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DNA FRAGMENTS AS MOLECULAR PROBES FOR THE DIAGNOSIS OF PLANT PARASITIC NEMATODES

by
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Summary. A restriction fragment extracted from *Meloidogyne artiellia* DNA was used as a molecular probe for the identification of several nematodes. The results obtained indicate the feasibility of using a specific DNA fragment in a molecular diagnostic test. Further, the results provide evidence that of the different *Meloidogyne* species tested, *M. artiellia* and *M. hapla* are closely related.

The difficulties encountered in distinguishing nematode species has led researchers to focus on novel approaches to taxonomy. Initially biochemical and serological methods were used (Misaghi *et al.*, 1974; Esbenshade and Triantaphyllou, 1987). More recently developments in molecular biology and biotechnology have being evaluated for their application in nematode identification. Our research has centred on finding a DNA restriction fragment to use as a diagnostic tool for nematode identification. As other research was concerned with some molecular biological aspects of *Meloidogyne artiellia* Franklin and on the *Xiphinema index* Thorne *et* Allen, these two species were used as nematode models.

Materials and methods

Nematode cellular DNA was extracted from whole nematodes or purified egg preparations by liquid nitrogen grinding technique, followed by two phenol extractions.

Total cellular DNA was also obtained from minilysate prepared from nematode eggs or adults according to the procedure of Costanzi and Gillespie (1987), but with the following modifications. Nematode samples were taken up in 50 µl of MSB buffer (0.20 M mannitol, 0.07 M sucrose, 0.05 M Tris HCL pH 7.5, 0.01 M EDTA, 200 µg/ml proteinase K), and then mechanically disrupted and incubated at 65°C for 30'-60'. A concentrated NaOH solution was added to achieve a 0.3 M final concentration and incubated at 70°C for 1 hour. Finally 1M (f.c.) ammonium acetate was added.

Similarly a gall macerate was prepared by collecting 1 or 2 galls from infected plant roots in 100 µl MSB buffer and using the same procedure described for the minilysates.

Restriction endonucleases were purchased from commercial sources and used according to manufacturers recommendations.

The probe was radiolabelled by nick translation and the hybridization was in 4xSET + 10% Denhardt's solution and 0.1% SDS at 42°C (1 x SET = 0.15 M NaCl, 0.03 M Tris HCl pH 7, 2 mM EDTA). Final washes were set up in 0.1 x SET + 0.1% SDS at 42°C.

Results and discussion

The use of specific DNA fragments as a molecular tool for the diagnosis of various nematodes has been evaluated previously. The use of mitochondrial DNA (mtDNA) in the mtDNA-based hybridization assays in nematode diagnosis has been investigated by Powers *et al.*, (1986) and Hyman *et. al.* (1990). However, recent observations on the extent of intra-specific variability of nematode mtDNA (Thomas and Wilson, 1990) indicate that a broad application of this technique may be limited. For this reason it was decides to concentrate on a nuclear DNA fragment, which is presumably more conserved than mtDNA in an intra-specific analysis.

In order to obtain a DNA fragment which could be useful in the diagnosis of various nematodes, attention was focused on the Eco R1 fragments obtained from *M. artiellia* total cellular DNA.

M. artiellia DNA was digested with the restriction enzyme Eco R1, and the fragment separated through agarose gel electrophoresis. The fragment pattern obtained is shown in Fig. 1. It can be seen that *M. artiellia* DNA is cleaved into many fragments of continuously decreasing size which do not appear as discrete bands but are present

all along the gel as a smear. Only a few bands are readily discernible as these fragments are present many times in the genome as ripetitive DNA. The sizes of these repeats were measured in comparison with DNA fragments of known size and the values were 8.500, 7.500, 3.800, 2.100 and 1.900 base pairs (bp). It is interesting to note that the presence of smaller size repeats of different species of *Meloidogyne* has been reported in the literature and it has been proposed that the differences in the restriction fragments can be used as a tool to identify nematode species (Curran *et al.*, 1985). Therefore, the fragments of 8.500 and of 7.500 could be a distinctive feature of *M. artiellia*.

