kb for Angleterre distinguished these two populations.

Satellite DNA as a potential field tool for *M. hapla* vs. *M. chitwoodi* discrimination. The species-specificity and reiteration of *M. hapla* satellite sequence allowed us to use it as a probe to differentiate two *M. hapla* isolates vs. two *M. chitwoodi* isolates, both originating from the Netherlands, directly on the biological

TABLE 1. Origin of *Meloidogyne* isolates used in this study

| *Meloidogyne* species	Origin
M. arenaria	Espiguette, France
M. arenaria	Monteux, France
M. chitwoodi	Rips, The Netherlands
M. chitwoodi	Valkx, The Netherlands
M. hapla	England
M. hapla	Frontignan, France
M. hapla	La Môle, France
M. hapla	Maasbree, The Netherlands
M. hapla	Gilze, The Netherlands
M. incognita	Taiwan
M. incognita	United States
M. javanica	La Réunion, La Réunion Island
M. javanica	Oualidia, Morocco
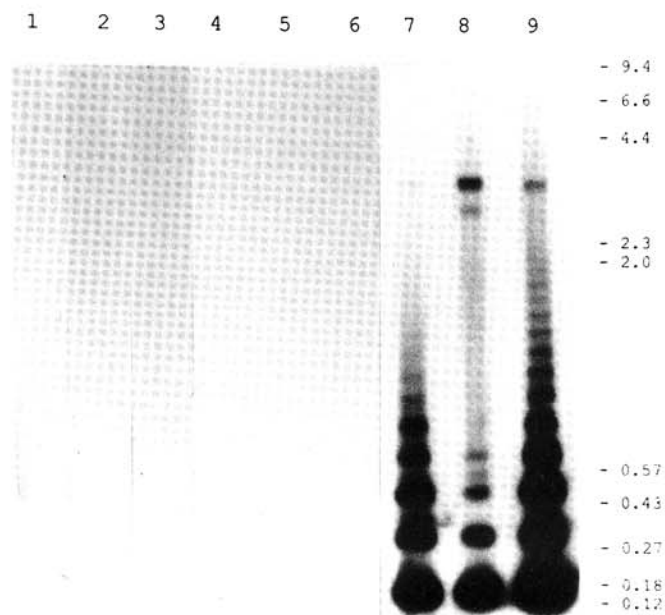
Fig. 1. Autoradiogram of a genomic Southern blot of nine *Meloidogyne* populations digested by *Sty*I and hybridized with ^{32}P-labeled *M. hapla* La Môle satellite DNA. *M. incognita* populations: lane 1, INCR2; lane 2, Taiwan. *M. arenaria* populations: lane 3, Espiguette; lane 4, Monteux. *M. javanica* populations: lane 5, La Réunion; lane 6, Oualidia. *M. hapla* populations: lane 7, La Môle; lane 8, Angleterre; lane 9, Frontignan. Numbers on the right refer to the size of molecular markers in kilobases.

material without any time-consuming DNA extraction procedures (Fig. 4). In squashed-nematode experiments, the tested populations of *M. hapla* gave a strong signal while the populations of *M. chitwoodi* never hybridized with the *M. hapla* satellite DNA. The intensity of the signal increased with the quantity of material (one female < five females < one egg mass). The galled root around the female did not prevent hybridization. The signal of *M. hapla* Maasbree was stronger than that of *M. hapla* Gilze.

The positive control, *M. hapla* La Môle, hybridized with its own satellite DNA. The negative control, *M. incognita* Taiwan, did not hybridize with this probe (Fig. 4). The hybridization of noninfected plant material was also negative (data not shown).

DISCUSSION

M. hapla La Môle satellite DNA generated a signal only with DNA from the five European populations tested. Therefore, this probe appeared to be species-specific. The *M. hapla* La Môle satellite probe also allowed us to distinguish among geographic populations. La Môle, Angleterre, and Frontignan gave very different patterns after digestion by *Alu*I and *Msp*I, and hybridization with *M. hapla* La Môle satellite DNA. In these cases,

the absence of regular ladder patterns suggests the accumulation of point mutations and unequal crossing-over that results in the generation or elimination of restriction sites within repeating units (3). This clear polymorphism is a direct consequence of the rapid evolution of this DNA fraction (4). If some polymorphism appeared between *M. hapla* populations digested with *Sty*I, a more pronounced polymorphism was shown after digestion by

