Two cloned DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis*

Paul R. BURROWS and Roland N. PERRY

Entomology and Nematology Department, AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ, England.

SUMMARY

A genomic library was produced from *Globodera pallida* and two DNA probes which differentiate *G. pallida* from *G. rostochiensis* have been isolated. The cloned fragments of DNA are of similar size equal to approximately 370 base pairs. The results are discussed in the context of the application of these techniques for the identification of *Globodera* species and pathotypes.

RÉSUMÉ

Deux sondes de DNA différenciant Globodera pallida *de* G. rostochiensis

Une banque génomique a été obtenue à partir de *Globodera pallida.* Deux sondes de DNA différenciant *G. pallida* de *G. rostochiensis* ont été isolées. Les fragments clonés sont de taille similaire et mesurent environ 370 paires de base. La discussion porte sur l'application de ce type de méthode à l'identification des espèces et pathotypes de *Globodera.*

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida,* are major pests of potatoes throughout the world. In the UK alone they can claim between 2-9 % of the national crop, a maximum cost equivalent of £54 million (Anon., 1986). The success of integrated control programmes depends on rapid and reliable identification of PCN and their pathotypes. Faced with the conservative morphology of PCN, taxonomists have turned to biochemical approaches (Fleming & Marks, 1982) of which the techniques of molecular biology are probably the most promising. Differences in genetic material are exploited directly and, by contrast to many of the expressed products, they are not influenced by developmental stage or environment.

Restriction endonuclease enzymes recognise specific nucleic acid sequences in DNA and cleave the DNA at these or adjacent sites. The number and size of the restriction fragments produced reflects the underlying genome. Fractionation of restriction fragments by electrophoresis reveals distinct bands originating from the digestion of highly repetitive DNA sequences. Restriction fragment length differences have been observed between several nematode groups (Curran, Baillie & Webster, 1985) and used to characterise four major species of *Meloidogyne* (Curran, McClure & Webster, 1986). Populations of *G. rostochiensis* and *G. pallida* have also been separated by restriction fragment patterns (Burrows & Boffey, 1986). Unfortunately, these highly repetitive fragments represent only a minute proportion of the restriction fragments produced; to exploit sequences which are much less frequent, they must be cloned. Cloned sequences have great potential as sensitive and discriminating probes for the detection and identification of nematode species and pathotypes. They can provide new insight into studies on taxonomy, population biology and genetics.

In this study a genomic library was prepared from *G. pallida* second stage juveniles and cloned DNA sequences which differentiated *G. pallida* from *G. rostochiensis* were identified. This work extends earlier studies on PCN (Burrows & Boffey, 1986) and forms part of a larger programme of research at Rothamsted on the use of DNA hybridization techniques for separation of *Globodera* spp, preliminary accounts of which have been reported (Burrows, 1987; Burrows & Perry, 1987).

Materials and methods

CYST MATERIAL

Populations of *Globodera* spp. were increased on the susceptible cv. Arran Banner in pot cultures. They were : *G. rostochiensis* (Pathotype RO 2; original material from Obersteinbach), *G. rostochiensis* (RO 5; Harmerz), *G. rostochiensis* (RO 1; Woburn); *G. pallida* (Pa 4; New Leake) and *G. pallida* (Pa 1; Plant Breeding Institute). Eggs and second stage juveniles (JJ 2) were collected and prepared for DNA extraction as detailed by Burrows and Boffey (1986).

Many of the subsequent methods were based on the laboratory manual of molecular biological techniques by Maniatis, Fritsch and Sambrook (1982).

DNA EXTRACTION

After extraction, JJ 2 (approximately 500 mg wet weight) were washed twice with DNA extraction buffer (Tris 0.1 M pH 8.5, EDTA 0.05 M, NaCl 0.1 M, 2 % SDS) pelleted in a microcentrifuge and resuspended in 0.5 ml extraction buffer. The suspension was ground under liquid nitrogen in a pestle and mortar before being transferred to a 30 ml glass centrifuge tube (corex) containing 1.55 ml extraction buffer and 5 mg proteinase K (Sigma) and incubated at 65° for 1 h with occasional gentle shaking.

