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Repetitive DNA and hybridization patterns demonstrate extensive variability between the sibling species *Globodera rostochiensis* and *G. pallida*

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SUMMARY

A method is described for the isolation of high molecular weight DNA from females of potato cyst nematodes. Restriction enzyme analyses of repetitive DNA revealed 30 bands specific for *Globodera rostochiensis* and 8 bands specific for *G. pallida*. None of the repetitive DNA bands resolved was common to both species. Hybridization patterns, obtained with a *Drosophila* β-tubulin probe, revealed 4 common bands, 3 bands specific for *G. rostochiensis* and 12 bands specific for *G. pallida*.

Key words: *Globodera rostochiensis*, *Globodera pallida*, DNA, hybridization patterns.

INTRODUCTION

Globodera rostochiensis (Woll.) Skarbilovich and *G. pallida* Stone are major pests of potatoes. They are morphologically so similar that until 1970 they were considered as strains of one species (Jones *et al.* 1970; Stone, 1973). In both species a number of pathotypes have been defined by their ability to overcome certain genes for resistance (Kort *et al.* 1978). The current international pathotype scheme recognizes 8 pathotypes, 5 within *G. rostochiensis* (Ro_1–Ro_5) and 3 within *G. pallida* (Pa_1–Pa_3).

Various electrophoretic techniques have been used to distinguish *G. rostochiensis* and *G. pallida* on the basis of differences in their proteins. Polyacrylamide disc electrophoresis of females revealed 14–18 major protein bands and marked differences between the two species (Trudgill & Carpenter, 1971; Greet & Firth, 1977). Isoelectric focusing of eggs revealed 40 protein bands and 23 enzymes, but only 1 protein band and 2 enzymes exhibited interspecific variation (Fox & Atkinson, 1984). High resolution two-dimensional gel electrophoresis resolved approximately 250 gene products and demonstrated that the sibling species differ in 70% of their polypeptides (Bakker & Bouwman-Smits, 1988).

Nucleic acid techniques have been used successfully to distinguish species within *Drosophila* (Dowsett & Young, 1982), *Caenorhabditis* (Emmons, Klass & Hirsch, 1979), *Trypanosoma* (Majiwa & Webster, 1987), *Neurospora* (Natvig & Jackson, 1987) and *Brugia* (McReynolds, Desimone & Williams, 1986). Also plant-parasitic nematodes have been discriminated at the DNA level: *Meloidogyne* (Curren, Baillie & Webster, 1985; Curren, McClure & Webster, 1986), *Bursaphelenchus*

(Bolla, Weaver & Winter, 1988) and *Heterodera* (Radice *et al.* 1988). Although *G. rostochiensis* and *G. pallida* have been differentiated by using restriction enzyme analyses (Burrows & Boffey, 1986), no appropriate data are available on the extent of genetic divergence.

In this report a reproducible method for total DNA isolation is described for females of potato cyst nematodes and DNA restriction patterns of the sibling species *G. rostochiensis* and *G. pallida* are compared.

MATERIALS AND METHODS

Nematodes

The *G. pallida* population HPL-1 (pathotype Pa_2, Veendam, the Netherlands) and the *G. rostochiensis* population MIER (pathotype Ro_1, Wageningen, the Netherlands) were maintained on *Solanum tuberosum* L. 'Eigenheimer', susceptible to all pathotypes. To produce young females, plants of cultivar Eigenheimer growing in pots (1 litre) filled with a sandy loam soil were inoculated with approximately 200 cysts. The pots were maintained in a growth chamber at 18 °C and 16 h daylength and roots with young females were harvested 35 days after inoculation. The females were gently washed from the roots and separated from root debris by sieving (0·75 mm pore diameter) followed by centrifuging in a 35% sucrose solution (1000 *g*, 5 min). The female nematodes were rinsed with water, any remaining root debris was removed by hand picking and the females were stored in 0·2 g aliquots at −80 °C until used.