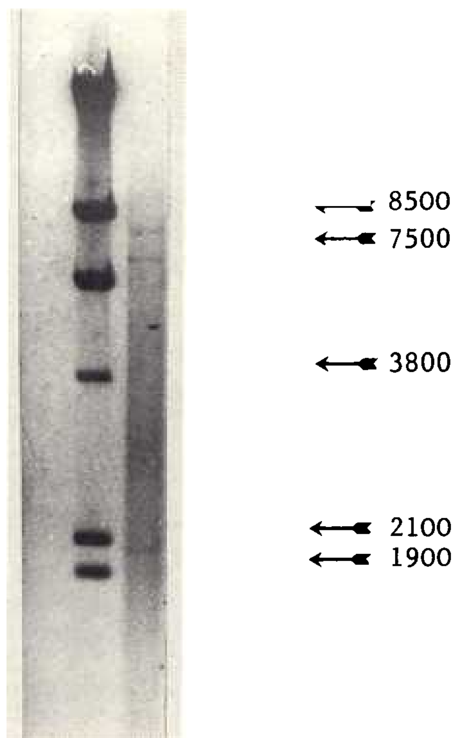

Fig. 1 - Ethidium bromide-stained agarose gel of Eco R1 restricted total DNA from *Meloidogyne artiellia*.
The *M. artiellia* DNA was digested with Eco R1 and electrophoresed on agarose gel. DNA fragments of known molecular weight were run in a parallel column.
The arrows indicate the repetitive DNA fragments and the relative size in base pairs is indicated.

Analysis of the Eco R1 fragments cloned shotgun in the plasmid pUC8, showed that a fragment of about 500 bp was the most abundant, although not evident in Fig. 1. It was therefore decided to use this fragment. The results of a preliminary experiment using various DNAs extracted from different sources apparently showed that all the DNA samples gave a positive signal when hybridized with the radioactive 500 bp fragment. However, when the filter was washed all the hybrids disappeared. The partial sequencing of the probe (Fig. 2) suggest that the short size of the fragment together with the high frequency of the bases A + T, determined the false positives observed and thus such a fragment is not suitable for our purpose.

It was therefore decided to try a different fragment and the best candidate seemed to be the fragment 2.100 bp long which is a repetitive DNA band in *M. artiellia*, clearly visible in Fig. 1.

The fragment of 2.100 base pairs from nuclear DNA of *M. artiellia* was isolated and cloned in bacterial cells, again using the vector pUC8 cleaved with Eco R1. This probe was used in a Southern hybridization experiment (Southern, 1975) with both *M. artiellia* and *X. index* restricted DNAs (Fig. 3). It can be seen that the fragment extracted from *M. artiellia* hybridizes only with the 2.100 bp fragment present in the digest of *M. artiellia* and with nothing else. This result demonstrates that the fragment selected can discriminate between *M. artiellia* and *X. index* and therefore tests were undertaken to ascertain whether such a fragment could recognize other *Meloidogyne* species.

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        10         20         30         40         50         60

TATAGGGCGA ATTCGAGCTC GGTACCCCTA CTAGAATGGA CAAGCCTTTT AAGAATTAAG

        70         80         90        100        110        120

TAGCTTTTTG TTTTATTTAA TTCTTGCTTT AATGTTTTTG GGGTTTGGCA TTGAAGTTTC

       130        140        150        160        170        180

AGGTGTAGGG GGTTGGGGGG GTTTGTTGAC AGATTTTCAG TATTATAATA TTATGTCTAC

       190        200

ACTATGATAT GCTCGGCTGT CTTGG
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Fig. 2 - Partial nucleotide sequence of the 500 bp probe. The fragment was obtained by a shotgun cloning in the vector pUC8 of the Eco R1 fragments of *M. artiellia*. The sequencing was carried out by the method of Sanger (Sanger *et al.*, 1977).

