Analysis of Repetitive DNA Sequences from Potato Cyst Nematodes and Their Use as Diagnostic Probes

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ABSTRACT

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The potato cyst nematode species *Globodera rostochiensis* and *G. pallida* pathotype Pa2/Pa3, as well as *G. pallida* pathotype Pa1, can be distinguished from each other by restriction fragment length polymorphisms using DNA probes, which are present at low frequencies in the nematode genome. By differential screening of nematode DNA we have isolated DNA sequences that are highly abundant in *G. rostochiensis, G. pallida*, or the *G. pallida* Pa1 pathotype. These DNAs can be used as probes to distinguish potato cyst nematodes by a simple dot blot procedure using single cysts. The genomic organization of these highly abundant sequences has been examined; some of them are found to exist in long tandem arrays of 200 copies or more.

Potato cyst nematodes, *Globodera rostochiensis* (Woll.) Behrens and *G. pallida* (Stone) Behrens, are major pathogens of potato. A number of pathotypes within each species were distinguished on the basis of their ability to multiply on different species of wild potato. Kort et al (20) recognized five *G. rostochiensis* pathotypes (Ro1–Ro5) and three *G. pallida* pathotypes (Pa1–Pa3) in Europe, although others called this pathotype classification into question (21). Evidence was presented for a gene-for-gene relationship between *G. rostochiensis* (Ro1 and Ro4) and the H1 resistance gene derived from *Solanum tuberosum* ssp. *andigena* CPC 1673 (17,19,22). Although the situation with *G. pallida* is less clear, the Pa1 pathotype may be distinguished by using potatoes containing the resistance gene H2 from *S. multidissectum*, to which it has no virulence (22). Major gene (H1) resistance to the Ro1 and Ro4 pathotypes of *G. rostochiensis* was incorporated into commercial potato varieties by conventional plant breeding. However, widespread use of these cultivars (e.g., Maris Piper and Pentland Javelin) led to an increase in the distribution of *G. pallida*, for which only partial resistance is presently available (14). Effective use of resistant cultivars and programs for nematode control are therefore critically dependent on accurate and reliable nematode identification.

Identification of potato cyst nematode species using morphological characters is a specialized technique because of inconsistent variation and the conservative morphology of the potato cyst nematode. Moreover, identification of morphologically indistinguishable pathotypes within species is impossible using physical characters alone.

Attempts have been made to distinguish between nematode species and pathotypes using monoclonal antibodies (26) and various protein electrophoretic techniques (2,11,12). These techniques are, however, not entirely satisfactory, because they are time consuming, and because the protein population in the nematode is subject to environmental and developmental variation. Furthermore, it was shown that protein variation between pathotypes is often less than that observed within pathotypes (1,29).

The use of DNA probes to distinguish between different plant-pathogenic viruses (4), bacteria (3), and fungi (13) is becoming more widespread, and there have also been reports of DNA probes to distinguish plant-pathogenic nematodes (reviewed in 6,16). Here we describe the development of DNA probes derived from repetitive sequences that are diagnostic for either *G. rostochiensis, G. pallida*, or the Pa1 pathotype of *G. pallida*. These clones provide sensitive and reliable probes for the direct identification of individual cysts of potato cyst nematode using a simple dot blot procedure. In addition, we have examined the genomic organization of these clones and present the first report of tandemly repeated sequences in the potato cyst nematode.

MATERIALS AND METHODS

Cyst material. The *G. rostochiensis* Ro1 populations were from Plant Breeding International (PBI), Cambridge, or from collections at the Department of Agriculture for Northern Ireland (DANI) (Ro1A1 and Ro1A2). The other *G. rostochiensis* pathotypes were from the DANI collection (Ro2, Ro3, and Ro4, all originally from the Netherlands, and Ro5, originally from West Germany). The *G. pallida* populations were Pa2/Pa3 (PBI) or were from the DANI collection: pathotype Pa1 (B1, B2, Scottish, B11, B21, B31, B41, Caer, Dunminning, and Portglenone), pathotype Pa2 (CA, WF, C, and Netherlands), and pathotype Pa3 (E1, E2, EC, FS7l, PJ58, FS8O, Bankhead, Netherlands, and Charvornay).

In addition, five other samples of *G. pallida* were obtained from H. J. Rumpenhorst at the Biologische Bundensanstalt, Münster, Germany. Cysts of *G. tabacum* ssp. *solanacearum* and *Punctodera punctata* were obtained from the CAB International Institute of Parasitology.

Pathotype identification of the potato cyst nematode populations was made using the scheme of Kort et al (20).

DNA manipulation. Restriction enzyme digestions, agarose gel electrophoresis, DNA cloning, Southern blotting, and other standard molecular biology procedures were performed essentially as described by Sambrook et al (24). Genomic libraries of Ro1 (PBI), Pa2/Pa3 (PBI), and Pa1 B1 (DANI) were constructed in the Bluescript plasmid vector (Stratagene, La Jolla, CA) and transformed into *E. coli* strain DH5α. The differential screening procedure was performed on ordered arrays of recombinant colonies blotted onto duplicate Hybond N filters (Amersham, Bucks, UK). To identify species-enhanced clones, one filter was probed with ^{32}P-labeled total genomic DNA from *G. rostochiensis* and the other with ^{32}P-labeled total genomic DNA from *G. pallida*. *G. rostochiensis* clones that hybridized with *G. rostochiensis*

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genomic DNA, but not with *G. pallida* genomic DNA, were considered to be enhanced in *G. rostochiensis*, and vice versa. The Pa1 clones were prepared in a similar manner by comparing Pa1B1 (DANI) and Pa2/Pa3 (PBI) genomic libraries.