Extraction of total DNA

Several different methods were tested for extracting the DNA. The method found most successful is described below. Approximately 0·2 g of frozen nematodes (about 8000 females) were ground in a mortar on ice in 500 μl of homogenization buffer consisting of 0·1 M Tris (pH 8·5), 0·1 M EDTA, 0·2 M NaCl, 50 mM DTT and transferred to a 1·5 ml microcentrifuge tube. To the homogenate 200 μl of 10% (w/v) SDS and 500 μg proteinase K (EC 3.4.21.14) were added and incubated for 5 min at room temperature and then for 3 h at 65 °C. The solution was extracted twice with an equal volume of redistilled phenol (saturated with 0·01 M Tris, pH 8·0, 0·001 M EDTA buffer). The first extraction lasted 30 min with occasional mixing by gentle inversion of the microcentrifuge tube and the second extraction was overnight with gentle shaking. After each extraction the mixture was centrifuged (8000 g at 4 °C for 3 min) and the aqueous layer transferred to another 1·5 ml microcentrifuge tube. Before centrifugation 0·5 vol. of chloroform:isoamyl alcohol (24:1) was added and the solutions were gently mixed. The first phenol layer, with nematode debris in it, was back-extracted overnight in the presence of 200 μl of homogenization buffer and treated as the second phenol layer. The combined aqueous layers of both extractions were treated with RNase A (EC 3.1.27.5) (30 min, 37 °C) and with 100 μg of proteinase K (1 h, 65 °C). To remove these enzymes, the solution was again extracted once with an equal volume of redistilled phenol and twice with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated from the aqueous layer by adding 0·1 vol. of sodium acetate (3 M, pH 4·8) and 0·6 vol. of isopropanol at −20 °C and incubated for 10 min at room temperature. The precipitated DNA was pelleted in a microcentrifuge (10000 g at 4 °C, 3 min), washed twice with 200 μl of 75% (v/v) ethanol at −20 °C, dried under vacuum and resuspended overnight in 50 μl of sterile H_2O at 4 °C. Finally 150 μl of 0·01 M Tris, pH 8·0, 0·001 M EDTA buffer was added and the DNA was stored at 4 °C.

Digestion with restriction endonuclease

DNA in the stock solution was precipitated by adding 2·5 vol. of 96% (v/v) ethanol at room temperature and leaving for 30 min on ice. The DNA was spun down (10000 g at 4 °C for 3 min) and the pellet rinsed with 75% (v/v) ethanol, dried and dissolved in a small volume of sterile H_2O. The DNA was digested with 15 U/μg DNA restriction enzyme (*Bam* HI, *Hind* III, *Pst* I or *Xho* I) at 37 °C for 4 h in buffer supplied by the manufacturer (Boehringer Mannheim); 4 μg of RNase A was also added. After digestion, 0·1 vol. of loading buffer,

0·25% (w/v) bromophenolblue, 0·25% (w/v) xylene cyanol and 25% (w/v) Ficoll (type 400) (Maniatis, Fritsch & Sambrook, 1982), were added and the sample was loaded on to a 0·8% agarose gel containing 0·5 μg/ml ethidium bromide. The electrophoresis was performed at 2 V/cm in 0·04 M Tris–acetate and 0·001 M EDTA as electrode buffer.

Southern hybridization

Electrophoresis was terminated after 18 h and DNA was transferred from the gel onto a blotting membrane (Zeta probe, BioRad) by the method of Southern (1975), except that 20 × SSPE (3 M NaCl, 0·2 M $NaH_2PO_4.H_2O$, 0·002 M EDTA, pH adjusted with NaOH to 7·4) was used instead of SSC. The DNA was fixed to the membrane by u.v.-radiation for 1·5 min and baking at 80 °C for 1 h. The membranes were incubated for 6 h at 42 °C by flooding them to a depth of 2 mm with a pre-hybridization solution containing 50% formamide, 0·2% (w/v) BSA, 0·2% (w/v) Ficoll, 0·2% (w/v) polyvinyl pyrrolidone, 1 M NaCl, 2% (w/v) SDS, 5% (w/v) dextran sulphate, 50 mM Tris, pH 8·0, and 200 μg/ml herring sperm DNA. Prior to adding to the pre-hybridization solution, the DNA from herring sperm was heated at 90 °C for 10 min and cooled on ice for 10 min. The probe was a *Kpn* I–*Bam* HI fragment (0·5 kb) from the β_4-tubulin gene from *Drosophila hydei* (a generous gift from Dr R. Brand) which was labelled with α-[^{32}P]dATP (specific activity 3000 Ci/mmol) according to Feinberg & Vogelstein (1983). Prior to adding to the pre-hybridization mixture the probe was denaturated at 90 °C for 10 min and cooled on ice for 10 min. The blot was hybridized overnight at 42 °C and washed with 2 × SSPE, 1% SDS at room temperature (2 × 10 min) and with 0·5 × SSPE, 0·2% SDS at 42 °C (1 × 30 min). The membrane was exposed under X-ray film (Kodak X-omat AR) at −70 °C with an intensifying screen for several days.

