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Identification of potato cyst nematodes using the polymerase chain reaction

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Summary – The polymerase chain reaction was used to amplify a region between the 5S rRNA and spliced leader RNA genes in *Globodera rostochiensis* and *G. pallida*. Isolates of *G. rostochiensis* consistently amplified a single 914 bp product and were distinguishable from *G. pallida* isolates which amplified 914 and 853 bp products or, in the case of most *G. pallida* Pa1 populations, a single 853 bp product. Concordant identifications of *G. pallida* and *G. rostochiensis* isolates were obtained when using 5S-SL PCR, PCN specific DNA probes and differential host plant tests. Distinguishing *G. pallida* Pa3 from *G. pallida* Pa1 was less certain as some PA1 isolates amplified both 914 and 853 bp products (typically a Pa3 reaction) yet hybridised with a Pa1 specific DNA probe. In the case of one of these isolates, host resistance tests indicated that the nematode population may be a Pa1-Pa3 mixture. Hybridisation experiments indicated that the DNA probe sequences were not homologous to the amplifiable 5S-SL sequences.

Résumé – ***L'identification des nématodes à kystes de la pomme de terre au moyen de la réaction d'amplification en chaîne par polymérase.*** – On a utilisé la réaction d'amplification en chaîne par polymérase (PCR) pour amplifier une région entre l'ARNr 5S et la séquence de tête épissée de gènes d'ARN (5S-SL) de *Globodera rostochiensis* et de *G. pallida*. On a pu distinguer les souches de *G. rostochiensis*, qui ont amplifié invariablement un seul fragment de 914 pb, des souches de *G. pallida*, qui ont amplifié des fragments de 914 bp et 853 bp, et aussi de la plupart des souches Pa1 de *G. pallida*, qui ont amplifié un seul fragment de 853 bp. L'identification des souches de *G. pallida* et de *G. rostochiensis* concordait bien lorsque l'on a utilisé soit la réaction PCR pour amplifier des fragments 5S-SL, soit des sondes d'ADN spécifiques aux nématodes à kystes de la pomme de terre, soit des tests sur plantes hôtes différentielles. La distinction entre les souches PA3 et PA1 de *G. pallida* a été peu précise car certaines souches de Pa1 ont amplifié des fragments de 914 bp et de 853 bp, une réaction typique de Pa3, alors qu'elles s'hybridaient avec une sonde d'ADN spécifique de Pa1. D'ailleurs les tests de résistances ont montré qu'une de ces populations de nématodes pouvait être un mélange de Pa1 et de Pa3. Les expériences d'hybridation moléculaires ont indiqué que les sondes d'ADN utilisées n'étaient pas homologues aux séquences 5S-SL.

Key-words : 5S rDNA, diagnostics, DNA, *Globodera*, nematodes, polymerase chain reaction, spliced leader.

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are targets of statutory and advisory programmes in most potato producing countries and a common feature of these programmes is their reliance on the accurate identification of PCN species and pathotypes (Canto Saenz & De Scurrah, 1977; Kort *et al.*, 1977). Diagnostic procedures based on morphology and host plant tests can be inaccurate or time consuming and problems resulting from the use of such tests are well documented (Stone, 1985; Trudgill, 1985). Attempts have been made to develop routine diagnostic tests involving alternative techniques including immunology, protein electrophoresis, isoelectric focusing and 2 D electrophoresis (Trudgill & Parrot, 1972; Bakker & Gommers, 1982; Fleming & Marks, 1983; Fox & Atkinson, 1984, 1986; Robinson *et al.*, 1993).

DNA analysis, particularly when based on the use of DNA probes or the polymerase chain reaction (PCR), offers both the sensitivity and genetic specificity required by advisors and phytosanitary workers (Burrows & Boffey, 1986; Marshall & Crawford, 1987; Burrows & Perry, 1988; De Jong *et al.*, 1989; Schnick *et al.*, 1990; Fleming *et al.*, 1993; Chacon *et al.*, 1994). Stratford *et al.* (1992) employed differential screening to isolate DNA sequences that are highly abundant in either *G. rostochiensis, G. pallida* or *G. pallida* pathotype Pa1. The different sequences were then used as *G. pallida, G. pallida* pathotype Pa1 and *G. rostochiensis* specific DNA hybridisation probes to distinguish PCN by a dot blot procedure involving single PCN cysts. These techniques have since been used routinely to characterise PCN field populations (Marshall, 1993).