Radioactive labeling and DNA hybridization. DNA was radiolabeled with [α^{32}P]dCTP by means of the Multiprime Labelling System (Amersham), and unincorporated nucleotides were removed by Sephadex G-50 chromatography. Filters were prehybridized for 2–4 h and hybridized overnight at 65 C in 1× HSB (0.6 M NaCl, 20 mM PIPES, and 4 mM EDTA, pH 6.8), 1× Denhardt's III (0.2% gelatin, 0.2% Ficoll 400, 0.2% PVP360, 2.5% sodium pyrophosphate, and 8% SDS), and denatured salmon sperm DNA (100 μg/ml).

Blots were washed twice for 10 min in 2× SSC (0.3 M NaCl and 30 mM sodium tricitrate) plus 0.1% SDS at room temperature and then once in 0.1× SSPE (18 mM NaCl, 1 mM sodium phosphate, and 0.1 mM EDTA, pH 7.7) plus 0.1% SDS at 65 C for 60 min.

Preparation of dot blot samples. Individual cysts were transferred to microfuge tubes and homogenized in 10 μl of sterile water with a close-fitting pestle (Kontes, Vineland, NJ). The suspension was made 0.83 M with NaOH and incubated at room temperature for 10 min. Aliquots of each sample were spotted onto replicate Hybond membranes (Amersham), which were neutralized twice on filter paper soaked in 1.5 M Tris-Cl, pH 7.2, 0.5 M NaCl and 1 mM EDTA. The membranes were air dried and fixed under UV light (301 nm) for 2 min.

Estimation of relative difference in copy number of species-enhanced clones. Each species-enhanced clone was digested with *Eco*RI, and serial sixfold dilutions were made. The dilutions were electrophoresed and duplicate blots were prepared. One blot was probed with ^{32}P-labeled genomic DNA from *G. rostochiensis*, and the other with DNA from *G. pallida*. The autoradiographs were examined by eye to determine which dilutions gave similar signal intensities. For example, clones enhanced in *G. rostochiensis* hybridized well to genomic DNA from *G. rostochiensis* but not *G. pallida*. The lowest dilution of clone probed with *G. rostochiensis* DNA that gave a similar signal to the undiluted clone probed with *G. pallida* was taken as an estimate of the relative copy number difference. Dilutions of an rDNA clone and a single-copy clone that hybridized equally to both species were used as controls. This estimation assumes that *G. rostochiensis* and *G. pallida* have the same genome size.

RESULTS

RFLP analysis of potato cyst nematode. To assess the degree of similarity at the DNA level between *G. rostochiensis* and *G. pallida*, we looked for restriction fragment length polymorphisms (RFLPs). Random genomic clones were prepared from *Eco*RI-digested *G. rostochiensis* DNA assigned into single-copy, moderately repetitive, and highly repetitive categories after electrophoresis of plasmid DNA, transfer to membranes, and probing with ^{32}P-labeled total genomic DNA. Single-copy clones (i.e., those with a relatively weak hybridization signal) were then used to probe blots of potato cyst nematode genomic DNA digested with different restriction enzymes. This revealed extensive RFLPs between *G. rostochiensis* and *G. pallida* (Fig. 1A). We also used single-copy probes to determine if differences could be detected between pathotypes of *G. pallida*. The Pa1 pathotype was clearly distinguishable from Pa2 and Pa3 with the pSCR-44 probe used (Fig. 1B). Others have found that isolates of Pa1 were also clearly different from Pa2 and Pa3, and different isolates of Pa1 were similar to each other when examined with a limited number of probes (25).

Isolation of diagnostic repetitive sequences from *G. rostochiensis* and *G. pallida*. Although RFLPs can be used to differentiate potato cyst nematode species and certain pathotypes, routine identification would be facilitated by the isolation of sequences that are significantly more abundant in one species than in the other. Such sequences could then be used as diagnostic probes to screen dot blot samples without the need for DNA extraction, digestion with restriction enzymes, and electrophoresis.

Since repetitive sequences should provide the most sensitive probes, we decided to search for sequences that were more abundant in *G. rostochiensis* than in *G. pallida* and vice versa. Random genomic libraries were constructed from *Eco*RI-digested *G. rostochiensis* and *G. pallida* DNA. Each library contained approximately 1,000 recombinants. Clones containing high-copy-number sequences that were enhanced in one species but not the other were obtained by differential screening procedures on colony blots using ^{32}P-labeled total DNA from *G. rostochiensis* or *G. pallida* as probes. Plasmid DNA from selected colonies was isolated, digested with restriction enzymes, electrophoresed, and transferred to membranes. The differential screening technique was then repeated on the blots and confirmed the presence of 64 *G. rostochiensis*-enhanced and 14 *G. pallida*-enhanced clones.

The majority (63) of *G. rostochiensis*-enhanced clones contained inserts of approximately 120 bp. To determine whether these clones were related, the insert from one clone (pTID-1) was gel-purified and used to probe a blot of all the clones that were of a similar size. A total of 60 clones hybridized under stringent conditions and so probably represent the same sequence (data not shown). However, three clones were of similar size to pTID-1 but did not hybridize. These were pNTID-30 and pNTID-39, which were enhanced in *G. rostochiensis*, and pNTID-45, which was enhanced in *G. pallida*. Another *G. rostochiensis*-enhanced clone, pRo-67, had a 2.7-kb insert. The *G. pallida*-enhanced clones were sorted into groups on a size basis, and one clone, pPa-60 (2.9 kb), was used for further analysis.

To estimate the difference in copy number of these sequences between *G. rostochiensis* and *G. pallida*, duplicate blots of serial sixfold dilutions of each clone were prepared. Each blot was then probed with total genomic DNA from either *G. rostochiensis* or *G. pallida*. The results in Table 1 indicate that the greatest differences in relative copy number occurred with pRo-67 and pPa-60.