The genetic distance was calculated according to Nei & Li (1979), $F = 2N_{xy}/(N_x + N_y)$, in which N_x and N_y are the total number of bands in genotype X and Y, respectively, and N_{xy} the number of common bands. The genetic distance D is 1-F.

RESULTS

Various methods were tested for isolating DNA from females of potato cyst nematodes. Neither the DNA extraction method of Emmons *et al.* (1979) nor those of Murray & Thompson (1980), Curran *et al.* (1985) or Burrows & Boffey (1986) produced long, restrictable DNA from *Globodera* females. This may have been because of the tannins from the cuticle of the females. All these extraction methods did not separate properly the tannins from the DNA.

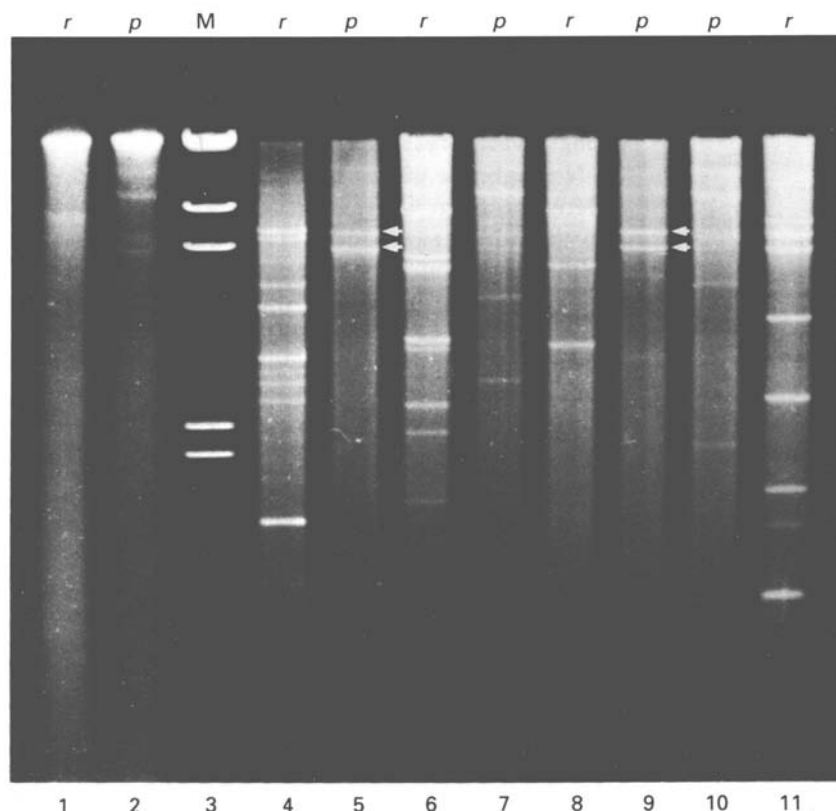The extraction method used here gives reasonable

Fig. 1. Repetitive DNA band patterns of *Globodera rostochiensis* (*r*) and *G. pallida* (*p*). Each lane carried 3 μg potato cyst nematode DNA. The 0·8 % agarose gel was stained with ethidium bromide and viewed under 230 nm transmitted u.v. irradiation. Arrows indicate bands present both in undigested and digested DNA. Lanes 1 and 2, undigested DNA; Lane 3, *Hind* III digested lambda DNA as a standard for molecular weight (bands are 23·0, 9·6, 6·6, 4·3, 2·3, 2·1 and 0·6 kb, respectively); Lanes 4 and 5, DNA digested with *Hind* III; Lanes 6 and 7, DNA digested with *Bam* HI; Lanes 8 and 9, DNA digested with *Pst* I; Lanes 10 and 11, DNA digested with *Xho* I.

