Molecular characterization of species and populations of *Meloidogyne* from various geographic origins with repeated-DNA homologous probes

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Summary — Sixteen populations from various geographic origins, mostly belonging to the four economical major species of *Meloidogyne*, were characterized by analyzing the restriction fragment length polymorphism obtained after digestion and hybridization of their total DNA with labelled homologous probes. These probes were repeated sequences isolated at random from a genomic library constructed with DNA of *M. incognita* " Côte d'Ivoire " juveniles. The results revealed taxonomically useful differences in the number and position of restriction fragments, both between species and populations. Moreover, we have observed species-specific profiles for the three main species, *M. incognita, M. arenaria* and *M. javanica*.

Résumé — *Caractérisation moléculaire d'espèces et de populations de* Meloidogyne *d'origines géographiques variées à l'aide de sondes homologues d'ADN génomique répété* — Seize populations de *Meloidogyne*, appartenant principalement aux quatre espèces d'importance économique majeure, ont été caractérisées par l'analyse du polymorphisme de restriction obtenu après digestion et hybridation de leur ADN total, avec des sondes homologues marquées radioactivement. Celles-ci sont des séquences répétées, isolées au hasard à partir d'une banque génomique construite avec l'ADN de larves de *M. incognita* « Côte d'Ivoire ». Les hybridations réalisées avec trois de ces sondes révèlent des différences au sein des profils de restriction, exploitables sur le plan taxonomique, tant au niveau intra- qu'interspécifique. De plus, des profils caractéristiques de chacune des principales espèces, *M. incognita, M. arenaria* et *M. javanica*, ont été mis en évidence.

Species of the genus *Meloidogyne* are among the most destructive plant-parasitic nematodes. Their unambiguous identification is essential for successful management practices which could be optimized if both species and populations are characterized. As the most common *Meloidogyne* species reproduce by parthenogenesis, reproductive isolation cannot be used as a criterion to define these species. Still now, characterization of *Meloidogyne* is mostly based on minor morphological and anatomical features of second stage juveniles, males and females (Eisenback, 1985). At this level, *Meloidogyne* species identification is a formidable and time-consuming task, even for well-qualified taxonomists. Biochemical studies have demonstrated that the major species of *Meloidogyne* can be differentiated by species-specific enzyme phenotypes revealed by polyacrylamide gel electrophoresis, e.g. the isoesterases (Dalmasso & Bergé, 1978) or the malate dehydrogenases (Esbenshade & Triantaphyllou, 1990). Intraspecific characterization can also be reached by this method but it consists in the analysis of minor bands inside proteic patterns (Janati *et al.*, 1982). Morphological features are subject to phenotypic variations (Whitehead, 1968; Franklin, 1979), while proteins, or any other expression product, only allow the investigation of 15 to 20 % of the genetic

material. Consequently, only a small fraction of the potentially useful variation between nematode species and populations is available for study by such techniques. Direct analysis of nematode nucleic acids avoids both of those problems.

Recently, the value of recombinant DNA techniques to identify *Meloidogyne* species has been demonstrated (see Hyman, 1990, for a review). 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 several restriction enzymes (Curran *et al.*, 1985). In this case, RFLP appears as bright bands that represent only the most repeated sequences in the genome. But the possible polymorphism in the less abundant sequences is not detectable this way and must be vizualised using labelled probes. Some nematode genera have been characterized at inter- or intraspecific level according to this approach, by analysis of their nuclear DNA (Curran & Webster, 1987; Burrows & Perry, 1988) or mitochondrial DNA (Powers *et al.*, 1986), but no results are available concerning the discrimination of *Meloidogyne* populations at intraspecific level with cloned genomic DNA probes.

In a preliminary study, we demonstrated a clear

polymorphism among *M. incognita* populations using a single homologous DNA probe (Castagnone-Sereno *et al.*, 1991). In this work, our purpose is to show precise and reliable characterization of sixteen populations of *Meloidogyne* from various geographic origins, at inter- and intraspecific levels by direct analysis of DNA using several homologous labelled probes. The results are discussed through their potential agronomic applications and their significance to the organization and evolution of the *Meloidogyne* genome.

Materials and methods

NEMATODE ISOLATES

Meloidogyne populations were maintained on potted tomatoes (*Lycopersicon esculentum* cv. St Pierre) in a greenhouse at 20-25 °C. The name and geographic origin of each population are reported in Table 1. " Pikine " and " Concarneau " are not yet described but presumed to be two new species according to their morphometrics and electrophoretic patterns. Eggs were extracted from infested roots and collected (Mc Clure *et*

Table 1. Name and geographic origin of *Meloidogyne* populations.

