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The use of a digoxigenin-labelled synthetic DNA oligonucleotide for the rapid and sensitive identification of *Meloidogyne incognita*

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Summary – Accurate identification of *Meloidogyne* species is essential for effective control and management strategies. Variable morphology and the existence of intraspecific races make most current diagnostic procedures difficult and unreliable. This work describes the use of a 30 base non-radioactive digoxigenin-labelled synthetic oligonucleotide to identify *M. incognita* in Slot Blot assays. The sensitivity of this approach is such that it is capable of detecting as little as 2 ng of *M. incognita* genomic DNA. Preliminary attempts to characterise the 50 base *M. incognita* specific probe on which this oligonucleotide was based indicated that it could represent approximately 1 % of the haploid genome of *M. incognita* and is repeated 17 000 times.

Résumé – ***Utilisation d'un oligonucléotide synthétique marqué à la digoxigénine pour l'identification rapide et aisée de* Meloigogyne incognita** – Le contrôle effectif et les stratégies de régulation des espèces de *Meloidogyne* requièrent une identification précise. La variabilité morphologique de ces nématodes et l'existence de races intraspécifiques rendent difficile l'usage de la plupart des techniques d'identification, et leurs conclusions douteuses. Cette étude décrit l'utilisation d'un oligonucléotide synthétique de 30 bases, non radioactif, marqué à la digoxigénine, pour identifier *M. incognita* dans des tests " slot blot ". La sensibilité de cette technique est telle qu'elle est capable de détecter une quantité aussi faible que 2 ng d'ADN génomique de *M. incognita*. Des essais préliminaires visant à caractériser les 50 bases de la sonde spécifique à *M. incognita* dont a été extrait l'oligonucléotide indiquent qu'elle est répétée 17 000 fois, et pourrait représenter environ 1 % du génome haploïde de *M. incognita*.

Key-words : Nematodes, *Meloidogyne,* identification, digoxigenin, oligonucleotide.

Reliable identification of *Meloidogyne* species is an essential prerequisite for effective management. Accurate diagnosis of root-knot nematodes has generally relied on measurements and observations of anatomical features such as perineal patterns and stylet structure (Eisenback *et al.*, 1980; Hartman & Sasser, 1985) or on host-range testing (Hartman & Sasser, 1985). However, variable morphology and the occurrence of intraspecific races that can only be identified by reproductive performance on defined hosts have confounded diagnostic procedures (Taylor & Sasser, 1983; Eisenback, 1985).

Proteins, carbohydrates and lipids have been used with mixed success to characterise *Meloidogyne* species and races (Hussey, 1979). More recently, serological methods that were once dismissed as too cross-reactive are enjoying a revival with the introduction of monoclonal antibody technology (Jones *et al.*, 1988; Hussey, 1989). This approach in particular promises to be very discriminating. Although useful, all these techniques have the risk of being specific to a particular stage in the nematode life cycle (Robinson *et al.*, 1989). Direct analysis of nematode nucleic acids avoids this problem. DNA probes provide an unambiguous, rapid and inexpensive method of diagnosis and have been used successfully for the diagnosis of animal, human and plant parasites (Burrows & Perry, 1988; Barker, 1989; Gárate *et al.*, 1990). Furthermore, synthetic oligonucleotides, which can be made cheaply in large amounts, offer important advantages as probes and these too have been employed successfully (McLaughlin *et al.*, 1985; Harnett *et al.*, 1989). Although assays using oligonucleotides are sensitive, they are most often based on the use of radioactive isotopes which have the disadvantages of short half-lives, radiation exposure to the investigator and waste disposal problems. Adoption of non-radioactive techniques would expand the usefulness of oligonucleotides by allowing routine use in the field.

Here we describe the use of a 30 base synthetic oligonucleotide, derived from a 50 base *M. incognita* specific probe described previously (Chacón *et al.*, 1991), coupled directly to digoxigenin via a spacer-arm, for the detection of *M. incognita* DNA.