Table 1. *Repetitive DNA bands and hybridization bands (kb) of a* Drosophila *β-tubulin probe obtained by treating total DNA from* Globodera rostochiensis (*r*) *and* G. pallida (*p*) *with restriction enzymes* (Common bands are italicized)

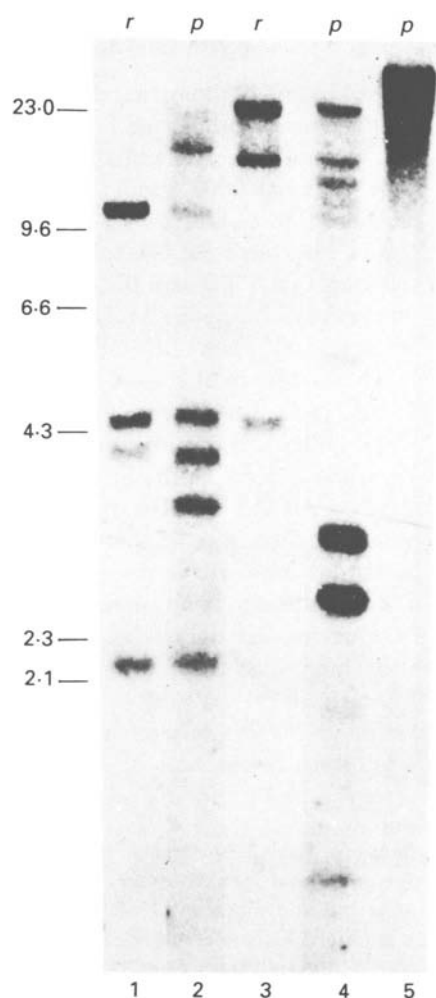| Restriction enzyme and species									
	Repetitive DNA bands								
r × *Hind* III	7·1	6·7	4·8	4·2	3·0	2·9	2·6	2·5	1·4
p × *Hind* III	0·8*	0·55*							
r × *Bam* HI	9·5	5·8	5·3	5·1	3·4	3·2	2·4	2·2	1·6
p × *Bam* HI	4·5	2·7							
r × *Pst* I	9·5	5·3	3·3						
p × *Pst* I	5·0*	3·0*							
r × *Xho* I	8·0	6·9	6·5	4·1	2·6	1·8	1·3	1·0	0·5*
p × *Xho* I	4·9*	2·15*							
	Hybridization bands								
r × *Xho* I	*10*	*4·4*	*3·8**	*2·2*					
p × *Xho* I	15	10*	*4·4*	3·7	3·3	*2·2*			
r × *Bam* HI	> 23	*14*	4·3						
p × *Bam* HI	> 23	*14*	12·5*	12*	11*	4·9*	3·1	2·8	2·0*
	1·4								

* Weak band.

Fig. 2. Restriction analysis of DNA of *Globodera rostochiensis* (*r*) and *G. pallida* (*p*). DNA fragments (3 μg each lane) were separated on a 0·8 % agarose gel and transferred to Zeta probe membrane. The membrane was then hybridized with a ^{32}P-labelled *β*-tubulin probe from *Drosophila hydei*. Lanes 1 and 2, DNA digested with *Xho* I; Lanes 3 and 4, DNA digested with *Bam* HI; Lane 5, undigested DNA. Molecular weight markers are indicated on the left (kb).

quantities (20 μg/100 mg wet weight of nematodes) of high molecular weight potato cyst nematode DNA which can be used for restriction analyses and hybridization experiments. Essential in our method is the second proteinase K treatment and DNA precipitation with isopropanol instead of ethanol. Isopropanol precipitation provides an adequate separation of the DNA from the tannins.

Treatment with the restriction enzymes *Bam* HI, *Hind* III, *Pst* I or *Xho* I revealed a number of bands on an agarose gel (Fig. 1) which represent multiple copies of repetitive DNA sequences. All restriction enzymes used showed different repetitive DNA sequence bands between *G. pallida* and *G. rostochiensis*. Most striking was the consistently greater number of bands obtained from *G. rostochiensis* compared with *G. pallida* (Table 1). Bands present

both in undigested and digested DNA were not taken into account (e.g. Fig. 1, Lane 2 and Lane 9).