The resulting viscous solution was extracted twice with redistilled phenol/chloroform 1:1 (phenol saturated with 1 M Tris, pH 7.5). Each extraction was incubated at 37° for 5-10 min before centrifugation (4 000 rpm) for 10 min. Each aqueous phase was carefully removed and the organic layer re-extracted with 2 ml Tris low-EDTA buffer (TLE : 0.01 M Tris, 0.1 mM EDTA, pH 8.0) and centrifuged as before. After pooling the aqueous phases in a 30 ml corex tube, sodium acetate was added (to a concentration of 0.2 M) followed by 2.5 volumes of precooled high grade absolute ethanol and left overnight at — 20°. After centrifugation (10 000 rpm for 30 min at 4°) to pellet the precipitated DNA, as much of the ethanol as possible was removed, without letting the pellet dry out, before the DNA was resuspended in 4.13 ml TLE.

DNA PURIFICATION

Nematode DNA was purified by centrifugation in 60 % CsCl, 10 mg ml^{-1} ethidium bromide, at 50 000 rpm for 14-16 h at 15°. The ethidium bromide was removed from the DNA by repeated extractions with butan-1-ol satured with Tris EDTA buffer (TE : 0.01 M Tris, 1 mM EDTA, pH 8.0). The DNA solution was dialised against 1 l TE for 2 h during which the TE was changed at least once. The DNA was then precipated and centrifuged as before. The resulting pellet was washed once in 75 % ethanol, centrifuged at 10 000 rpm for 15 min at 4° and then resuspended in TLE. This gave a typical yield of 150-200 µg of clean, high molecular weight DNA.

CLONING

Genomic DNA from *G. pallida* (New Leake) was digested to completion with Hind III (Bethesda Research Laboratories, BRL) and 4 ng ligated into phosphatase-treated plasmid pUC-9. The ligation mixture contained 1 Weiss unit of T 4 ligase (BRL) and ligation buffer giving a final concentration of 50 mM Tris HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol and 5 % w/v polyethyleneglycol — 8 000. Total volume was adjusted to provide an approximate molar ratio of 5:1, vector : nematode DNA. This was incubated overnight at 15°.

The ligation mixture was used to transform competent *E. coli* (JM 83). Bacterial cells with chimeric plasmids containing nematode DNA produced white colonies in the presence of 5-chloro-4-bromo-3-indolyl β-D-galactoside (X-gal) while those without nematode inserts produced blue colonies. White recombinants were removed to LBA master plates (bacto tryptone 10 g l^{-1}, bacto yeast extract 5 g l^{-1}, NaCl 5 g l^{-1}, pH 7.2 with 1,5 % agar and 100 µg ml^{-1} ampicillin) and stored at 4°.

SCREENING THE GENOMIC LIBRARY

Screening of the genomic library was done in two parts. Firstly, the recombinant colonies containing fragments specific to *G. pallida* were selected using *in situ* hybridization techniques and, secondly, chimeric plasmids were then prepared from any *G. pallida* specific clones and subjected to dot blot hybridization. Nematode DNA fragments were also cut out of the plasmids and their sizes estimated by polyacrylamide gel electrophoresis.

Radio-labelling nematode DNA and plasmids

The incorporation of [α^{-32}P] dATP was accomplished by nick-translation; unincorporated nucleotides were removed from the DNA by chromatography through a Sephadex G 50 column.

In situ *hybridization*

Nitrocellulose filters (82 mm diameter) were laid on the surface of LBA plates. Using a sterile toothpick, 46 recombinant colonies were placed onto the surface of each filter in a grid formation. Master plates containing the antibiotic but not the filter were simultaneously prepared. The plates were inverted and grown overnight at 37°. A filter containing JM 83 without pUC was similarly processed as a control.

For each nitrocellulose filter, five filter paper (Whatman No. 1) pads were prepared and placed in the lids of disposable plastic Petri dishes. Each pad comprised five sheets of filter paper and the first pad was saturated with 10 ml 10 % SDS, the second with 10 ml 0.5 M NaOH, the third with 10 ml 1 M Tris HCl (pH 7.4) and the remaining two pads were each saturated with 10 ml 1 M Tris HCl (pH 7.4), 1.5 M NaCl. Each nitrocellulose filter was removed from the agar and, with colonies uppermost, was placed on the 10 % SDS pad for 3 min. The filter was then transferred to each of the remaining four pads in turn for 5 min per pad. The filters were then sandwiched between two sheet of filter paper (Whatman 3 mm) and gently pressed before the top sheet was peeled off and the nitrocellulose filters left to air-dry for 1 h then baked at 80° under vacuum for 2-3 h.