It has been shown recently that the 5S rRNA and trans spliced leader (SL) RNA genes are present in tandem repeats in the genomes of both *G. rostochiensis* and *G. pallida* (Stratford & Shields, 1994). The near perfect conservation of the 5S and SL sequences in these species permitted the design of PCR primers which enabled amplification of the intergenic region and variation in the length of the intergenic sequence was

used to distinguish isolates of *G. rostochiensis, G. pallida* and *G. pallida* pathotype Pa1 (Stratford & Shields, 1994).

Here we attempt to evaluate the efficacy of 5S-SL PCR analysis for PCN diagnostics by testing it against a wide range of PCN isolates from South America, Europe and other parts of the world and assessing its concordance with both the dot blot procedure and standard PCN pathotyping tests based on nematode reproduction on differential *Solanum* clones. As any diagnostic test should be specific to the target species, specificity of 5S-SL PCR was also determined by testing for amplification against some common fungal contaminants occurring in PCN cysts.

Materials and methods

Potato cyst nematode isolates

PCN isolates were cultured on cv. Désirée either at Plant Breeding International (PBI) Cambridge or the Nematology Laboratory, Department of Agriculture for Northern Ireland.

Nematode and fungal DNA extraction

Nematode DNA extraction and purification was performed as previously described (Stratford *et al.,* 1992; Stratford & Shields, 1994). DNA from fifteen isolates of fungi detected in PCN cysts and sub-cultured onto malt agar (Kissock, 1993) was extracted by boiling 20 mg of fungal hyphae in 50 µl H_2O at 100 °C for 10 min. Isolates were identified as members of the *Gliocladium, Penicillium, Aspergillus, Humicola, Cylindrocarpon* and *Beauveria* genera and aliquots (5 µl) of these DNA solutions were used directly in PCR reactions.

5S-SL polymerase chain reaction

PCR was performed as described by Stratford and Shields (1994). Oligonucleotide primers were designed to be complementary to the 5S rRNA sequence (5S primer) 5'-CGCGGATCCTTACGACCATAC-CACG-3' and the SL RNA sequence (SL primer) 5'-CTCAAACTTGGGTAATTAAACC-3'. PCR positive controls for the fungal DNA isolates were performed using fungal ribosomal RNA gene primers based on published *Neurospora* sequence (Chambers *et al.,* 1986). PCR reactions (25 µl total volume) contained either 5-10 ng of PCN DNA or 1-2 µl of cyst homogenate (see below), 200 µM of each dNTP, 125 ng of each primer, 0.3 units of Thermalase (IBI) in Thermalase buffer (IBI) (50 mM KC1; 10 mM Tris-HC1; pH 8.3); 1.5 mM magnesium chloride; 0.01 % Tween 20; 0.01 % gelatin (w/v); 0.01 % tergitol NP-40). The mixture was overlaid with paraffin oil and subjected to 30 cycles of amplification (30 s at 94 °C, 1 min at 40 °C, 2 min at 72 °C). Amplified products were visualised on 1.4 % agarose gels containing Ethidium Bromide (Sambrook *et al.,* 1989).

Pathotype specific DNA probes and dot blot tests

The preparation of radiolabelled probes corresponding to the *G. rostochiensis, G. pallida* and *G. pallida* pathotype Pal diagnostic clones was carried out as described by Stratford *et al.* (1992). Target DNA for dot blot tests and assessments of concordance with 5S-SL PCR was prepared by homogenising a single PCN cyst in 40 µl H_2O, with 2 µl of this homogenate used in PCR tests and the remainder made 0.83 M with NaOH and incubated at room temperature for 10 min. Aliquots (5 µl) of this were spotted onto replicate Hybond membranes (Amersham) and replicate membranes hybridised with P^{32} labelled probes [*G. rostochiensis* (pRo-67), *G. pallida* Pa2/3 (pPa-60), *G. pallida* Pal (pB10-1)] or a probe complementary to a ribosomal DNA clone (Whale-1) which acted as a control for DNA loading) Stratford *et al.,* 1992).

Pathotyping of differential *Solanum* clones

Populations were assigned to specific pathotypes using standard testing procedures (Zaheer *et al.,* 1993). Tests were carried out under glass in 15 cm pots containing 800 g steam sterilised sandy loam at five times replication. Egg and juvenile contents of PCN cysts were determined (Shepherd, 1986) and inocula prepared in 200 µm mesh nylon bags to give an initial nematode density *(Pi)* of 15-20 eggs/g soil. After plants had senesced, final egg density *(Pf)* was determined (McKenzie & Turner, 1987) and the nematode multiplication rate *(Pf/Pi)* calculated.

