Fundam. appl. Nematol., 1993, **16** (2), 115-120

Isolation and use of a species-specific clone for the identification of the rhabditid entomopathogenic nematode *Steinernema feltiae* (Filipjev, 1934)

Alexander P. Reid and William M. Hominick

Imperial College of Science, Technology and Medicine, Department of Biology, Silwood Park, Ascot, Berks, SL.5 7PY UK

Accepted for publication 20 May 1992.

Summary – Steinernematid nematodes isolated from British soils were shown to consist of five distinct ribosomal DNA RFLP types (Reid & Hominick, 1992). Total genomic DNA hybridization patterns suggested the five types to belong to two species. One, identified as *Steinernema feltiae (= bibionis)* consisted of two RFLP types, while the other three belonged to an undescribed but morphologically similar species. Genomic DNA from the standard *S. feltiae* RFLP type (type A 1) was cloned into the phage λ gt 10 and the resulting library screened for species-specific clones. A likely candidate was subcloned into pUC18 to give the plasmid described in this study, pSf 106. This plasmid contains 1.75 copies of a 161 base pair Hinf I tandem repeating unit which comprise 10 % and 0.5 % of the A 1 and A 2 RFLP type genomes respectively. The high copy number of this sequence within the genome of A 1 RFLP types enables the DNA from a single infective stage nematode to be detected in a dot blot assay.

Résumé – ***Isolement et utilisation d'un clone distinguant les espèces pour l'identification du nématode Rhabditide entomopathogène* Steinernema feltiae *(Filipjev, 1934)*** – Cinq types de RFLP ont été caractérisés dans l'ADN ribosomal de nématodes Steinernématides isolés de sols de Grande-Bretagne (Reid & Hominick, 1992). Les schémas d'hybridation de l'ADN génomique total suggèrent que les cinq types appartiennent à deux espèces. Deux types de RFLP sont caractéristiques de *Steinernema feltiae (= bibionis)* tandis que les trois autres appartiennent à une espèce non décrite, mais morphologiquement similaire. L'ADN génomique du type de RFLP standard de *S. feltiae* (type A 1) a été cloné dans le phage λ gt 10 et la collection de clones a été évaluée pour repérer les clones spécifiques. Un clone candidat a été sous-cloné dans pUC 18 pour donner le clone pSf 106 décrit dans cette étude. Ce plasmide contient 1,75 copie d'une séquence Hinf I de 161 bp répétée en tandem qui comprend 10 % et 0,5 % des génomes de type de RFLP A 1 et A 2. Le nombre élevé de copies de cette séquence dans le génome de type de RFLP A 1 a permis de détecter l'ADN d'un seul specimen du nématode au stade infectieux par un test de « dot blot ».

Key-words : *Steinernema*, ribosomal DNA.

Entomopathogenic nematodes of the genus *Steinernema* are obligate and lethal parasites of insects. For this reason they are being used as biological control agents for a number of economically important crop pests. The nematodes act by gaining entry to the host insect, usually via natural openings such as the mouth, anus or spiracles, entering the haemocoel and releasing a symbiotic bacterium of the genus *Xenorhabdus*. It is thought that the proliferation of these bacteria kills the host, normally within 48 h. Over the next 2 to 3 weeks the nematodes go through several generations reproducing sexually until finally the cadaver is filled with potentially thousands of the long-lived infective stage known as dauerlarvae.

