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The use of repeat sequence primers for investigating genetic diversity between populations of potato cyst nematodes with differing virulence

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Summary – A number of biochemical and molecular techniques have been used to investigate genetic variability in potato cyst nematodes with a view to finding markers related to pathotypes or to find groupings that relate to biological characteristics. In this study simple sequence repeat (SSR) primers have been used on a number of *Globodera rostochiensis* and *G. pallida* populations. Eight such SSR primers were examined of which three gave reproducible results. The data obtained from these clearly separated the two species and allowed discrimination between the populations within each species. Some of the groupings obtained related to the geographical origin of the populations rather than their virulence characteristics.

Résumé – *Utilisation d'amorces en séquence répétitive pour l'étude de la diversité génétique entre populations de nématodes à kystes parasites de la pomme de terre ayant des virulences différentes* – De nombreuses techniques biochimiques et moléculaires ont été utilisées pour l'étude de la variabilité génétique des nématodes à kystes de la pomme de terre en vue de trouver des marqueurs liés aux pathotypes ou de définir des groupements à caractéristiques biologiques. Au cours de la présente étude ont été utilisées des amorces en séquence répétitive simple (SSR) appliquées à des populations de *Globodera rostochiensis* et *G. pallida*. Huit de ces SSR ont été testées dont trois donnent des résultats reproductibles. Les résultats obtenus permettent une séparation nette des deux espèces de même qu'une différenciation entre populations d'une même espèce. Certains des groupements obtenus paraissent plus liés à l'origine géographique qu'aux caractéristiques de virulence.

Key-words : *Globodera* spp., PCR, simple sequence repeats, virulence, genetic diversity.

Various biochemical techniques have been used to study population diversity in potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) including isozymes and RFLP's (Fleming & Marks, 1983; Fox & Atkinson, 1984; Phillips *et al.*, 1992). In both approaches there is a requirement for a relatively large quantity of material to provide enough protein or DNA for the analysis. Methods employing small amounts of DNA can have advantages and polymerase chain reaction (PCR) based procedures meet this requirement. In studies of *Xiphinema* and *Meloidogyne* species, primers that amplify specific areas of the genome had been used by Vrain *et al.* (1992) and Powers and Sandall (1990), whereas Bakker *et al.* (1993), Xue *et al.* (1993), Cenis (1993) and Caswell-Chen *et al.* (1992) used randomly amplified polymorphic DNA (RAPD's) (Williams *et al.*, 1990) or arbitrarily primed PCR (Welsh & McClelland, 1990) on *Globodera, Meloidogyne* and *Heterodera* species DNA. In using the latter two techniques care must be exercised in the use of RAPD's both in their interpretation but especially in the procurement of reproducible data (Hadrys *et al.*, 1992; Black, 1993).

Arbitrary primers based on short tri- or tetrameric repeats were used by Perring *et al.* (1993) to distinguish species of whitefly and Van der Knapp *et al.* (1993) also used them to distinguish between bacterial-feeding nematodes. These primers are fifteen to twenty bases long and annealing temperatures of 52 or 60 °C are used in the PCR reaction. Both these factors are likely to increase the specificity of the reaction when compared to shorter 10 mer primers where lower annealing temperatures must be used.

In this study primers based on repeat sequences were investigated as a means of studying genetic variation in PCN whilst at the same time the sequence data available from *Caenorhabditis elegans* was also examined to determine what repeat sequences are likely to be found in nematodes.

Materials and methods

All sequences from *Caenorhabditis elegans* in the EMBL databank were searched for some of the possible di-, tri- and tetranucleotide repeats using the Findpatterns programme in the Sequence Analysis Software from the Genetics Computer Group (Devereux *et al.*, 1984). Obvious duplications and poly (a) tails of cDNA clones were omitted. The search was conducted in October 1994 when the database held 4.4×10^6bp of *C. elegans* sequence (genome size of *C. elegans c.* 10^8bp).

NEMATODE POPULATIONS AND DNA EXTRACTION

Seven populations of *G. rostochiensis* from Europe and fifteen populations of *G. pallida* from Europe and South America were studied (Table 1). DNA was extracted using the procedure described by Phillips *et al.* (1992).