Materials and methods

Nematode populations

Populations of *Meloidogyne* were maintained at AFRC Institute of Arable Crops Research (IACR) Rothamsted and had the following origins : *M. incognita* Race 1 (originally from North Carolina State University : NCSU) (NCSU n° 78), *M. incognita* Race 2 (NCSU n° 1135), *M. incognita* Race 3 (NCSU n° 285), *M. incognita* Race 4 (NCSU n° 401), *M. javanica* (NCSU ex, USA), *M. arenaria* (NCSU n° 352), *M. graminicola* (NCSU, Bangladesh) and *M. hapla* (NCSU, Wageningen). Two other plant parasitic nematodes genera were used, *Heterodera glycines, H. trifolii* and *Globodera rostochiensis* Ro_1, *G. rostochiensis* $Ro_{2/3}$, *G. pallida* and *G. tabaccum* all from the cyst nematode collection at Rothamsted. Eggs and second stage juveniles were collected and prepared for DNA extraction as detailed (Chacón *et al.*, 1991).

Extraction of DNA

DNA was prepared from 0.2-0.5 ml of packed juveniles essentially as described by Simpson *et al.* (1982), with the exception that the purification step by CsCl centrifugation was replaced by phenol extraction and ethanol precipitation (Sambrook *et al.*, 1989).

Synthesis of oligonucleotides

Four oligonucleotides were prepared on an Applied Biosystems 380 B instrument (used according to the manufacturer's instructions). The oligonucleotides had the following sequences : MR1 n° 15.2.1 (5'-TGTAGACAGTAGGGGAAACTA-3'), MR1 n° 15.2.2 (GACCCCCCAAATGACCCCCCAACCCCCCCA-3'), MR1 n° 15.2.3 (5'-ATGACCCCCCAACCCCCCCCAG-3'), MR1 n° 15.2.4 (5'-GTTGTTCCCCCCGCGCCCACG-3'). Deprotection was carried out using ammonia solution at 55 °C for 16 h. The product was precipitated at – 70 °C, for 20 min, with 0.3 M sodium acetate.

Slot blot technique

DNA samples (1000 to 1 ng) from the above mentioned *Meloidogyne, Globodera* and *Heterodera* species were denatured by the addition of 200 µl of 0.3 M NaOH followed by incubation in a water bath at 80 °C for 10 min and cooled on ice. Neutralisation of the samples were carried out at room temperature with 300 µl of a neutralising solution (0.2 M Tris-HCl pH 7.4, 0.25 M HCl, 12.5 × SSC). The neutralised DNA samples (500 µl) were applied to nitrocellulose filters (Schleicher & Schuell) using a Shleicher and Schuell manifold slot blot system, according to the manufacturer's instructions. After blotting filters were air dried and baked on a vacuum oven at 80 °C for 2 h. At this stage they could either be used immediately, or stored desiccated at 4 °C for up to 6 months.

Incorporation of digoxigenin or ^{32}P into the *M. incognita* oligonucleotides

The oligonucleotides derived from the 50 base *M. incognita* specific probe (Chacón *et al.*, 1991) were labelled at the 3'-end via a spacer-arm by incorporation of a single digoxigenin-labelled dideoxyuridine-triphosphate (Dig-ddUTP), using the Dig-Oligonucleotide 3'-end labelling kit from Boehringer Mannheim and following the manufacturer's directions.

The specific oligomer was ^{32}P end-labelled using ^{32}P-dATP and T4 Kinase (Maxam & Gilbert, 1980) to an specific activity 3.2×10^5 Bq/ml.

Detection of *M. incognita* DNA with a digoxigenin labelled probe

Prehybridisation and hybridisation of the filters were performed in sealed plastic bags only slightly larger than the filters. Washing the filters required larger volumes of solution and could be carried out satisfactorily in plastic trays.

Nitrocellulose filters were placed in a plastic bag containing 10 ml of prehybridisation buffer (5 × SSC, 4 × Denhardt's, 0.1 % N-lauroylsarcosine, 0.02 % SDS, 50 µg/ml of denatured salmon sperm DNA) and incubated at 68 °C for 1 h. Prehybridisation solution was replaced by 2 ml of the same solution. The probe was heated at 100 °C for 5 min and added to the 2 ml of prehybridisation solution to give a final concentration of 50 pmol/ml. The bag was sealed and incubated at 52 °C for 6 h with gentle shaking. After hybridisation the filters were washed 2 × 5 min in 2 × SSC, 0.1 % SDS at 52 °C and 2 × 5 min in 0.1 × SSC, 0.1 % SDS at 52 °C.