Steinernematid nematodes are widespread throughout the world, having been isolated in Europe, Australia and the Americas. In their soil survey of the United Kingdom, Hominick and Briscoe (1990*a*) found steinernematid nematodes in 196 of 403 soil samples tested by the *Galleria* trap method (Bedding & Akhurst, 1975). These were subsequently shown to consist of five distinct types according to restriction fragment length polymorphisms (RFLPs) within their ribosomal DNA repeat units (Reid & Hominick, 1992). Total genomic DNA hybridization patterns showed the five types belonged to two species. One was identified as *S. feltiae (= bibionis)* (Filipjev, 1934) and consisted of two RFLP types (designated A 1 and A 2), while the other three belonged to an undescribed species (designated B 1 to B 3) which is morphologically similar to *S. feltiae* (Reid & Hominick, 1992). The A 1 *S. feltiae* RFLP type proved to be the most frequently observed type in the UK with a prevalence of 56.4 %. The standard A 1 type was isolated from site 76 (Ordnance survey reference SU 568 729) (Hominick & Briscoe, 1990*a*). When added to its RFLP variant (type A 2), the prevalence of *S. feltiae* in British soils was 70.0 %. The standard for the B 1 type has been designated Nashes isolate (Ordnance survey reference SU 945 692) (Reid & Hominick, 1992). A method that could quickly and reliably identify new nematode isolates was required so that the minoritory of nematodes belonging to the B-types, or other species isolated subsequently could be pinpointed for further study to determine their potential as control

agents. One such method is to use species-specific clones isolated from libraries derived from the genomic DNA of an organism. Indeed, clones that can identify very small amounts of material, even if it is heavily contaminated with foreign DNA, have been isolated from a variety of parasite genomes such as those for the identification of *Leishmania* species in cutaneous lesions (Wirth & McMahon-Pratt, 1982) and for the detection of the filarial parasite *Brugia malayi* in its vector (Sim *et al.*, 1986). This paper describes the isolation and characterisation of a DNA clone from *S. feltiae (= bibionis)*, and its use to identify positively infective juveniles of this species.

Materials and methods

Details of the methods used for nematode DNA extraction, Southern blotting and the hybridization of radioactively labelled clones can be found in Maniatis *et al.* (1989) and Reid and Hominick (1992).

Total genomic DNA from the standard UK *S. feltiae (= bibionis)* site 76 strain was randomly-sheared by sonication, blunt-ended and cloned into the Eco RI site of λ gt 10 using a cloning kit obtained from Amersham International as per their instructions. A portion of the library was plated out on LB agar plates (Maniatis *et al.*, 1989) and replica nitrocellulose lifts taken. These filters were then hybridized with radioactively labelled total genomic DNA from either *S. feltiae* site 76 or *S. carpocapsae* (Weiser, 1955) All strain. Several clones were selected that hybridized strongly with the *S. feltiae* DNA but not with the *S. carpocapsae* DNA. These clones were then replated in an ordered array and replica filters again taken. In this case, the filters were hybridized with the same two DNAs as in the initial screen, and also with the total genomic DNA from the undescribed *Steinernema* species (Nashes isolate) and the rDNA clone from *Caenorhabditis elegans*, pCe 7 (Files & Hirsh, 1981). A number of clones were selected that hybridized only to the DNA from *S. feltiae* site 76. DNA from these clones was then hybridized to Southern blot filters of restriction enzyme digested DNA from the two species found in the UK and the filters exposed for 2 to 3 weeks to ensure that they did not hybridize with the DNA of the undescribed species.

Once a suitable clone (no. 106) was identified by this method, the insert was cut out of the recombinant λ DNA and subcloned into the Eco RI site of both the plasmid pUC 18 and the single-stranded phage M 13 mp 18 (Maniatis *et al.*, 1989). Both recombinant plasmid and phage DNA were used to transform *E. coli* SURE cells (Stratagene) by the method of Chung *et al.* (1989). Once recombinant colonies were identified, DNA was prepared from them according to methods in Maniatis *et al.* (1989).

The nucleotide sequence of the *S. feltiae* species-specific clone (designated pSf 106 in this laboratory) was determined by using a multiwell microtitre plate sequencing kit supplied by Amersham. The plasmid was sequenced in both forward and reverse directions and the sequence obtained confirmed by analysis of the M 13 clone.