Results

Specificity of 5S-SL PCR to nematode DNA

5S-SL amplifications using fungal DNA as template were all unsuccessful, with the primers apparently failing to anneal to the fungal DNA under the PCR conditions used. In controls using the fungal ribosomal primers, products of the expected size were amplified in all isolates (data not shown).

Use of 5S-SL PCR as a diagnostic tool for potato cyst nematodes

Results of 5S-SL amplifications are shown in Figure 1 with *G. rostochiensis* amplifying a single major product 914 bp in size and *G. pallida* amplifying two products, one being similar in size to the *G. rostochiensis* 914 bp product and the other 853 bp in length. Amplifications using eggs and juveniles resulted in the same products as generated by purified DNA. Smaller and fainter amplification products were sometimes seen and may correspond to the shorter version of the basic repeat unit identified by Stratford and Shields (1994).

Products generated during 5S-SL amplifications and pathotype assessments of nineteen *G. rostochiensis* and twenty *G. pallida* isolates are listed in Table 1. In all *G.*

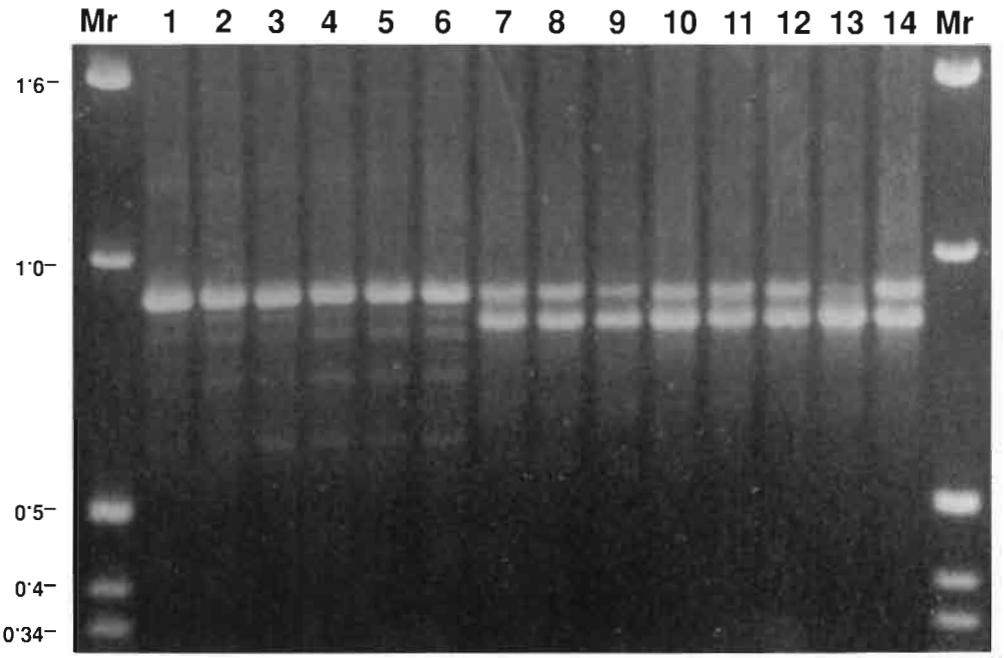

Fig. 1. *5S-SL amplification products generated using bulk DNA extracted from* Globodera rostochiensis *(lanes 1-6) and* G. pallida *(lanes 7-14). Lane 1 : Ro1 PBI, lane 2 : Ro1 Dundonald, lane 3 : Ro2 DB 1, lane 4 : Ro3 C 156, lane 5 : Ro4 F 520, lane 6 : Ro5 Harmerz, lane 7 : Pa2/3 PBI, lane 8 : Pa3 Scotland, lane 9 : Pa3 England, lane 10 : Pa3 Moneynick, lane 11 : Pa2 England, lane 12 : Pa1 Glarryford, lane 13 : Pa1 Dunminning, lane 14 : Moneynick × Glarryford cross. Molecular weight markers (kb) are shown (Mr).*