Fig. 3. Autoradiogram of a genomic Southern blot of three *Meloidogyne hapla* populations digested by *Alu*I and *Msp*I and hybridized with ^{32}P-labeled *M. hapla* La Môle satellite DNA: lane 1, La Môle; lane 2, Frontignan; lane 3, Angleterre. Numbers on the right refer to the size of molecular markers in kilobases.
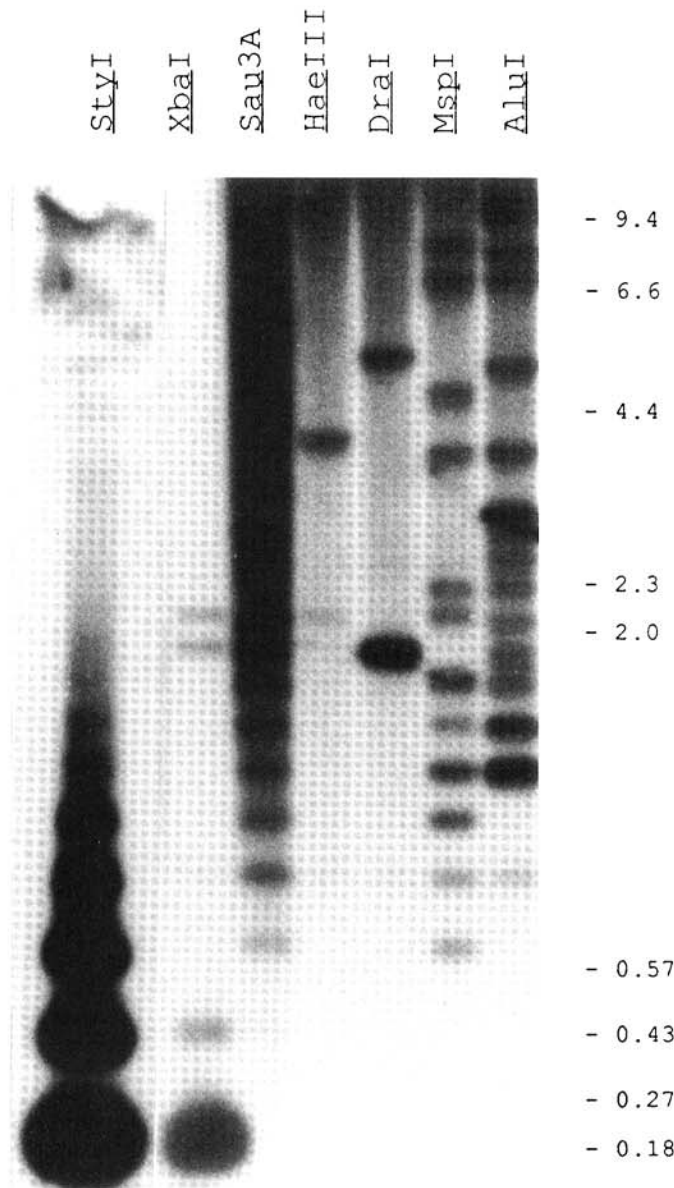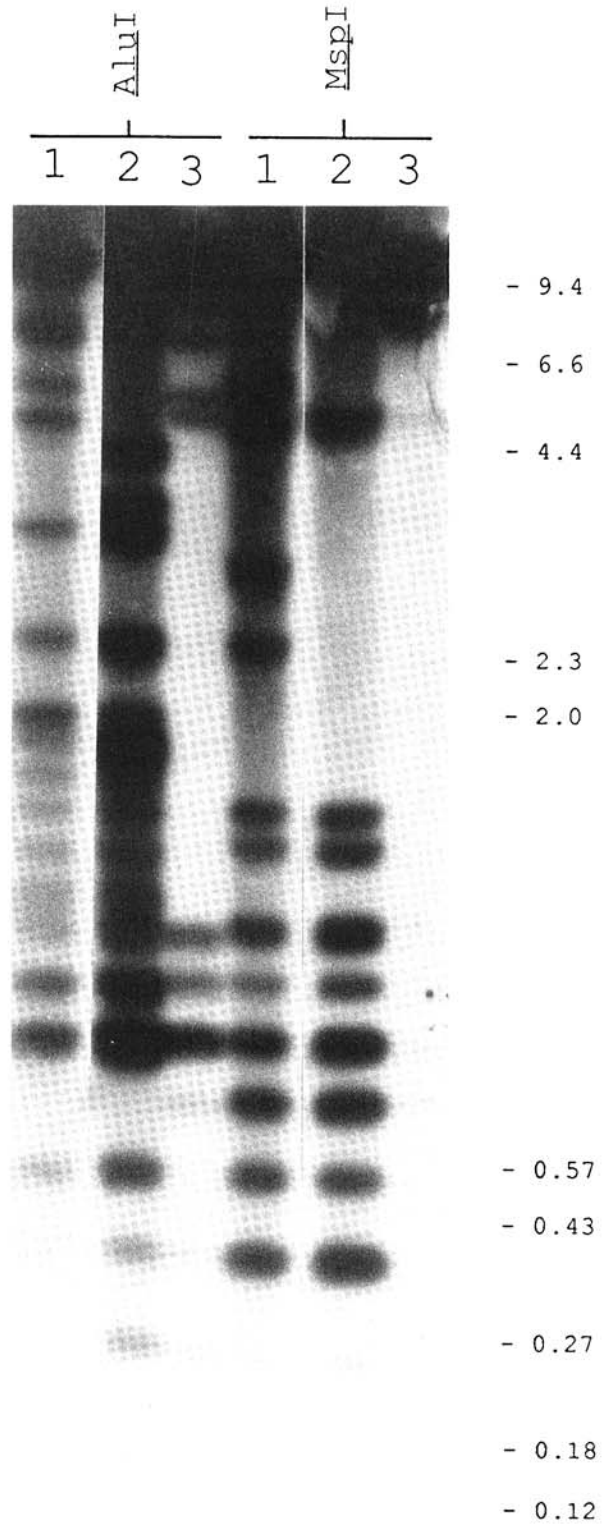
Fig. 2. Autoradiogram of a genomic Southern blot of *Meloidogyne hapla* La Môle digested by endonucleases indicated above lanes and hybridized with ^{32}P-labeled *M. hapla* La Môle satellite DNA. Numbers on the right refer to the size of molecular markers in kilobases.