Dot blots of genomic DNA were prepared by diluting the DNA to the required concentration, denaturing at 95 °C for 10 min and then quenching on ice. An equal volume of 20 × SSC was added to the samples which were then dotted onto nylon membranes (Amersham) equilibrated with 10 × SSC in a Bio Rad dot blotting apparatus as per the manufacturers instructions. For the dot blots of nematode extracts, infective stage nematodes were counted into separate siliconized tubes under a dissecting microscope. The nematodes (numbering 1, 2, 5 or 10) were digested at 37 °C for 4 h in 20 µl of extraction buffer (0.1 M Tris, pH 8.0, 0.2 M NaCl, 0.05 M EDTA, 1 % Triton X-100, and 2 mg/ml proteinase K). Triton X-100 was used instead of SDS because at high salt concentrations and low temperatures SDS precipitates, making dot blotting by vacuum virtually impossible. The nematode extract samples were dotted onto the membranes by the same method as used for the genomic DNA samples. The membranes were hybridized and washed under the same conditions as those for Southern blots (Reid & Hominick, 1992).

Results

Specificity of pSf 106

Replica Southern blots of DNA from RFLP types A 1, A 2, B 1 and B 2 (Reid & Hominick, 1992), cut with Eco RI and Hind III, were prepared. These were hybridized to radioactively labelled pSf 106. The filters were washed to varying degrees of stringency. The lack of hybridization of pSf 106 to the genomic DNA from *Steinernema* n. sp. (Nashes isolate) and its variant (Fig. 1 A, lanes B 1 and B 2), even at the lowest stringency wash (2 × SSC, 65 °C, 15 min), shows the high specificity of this clone for *S. feltiae* DNA. The ladder of bands in the two variants of *S. feltiae* (Fig. 1 B, lanes A 1 and A 2) confirms that pSf 106 hybridizes to highly repetitive elements within the genome of these nematodes. The presence of DNA in lanes B 1 and B 2 was confirmed by hybridizing the same filter with the rDNA clone pCe 7 (Fig. 1 C). These produced the characteristic patterns for B 1 and B 2, described elsewhere (Reid & Hominick, 1992).

Nucleotide sequence of pSf 106

The nucleotide sequence of the *S. feltiae* species-specific clone pSf 106, which contained an insert of 277 bp, showed that there were Hinf I and Taq I recognition sites 161 bp apart (Fig. 2). This indicates that the clone is present in a tandem array in the genome of the nematode. This therefore means that pSf 106 contains approximately 1.75 copies of the 161 bp repeat. Analysis

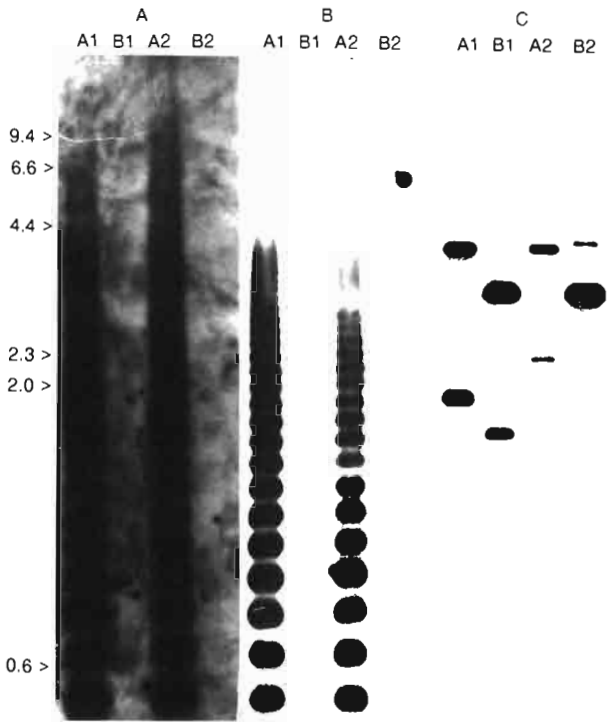

Fig. 1. The clone pSf 106 was hybridized to a Southern blot of Eco RI/Hind III digested DNA and was then exposed after each stringency wash. A types; *S. feltiae (= bibionis).* B types; *Steinernema* n. sp. A 1, site 76; A 2, site 107; B 1, Nashes isolate; B 2, site 216. Panel A, washed to low stringency (exposed after the first wash, see text for details). Panel B, washed to high stringency (exposed after the final wash, see text for details). Panel C, the same filter as Fig. 1 A and B., hybridized with the ribosomal clone pCe 7 showing the presence of DNA in lanes B 1 and B 2. The positions of the λ Hind III markers are shown in kb.