Code	Name	Origin	Species
1	Côte d'Ivoire	Ivory Coast	*M. incognita*
2	Taiwan	Taiwan	*M. incognita*
3	Valbonne	France	*M. incognita*
4	Guadeloupe	French West Indies	*M. incognita*
5	Race 1 NSCU 83	USA	*M. incognita*
6	Race 2 NSCU E1135	USA	*M. incognita*
7	Calissane	France	*M. incognita*
8	Monteux	France	*M. arenaria*
9	Espiguette	France	*M. arenaria*
10	Ain Taoujdate	Morocco	*M. arenaria*
11	Oualidia	Morocco	*M. javanica*
12	La Reunion	La Reunion Island	*M. javanica*
13	Pikine	Sénégal	undescribed
14	Concarneau	France	undescribed
15	La Môle	France	*M. hapla*
16	Séville	Spain	*M. hispanica*

al., 1973). They were suspended in 0.3 M NaCl, 0.7 % streptomycin sulfate solution and placed on 10 µm-pore-sieve at 20 °C for a week. The saline solution was replaced by 0.7 % streptomycin sulfate solution and egg hatching started three days later and lasted for about two weeks. Second stage juveniles were washed on a 0.5 µm-pore-sieve, pelleted in a microcentrifuge and stored at − 80 °C until use.

DNA EXTRACTION

An aliquot of each nematode isolate (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 % SDS, 0.5 % triton), according to a phenol/chloroforme procedure (Maniatis *et al.*, 1982), precipitated by adding 2.5 volumes of 100 % ethanol and resuspended in TE (0.01 M Tris pH 8.0, 0.001 M EDTA) before storage at − 80 °C.

SOUTHERN BLOTS AND HYBRIDIZATION

DNAs of *Meloidogyne* populations, bacteriophage clones and plasmid clones were digested using restriction endonucleases (Boehringer Mannheim). RNase A treatment (0.5 mg/ml) was conducted after digestion. The fragments of DNA were separated in 1 % agarose gel by electrophoresis. DNAs were then transferred onto nylon membranes (Hybond N +, Amersham) according to a modified Southern procedure (Southern, 1975; Smith & Summers, 1980). Labelling was realized by nick translation (Rigby *et al.*, 1977) or random oligonucleotide primer method (Feinberg & Vogelstein, 1983) with ^{32}P dCTP. Hybridizations were conducted at high stringency (6X SSC, 5X Denhart's, 0.5 % SDS, 65 °C) during twelve hours and revealed by autoradiography (X-Ray Film, − 80 °C, with intensifying screen).

HOMOLOGOUS REPEATED PROBES ISOLATION

The genomic library was prepared in bacteriophage λ EMBL 3 with DNA of *M. incognita* " Côte d'Ivoire " juveniles as follows. One µg of λ EMBL3 DNA was precipitated together with 0.4 µg of 15 to 25 kilobases (kb) fragments produced by partial digestion of nematode genomic DNA with Sau3A restriction enzyme. The ligation was done at room temperature for 4 hours. After ethanol precipitation, ligated DNA was packaged using a λ DNA *in vitro* packaging kit (Amersham). The resulting phages were plated on *Escherichia coli* strains Q 359 and C 600. This library was screened with the labelled DNA of *M. incognita* " Côte d'Ivoire " by *in situ* hybridization (Benton & Davis, 1977). After autoradiography, 40 clones that contained fragments of weak, medium and high repetitivity were selected at random on the basis of the intensity of the signal produced. To identify potential probes, each bacteriophage DNA was digested to completion with BamHI, EcoRI and HindIII endonucleases, fractionated by 1 % agarose gel electrophoresis and transferred onto nylon membranes. After hybridization of the filters with labelled genomic DNA of the *Meloidogyne* " Côte d'Ivoire " population, seven fragments were chosen to be used as probes. These fragments were recovered from gel by electrophoresis through DEAE paper (Dretzen *et al.*, 1981).

SUB-CLONING STRATEGY

The fragments of interest were amplified by sub-cloning (Maniatis *et al.*, 1982). Phagous DNAs that contained the fragments used as probes were ligated to plasmid vector pUC 18. Plasmids that integrated DNA inserts were amplified in *Escherichia coli* strain NM 522. Transformed cells were selected on ampicillin-agar growth media (100 µg/ml) in presence of X-Gal (25 mg/ml) and IPTG (25 mg/ml). The inserts of re-combinant plasmids were identified by restriction with appropriate endonucleases.