rostochiensis isolates, the single 914 bp product was amplified. Typically, the 914 bp and 853 bp products were generated in *G. pallida,* but some variation was observed within the amplification products of this species. Specifically in the case of three *G. pallida* pathotype Pa1 isolates (Portglenone, Garvaghy and DAFS B), where only the 853 bp product was amplified. Two other *G. pallida* pathotype Pa1 isolates (Glarryford and Dunminning) amplified both the 914 and 853 bp products (see Fig. 1, lanes 12 - 13 and Table 1) though on gels the 914 bp product was generally less intensely stained than the 853 bp product. The possibility that amplification of the single 853 bp product is diagnostic for *G. pallida* Pa1 and the presence of 914 bp product in the Glarryford and Dunminning isolates is due to mixing with *G. pallida* Pa3, is explored in the next section.

Concordance of differential host plant tests, DNA probes and 5S-SL PCR

Individual cysts from populations of *G. rostochiensis* Ro1, *G. pallida* Pa3 and two Pa1 isolates were selected to assess the concordance of the dot blot and PCR procedures. Pal Garvaghy, was chosen as it was similar to the majority of the Pa1 populations tested, in that its DNA amplified to give the single 853 bp product. A second Pa1 population, Pa1 Dunminning, gave DNA which in addition to the 853 bp product, also amplified a weak 914 bp product (Fig. 1, lane 13). Individual cysts from each population were homogenised and aliquots spotted out on nitrocellulose membranes before testing with the pathotype specific probes. A further aliquot was used for 5S-SL PCR. The results of PCR and radioactive probing were completely concordant for *G. rostochiensis, G. pallida* Pa3, and *G. pallida* Pa1 Garvaghy (Fig. 2 panels A-C). For the Dunminning population, occasional individual cysts gave two PCR producs but tested as Pa1 with the DNA probes (Fig. 2, panel D, sample 5). The PCN specific DNA probes failed to hybridise to amplified 5S-SL products (data not shown).

Southern blots of the gels shown in Fig. 2 using a cloned 5S to SL repeat monomer as a probe showed that DNA amplified from all *G. pallida* isolates regardless of pathotype, showed both large and small bands. Blots of genomic DNA from Pa1 isolates also revealed two bands, however the high molecular weight version was invariably less intense than the lower (data not shown). This indicates that the larger molecular weight version of the 5S-SL repeat is present in *G. pallida* Pa1 but does not amplify well in PCR.

Multiplication rates on differential host plants for the Pa1 Dunminning and Pa1 Garvaghy isolates, along

Table 1. *PCR amplification products generated with SL - 5S primers and DNA from 39 potato cyst nematode isolates.*

Population	Origin	Species	Pathotype	PCR Product	
				914 bp	853 bp
Dundonald	N. Ireland	*G. rost.*	Ro1	+	
Ballyvoy	N. Ireland	*G. rost.*	Ro1	+	
Strabane	N. Ireland	*G. rost.*	Ro1	+	
Talybont	England	*G. rost.*	Ro1	+	
Arequipo	Peru	*G. rost.*	R 1 A	+	
DB 1	Netherlands	*G. rost.*	Ro2	+	
B 21	Netherlands	*G. rost.*	Ro2	+	
C 156	Netherlands	*G. rost.*	Ro3	+	
Morochata	Bolivia	*G. rost.*	R 3 A	+	
F 520	Netherlands	*G. rost.*	Ro4	+	
Harmerz	Germany	*G. rost.*	Ro5	+	
Bol 1	Bolivia	*G. rost.*	Ro5	+	
Desaguadero	Peru	*G. rost.*	R 2 A	+	
Copo	Peru	*G. rost.*	R 1 A	+	
Harghita	Romania	*G. rost.*	Ro1	+	
Benguet	Phillipines	*G. rost.*	?	+	
Tlaxeala	Mexico	*G. rost.*	?	+	
Aust 1	Australia	*G. rost.*	?	+	
Casani	Peru	*G. rost.*	R 2 A	+	
Portglenone	N. Ireland	*G. pall.*	Pa1		+
Glarryford	N. Ireland	*G. pall.*	Pa1	+	+
Dunminning	N. Ireland	*G. pall.*	Pa1	+	+
Garvaghy	N. Ireland	*G. pall.*	Pa1		+
DAFS B	Scotland	*G. pall.*	Pa1		+
Ballynamagna	N. Ireland	*G. pall.*	Pa2/3	+	+
Moneynick	N. Ireland	*G. pall.*	Pa2/3	+	+
Carmacmoin	N. Ireland	*G. pall.*	Pa2/3	+	+
Bovedy	N. Ireland	*G. pall.*	Pa2/3	+	+
Otuzco	Peru	*G. pall.*	P 5 A	+	+
Huancayo	Peru	*G. pall.*	P 4 A	+	+
E 390	Netherlands	*G. pall.*	Pa2/3	+	+
F 8	Falkland Is.	*G. pall.*	Pa2/3	+	+
Ichu Zallin	Peru	*G. pall.*	P 5 A	+	+
Pilayo	Peru	*G. pall.*	P 5 A	+	+
Tiabaya	Peru	*G. pall.*	P 5 A	+	+
Andenes	Peru	*G. pall.*	P 5 A	+	+
Chocan	Peru	*G. pall.*	P 4 A	+	+
San Marcos	Peru	*G. pall.*	P 6 A	+	+
Copacabana	Peru	*G. pall.*	P 2 B	+	+