of the nucleotide sequence shows that there is 94 % homology between the two repeated sections present. There is also an Alu I site within the clone, but the position of this recognition site makes it impossible to determine whether there is also a 161 bp repeat for this enzyme.

Comparison of pSf 106 related sequences between RFLP types A 1 and A 2

Genomic DNA from the two *S. feltiae* RFLP types (A 1 and A 2) was digested with one of Alu I, Hinf I or Taq I and the fragments electrophoretically separated on 1.5 % (w/v) agarose gels. The resulting Southern blots were hybridized with pSf 106 and washed to high stringency (0.1 × SSC, 0.1 % SDS, 65 °C, 30 min) (Fig. 3). In the A 1 RFLP variant, strong bands corresponding to the 161 bp monomer were observed for the Hinf I and Taq I digests and there were also higher molecular weight bands corresponding to dimers, trimers

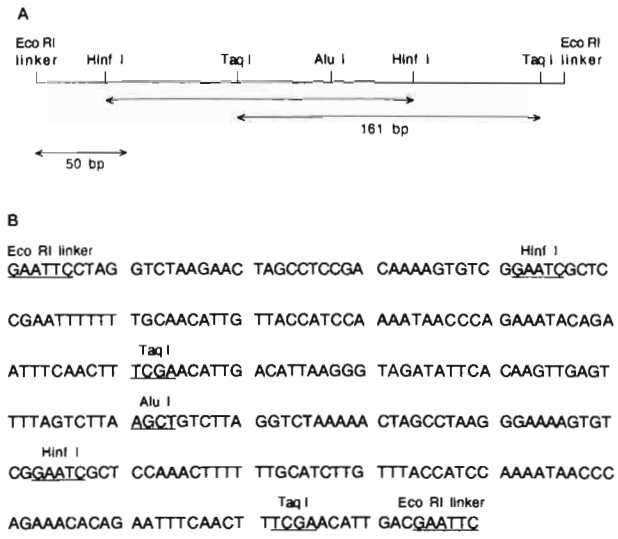

Fig. 2. A : Restriction map of pSf 106. The recognition sites for the enzymes Hinf I and Taq I are 161 bp apart, indicating that there is a tandem repeat of this length in the genome of *S. feltiae (= bibionis);* B : The nucleotide sequence of pSf 106. The positions of the restriction enzyme cut sites are shown as lines below the sequence with the names of the enzyme above. The Eco RI linkers have also been included although it should be noted that these are not present in the genomic DNA.

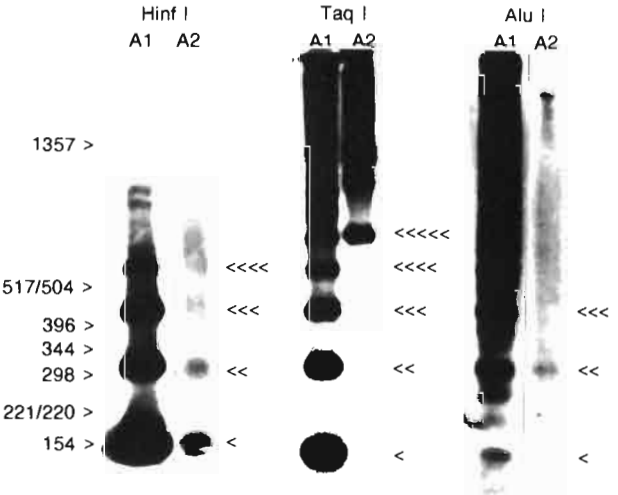

Fig. 3. Genomic DNA from the two RFLP variants of *S. feltiae (= bibionis).* A 1, site 76; A 2, site 107. The DNAs were digested with the restriction enzymes as shown and the filter hybridized with pSf 106. The key to the bands is as follows; >, basic 161 bp unit; >>, dimer; >>>, trimer. The positions of the pBR 322 Bam HI/Hinf I markers corresponding to the gel containing the Hinf I digests are shown in base pairs.