Washed filters were incubated in blocking buffer (4 × Denhardt's, 3 % BSA) at room temperature for 30 min. A preformed complex of antibody conjugate to the chromogenic enzyme alkaline phosphatase was then added in a dilution (1 : 5000) and incubated at room temperature for 30 min. The filters were washed at room temperature 2 × 15 min in washing buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and finally rinsed briefly in staining buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Bound alkaline phosphatase was detected by staining the filters in freshly made solution of nitroblue tetrazolium (NBT) and 5-bromo-4 chloro-3-indolyl phosphate (BCIP) (0.33 mg/ml NBT, 0.175 mg/ml BCIP in staining buffer). Colour was allowed to develop in the dark for 5 min to 3 h. Once developed, the filters were rinsed in distilled water and allowed to air dry.

Determination of copy number by using ^{32}P labelled 30 base (MR1 n° 15.2.2) specific probe

The copy number of the specific oligonucleotide was determined by screening slot blots of serially diluted *M. incognita* Race 1 DNA and pMR1 n° 15 plasmid (Chacón *et al.*, 1991) with the ^{32}P end-radiolabelled 30 base

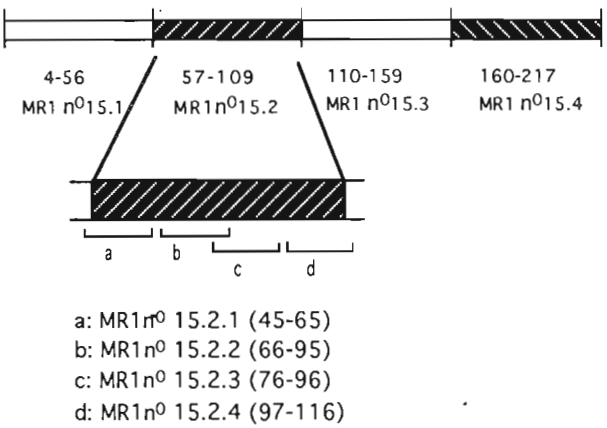

Fig. 1. Schematic distribution and selection of *Meloidogyne incognita* synthetic oligonucleotides along the MR1 n° 15.2 probe (Chacón *et al.*, 1991).

oligonucleotide. Prehybridisation, hybridisation and washings of the filters were performed as described by Chacón *et al.*, (1991).

Results

Detection of *M. incognita* DNA with digoxigenin-labelled DNA oligonucleotides

The ability of the digoxigenin labelled MR1 n° 15.2.2 oligonucleotide to detect the presence of *M. incognita* DNA in slot blot assays was demonstrated after 30 min of colour development (Fig. 2 a-d). Cross reaction with the morphologically similar species *M. javanica, M. arenaria, M. graminicola* and *M. hapla* (Fig. 2 e-h) or host DNA (data not shown), was not observed and no colour development was observed with DNA from the most distantly related species from the genera *Globodera* and *Heterodera* (Fig. 2 i-n).

The rest of the oligonucleotides (Fig. 1) (MR1 n° 15.2.1, MR1 n° 15.2.3 and MR1 n° 15.2.4) failed to recognize the DNAs assayed (data not shown).

Comparison of sensitivity of (MR1 n° 15.2.2) labelled with digoxigenin or ^{32}P

In order to further characterise (MR1 n° 15.2.2) serial dilutions (60 to 1 ng) of *M. incognita* Race 1 total DNA were bound onto nitrocellulose and hybridised with the Dig-ddUTP labelled oligonucleotide. After 3 h of color developing MR1 n° 15.2.2 could detect as little as 2 ng of purified DNA (Fig. 3 A).