Table 1. *Populations of potato cyst nematode.*

Code	Species	Pathotype	Origin
British A	*G. rostochiensis*	Ro1	U.K.
A43	*G. rostochiensis*	Ro1	Netherlands
C257	*G. rostochiensis*	Ro3	Netherlands
C258	*G. rostochiensis*	Ro3	Netherlands
F536	*G. rostochiensis*	Ro4	Netherlands
G1510	*G. rostochiensis*	Ro5	Netherlands
Harmetz	*G. rostochiensis*	Ro5	Germany
D375	*G. pallida*	Pa2	Netherlands
P2-22	*G. pallida*	Pa2	Netherlands
Coll. 1077	*G. pallida*	Pa3	Netherlands
VP 74-768-20	*G. pallida*	Pa3	Netherlands
VP 75-884-4	*G. pallida*	Pa3	Netherlands
Rookmaker	*G. pallida*	Pa3	Netherlands
E408	*G. pallida*	Pa3	Netherlands
BBA2	*G. pallida*	Pa2	Germany
P4a	*G. pallida*		S. America
P5a	*G. pallida*		S. America
Luffness	*G. pallida*	Pa3	Scotland
Friskney	*G. pallida*	Pa2	England
Yapham	*G. pallida*	Pa3	England
Newton	*G. pallida*	Pa3	England
Bedale	*G. pallida*	Pa3	England

PCR REACTIONS

Eight oligonucleotide primers were used. They comprised simple repeated di-, tri- or tetrameric repeats as follows:
5′-dCACACACACACACACA-3′;
5′-dGTGGTGGTGGTGGTG-3′;
5′-dCAGCAGCAGCAGCAG-3′ (Rodriguez & Yoder, 1991); 5′-dAGGAGGAGGAGGAGG-3′;
5′-dTCCTCCTCCTCCTCC-3′ (Gupta & Filner, 1991); 5′-dGACAGACAGACAGACA-3′ (Weising *et al.*, 1989); 5′-dGGATGGATGGATGGAT-3′ and 5′-dGATAGATAGATAGATA-3′. These are designated $(CA)_8$ $(GTG)_5$, $(CAG)_5$, $(AGG)_5$, $(TCC)_5$, $(GACA)_4$, $(GGAT)_4$, and $(GATA)_4$, respectively in the text.

Table 2. *SSR primers studied, their TM values, annealing temperatures and summary results.*

	TM°C	Annealing Temp. °C	
$(CA)_8$	48	52-60	Smear
$(GTG)_5$	50	60	Inconsistant bands
$(GAG)_5$	50	60	Bands
$(AGG)_5$	50	52	Bands
$(TCC)_5$	50	42	Bands
$(GGAT)_4$	48	52	Inconsistant bands
$(GACA)_4$	48	52	Inconsistant bands
$(GATA)_4$	40	40-52	No products

Table 3. *The number of simple sequence repeats is recorded in the EMBL database for* Caenorhabditis elegans.

Number of repeats:	4-5	6-7	8-9	10-11	12-13	14-15	16-17	18-19	20-21	> 22	Total
AG/TC			21	6	1	1				2	31
AT/TA			5	8	4	1	1				19
AC/GT			9	2	1		1			1	14
GAA/CCT	27	2	4		1						34
TAA/ATT	12	4									16
ATC/TAG	12	1									13
ACC/TGG	4	1									5
AAC/TTG	2		1								3
ACG/TGC	2										2
ACT/TGA	1										1
AGC/TCG	1										1
AGG/TCC	1										1
AAAT/ TTTA	10	1									11

ACTC, AGAT, AGGC, AAAG and AAGT were found to each occur once with four repeats.

Amplification reactions including 6 ng of DNA in 50 µl of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µm dATP, dCTP, dGTP, and dTTP and one unit of Boehringer Taq polymerase. Amplifications for 45 cycles of 90 s at 94 °C with an annealing temperature of between 45 and 60 °C for 1 min and extension at 72 °C for 2 min with maximum ramp times between each phase were performed to find the most reproducible results. The final annealing temperatures used for each primer are given in Table 2. The products from these PCR reactions were separated by electrophoresis in TBE buffered 1.5 % agarose gels (Sambrook *et al.*, 1989) and the products visualized with ethidium bromide and UV illumination. To assess reproducibility each reaction was repeated on three separate occasions.

Results

Database search

The search of the EMBL database revealed that the most abundant repeats were dinucleotides the majority of which were no more than 22 bp in length (Table 3).