and oligomers of the basic 161 bp unit. The Alu I lane showed little hybridization at 161 bp, indicating that the Alu I site is rare in the single 161 bp unit. However a number of dimers were present. It should be noted that the slight variation in migration distances of the bands produced by the various enzymes is due to minor differences in the length of the runs of the respective gels.

The patterns for the A 2 RFLP variant were quite different (Fig. 3). A lower level of signal was obtained, which could be explained by a reduced copy number for this sequence in the genome of this RFLP type. The 161 bp band produced by Hinf I digestion was present in the A 2 variant as was the dimer. Other multiples of the basic unit are probably also present, but the lower copy number made it impossible to see them on this autoradiogram. When the genomic DNA was digested with Taq I, the repeating unit appeared to be present as a penta-mer in its smallest form. With Alu I, the repeating unit was also larger in this variant than in the A 1 RFLP type. This is an indication of other differences between the two RFLP types of *S. feltiae* adding to those already documented between their rDNA repeat units and their total genomic DNA patterns on Southern blots (Reid & Hominick, in press).

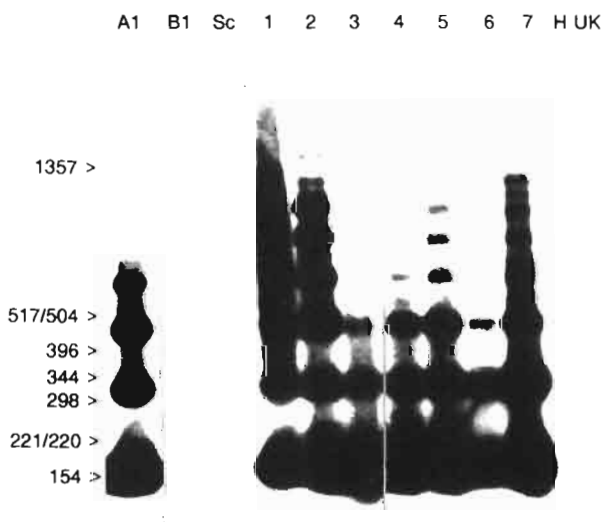

Fig. 4. The genomic DNA from a selection of A 1 RFLP types (lanes 1-7) as digested with Hinf I and hybridized with pSf 106. Lane A 1, *S. feltiae (= bibionis)* (site 76); B 1, *Steinernema* n. sp. (Nashes isolate); Sc, *S. carpocapsae* All; H UK, *Heterorhabditis megidis* (site 211); 1, T 319; 2, site 2; 3, site 42; 4, site 157; 5, site 15 D; 6, site 18 D; 7, site 38 D. The last three are all from a survey of the Netherlands (Hominick and Briscoe, unpublished data). The positions of the pBR 322 Bam HI/Hinf I markers are shown in base pairs.

Consistency of pSf 106 related sequences within the A 1 RFLP type

Genomic DNA from a number of other A 1 isolates was digested with Hinf I, blotted and probed as before. The DNA on this gel was obtained from isolates collected throughout the UK as well as from the Netherlands (Hominick & Briscoe, 1990*a*, 1990*b;* unpubl.). The Australian *S. feltiae* standard isolate (T 319; Akhurst, 1983) was also probed with pSf 106 as were the B 1 RFLP type, *S. carpocapsae* All strain and a *Heterorhabditis* isolate from the UK. All of the *S. feltiae (= bibionis)* samples (lanes 1-7) displayed the 161 bp band as well as a number of lower intensity bands corresponding to dimers, trimers and oligomers (Fig. 4). This suggests that the 161 bp repeat is highly conserved within the genome of the A 1 RFLP types. Differences seen between lanes 1-7 in Figure 4 are due to variations in the amount of DNA loaded in each lane. No hybridization was observed for the B 1 RFLP type, *S. carpocapsae* All strain or the UK *Heterorhabditis* isolate.