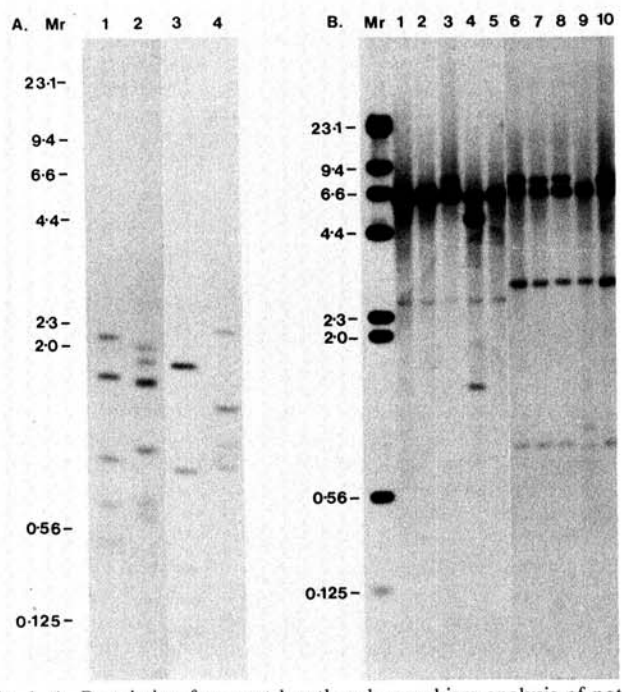

Fig. 1. A, Restriction fragment length polymorphism analysis of potato cyst nematode species. *Dra*I-digested genomic DNA from *Globodera rostochiensis* Ro1 PBI (lanes 1 and 3) and *G. pallida* Pa2/Pa3 PBI (lanes 2 and 4) blotted and probed with single-copy clones pSCR-44 (lanes 1 and 2) and pSCR-104 (lanes 3 and 4). **B,** RFLP analysis of *G. pallida* pathotypes. Genomic DNA from *G. pallida* pathotypes digested with *Hind*III (lanes 1–5) or *Pst*I (lanes 6–10), blotted and probed with pSCR-44. DNA was from Pa2 CA (lanes 1 and 6), Pa3 C (lanes 2 and 7), Pa3 E2 (lanes 3 and 8), Pa1 B2 (lanes 4 and 9), and Pa2/Pa3 PBI (lanes 5 and 10). Molecular weight markers (M_r) are shown (kb).

The quantitation of the relative amounts of repetitive DNA assumes that the sequences detected with a given probe in both nematode species are the same or very nearly so. If they are not, then hybridization with a probe isolated from the heterologous nematode will be inefficient with the stringencies used and lead to an overestimate of the relative copy number. While we can be reasonably certain that the relative copy number difference of the pTID-1 sequence itself is 200-fold higher in *G. rostochiensis*, it does not exclude the possibility that multiple copies of a sequence more distantly related to pTID-1 exist in *G. pallida*. In fact, we have isolated from *G. pallida* a sequence that is 85% identical to the *G. rostochiensis* pTID-1 (data not shown); it is not clear if this sequence would have been detected with the pTID-1 probe under the hybridization conditions we used for copy number estimation. Similar caveats exist for the calculations of the relative copy numbers of the other repeated sequences in the two nematode species.

Use of diagnostic probes. To determine whether the species-enhanced repetitive sequences that we had isolated could be used as rapid diagnostic probes, we devised a simple dot blot procedure for use on individual cysts without the need to purify DNA. Duplicate filters were prepared; one was probed with pRo-67, and the other with pPa-60. These probes were selected because they showed the greatest difference in copy number (Table 1). As shown in Figure 2, pRo-67 hybridizes with all *G. rostochiensis* populations tested but not with *G. pallida* (pRo-67 also hybridizes with samples of Ro2, Ro3, Ro4, and Ro5 but not with any *G. pallida* population tested; data not shown). Similarly, pPa-60 specifically cross-hybridizes with *G. pallida* populations (Fig. 2). A total of 38 *G. pallida* populations were tested: 10 Pa1, 10 Pa2, 16 Pa3, and 2 Pa2/Pa3 populations. All produced a positive signal. No cross-reaction with *G. t. solanacearum* and *P. punctata* cysts was observed with the pRo-67 or pPa-60 probe, indicating that the probes are highly specific for potato cyst nematode (data not shown). Both species-specific probes readily detected 30 ng of purified potato cyst nematode DNA after overnight exposure of the blot (data not shown). We did not test the sensitivity of the probes further.

Genomic structure of species-enhanced probes. We investigated the structure of the series of small (120- to 140-bp) *Eco*RI clones. The insert from pTID-1 was gel-purified and used to probe a blot containing digested and undigested genomic DNA from *G. rostochiensis* and *G. pallida* (Fig. 3A). The most prominent band in the *G. rostochiensis* *Eco*RI and *Hin*dIII tracks was approximately 120 bp, which was the same size as the pTID-1 *Eco*RI insert. This indicated that the *Eco*RI site in the repeat unit was well conserved in the genomic DNA. The presence of a 120-bp fragment in the *Hin*dIII digest suggested that there was a *Hin*dIII site within the 120-bp repeat unit and that it was arranged in at least one tandem array. The presence of an internal *Hin*dIII site was confirmed by sequence analysis of pTID-1 (Fig. 4). A small amount of a 240-bp fragment was also seen (Fig. 3A), and longer exposure revealed a regular ladder pattern with average increments of 120 bp. The majority of the signal in the *Eco*RV, *Dra*I, and *Bam*HI restriction digests of *G. rostochiensis* (Fig. 3A, tracks 3–5) was in an equivalent position to the undigested DNA (track 6) and migrated with the 23-kb marker. This suggested that the repeat unit does not cut with these enzymes, and the sequence of pTID-1 showed no sites for these enzymes (Fig. 4). Similar results were observed for *G. pallida*, although the copy number of the repeat unit was much reduced, and much longer exposure times were required.