with comparative data for *G. rostochiensis* Ro1, *G. pallida* Pa3 and additional Pa1 isolates are presented in Table 2. Notably Pa1 Dunminning, which on DNA dot blots was identified as Pa1 but on PCR showed the weak presence of the 914 bp amplification product (Fig. 2), tested as a mixture of Pa1 and Pa3 on the basis of multiplication on the Pa1 differential *S. multidissectum* P 55/7. Other Pa1 isolates, including Pa1 Garvaghy, all amplified the single 853 bp band and tested as pure Pa1 on P 55/7 (Pf/Pi < 1.0) and in single cyst dot blot tests.

Discussion and conclusions

We have previously described the isolation of DNA probes that are diagnostic for *G. rostochiensis, G. pallida* and the Pa1 pathotype of *G. pallida* (Stratford *et al.*, 1992). In this paper we have demonstrated that PCR primers based on 5S and SL sequences can also be used for this purpose, with apparent concordance between 5S-SL PCR and DNA probes in the analysis of single cysts of *G. rostochiensis, G. pallida* Pa3 and the majority of isolates of *G. pallida* Pa1. The DNA diagnostic

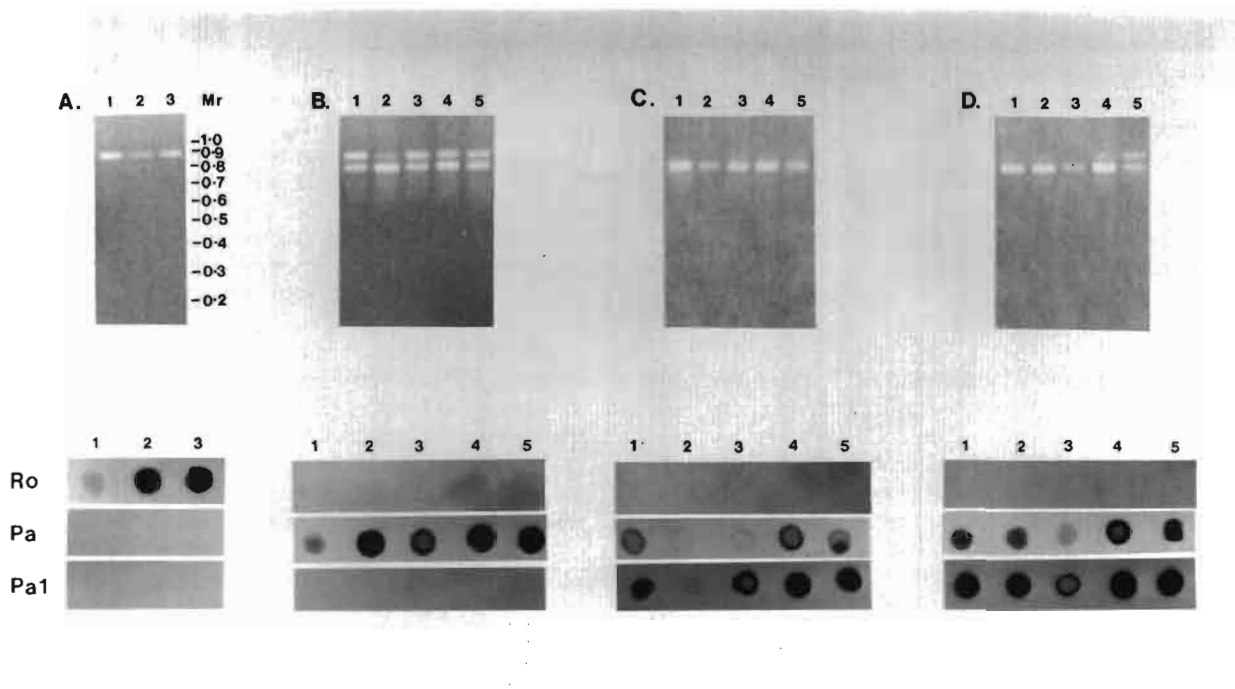

Fig. 2. *Comparison of analysis of single potato cyst nematode cysts by 5S-SL PCR and dot blots. Panel A :* Globodera rostochiensis *Ro1, Panel B :* G. pallida *Pa2/3, Panel C :* G. pallida *Pa1 Garvaghy; Panel D :* G. pallida *Pa1 Dunminning.* (Individual cysts were homogenised and aliquots subjected to PCR (top row) or dot blotted and probed with *G. rostochiensis, G. pallida* and *G. pallida* Pa1 specific probes. The numbered gel tracks correspond to those of the dot blotted samples.)

Table 2. *1993 pathotype assessments for nine populations of potato cyst nematode : data expressed as* Pf/Pi *(mean of five replicates).*

Population	Clone and resistance code (Kort et al. 1977)				Pathotype Assessment
	Desiree (non-res)	M. Piper (Ro1,4)	P 55/7 (Pa1)	62.33.3 (Pa1, 2 + Ro1-4)	
G. rostochiensis, Ballyvoy	52.3 *a*	0.1 *a*	11.5 *a*	0.9 *a*	Ro1
G. pallida, standard Pa3	26.9 *ab*	37.5 *b*	25.0 *a*	25.1 *c*	Pa3
G. pallida, E 390	45.5 *a*	65.2 *c*	30.1 *a*	43.6 *c*	Pa3
G. pallida, Huancayo	24.6 *ab*	13.1 *b*	11.8 *a*	7.9 *b*	Pa3
G. pallida, Carmacmoin	50.9 *a*	39.9 *bc*	17.6 *a*	12.6 *bc*	Pa3