Quantitative analysis of pSf 106 and identification of dauerlarvae

Dilutions of DNA from *S. feltiae* RFLP types A 1 and A 2, and pSf 106 were dot blotted onto nylon membranes and hybridized with the gel purified insert cut from pSf 106. The signals obtained from the two RFLP types were of different intensities (Fig. 5). For the A 1 type, the 5 ng spot was of a similar intensity to the 5 ng spot of pSf 106. The plasmid pUC 18 is approximately 2.7 kb in size and the insert DNA is 277 bp in length, so that approximately 10 % of the plasmid is insert

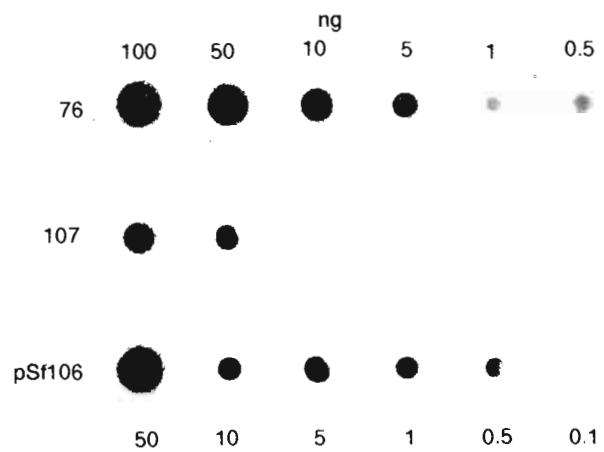

Fig. 5. Dot blot of dilutions of genomic DNA from 76, *S. feltiae (= bibionis)* (site 76) the A 1 RFLP type; 107, *S. feltiae (= bibionis)* (site 107) the A 2 RFLP type; and, pSf 106, the plasmid pSf 106. The dilutions are shown in ng and the filter was hybridized with the gel excised insert from pSf 106. N. B. the dilutions for the genomic DNAs are shown above the blot and the dilutions for the plasmid pSf 106 are below the blot.

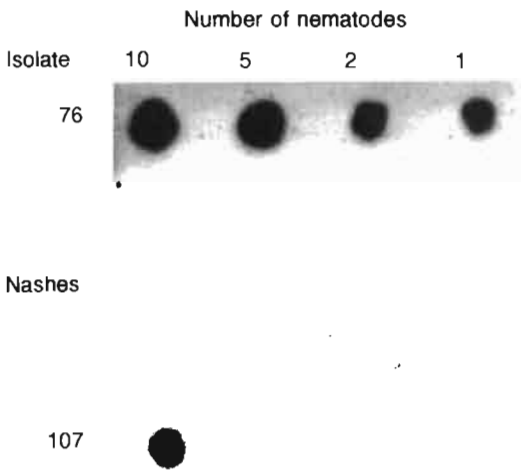

Fig. 6. Dot blot of the DNA extracted from infective stage nematodes. 76, *S. feltiae (= bibionis)* (site 76), the A 1 RFLP type; Nashes, *Steinernema* n. sp. (Nashes isolate), the B 1 RFLP type; and, *S. feltiae (= bibionis)* (site 107), the A 2 RFLP type. The filter was hybridized with pSf 106. The numbers correspond to the numbers of infective stage nematodes in each dot.