A similar pattern was observed with the *G. rostochiensis*-enhanced clones pNTID-30 and pNTID-39; again the most prominent band in the *Eco*RI digest was approximately 120 bp. In addition, blots probed with pNTID-30 showed that this repeat was also fully excised with *Dra*I (Fig. 3B, track 4), demonstrating that the repeat unit contained both *Eco*RI and *Dra*I sites. This was confirmed by sequencing pNTID-30 (Fig. 4). The majority of the signal in the lanes of DNA digested with the other enzymes comigrated with undigested DNA at approximately 23 kb, which suggests that most of the repeat units are nearly 200 elements long and do not contain sites for these enzymes. The presence of faint bands in some of the tracks in the region of 2 kb and upward (for instance, the *Dra*I digest probed with pNTID-39, Fig. 3C, track 4, or other digests probed with pTID-45, Fig. 3D, tracks 7–9, 11, and 12) might indicate that some of the repeats of about 20 units and more are dispersed in regions of the genome flanked by sites for these enzymes. Alternatively, variation of sequence within very much longer repeats might lead to the occasional creation of sites for these enzymes.

Blots probed with the *G. pallida*-enhanced sequence pNTID-45 (Fig. 3D) showed that the majority of the signal in the *Dra*I track migrated at 130 bp (Fig. 3D, track 10), and longer exposures of the blot revealed the presence of a 130-bp fragment in the *Eco*RI track. This shows that this sequence also exists in long tandem repeats of a 130-bp unit, the majority having a *Dra*I site and the minority having an *Eco*RI site as well. The cloned pNTID-45 falls into the latter category (Fig. 4).

The sequence of the small *Eco*RI repeat clones pTID-1, pNTID-30, pNTID-39, and pNTID-45 was determined (Fig. 4). No significant homologies were detected either between clones or with sequences on the GenBank Database (December 1990 edition) using the DNASTAR package (DNA STAR, Inc., Madison, WI).

Clones pRo-67 and pPa-60 were also used to probe similar genomic blots and revealed complex banding patterns in *G. rostochiensis* and *G. pallida* tracks, respectively (Fig. 5A and B). In addition, the bands in the undigested DNA (tracks 6 and 12) migrated faster than the undigested DNA band when visualized by ethidium bromide (not shown) or when probed with the small *Eco*RI repeat clones (see Fig. 3).

Isolation of a diagnostic repetitive sequence from the Pa1 pathotype of *G. pallida*. Since the Pa1 pathotype of *G. pallida* was clearly distinguished from the Pa2 and Pa3 pathotypes by RFLP analysis (Fig. 1B), we decided to try and isolate diagnostic repetitive sequences. New plasmid genomic libraries were constructed from Pa1 and Pa2/Pa3 by the use of four different restriction enzymes (*Eco*RI, *Pst*I, *Hin*dIII, and *Bam*HI). Each pathotype library consisted of approximately 3,500 recombinants. Clones containing high-copy-number sequences that were enhanced in one pathotype but not in the other were obtained by differential screening procedures on colony blots. Plasmid DNA from selected colonies was isolated, cut with restriction enzymes, electro-

TABLE 1. Size and estimated relative difference in copy number[a] of clones enhanced in potato cyst nematode species

Clone	Size	Relative copy number in hybridization with:	
		Globodera rostochiensis	*G. pallida*
pRo-67	2.7 kb	1,300–7,700	1
pPa-60	2.9 kb	1	1,300–7,700
pTID-1	116 bp	200	1
pNTID-30	119 bp	6	1
pNTID-39	124 bp	1,300	1
pNTID-45	130 bp	1	>6

[a] Relative copy number is assessed by relative hybridization intensities of total labeled nematode DNA to serial dilutions of the species-enhanced clones.

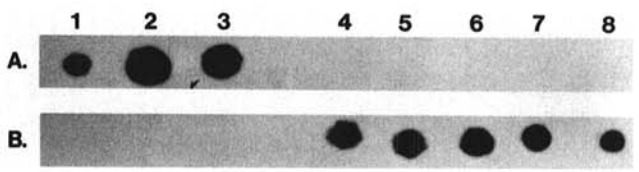

Fig. 2. Distinction between *Globodera rostochiensis* and *G. pallida* using single-cyst dot blots. Dot blots of single potato cyst nematode cysts probed with **A,** the *G. rostochiensis*-enhanced clone pRo-67, or **B,** the pallida-enhanced clone pPa-60. Cysts were from *G. rostochiensis* isolates Ro1 A2 (1), Ro1 A2 (2), and Ro1 PBI (3) and *G. pallida* isolates Pa1 B2 (4), Pa2 CA (5), Pa3 E2 (6), Pa3 C (7), and Pa2/Pa3 PBI (8).

phoresed, and transferred to membranes. The differential screening technique was then repeated on the blots and confirmed the presence of six Pa1-enhanced clones. Each had a 450-bp *Hin*dIII insert with a similar restriction pattern and so probably represented the same sequence. No Pa2/Pa3-enhanced clones were isolated.