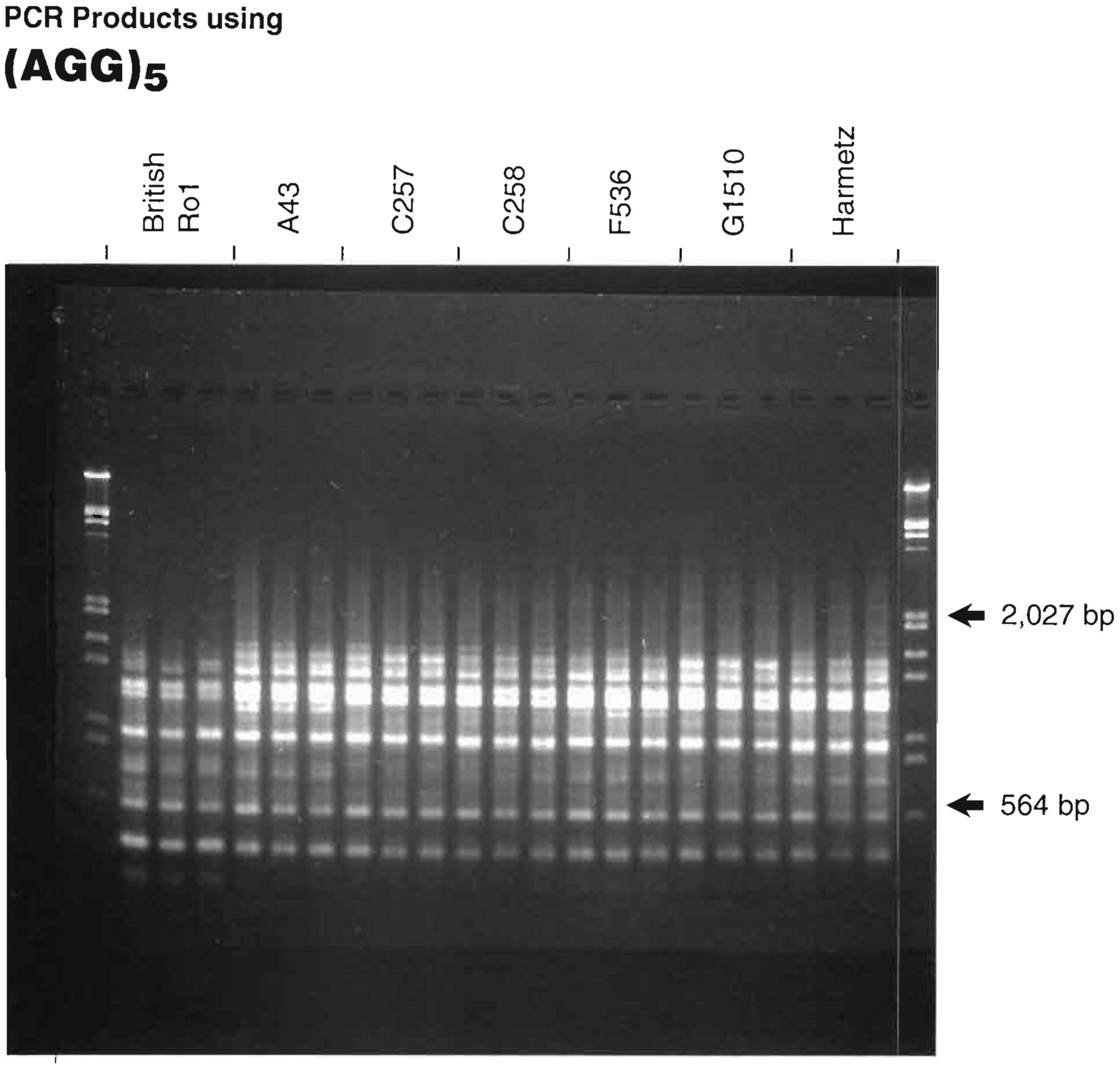