DNA. As the 5 ng spots of the genomic DNA and pSf 106 are of equal intensities, this clone must comprise about 10 % of the genome of this nematode. Assuming a genome size of 8×10^7 bp i.e. similar to that of *Caenorhabditis elegans* (Sulston & Brenner, 1974), then the 161 bp unit would be present in approximately 50 000 copies (10 % of $8 \times 10^7 = 8 \times 10^6$ bp, 8×10^6 divided by 161 bp = 50 000). This figure is comparable to ones obtained from sequences that have been isolated from other parasitic organisms such as the Hha I repeat from *Brugia malayi* (McReynolds *et al.*, 1986). By contrast, the A 2 variant contains fewer sequences homologous to pSf 106, so that the 100 ng spot of the genomic DNA is of similar intensity to the 5 ng spot of pSf 106. This means that about 0.5 % of the genome of this variant is homologous to pSf 106 which suggests a copy number of around 2500. This significant difference in the amounts of repetitive DNA does not appear to prevent cross-breeding of the two types, although neither will cross with members of the undescribed *Steinernema* species (Fan & Hominick, unpubl.). However, such differences may cause some incompatibility and because the cross breeding tests were of a preliminary nature it is now important to perform more extensive tests to determine the efficiency of mating.

When pSf 106 was hybridized to dot blots of extracts of 1-10 dauerlarvae, it was possible to detect a single nematode of the A 1 RFLP type after an overnight exposure and 10 nematodes of the A 2 variant (Fig. 6). Longer exposures were required to detect 5 nematodes of the A 2 RFLP type. No hybridization was detected with nematodes of the B 1 RFLP type even after lengthy exposures of several weeks.

Discussion

Use of the clone pSf 106 (the first documented species-specific clone for the genus *Steinernema)* can greatly reduce the amount of time needed for identification of unknown nematode isolates by DNA/DNA hybridization techniques. The normal procedure involves exposing soil samples to larvae of the wax moth *Galleria mellonella* (Bedding & Akhurst, 1975). Once the larvae have died, a wait of two to three weeks for the emergence of a new generation of infective stages is required, and often there are insufficient nematodes for DNA extraction. Then, a further set of *Galleria* larvae need to be infected with the resulting additional wait. Once sufficient nematodes are obtained, the processes of DNA extraction, restriction enzyme digestion, gel electrophoresis and Southern blotting can take a number of days. By contrast, only 1-5 nematodes are required for a positive/negative identification of an isolate if pSf 106 is used in a dot blot assay. Also, a large number of isolates can be tested quickly and cheaply compared to the time, labour and expense required for Southern blot RFLP analysis. Presence of a particular species in a soil sample could be confirmed by using standard soil extraction techniques (see Southey, 1986) and then performing the dot blot assay on the mixed community of nematodes.

Use of this clone avoids problems related to the use of morphological criteria for identification. The problem associated with the lack of material is as limiting for morphological studies as it is for DNA-based approaches. Because of the variability in quantitative characters, both dauer larvae and adult males must be obtained in numbers sufficient to make identification reliable. The males can only be obtained by infecting a suitable host in the laboratory and dissecting after an appropriate time. Relying on morphology for identification is further complicated when attempting to differentiate morphologically similar nematodes, as was the case in the UK. Use of a species-specific clone like the one described above leaves no doubt as to the identity of the nematodes. Production of such clones offers great potential for identifying the morphologically conservative species within the genus *Heterorhabditis*. To this end, Curran (pers. comm.) has produced a clone specific for *H. megidis*. Others will no doubt follow.

Examination of the nucleotide sequence of pSf 106 showed the clone to contain 1.75 copies of a 161 bp tandemly repeated unit. The 161 bp unit is 65 % A-T rich, a figure similar to those obtained from clones isolated from other nematodes (McReynolds *et al.*, 1986). This value is also consistent with the total A-T content for *C. elegans* of 65 % (Sulston & Brenner, 1974). There is 94 % homology between the two repeated sections of

pSf 106, indicating the highly conserved nature of the repeat unit within the genome of *S. feltiae*.

The ability to identify single infective stages will prove to be of great use in ecological and population studies. Curran (1990) makes the point that this would be possible using PCR (polymerase chain reaction) technology but that this method would not be cost effective. Specific clones are cost effective and we intend to use clone pSf 106 for such studies in the near future.