Use of Pa1-enhanced sequence as a diagnostic probe. One Pa1-enhanced clone (pB10-1) was selected, and the insert was used to probe dot blot samples of cysts (Fig. 6A). Replicate blots were also probed with pWHALE-1 (Fig. 6D), which contains sequences homologous to rDNA (*unpublished observations*); pRo-67 (Fig. 6C); and pPa-60 (Fig. 6B). All Pa1 isolates tested strongly hybridized with pB10-1. No cross-reaction to *G. rostochiensis* was observed. However, long exposures revealed that some Pa2 and Pa3 populations weakly hybridized with pB10-1. This suggests that sequences with homology to pB10-1 are present in DNA isolated from non-Pa1 pathotypes, albeit at a significantly reduced level. Since this hybridization was observed even when individual Pa2/Pa3 cysts were probed, it is unlikely to be caused by contamination with Pa1. When such samples were probed with pWHALE-1 or pPa-60, strong signals were obtained, indicating that the reduction in intensity of Pa2/Pa3 cysts probed with pB10-1 is not a reflection of the amount of DNA in each cyst. In blind tests using 10 Pa1, 10 Pa2, and 16 Pa3 isolates, all Pa1 isolates were correctly identified. Hence, pB10-1 is suitable for use as a Pa1 diagnostic probe, provided duplicate blots are made, and the pB10-1 signal is similar to that obtained with pWHALE or pPa-60.

Genomic organization of the Pa1 diagnostic probe. To investigate the genomic organization of the Pa1 diagnostic clone, we probed blots of genomic DNA from two Pa1 and four non-Pa1 populations (Fig. 7). The most prominent band with the Pa1 DNA digested with *Hin*dIII was approximately 450 bp, indicating that the repeat unit was excised with *Hin*dIII. The majority of signal in the lanes with DNA digested with *Eco*RI and *Dra*I migrated with undigested DNA. This indicates that most of the repeat units did not excise with these enzymes and are, therefore, organized in a long tandem array. A ladder of up to 16 multiples of the basic 450-bp *Hin*dIII fragment can be seen (albeit at different intensities) on longer exposure of the blot. Whether this is due to partial digestion or the occasional absence of a *Hin*dIII site in the array was not investigated. In the *Dra*I digests of Pa1 DNA, a fraction of DNA ran in positions approximately equivalent to five to six repeat units, which could mean that a subfraction of the arrays had *Dra*I sites at this frequency. The Pa2/Pa3 samples on the same blot had either negligible hybridization (Pa3 E2, Fig. 7C) or much reduced hybridization. This is consistent with either a very much reduced copy number (or the presence of a sequence distantly related to B10-1) in the Pa2/Pa3 samples or a slight contamination of the Pa2/Pa3 cysts with Pa1 cysts. The observation that dot blots of individual cysts of some Pa2/Pa3 weakly hybridized to the B10-1 probe (see above) would support the former interpretation, although the amount of B10-1-hybridizing sequence in Pa2/Pa3 would have to vary from isolate to isolate to explain the variable hybridization seen on genomic blots (Fig. 7C–F).

DISCUSSION

In this report, we describe the isolation of repetitive elements from potato cyst nematode species and pathotypes that provide molecular tools for pathogen identification. Potato cyst nematode

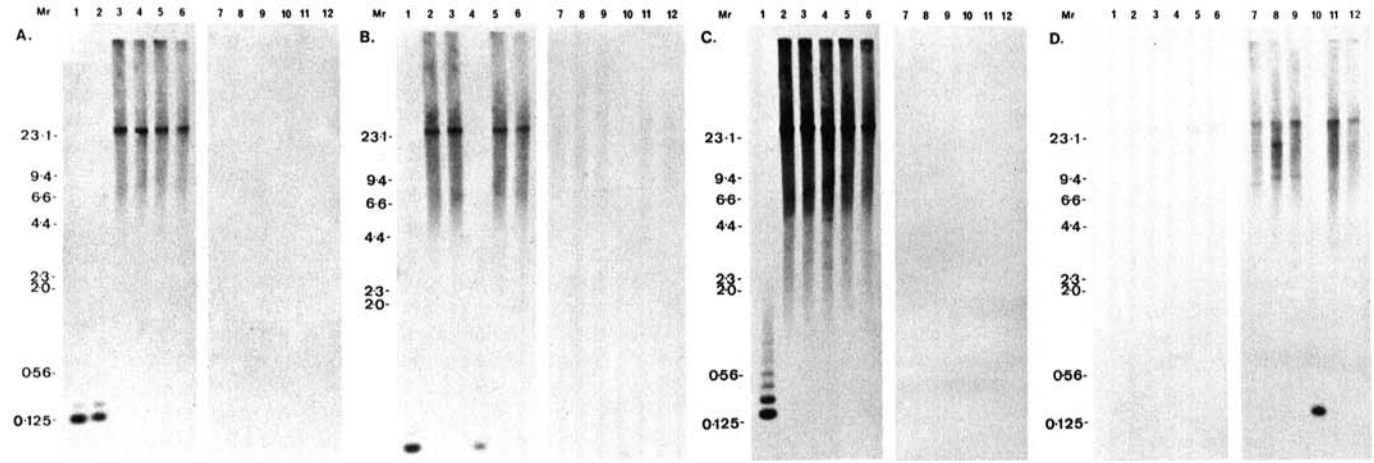

Fig. 3. Genomic structure of small *Eco*RI-repetitive clones. Genomic DNA from *Globodera rostochiensis* Ro1 PBI (lanes 1–6) and *G. pallida* Pa2/Pa3 PBI (lanes 7–12), blotted and probed with **A,** clones pTID-1; **B,** pNTID-30; **C,** pNTID-39; or **D,** pNTID-45. DNA was digested with *Eco*RI (lanes 1 and 7) *Hin*dIII (lanes 2 and 8), *Eco*RV (lanes 3 and 9), *Dra*I (lanes 4 and 10), or *Bam*HI (lanes 5 and 11) or was undigested (lanes 6 and 12). Molecular weight markers (M_r) are shown (kb).