*Alu*I and *Msp*I. La Môle and Frontignan profiles were quite different but closer to each other than they were to Angleterre profiles. From these data, two hypotheses could be developed: 1) this result is linked to the geographical localization of the tested isolates (Frontignan and La Môle are from geographically close origins and therefore could have derived from the same ancestral population), and 2) a relation exists between this result and the modes of reproduction of these populations (Angleterre is a parthenogenetic mitotic population while La Môle and Frontignan reproduce by meiotic parthenogenesis). These modes of reproduction certainly correspond to different mechanisms and rates of evolution, which could explain the result obtained. In fact, it would be possible to solve this problem if we could test French mitotic populations or English meiotic ones. Nevertheless, the fact that the *M. hapla* satellite DNA hybridized to DNA from a strictly mitotic parthenogenetic population of *M. hapla* as well as to DNA from facultative meiotic parthenogenetic populations strongly indicates that it might hybridize to DNA from all *M. hapla* populations. Moreover, the fact that the satellite DNA from *M. hapla* hybridized with both meiotic and mitotic parthenogenetic *M. hapla* species that are thought to have diverged a long time ago indicates the stability of the polymorphisms observed and thus the real utility of using this DNA fraction for population discrimination. Furthermore, it is well documented that satellite DNA can serve as an excellent tool for taxonomic studies and molecular diagnostic (see 2 for review).

Distinguishing *M. hapla* from *M. incognita, M. arenaria,* and *M. javanica* is not of significant interest because these four species are easily identifiable. Furthermore, *M. hapla* occurs under cooler climate than these three other species. However, it is of agronomic interest to discriminate between *M. hapla* and *M. chitwoodi* since only one of these two sympatric species is very damaging to potato crops in the temperate climates of the northwestern United States and of Europe. To develop a reliable and rapid diagnostic assay to discriminate these two species, we set up a squashed-nematode experiment using the satellite DNA from *M. hapla* as a specific probe. This assay allowed us to unambiguously separate *M. hapla* from *M. chitwoodi*. The variation noticed in the hybridization signal intensity between the two *M. hapla* isolates from the Netherlands (Fig. 4) probably reflects some variations in the experimental conditions instead of a different genetic background between these two populations. The principal advantage of this procedure is to avoid time-consuming DNA extraction procedures. Because of the high-power of sequence detection of the satellite DNA, this sequence is able to detect one individual in a simple squashed-nematode experiment, even in root tissues. Due to the high resolution of such a squash blot hybridization, this satellite DNA could then be developed into a specific non-radioactive probe. Moreover, as the experimental procedure is very easy and not time-consuming, it should be possible to introduce it into field work without the need for a well-equipped laboratory. From this study, it appears that cloned satellite DNA theoretically represents a powerful tool to demonstrate polymorphism between closely related species for which specific markers are needed.

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	1 female	5 females	1 egg mass	1 gall
M. incognita Taïwan				
M. hapla La Môle	●	●	●	●
M. chitwoodi Rips				
M. chitwoodi Valks				
M. hapla Maasbree	●	●	●	●
M. hapla Gilze	●	●	●	●

Fig. 4. Squashed-nematode experiments. Squashed *Meloidogyne hapla* and *M. chitwoodi* material was hybridized with ^{32}P-labeled *M. hapla* La Môle satellite DNA.

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