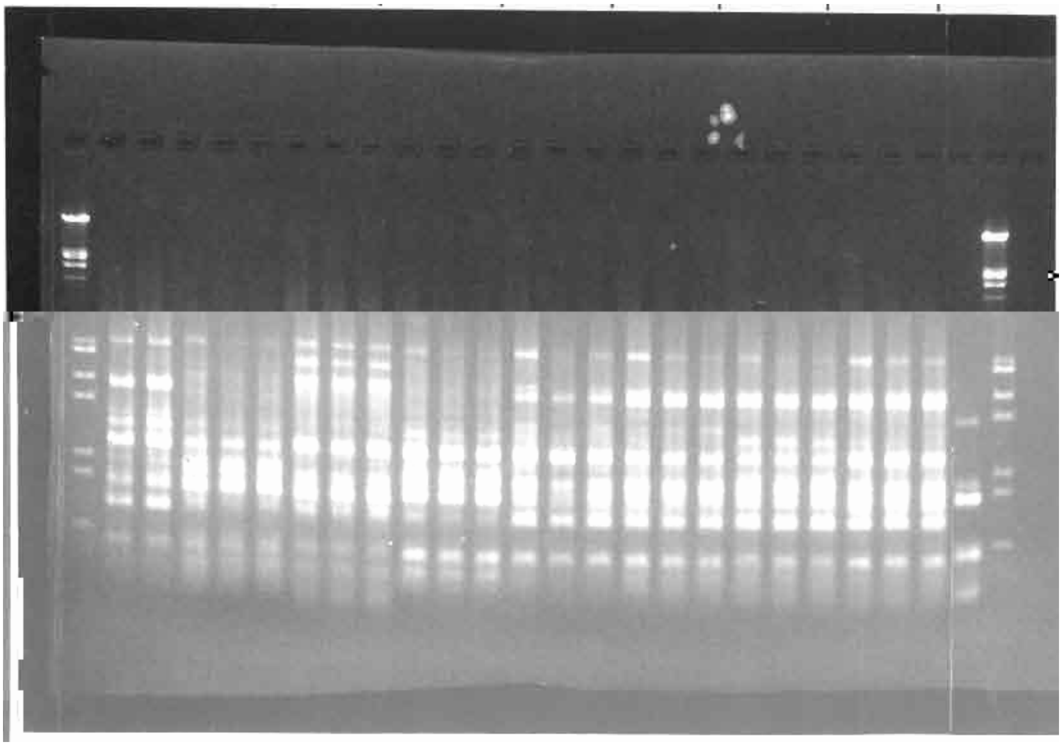