To compare the sensitivity of the digoxigenin labelling with the radioisotopic labelling an identical blot was hybridised with the ^{32}P labelled MR1 n° 15.2.2. After four washings at 52 °C, the filter was exposed to a X-ray film with an intensifying screen for 24 h. The results

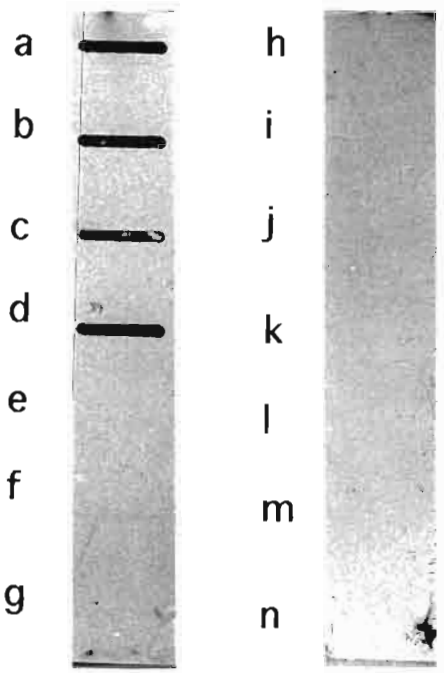

Fig. 2. Specificity studies of MR1 n° 15.2.2 digoxigenin la belled synthetic oligonucleotide by genomic DNA hybridisation : A sample of 500 ng of each of the following genomic DNAs were denatured, neutralised and applied directly to nitrocellulose by Slot Blot : a : *Meloidogyne incognita,* Race 1; b : *M. incognita,* Race 2; c : *M. incognita,* Race 3; d : *M. incognita,* Race 4; e : *M. javanica;* f : *M. arenaria;* g : *M. graminicola;* h : *M. hapla;* i : *Heterodera glycines;* j : *H. trifolii;* k : *Globodera rostochiensis* R_{01}; l : *G. rostochiensis* $Ro_{2/3}$; m : *G. pallida* and n : *G. tabaccum.* Hybridisation was carried out using Dig-ddUTP end-labelled MR1 n° 15.2.2 oligomer. Washings and development were as described in the Materials and Methods.

shown in Figure 3 B demonstrate that under these conditions the radiolabelled probe will detect half of the amount detectable with the digoxigenin probe.

Copy number

To determine the copy number of the 30 base sequence (MR1 n° 15.2.2) within the *M. incognita* genome, slot blots containing serially diluted *M. incognita* Race 1 genomic DNA and pMR1 n° 15 plasmid DNA were probed with the MR1 n° 15.2.2 ^{32}P end-radiolabelled sequence. The optical densities of the resultant autoradiograph signals after 24 h exposure were measured using a laser densitometer. The results (Fig. 4) indicated that the 30 base sequence (MR1 n° 15.2.2) represents approximately 1 % of the genome of the parasite. Assuming a haploid genome size of *Meloidogyne* of 5.1×10^7 base pairs (Pableo *et al.*, 1988) we estimate that MR1 n° 15.2.2 is represented 17 000 times.

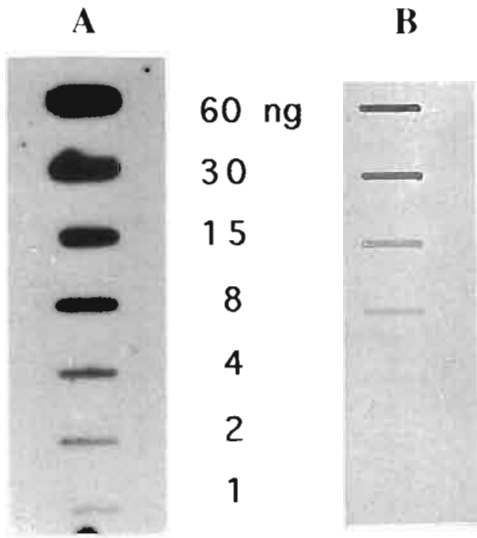

Fig. 3. Comparison of sensitivity of MR1 n° 15.2.2 oligonucleotide labelled with Dig-ddUTP and ^{32}P : Duplicated dilutions (60-1 ng) of *Meloidogyne incognita,* Race 1 DNA were bound to nitrocellulose by Slot Blot. One of the filters (A) were probed with MR1 n° 15.2.2 digoxigenin labelled oligomer. Washings and development was as described in the Materials and Methods. The second filter (B) was probed with MR1 n° 15.2.2 ^{32}P-radiolabelled (3.2×10^5 Bq/ml). Following washings the filter was air-dried and subjected to autoradiography for 24 h.