DNA hybridization

The nitrocellulose filters were placed in a plastic bag containing pre-hybridization fluid (5 X Denharts, 5 X SSPE (0.9 M NaCl, 0.05 M $NaHPO_3$ pH 8.3, 5.0 mM EDTA), 0.1 % SDS and 100 µg ml^{-1} sheared and denatured salmon sperm DNA). After sealing, the bag was incubated at 65° for 1 h before the fluid was removed and replaced with hybridization solution (5 X Denharts, 5 X SSPE, 0.1 % SDS, 100 µm ml^{-1} denatured salmon sperm DNA). Radio-labelled *Hind III* genomic digest of the appropriate population of *G. rostochiensis* and *G. pallida* was denatured by boiling for 5 min and then added to the filter bag. Activity of about 1 X 10^6 counts min^{-1} ml^{-1} of hybridization solution gave adequate results. Hybridization was carried out overnight at 65° with gentle shaking.

Washing

The filters were washed three times for 20 min in 200 ml 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1 % SDS and sandwiched between two pieces of " cling-film " in a cassette and left to expose X-ray film (Fuji) at — 80° for 48 h.

PLASMID PREPARATION

Chimeric plasmids were prepared from the colonies selected by *in situ* hybridization using the boiling method for small scale isolation of plasmid DNA (Maniatis *et al.*, 1982). The technique was modified slightly : after removal of the chromosomal DNA, the plasmid preparations were extracted with phenol/chloroform and then plasmid DNA was precipitated overnight and pelleted in a microcentrifuge. Pellets were washed with 75 % ethanol, centrifuged and re-suspended in 90 µl TE. RNA was removed by treatment with DNase-free RNase before the preparations were again extracted with phenol/chloroform, precipated with ethanol overnight and pelleted in a microcentrifuge. The clean plasmids were re-suspended in 50 µg TE.

DOT BLOT

Genomic DNA (5 µg) from the three populations of *G. rostochiensis* and two populations of *G. pallida* detailed above was spotted onto 82 mm diameter nitrocellulose filters. When dry, the DNA was denatured (1.5 M NaCl; 0.5 M NaOH) for 5 mn, then neutralised (3 M sodium acetate) for 5 mn. The filters were air dried and then baked at 85° under vacuum for 1 h. The bound genomic DNA was probed with ^{32}P-labelled chimeric plasmids and ^{32}P-labelled pUC-9. DNA/DNA hybridization, washing and autoradiography were carried out as before.

POLYACRYLAMIDE ELECTROPHORESIS

Plasmid DNA (2 µg) was digested to completion by Hind III and the fragments separated by electrophoresis (200 V, 40 mA) on a vertical gel (20 % acrylamide, 1 % bis) using a TBE buffer system (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). The gel was stained with ethidium bromide (2 µg ml^{-1} TBE) and viewed on an ultraviolet transilluminator.

Results

Cloning the genomic DNA from *G. pallida* (New Leake) into a plasmid vector produced a library containing 206 viable recombinant colonies. Thus, with 46 colonies on each 82 mm nitrocellulose filter, the *in situ* hybridization examination was performed on just five med Pa 15 and Pa 16) that failed to hybridize with DNA from the populations of *G. rostochiensis* but hybridized strongly with both populations of *G. pallida.* The control spots of *E. coli* JM 83 DNA did not hybridize with any nematode DNA probes.

Verification of the *in situ* hybridization result for Pa 15 and Pa 16 was obtained using a dot blot hybridization. The autoradiograph (Fig. 1 *a*) shows hybridization of the chimeric plasmids with *G. pallida* DNA but not with *G. rostochiensis.* Radiolabelled pUC-9 failed to hybridize with any of the nematode DNA.

Polyacrylamide gel electrophoresis was used to determine fragment size. The results (Fig. 1 *b*) indicate that the cloned nematode DNA fragments from Pa 15 and Pa 16 are of approximately the same size i.e. between 360 and 380 base pairs. Although Pa 16 appears slightly larger than Pa 15 it is possible that they are basically the same fragment or at least possess a very similar base sequence.