Results

Seven probes were isolated, labelled and tested on Southern blots of the Bam HI-digested DNAs of *Meloidogyne* populations. Three of them that consisted of mid-repetitive sequences according to the radioactive signal yielded on the autoradiograms (data not shown), were sub-cloned. They were named pMiK4, pMiK13 and pMiK18 and were respectively restricted by Eco RI/Bam HI, Eco RI/Bam HI and Eco RI/Hind III. Their sizes, determined by comparison to known size marker

DNA, were 1.7 kb for pMiK4, 0.9 kb for pMiK13 and 0.8 kb for pMiK18. The hybridizations were performed twice with twelve populations of *Meloidogyne* for pMiK4 and with sixteen populations for pMiK13 and pMiK18. These fragments, used as probes, showed inter- and intraspecific polymorphism expressed in number and position of bands in the restriction profiles (Fig. 1). The reading of these profiles was possible between about 0.1 and 9 kb.

The hybridization with pMiK4 showed two bands common to *M. incognita*, *M. javanica*, *M. arenaria* and *M. hispanica* at 1.6 and 3 kb, and allowed the species to be distinguished by the presence of, for example, a characteristic band for *M. arenaria* at 3.5 kb, one for *M. incognita* at 2 kb, one for *M. javanica* at 0.7 kb and another at 1.5 kb for *M. hispanica* (Fig. 1). *M. hapla* and the undescribed isolates, " Pikine " and " Concarneau ", exhibited very different profiles from that of other species. *M. hapla* had a very poor profile in regard to the number of bands (only one at 4 kb) and the intensity of hybridization. The restriction pattern obtained using pMiK13 was closed to that obtained with pMiK4 in the number and position of the bands (Fig. 2). It also
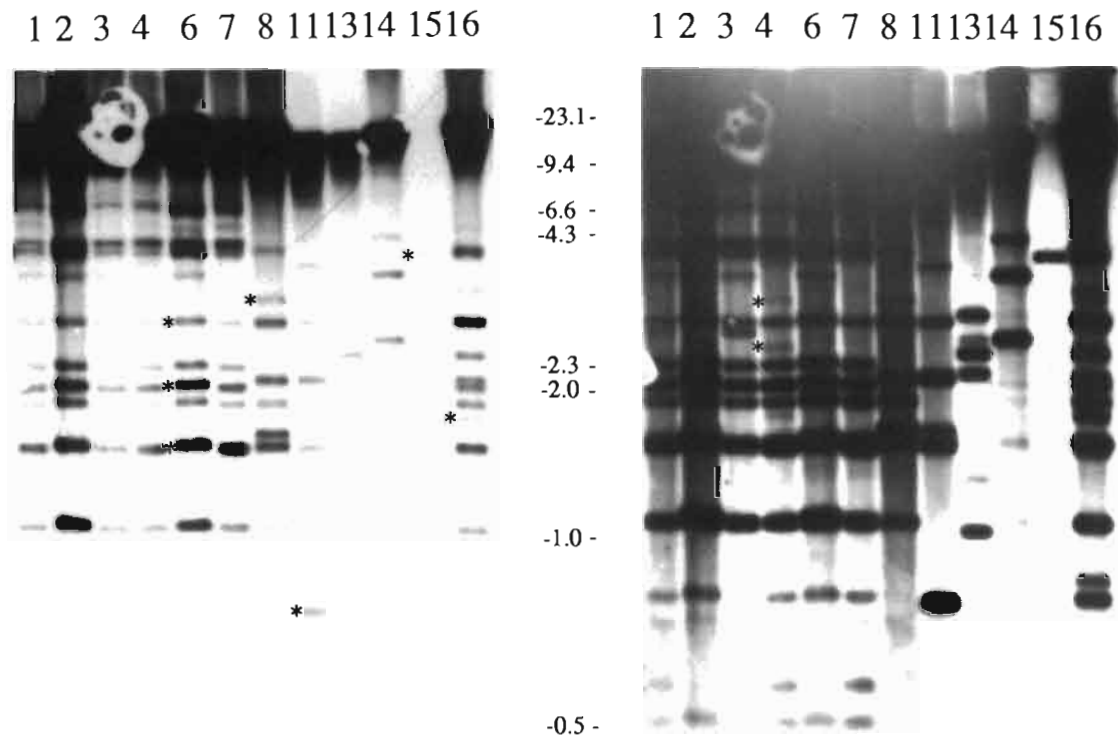