pTID-1: gaattcttatgatagcaaaattgagcgttagaaaaaaatttgtgggtcaatagcattgatgccgaacaaattaggggaagcttgggacagttcacggaagagatagtggatttttt
EcoRI HindIII

pNTID-30: gaattcttagcctaatttttcccaaagaatgacggctaaccaaacatttaaatttatgtcccctaaattacccgttttcagccgctaaaaaacgactatctcttttcagttagcaaccg
EcoRI DraI

pNTID-39: gaattcctactctaaaaaagttttcaagtgtaacttttttgcaatattttcaggattaatatatctgataagggatattagtgggtttcttcaaaattgagctccgcagaaggtaaagaaaaaa
EcoRI

pNTID-45: gaattccttaacgggcaataagcagcccctaattttcccaacaaaacgggaaaggtaggtaattaatattttaaaagtaattataatttcgatatttaggagggtggggagcccatataaaatgctaatt
EcoRI DraI

Fig. 4. Nucleotide sequence of the small *Eco*RI repeat clones pTID-1, pNTID-30, pNTID-39, and pNTID-45. Relevant restriction sites are underlined.

species (7,9) and pathotypes of *G. pallida* (25) have been distinguished by RFLPs, and our data from the use of the single-copy probes pSCR-104 and pSCR-44 (Fig. 1) confirm this extensive polymorphism. However, rapid identification of potato cyst nematode species and pathotypes requires the development of diagnostic probes. These consist of sequences present in one pathogen but not the other and can be used with simple dot blot procedures to give unambiguous results. We have isolated three clones (pRo-67, pPa-60, and pB10-l) that provide sensitive and reliable probes that are specific for *G. rostochiensis, G. pallida*, and the Pal pathotype of *G. pallida*. Previously, a 370-bp *Hin*dIII fragment was isolated that specifically hybridized with DNA extracted from *G. pallida* (8). The probes we describe are effective directly on squashed cysts spotted onto a filter in a very simple procedure with no requirement to extract DNA. Hence, identification of potato cyst nematode species and pathotypes may now be carried out both rapidly and reliably. The ability to use single cysts with these probes means that mixed populations can be identified (*unpublished observations*). Assessment of the proportions of potato cyst nematode species and pathotypes may be crucial for the success of a diagnostic test, since field infestations are often mixtures.

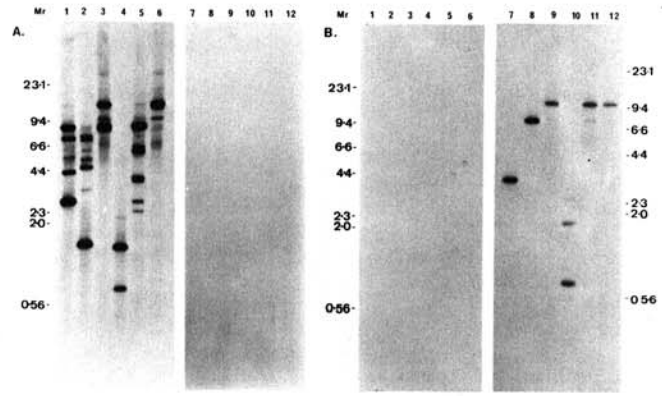

Fig. 5. Genomic structure of clones enhanced in potato cyst nematode species. Genomic DNA from *Globodera rostochiensis* Ro1 PBI (lanes 1–6) and *G. pallida* Pa2/Pa3 PBI (lanes 7–12), blotted and probed with **A,** the *G. rostochiensis*-enhanced clone pRo-67, or **B,** the *G. pallida*-enhanced clone pPa-60. DNA was digested with *Eco*RI (lanes 1 and 7), *Hin*dIII (lanes 2 and 8), *Eco*RV (lanes 3 and 9), *Dra*I (lanes 4 and 10), or *Bam*HI (lanes 5 and 11) or was undigested (lanes 6 and 12). Molecular weight markers (M_r) are shown (kb).

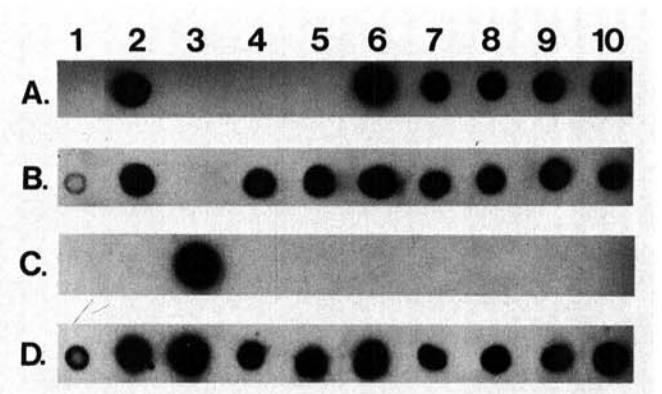

Fig. 6. Dot blot analysis of *Globodera pallida* pathotypes. Four cysts of each potato cyst nematode population were homogenized and probed with **A,** the *G. pallida* pathotype Pal enhanced clone pB10-l; **B,** the *G. pallida*-enhanced clone pPa-60; **C,** the *G. rostochiensis*-enhanced clone pRo-67; and **D,** a potato cyst nematode rDNA clone pWHALE-1. Cysts were Pa2 Netherlands (1), Pal B1 (2), Ro1 PBI (3), unidentified potato cyst nematode from PBI (4 and 5), Pal B41 (6), Pal B2 (7), Pal B1 (8), Pal Portglenone (9), and Pal Dunminning (10).