Fig. 1. *PCR Products from* Globodera rostochiensis *and* G. pallida *populations using the (GACA)$_4$ primer. The marker used was Lambda DNA cut with HindIII and EcoRI.*



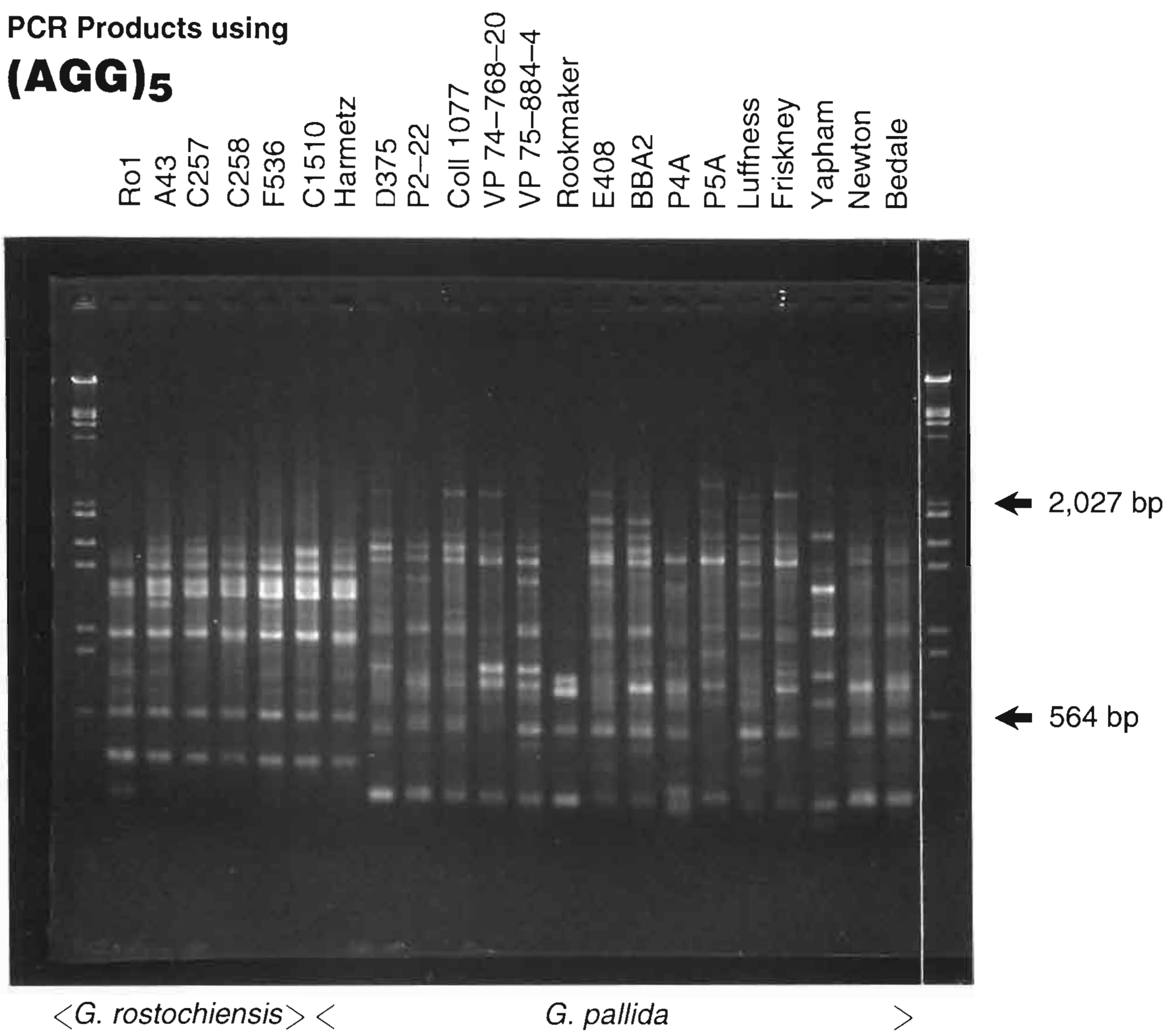


Fig. 3. *PCR products from* Globodera rostochiensis *and* G. pallida *populations using the* $(AGG)_5$ *primer.*

species did not appear to be related to virulence or pathotype but in some instances reflected geographic origin. Within *G. rostochiensis* the British Ro1 population was most distinct from all the other populations from the Netherlands and Germany (including the Ro1 population [A43]). Within the remainder the Ro5 (G1510) was grouped with the Ro3 (C257) whilst the Ro4 (F536) was grouped with the other Ro3 (C258) and the Ro5 (Harmetz) and thus there was no obvious clustering relating to pathotype. Within the *G. pallida* there are some groupings related to geographic origin. The P5A population from South America is the most distinct (< 50 % similarity). The group of Friskney, Yapham, Bedale and Newton populations are from England (92.7-81 % similarity) whilst the populations from the Netherlands are more diverse (90.5-62.2 % similarity). This latter cluster includes two groups namely P2.22, Coll. 1077, VP 75-884-4 and D375, E408 as well as the Rookmaker populations and BBA2 (from Germany). The remaining populations, Luffness, Vp 74-768-20 and P4A, are also dissimilar (< 65 % similarity).

Discussion

This study has shown that this type of SSR primer can have utility in examining the genetic variation in PCN. Not all primers were of equal value, however, and thus the greater length of the primers and higher anneal-