Acknowledgements

We thank our colleagues Dr. J. Bradley and Dr. Y. Kuo for their help and advice throughout the work documented here. Our thanks also to Mr. B. Briscoe for maintaining and rearing the nematodes examined in this study, to Dr. R. Akhurst (CSIRO, Canberra) for providing the T 319 isolate and to Dr. J. Sulston (MRC, Cambridge) for providing the *C. elegans* clone pCe 7. This work was supported by a grant from the Agricultural and Food Research Council.

References

AKHURST, R. J. (1983). *Neoaplectana* species : specificity of association with bacteria of the genus *Xenorhabdus*. *Exp. Parasitol.*, 55 : 258-263.

BEDDING, R. A. & AKHURST, R. J. (1975). A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica*, 21 : 109-110.

CHUNG, C. C., NIEMELA, S. L. & MILLER, R. H. (1989). One step preparation of competent *E. coli :* transformation and storage in the same solution. *Proc. natn. Acad. Sci. USA*, 86 : 2172-2175.

CURRAN, J. (1990). Molecular techniques in taxonomy. *In :* Gaugler, R. & Kaya, H. K. (Eds). *Entomopathogenic nematodes in biological control*, Boca Raton, Fl., CRC Press : 63-74.

FILES, J. G. & HIRSH, D. (1981). Ribosomal DNA of *Caenorhabditis elegans*. *J. mol. Biol.*, 149 : 223-240.

FILIPJEV, I. N. (1934). Miscellanea Nematologica 1. Eine neue Art der Gattung *Neoaplectana* Steiner nebst Bermerkungen über die systematische Stellung der letzteren. *Mag. Parasitol. Inst. Zool. Acad. USSR*, 4 : 229-239.

HOMINICK, W. M. & BRISCOE, B. R. (1990*a*). Occurrence of entomopathogenic nematodes (Rhabditida : Steinernematidae and Heterorhabditidae) in British soils. *Parasitology*, 100 : 295-302.

HOMINICK, W. M. & BRISCOE, B. R. (1990*b*). Survey of 15 sites over 28 months for entomopathogenic nematodes (Rhabditida : Steinernematidae). *Parasitology*, 100 : 289-294.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1989). *Molecular cloning : A laboratory manual.* 2nd. Ed., Cold Spring Harbor Publications : 9.1-9.62.

MCREYNOLDS, L. A., DESIMONE, S. M. & WILLIAMS, S. A. (1986). Cloning and comparison of repeated DNA sequences from the human filarial parasite *Brugia malayi* and the animal parasite *Brugia pahangi*. *Proc. natn. Acad. Sci. USA*, 83 : 797-801.

REID, A. P. & HOMINICK, W. M. (1992). Restriction fragment length polymorphisms within the ribosomal DNA repeat unit of British entomopathogenic nematodes (Rhabditida : Steinernematidae). *Parasitology* (in press).

SIM, B.K.L., MAK, J. W., CHEONG, W. H., SUTANTO, I., KURNIAWAN, L., MARWOTO, H. A., FRANKE, E., CAMPELL, J. R., WIRTH, D. F. & PEISSENS, W. F. (1986). Identification of *Brugia malayi* in vectors with a species-specific DNA probe. *Am. J. trop. Med. Hyg.*, 35 : 559-564.

SOUTHEY, J. F. (1986). *Laboratory methods for work with plant and soil nematodes. 6th. Ed.* London, HMSO : 2-30.

SULSTON, J. E. & BRENNER, S. (1974). The DNA of *Caenorhabditis elegans*. *Genetics*, 77 : 95-104.

WEISER, J. (1955). *Neoaplectana carpocapsae* n. sp. (Anguillulata, Steinernematidae), novy cizopasnik housenik obalece jablecného, *Carpocapsae pomonella* L. *Vestn. Česk. Spol. Zool.*, 19 : 44-52.

WIRTH, D. F. & MCMAHON-PRATT, D. (1982). Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. *Proc. natn. Acad. Sci. USA*, 79 : 6999-7003.