Discussion

This work has resulted in the construction of a genomic DNA library from *G. pallida* and has identified two probes which differentiate *G. pallida* from *G. rostochiensis.* Clearly, the specificity of the cloned fragments must be tested against more populations of both species before they can be considered as diagnostic. As well as this further screening, restriction analyses of Pa 15 and Pa 16 could provide valuable information, allowing future modification of the probes to increase affinity or improve specificity if required.

G. rostochiensis and *G. pallida* are morphologically distinct from each other, especially when compared with the differences in more cryptic cyst nematode species complexes. In addition, electrophoresis and isoelectric focusing studies on total soluble proteins from PCN have revealed marked differences between the two species (Fleming & Marks, 1982; Fox & Atkinson, 1984). These phenotypic differences only reflect underlying variation in the DNA and it is, therefore, not surprising that two probes that are potentially species-specific have

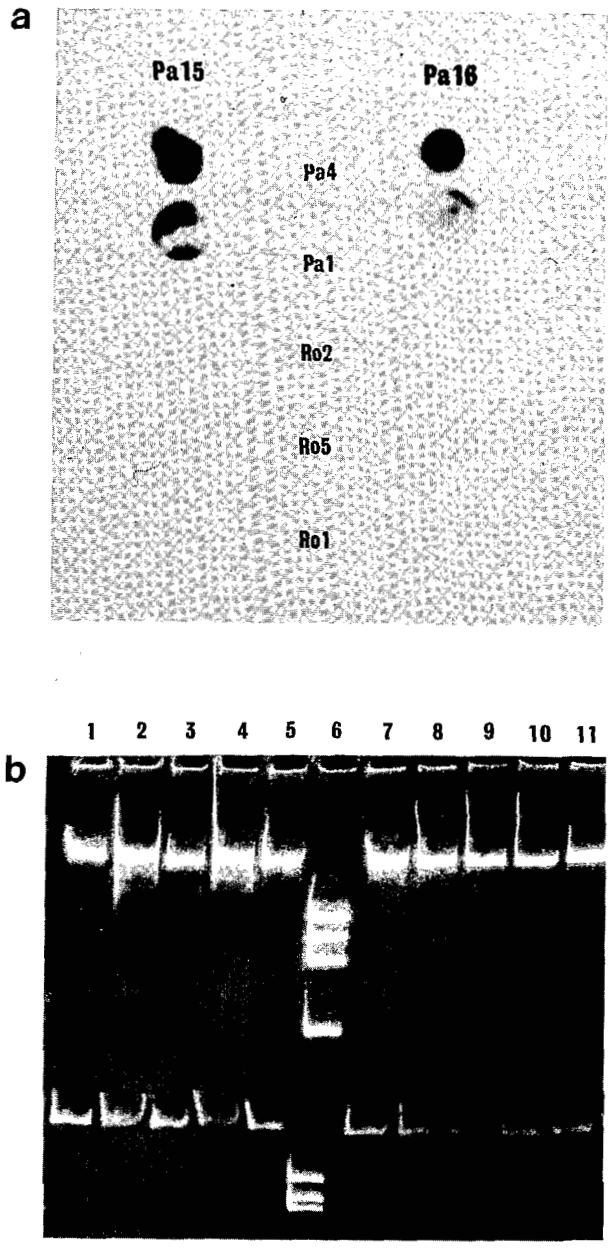

Fig. 1. a : Dot blot autoradiograph showing hybridization of radio labelled chimeric plasmids from Pa 15 and Pa 16 to *G. pallida* DNA (Pa 4 and Pa 1) but not to three populations of *G. rostochiensis* DNA (Ro 2, Ro 5 and Ro 1); b : Size determination of DNA probes using polyacrylamide gel electrophoresis. Lanes 1 to 5 : chimeric plasmid prepared from Pa 16 digested with Hind III (1 µg per lane). Lane 6 : DNA fragment size marker; Ø X-174 phage DNA (1 µg) digested with Hae III giving fragment sizes (kb) from origin 1 353, 1 078, 872, 603, 310 (281, 271; running to same point), 234. Lanes 7 to 11 : chimeric plasmid prepared from Pa 15 digested with Hind III (1 µg per lane).

been isolated from a relatively small genomic library. Extension of this approach to PCN pathotypes and other cyst nematode complexes is likely to demand more extensive screening of larger and more comprehensive libraries. The use of recombinant DNA techniques is probably more suitable than other biochemical approaches for differentiating pathotypes and species complexes as DNA based identification involves analysis of the complete genome rather than the 15-20 % indirectly examined by methods such as electrophoresis (Curran & Webster, 1987).