Potato cyst nematode populations containing the Pal pathotype are confined largely to Northern Ireland but are detected infrequently in Scotland and England (27). Within the province, Pal populations are restricted to a relatively small zone (some 40 km in diameter) centered on the town of Portglenone, which may represent the original focus of infestation following the introduction of Pal into the country (29). The origins of Pal are uncertain, the pathotype not having been detected in South America, the original source of all European potato cyst nematode populations. The possibility that Pal populations simply result from a mutation in a single avirulence gene (corresponding to the H2 resistance gene) combined with the founder effect would be at variance with the present findings, which reveal substantial and consistent differences in the DNA from Pal and Pa2/Pa3 revealed by RFLPs (Fig. 1) and the B10-1 probe (Figs. 6 and 7). More likely, Pal originated from a distinct (and as yet undetected) potato cyst nematode line in South America. The data of Stone et al (27) suggest that Pal may be present in many potato cyst nematode populations in Britain, albeit at low levels. The isolation of the Pal-specific pB10-1 probe will permit agricultural advisors to more accurately characterize *G. pallida* populations and to monitor any changes in pathotype composition resulting from the use of resistant and partially resistant potato varieties. In addition, they will also allow plant breeders to accurately determine the composition of potato cyst nematode populations used in selection of potato clones for resistance.

When undigested potato cyst nematode DNA was electrophoresed, blotted, and probed with pRo-67 and pPa-60, the bands migrated more rapidly (at approximately 12–17 kb) than total undigested DNA (approximately 23 kb) visualized with ethidium bromide (Fig. 5). This suggests that the probes might be derived from mitochondrial DNA. The sum total of the molecular weights of the bands revealed by probe pRo-67 (Fig. 5A) would, however, constitute a significant proportion of the 14- to 32-kb size characteristic of nematode mitochondrial DNA (15), leaving little room for the genes governing known mitochondrial functions. This would imply that pRo-67 hybridized to a dispersed repeat, the location of which varied in different mitochondrial DNAs of the population, or was inserted in a polymorphic region. Repeats of this type exist in the mitochondrial DNA of the nema-

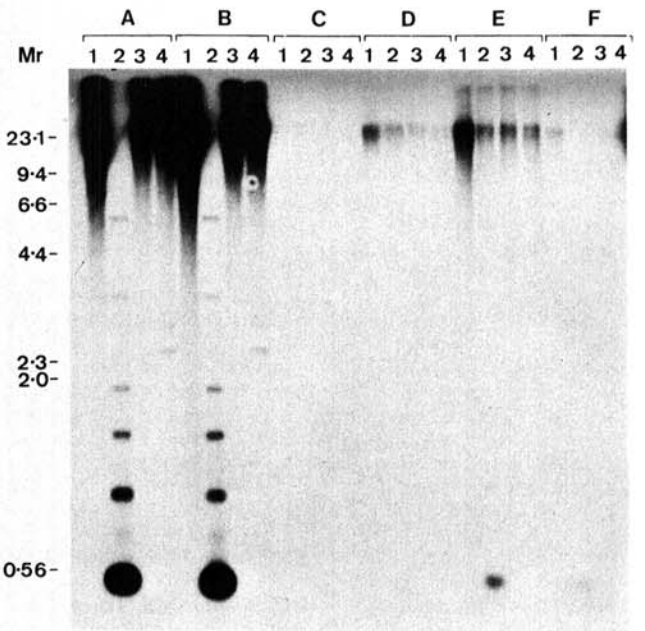

Fig. 7. Genomic structure of Pal-enhanced clone pB10-1. Genomic DNA from *Globodera pallida* pathotypes, blotted and probed with the Pal-enhanced clone pB10-1. DNA was undigested (lane 1) or digested with *Hin*dIII (lane 2), *Eco*RI (lane 3), or *Dra*I (lane 4). DNA was from **A,** Pal B2, **B,** Pal Scotland, **C,** Pa3 E2, **D,** Pa2/Pa3 PBI, **E,** Pa2 Ca, or **F,** Pa3 EC. Molecular weight markers (M_r) are shown (kb).

tode *Romanomermis culicivorax* (15). The simpler hybridization pattern observed with the probe pPa-60 (Fig. 5B) could also be consistent with a mitochondrial origin; on longer exposures of the blot, bands consistent with both open and closed circles can be seen in the undigested DNA track. If the sequences were indeed from mitochondria, it is surprising that such large probes do not cross-hybridize between the sister nematode species. However, without further work, the precise origin of these sequences remains unclear. Mitochondrial DNA is very abundant and highly variable, because it appears to evolve 10–100 times faster than nonrepetitive DNA (5). These features make it particularly suitable for generating specific and sensitive probes. Total mitochondrial DNA was used to detect *Meloidogyne* root galls by means of dot blots (23), and RFLPs were visualized by ethidium bromide staining of mitochondrial DNA from many nematode species (15).

We have presented evidence that the species-enhanced small *Eco*RI repeat family (pTID-1, pNTID-3O, pNTID-39, and pNTID-45) and the Pa1-enhanced pB10-1 sequences are arranged in tandem repeats. Tandemly repeated sequences are widespread in eukaryotic genomes and have been described in plants and many animal species, including the nematodes *Panagrellus silusiae* and *Ascaris lumbricoides* (10,28). Tandemly repeated DNA can be subdivided into two types. The first comprises repeated elements that are present in related species but have considerably different copy numbers, whereas the second type of sequence is highly species-specific. The small *Eco*RI repeat families pTID-1 and pB10-1 sequences described here would appear to belong to the first category, because each repeat unit was detectable in all isolates examined but had been specifically amplified in one particular species or pathotype. This amplification could have arisen by unequal crossing-over events or by slipped strand mispairing. When genomic DNA was digested with enzymes that did not cut within the repeat unit and probed with the small repeated clone, the DNA migrated to a similar position to that of undigested DNA and the 23-kb marker (Fig. 3). Since the repeat units are between 123 and 136 bp in length, there must be at least 170–187 copies in each tandem array (23 kb divided by 123–136 bp). Similar calculations can be applied to the other repeat units.

It has been suggested that tandem repeat structures may play a role in defining fertility barriers by limiting alien chromosome pairing (10). Further investigation of the organization of repetitive DNA within the genome of potato cyst nematode might enhance our understanding of the origin and evolution of closely related species and pathotypes.