Southern blots of digested nematode DNA hybridized with a ^{32}P-labelled *Drosophila β₄*-tubulin probe revealed various species-specific bands (Fig. 2). Not only the position and intensity of the bands differed between *G. rostochiensis* and *G. pallida*, but also the number of bands. Hybridization with a *β*-tubulin probe from the nematode *Haemonchus contortus* gave comparable hybridization patterns (results not shown). Weak bands (e.g. Fig. 2, Lane 2, the 10 kb band and Lane 4 the 1·4 kb band) may result from nucleotide divergence between the *Drosophila β*-tubulin gene from which the probe is obtained and the *Globodera β*-tubulin genes.

The overall genetic distance (*D*) between *G. rostochiensis* and *G. pallida* was estimated at 0·87. This estimate was based on both the repetitive bands and the bands obtained with the *β₄*-tubulin probe (Table 1).

DISCUSSION

As summarized by Fox & Atkinson (1986) a variety of biochemical techniques have been used to differentiate and identify nematode species. Using these methods, several authors have reported distinct protein differences between the sibling species *G. rostochiensis* and *G. pallida* (e.g. Bakker, 1987; Schots *et al.* 1987; Fox & Atkinson, 1984). In the present study striking differences at the DNA level have been demonstrated between females of the two potato cyst nematode species. Intraspecific variations at the level of DNA, focused on populations of pathotypes, are under investigation.

The differences observed in the number of repetitive DNA bands between *G. rostochiensis* and *G. pallida* suggest a dissimilar organization in the number, size and/or arrangement of repetitive DNA sequence families. Striking differences between sibling species have been reported before (McLain, Rai & Fraser, 1987; Majiwa & Webster, 1987; Cameron *et al.* 1988). In *D. melanogaster* the amount of repetitive DNA is 7-fold greater than in the sibling species *D. simulans* (Dowsett & Young, 1982). Another explanation, not mutually exclusive, may be the higher heterozygosity index of the *G. pallida* population, as was established by two-dimensional gel electrophoresis of total protein (Bakker, 1987). This index may also be representative for repetitive DNA. Multiple alleles in a population will result in fewer fragments of the same length and these fragments may be invisible on an ethidium bromide stained agarose gel.

The tubulins are evolutionary conserved proteins. Tubulins from a wide variety of eucaryotic sources migrate with comparable mobilities on one- and two-dimensional gels and tubulins from these diverse sources will form co-polymers (Snyder &

McIntosh, 1976). It is therefore not surprising that under non-stringent conditions a *Drosophila* β-tubulin probe hybridizes with *Globodera* β-tubulin genes or related sequences. Multiple copies of tubulin genes occur in most eucaryotes investigated to date and vary from 2 to 20 copies/haploid genome (Landfear, McMahon-Pratt & Wirth, 1983; Cleveland *et al.* 1980; Tomashaw *et al.* 1983). Our results indicate that *G. rostochiensis* and *G. pallida* are not exceptions in this generalization; both species seem to have multiple copies of the β-tubulin gene. The larger number of bands observed in the *G. pallida* population does not necessarily imply that this population has more copies of the β-tubulin gene than the *G. rostochiensis* population, but it may result from the higher heterozygosity index of this population.

The observation that the large majority of the hybridization bands and all repetitive DNA bands obtained in our study are species specific is in striking contrast with the morphological similarity between *G. rostochiensis* and *G. pallida*. These large differences suggest that the two species are considerably more distinct than is evident from their conserved morphology. These data are corroborated by large differences obtained with two-dimensional gel electrophoresis of proteins (Bakker & Bouwman-Smits, 1988). Such contrasts between morphological and molecular data are probably not rare in the phylum Nematoda (Emmons *et al.* 1979; Butler *et al.* 1981).

Hybridization techniques and restriction enzyme analyses are powerful tools for biochemical taxonomy, because, in contrast with electrophoresis of proteins, numerous characters can be measured. Moreover, these techniques sample often the non-coding regions of the genome. The non-coding regions, mostly repetitive DNA, are subjected to rapidly evolutionary changes and display not only extensive interspecific variation, but also intraspecific variation (McLain *et al.* 1987). The techniques employed here may therefore also be useful in tracing genetic differences between conspecific potato cyst nematode populations. Furthermore, the use of molecular biological techniques is a promising route towards the identification of virulence genes in potato cyst nematodes and pathotyping field populations.

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