Eukaryote DNA can be divided conveniently into three groups (Lewin, 1982). The first is single/low copy DNA, mainly comprising protein coding sequences. The second group is moderately repetitive DNA, some families of which are dispersed throughout the genome while others are arranged in tandem repeats; members of functional genes, such as those for ribosomal RNA, transfer RNA, histones and immunoglobulins, can be placed in this group. The third group is highly repetitive DNA, repeated 10^6 or more times, which is rather simple and usually not transcribed. The use of plasmids as cloning vectors is more efficient when the DNA fragments to be inserted are small (< 10 kb). However, fragments derived from moderately and highly repetitive DNA are likely to be represented in the final genomic library primarily because of their relative abundance in the restriction digest. This is not necessarily disadvantageous, as repetitive DNA fragments are known to be useful for differentiating nematode species and populations (Curran *et al.*, 1985, 1986; Burrows & Boffey, 1986).

Recent 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). The observed heterogeneity in repeated DNA families between species enhances their potential for differentiating nematode species. There is no evidence at present to suggest that the cloned fragments Pa 15 and Pa 16 are derived from repetitive DNA; future work will aim to resolve this aspect.

Plant nematologists have been slow to exploit the great potential offered by DNA technology. These techniques will not only be of great value to taxonomy but could provide fresh insight into studies on genetics and population biology of plant-parasitic nematodes. The practical value of these methods and their future use will depend on developing and improving techniques to allow analysis of small amounts of nematode material. Adopting non-radioactive detection

methods could facilitate the wide use of simple laboratory or field based nematode identification kits.

References

ANON (1986). *The future with Rothamsted.* Lawes Agricultural Trust : Harpenden, England, 28 p.

BURROWS, P. R. (1987). A cloned DNA fragment specific to *Globodera pallida. Assoc. appl. Biol. Meeting,* London, 1987.

BURROWS, P. R. & BOFFEY, S. A. (1986). A technique for the extraction and restriction endonuclease digestion of total DNA from *Globodera rostochensis* and *G. pallida* second stage juveniles. *Revue Nématol.,* 9 : 199-200.

BURROWS, P. R. & PERRY, R. N. (1987). In search of species and pathotype specific DNA sequences of *Globodera rostochiensis* and *G. pallida. British Society for Parasitology Meeting,* Edinburgh, 1987 : 53-54.

CURRAN, J., BAILLIE, D. L. & WEBSTER, J. M. (1985). Use of restriction fragment length differences in genomic DNA to identify nematode species. *Parasitology,* 90 : 137-144.

CURRAN, J., MCCLURE, M. A. & WEBSTER, J. M. (1986). Genotypic analysis of *Meloidogyne* populations by detection of restriction fragment length differences in total DNA. *J. Nematol.,* 18 : 83-86.

Accepté pour publication le 28 janvier 1988.

CURRAN, J. & WEBSTER, J. M. (1987). Identification of nematodes using restriction fragment length differences and species-specific DNA probes. *Can. J. Pl. Pathol.,* 9 : 162-166.

DOVER, G. A., BROWN, S., COEN, E., DALLAS, J., STRACHAN, T. & TRIC, M. (1982). The dynamics of genomic evolution and species differentiation. In : Dover, G. A. & Flavel, R. B. (Eds) *Genome evolution.* New York, Academic Press : 344-372.

FLEMING, C. C. & MARKS, R. J. (1982). A method for the quantitative estimation of *Globodera rostochiensis* and *G. pallida* in mixed species samples. *Rec. agric. Res.,* 30 : 67-70.

FOX, P. C. & ATKINSON, H. J. (1984). Glucose phosphate isomerase polymorphism in field populations of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida. Ann. appl. Biol.,* 104 : 503-509.

LEWIN, R. (1982). Repeated DNA, still in search of a function. *Science,* 217 : 621-623.

MACGREGOR, H. C. (1982). Big chromosomes and speciation amongst amphibia. In : Dover, G. A. & Flavell, R. B. (Eds) *Genome evolution.* New York, Academic Press : 325-341.

MANIATIS, T., FRITSCH, E. J. & SAMBROOK, J. (1982). *Molecular cloning, a laboratory manual.* Cold Spring Harbor, New York, 545 p.