The fragment was used as a probe in a dot-blot experiment. Increasing amounts of purified nematode DNA were spotted on a nitrocellulose filter. On the same filter total cellular DNA obtained from minilysate from *Cephalobus persegnis* Bastian, *Xiphinema diversicaudatum* (Micoletzky) Thorne, *X. index*, *M. javanica* (Treub) Chitw., *M. incognita* (Kofoid *et* White) Chitw., *M. arenaria* (Neal) Chitw., *M. hapla* Chitw. and *M. artiellia*, was also spotted. Finally crude macerates from galls from *Rhizobium* and from *M. hapla* were also spotted. The filter was hybridized with the labelled 2.100 bp probe isolated from *M. artiellia* and the results are reported in Fig. 4. Surprisingly, the purified DNAs gave an aspecific signal, even with *X. index* DNA in spite of the results reported in Fig. 3. This was probably due to the very high concentration of DNA. However, an important conclusion can be drawn from this experiment. It can be seen from the second row of Fig. 4 that the probe hybridized with all the *Meloidogyne* minilysates: the strongest signal was obtained with the *M. artiellia* minilysate, but hybridization also occurred with the others minilysates, although to a lesser extent. This observation strongly suggests that the other *Meloidogyne* species also contain the same repeats. Furthermore no hybridization was detected with *X. index* or *X. diversicaudatum*, and the trace around the *C. persegnis* spot was probably due to contamination.

The results obtained by using gall macerates can also be of practical use. It can be seen in the third row (Fig. 4) that the *Rhizobium* gall macerate does not hybridize at all, while the gall macerate from *M. hapla* gives a positive signal. Furthermore, this signal depends on the amount of galls spotted, being very strong in the case of two galls. In other similar experiments gall macerates derived from *Rhizobium*, from *X. index*, and other *Meloidogyne* species were roughly purified by a centrifugation and then spotted on the filter. Again, in the case of galls derived from *M. hapla* a strong signal of hybridization with the *M. artiellia* probe was observed (result not shown), indicating that the fragment is species- specific and that it is present in all the *Meloidogyne* tested, but probably in different amounts.

Fig. 3 - Southern hybridization of 2.100 bp *M. artiellia* probe, using DNA extracted from *Xiphinema index* and *M. artiellia*.
Total DNA was extracted from *X. index* and *M. artiellia*, digested with Eco R1 and electrophoresed on agarose gel. The DNA fragments were transferred to filter paper, the probe was labelled *in vitro* and hybridized as reported by Southern (1975). A) *X. index*. B) *M. artiellia*.

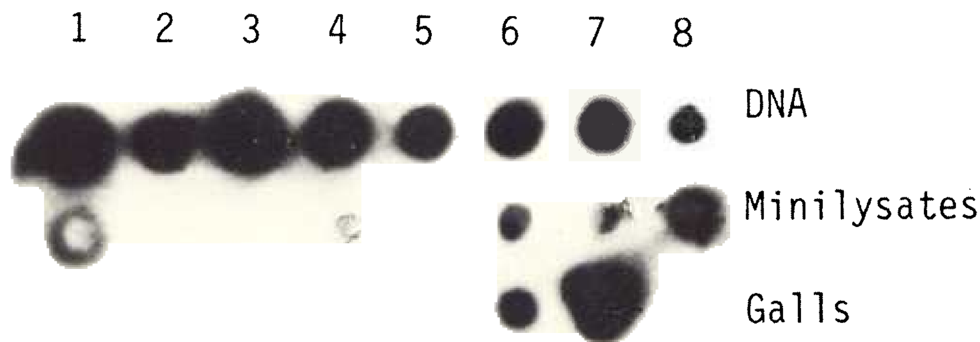

Fig. 4 - Dot blot hybridization of 2.100 bp probe.
In the first row DNA extracted from different sample was spotted. 1) pUC8 DNA, 2) *A. lixula* mtDNA fragment. 3) *X. index* 1 μg DNA. 4) *X. index* 400 ng. 5) *X. index* 100 ng. 6) *M. artiellia* 400 ng. 7) *M. artiellia* 150 ng. 8) *M. artiellia* 30 ng.
In the second row minilysates obtained from different nematodes were spotted. 1) *Cephalobus persegnis*. 2) *X. diversicaudatum*. 3) *X. index*. 4) *M. javanica*. 5) *M. incognita*. 6) *M. arenaria*. 7) *M. hapla*. 8) *M. artiellia*.
In the third row: Galls from infected roots were lysed and spotted 6) Single root gall from *M. hapla* 7) Two galls from *M. hapla*. 8) Root galls from *Rhizobium*.

Furthermore, *M. hapla* seems to be the most closely related to *M. artiellia*.

The results presented in this paper indicate the feasibility of using a nuclear DNA fragment for molecular diagnosis, but also indicate that care should be taken in the choice of the fragment.

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