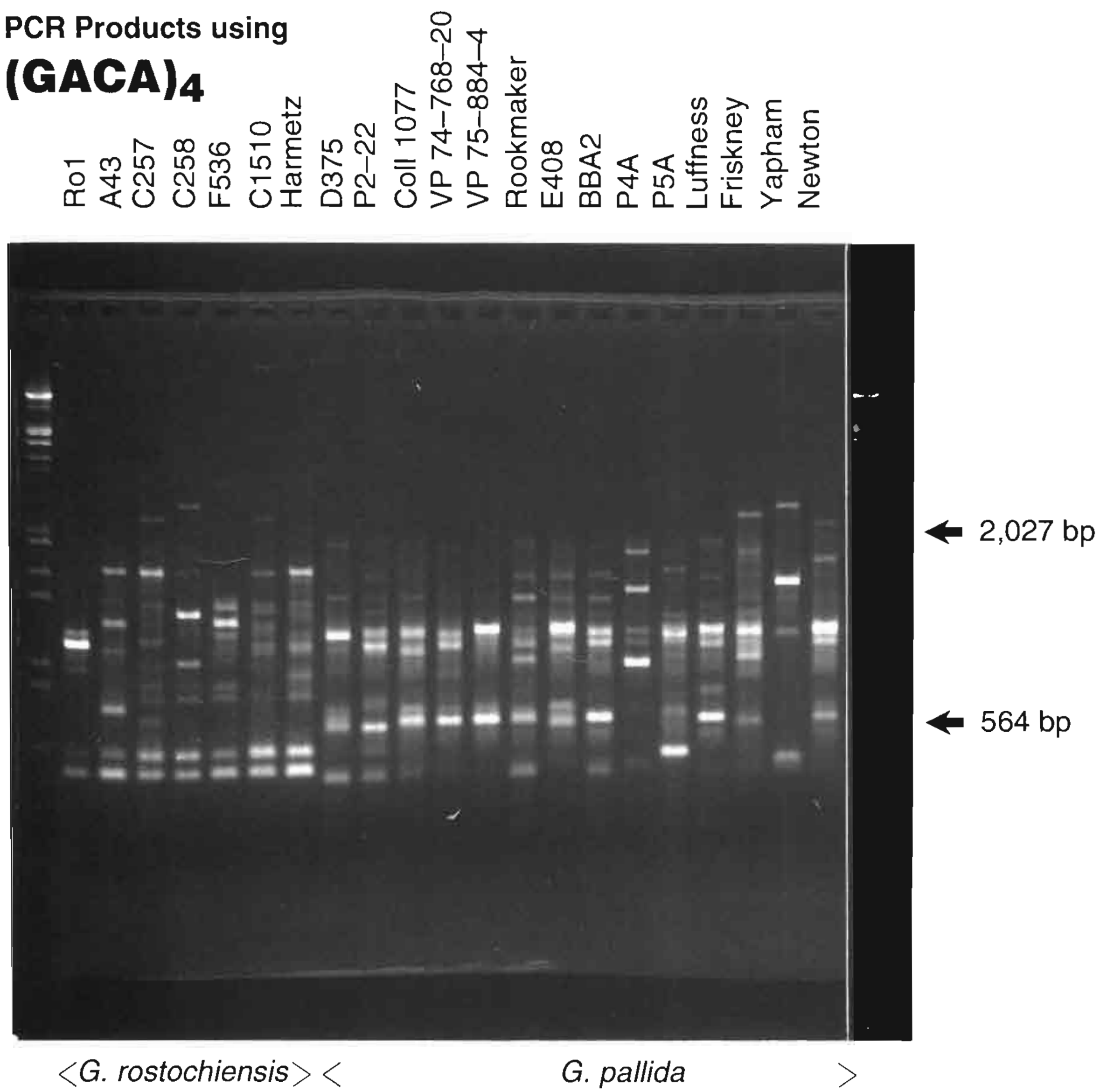


Fig. 4. *PCR products from seven* Globodera rostochiensis *populations using the* $(AGG)_5$ *primer showing replicate reactions.*

ing temperatures used does not necessarily lead to either greater specificity in priming or reproducibility. The SSR primers that were consistent here appeared to be less sensitive than 10mer primers used in RAPD analyses (Harrower, pers. comm.) to small changes in reaction conditions and the patterns were produced repeatedly.

Only one dimer primer was investigated and it amplified too many fragments to be useful. The most effective were the trimeric repeats with a GC content of 66 % other than $(GTG)_5$. The results presented here are a further indication of the wide utility that these primershave in a range of biological organisms such as plants, fungi and insects (Gupta & Filner, 1991, Rodriguez & Yoder, 1992 and Perring *et al.*, 1993, respectively). The tetramer primers proved difficult to use. The *C. elegans* database search showed a relative lack of tetramer repeats and if it could be assumed that plant parasitic nematodes are similar in this respect then our lack of success could be explained. However the data presented here cannot be used to support or refute this assumption partly because an exhaustive testing of all possible tetramer primers was not undertaken. The relative scarcity of these tetramer repeats in a database does not preclude their existence and indeed Van der Knapp *et al.*