LITERATURE CITED

1. Bakker, J. 1987. Protein variation in cyst nematodes. Ph.D. thesis. Agricultural University, Wageningen, Netherlands.
2. Bakker, J., and Bouwman-Smits, L. 1988. Genetic variation in polypeptide maps of two *Globodera rostochiensis* pathotypes. Phytopathology 78:894-900.
3. Barry, T., Powell, R., and Gannon, F. 1990. A general method to generate DNA probes for micro-organisms. Bio/Technology 8:233-236.
4. Boulton, R. E., Jellis, G. J., Baulcombe, D. C., and Squire, A. M. 1986. The application of complementary DNA probes to routine virus detection, with particular reference to potato viruses. Pages 41-53 in: Developments and Applications in Virus Testing. R. A. C. Torrance and L. Torrance, eds. Lavenham Press, Wellesbourne, U.K.
5. Brown, W. M., George, M., Jr., and Wilson, A. C. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. 76:1967-1971.
6. Burrows, P. R. 1990. The use of DNA to identify plant parasitic nematodes. Nematol. Abstr. 59:1.
7. Burrows, P. R., and Boffey, S. A. 1986. A technique for the extraction and restriction endonuclease digestion of total DNA from *Globodera rostochiensis* and *Globodera pallida* second stage juveniles. Rev. Nematol. 9:199-200.
8. Burrows, P. R., and Perry, R. N. 1988. Two cloned fragments which differentiate *Globodera pallida* from *G. rostochiensis*. Rev. Nematol. 11:441-445.
9. De Jong, A. N., Bakker, J., and Gommers, F. J. 1989. Repetitive DNA and hybridisation patterns demonstrate extensive variability between the sibling species *Globodera rostochiensis* and *G. pallida*. Parasitology 99:133-138.
10. De Kochko, A., Kiefer, M. C., Cordesse, F., Reddy, A. S., and Delseny, M. 1991. Distribution and organisation of a tandemly repeated 352bp sequence in the oryzae family. Theor. Appl. Genet. 82:57-64.
11. Fleming, C. C., and Marks, R. J. 1983. The identification of the potato cyst nematode *Globodera rostochiensis* and *G. pallida* by isoelectric focusing of proteins on polyacrylamide gels. Ann. Appl. Biol. 103:277-281.
12. Fox, P. C., and Atkinson, H. J. 1984. Isoelectric focusing of general protein and specific enzymes from pathotypes of *Globodera rostochiensis* and *G. pallida*. Parasitology 88:131-139.
13. Goodwin, P. H., English, J. T., Neher, D. A., Duniway, J. M., and Kirkpatrick, B. C. 1990. Detection of *Phytophthora parasitica* from soil and host tissue with a species-specific DNA probe. Phytopathology 80:277-281.
14. Gratwick, M., ed. 1989. Potato Pests. Ministry of Agriculture, Fisheries and Food Reference Book 187. Her Majesty's Stationery Office, London.
15. Hyman, B. C. 1988. Nematode mitochondrial DNA: Anomalies and applications. J. Nematol. 20:523-531.
16. Hyman, B. C. 1990. Molecular diagnosis of *Meloidogyne* species. J. Nematol. 22:24-30.
17. Janssen, R. 1989. Genetics of virulence in potato cyst nematode. Ph.D. thesis. University of Agriculture, Wageningen, Netherlands.
18. Jones, F. G. W., Carpenter, J. M., Parrott, D. M., Stone, A. R., and Trudgill, D. L. 1970. Potato cyst nematode: One species or two? Nature 227:83-84.
19. Jones, F. G. W., and Parrott, D. M. 1965. The genetic relationships of pathotypes of *Heterodera rostochiensis* Woll. which reproduce on hybrid potatoes with genes for resistance. Ann. Appl. Biol. 56:27-36.
20. Kort, J., Ross, H., Rumpenhorst, H. J., and Stone, A. R. 1977. An international scheme for identifying and classifying pathotypes of potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. Nematologica 23:333-339.
21. Nijboer, H., and Parlevliet, J. E. 1990. Pathotype-specificity in potato cyst nematodes, a reconsideration. Euphytica 49:39-47.
22. Parrott, D. M. 1981. Evidence for gene-for-gene relationships between resistance gene H1 from *Solanum tuberosum* ssp. *andigena* and a gene in *Globodera rostochiensis*, and between H2 from *S. multidissectum* and a gene in *G. pallida*. Nematologica 27:372-384.
23. Powers, T. P., Platzer, E. G., and Hyman, B. C. 1986. Species-specific restriction site polymorphism in root-knot nematode mitochondrial DNA. J. Nematol. 18:288-293.
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Schnick, D., Rumpenhorst, H. J., and Burgermeister, W. 1990. Differentiation of closely related *Globodera pallida* (Stone) populations by means of DNA restriction fragment polymorphisms (RFLPs). J. Phytopathol. 130:127-136.
26. Schots, A., Hermsen, T., Shouten, S., Gommers, F. J., and Egberts, E. 1989. Serological differentiation of the potato-cyst nematodes *Globodera pallida* and *G. rostochiensis*: II. Preparation and characterisation of species specific monoclonal antibodies. Hybridoma 8:401-413.
27. Stone, A. R., Holliday, J. M., Mathias, P. L., and Parrott, D. M. 1986. A selective survey of potato cyst-nematode pathotypes in Great Britain. Plant Pathol. 35:18-24.
28. Warren, T., and Pasternak, J. J. 1987. A related moderately repetitive DNA family in the nematodes *Ascaris lumbricoides* and *Panagrellus silusiae*. Nucleic Acids Res. 16:10833-10847.
29. Zaheer, K. 1991. Virulence and biochemical systematics of potato cyst-nematodes (potato cyst nematode). Ph.D. thesis. The Queen's University of Belfast, Northern Ireland.