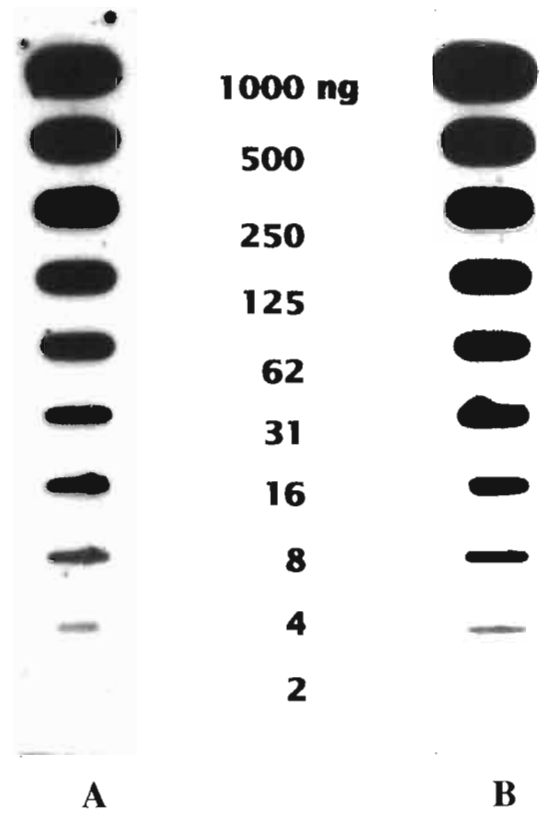

Fig. 4. Determination of copy number of MR1 n° 15.2.2 within the *Meloidogyne* genomic DNA : Serial dilutions (1000-2 ng) of *M. incognita,* Race 1 genomic DNA (A) and pMR1 n° 15 plasmid DNA (B) were bound onto nitrocellulose by Slot Blot. The membranes were probed with MR1 n° 15.2.2 end-radiolabelled oligonucleotide (3.2×10^5 Bq/ml). Following washings the filters were air-dried and subjected to autoradiography for 24 h.

Discussion

Classical methods for the diagnosis of *M. incognita* are most often based on the microscopic examination of morphological features of females. This is a laborious and time consuming exercise which has lead to the search for alternative technique such as the development of DNA probes. This is based on the premise that within any organism, there are unique DNA sequences which differentiate such organisms from closely related species. In *M. incognita* the use of the MR1 n° 15.2 specific oligonucleotide (Chacón *et al.,* 1991) is very amenable for use as a DNA probe since it shows no hybridisation to related species or hosts. However, the use of repetitive short oligonucleotide sequences renders the system more sensitive since there is an absence of unnecessary DNA making the detection more rapid and reduces the cost of synthesis.

The large number of times that MR1 n° 15.2.2 is present within the parasite genome has lead us to consider this sequence as highly repetitive DNA, which is usually not transcribed. Studies in genomic evolution indicate that frequent and rapid changes in repetitive DNA may be involved in speciation (MacGregor, 1982). Tandem and dispersed repeated DNA families have demonstrated a high degree of homogeneity within species with much greater variation between species. This phenomenon, known as concerted evolution, has been well documented but it is difficult to explain in terms of natural selection or drift (Dover *et al.,* 1982).

Many investigators have used specific repeated sequences in both cloned and synthetic forms as a means of identification, but in most cases radioactive detection methods have been employed (Barker, 1989). Non-radioactive detection of probes is often considered too insensitive for demanding diagnostic needs and in some case the sensitivity is ten fold less than reported for the radioactive equivalent (McLaughin *et al.,* 1985). However in the research reported here we have used the repeat sequence MR1 n° 15.2.2 as a synthetic oligomer labelled with digoxigenin and the compared results obtained with the ^{32}P radioactive equivalent only differs in 1 ng. We believe that this probe is sensitive enough for diagnostic purposes to reveal repetitive sequences in crude DNA preparations.

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