Fig. 1. Hybridization of Southern blot of the Bam HI-digested DNAs of twelve *Meloidogyne* populations with pMiK4. — Left : Exposure time 24 h. — Right : Exposure time 7 days. (Molecular weight markers are indicated in kilobases [kb]. Numbers correspond to *Meloidogyne* populations as reported in Table 1. All bands mentionned in results are indicated by a star.)
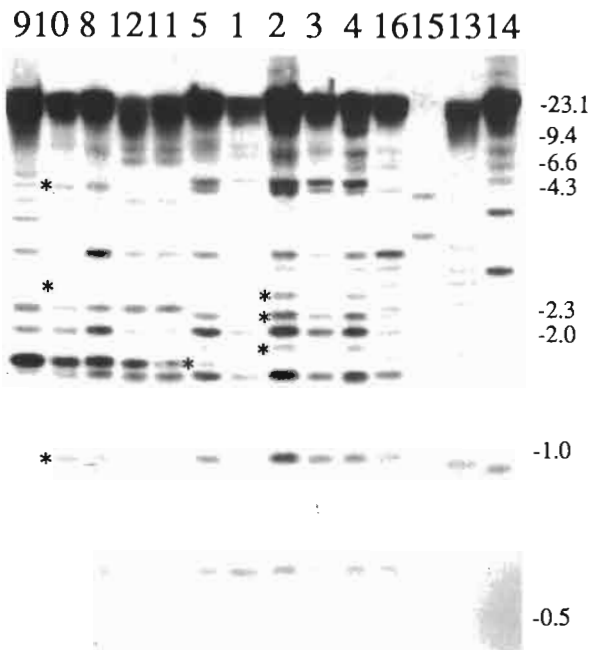
Fig. 2. Hybridization of Southern blot of the Bam HI-digested DNAs of fourteen *Meloidogyne* populations with pMiK13. (Molecular weight markers are indicated in kilobases [kb]. Numbers correspond to *Meloidogyne* populations as reported in Table 1. All bands mentionned in results are indicated by a star.)
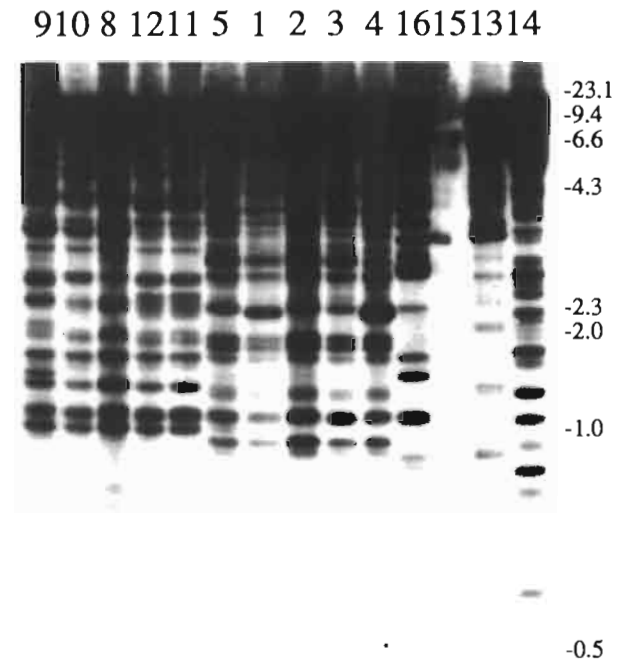

Fig. 3. Hybridization of Southern blot of the Bam HI-digested DNAs of fourteen *Meloidogyne* populations with pMiK 18. (Molecular weight markers are indicated in kilobases [kb]. Numbers correspond to *Meloidogyne* populations as reported in Table 1. All bands mentionned in results are indicated by a star.)

permitted separation of *M. arenaria* from *M. javanica* with bands at 0.8 and 4.3 kb in the *M. arenaria* profile. *M. incognita* profiles showed specific bands at 1.8 and 2.3 kb. Previous observations about *M. hapla* and the undescribed isolates remained valuable with pMiK13 hybridization. The observation of pMiK18 pattern led to the same conclusions than those deduced from the pMiK4 and pMiK13 ones, but this hybridization brought out a pronounced similarity between *M. arenaria* and *M. javanica* profiles (Fig. 3).

Even if all the possible probe-against-populations combinations are not shown on the figures, pMiK4, pMiK13 and pMiK18 hybridized with all tested populations. Two or more populations were assayed for *M. incognita*, *M. arenaria* and *M. javanica* and a set of species-specific bands was observed for each species. For *M. incognita*, it was constituted by ten bands with pMiK4, thirteen with pMiK13 and eighteen with pMiK18. However, in most cases, each population showed its own profile with differential bands between populations of one species. In those sets of differential bands, one-population-specific bands could be distinguished from bands which were common to several but not all populations. For example, on pMiK4 hybridization, " Guadeloupe " was the only *M. incognita* popu-

lation to have bands at 2.4 and 2.8 kb. On pMiK13 hybridization, " Ain Taoujdate " was the only *M. arenaria* isolate to have a 2.5 kb band, and only " Race 1 ", among *M. incognita* populations, showed a strong band at 1.6 kb. The polymorphism between populations revealed by pMiK18 was difficult to analyze because of the large number of bands exhibited by the hybridization pattern. However, some results were available with this probe : *M. hapla* weakly hybridized and the *M. javanica* tested populations seemed to have the same profile.

Discussion

Southern blots of the digested DNAs of *Meloidogyne* populations, probed with pMiK4, pMiK13 or pMiK18, revealed clear inter- and intraspecific polymorphisms and thus confirmed previous preliminary observations (Castagnone-Sereno *et al.*, 1991). Due to the repetitive nature of the probes, the autoradiograms exhibited extensive RFLP, especially the one obtained by pMiK18 hybridization. Even if a large number of bands complicates the reading of the profiles, it emphasizes the validity of the results, easier to analyse. In this connexion, the choice of mid-repetitive sequences to analyse

DNA polymorphism if of obvious interest. With respect to species identification, our results are in agreement with the previous morphological and biochemical studies. Considering their hybridization profiles, the undescribed isolates from Pikine and Concarneau seem to belong to two new species, this hypothesis is emphasized by their morphometrics (Germani, pers. com.) and isoesterase patterns (Dalmasso & Bergé, 1983; Berthou *et al.,* 1990).

RFLP is becoming a useful tool for the accurate characterization of plant pathogens, not only to identify nematodes but also microorganisms like fungi (Hamer *et al.,* 1989; Manicom *et al.,* 1990) or bacteria (Leach *et al.,* 1990). This recombinant DNA-based approach has several distinct advantages over previously described techniques. Because of the direct analysis of the genotype, it avoids all diagnosis bias due to phenotypic variations and opens a large field for inquiry. In this connexion, all our autoradiograms gave a strong signal obtained from unresolved high molecular weight fragments located at the top of the gels. Polymorphism can also exist in that region that could be revealed by recently developed pulsed field electrophoretic methods (Carle *et al.,* 1986; Gardiner *et al.,* 1986). The hybridization techniques we used, with anonymous DNA fragments, are inherently capable of great discrimination power, showing clear polymorphism between populations of one species, which is an original result for the genus *Meloidogyne.*

This kind of results could be directly applied to agronomy by the production of kits for rapid and reliable identification of *Meloidogyne* species and populations, envisionned after validation of diagnostic DNA fragments and standardisation of procedures. We could also isolate fragments identified as markers of biological features e.g. the virulence against the resistance-genes of plants.

In another field of investigation, hybridizations of total DNA with labelled probes might give precious informations about the phylogeny of *Meloidogyne.* Precise analysis of restriction patterns could allow us to establish interspecific phylogenetic relationships in this taxon. For example, it is obvious that *M. hapla,* which hybridizes weakly with all the probes, is phylogenetically distant from other species. This statement is confirmed by the fact that *M. hapla* reproduces by facultative meiotic parthenogenesis, instead of the obligatory mitotic parthenogenesis of the other tested species.

When two or more populations of one species were assayed, in each case, another original result is observed, consisting of a set of species-specific bands that permitted to define a species-specific profile for *M. incognita* and to envisage the existence of such profiles for *M. arenaria* and *M. javanica.* To an evolutionary extent, the species-specific profiles are quite exciting. That result is probably linked with the parthenogenetic mode of reproduction of the tested species. In fact, it seems that the genome of parthenogenetic species changes in particular ways that permit these organisms to evolve as amphimictic species do (King & Schall, 1990). These particular mechanisms of genetic variation are not yet well known. They could result from transposable elements activity (Richards, 1989), chromatids exchanges (Tartof, 1974; Szostak & Wu, 1980) and/or amplification of repeated sequences i.e. the ribosomal DNA (Bostock, 1986). Mechanisms of regulation also exist that prevent unadapted evolution (Dover, 1982; Dover *et al.,* 1982). Nevertheless, genomic modifications should preserve a form of stability in the genome of *Meloidogyne* which is revealed by the species-specific profile. Analysis with homologous probes of DNA of some obligatory amphimictic (i.e. *M. carolinensis*) and meiotic parthenogenetic species (i.e. *M. hapla, M. naasi*), should provide more information in that particular area. The possible linkage between the species-specific profile and the mode of reproduction could then be demonstrated.

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