G. pallida, Garvaghy	28.6 *a*	16.9 *b*	0.4 *c*	11.9 *bc*	Pa1
G. pallida, Dunminning	23.1 *ab*	12.9 *b*	2.9 *b*	14.9 *c*	Pa1 + Pa3
G. pallida, Portglenone	20.9 *b*	8.9 *b*	0.8 *c*	7.4 *b*	Pa1
G. pallida, DAFS B	25.9 *ab*	34.1 *b*	0.3 *c*	21.5 *c*	Pa1
Mean	33.2	25.4	11.2	16.2	

Within each clone, data followed by the same letter are not significantly different ($P < 0.05$) based on the Studentised Range (Q) method (Snedecor & Cochrane, 1967).

probes do not hybridise to amplified 5S-SL sequences and therefore the two techniques appear to be based on the detection of different genetic elements within PCN genomes. The speed and convenience of the PCR test, together with the fact that it is non-radioactive, makes it the method of choice for the rapid and reliable characterisation of potato cyst nematode infestations in the field. A further advantage of 5S-SL PCR over other amplification techniques such as random amplified polymorphic DNA PCR (Chacon *et al.,* 1994), is the specificity of the 5S-SL primers to PCN DNA and the failure to amplify fungal DNA, a common contaminant of PCN cysts.

The identification of individual PCN cysts, juveniles and eggs is an important tool for the management of these species and for the support of statutory certification schemes. The validation of the 5S-SL PCR test across a wide range of PCN isolates from South America, Europe and elsewhere confirms that it can also be used with confidence for the identification of PCN in imported plant products. Furthermore, the detection of intraspecific variation within 5S-SL sequences suggests that this region of nematode DNA may provide additional information for those interested in the subspecific systematics of plant parasitic nematodes in general and *Globodera* in particular.

PCN pathotypes are recognised on the basis of their reproduction on differential *Solanum* genotypes containing a range of resistance genes (Canto Saenz & De Scurrah, 1977; Kort *et al.,* 1977). In the case of *G. pallida* Pa3, these isolates differ from Pa1 by their ability to multiply on *S. multidissectum* which contains the resistance gene H_2. The Pa1 pathotype of *G. pallida* forms a distinctive grouping largely confined to a discrete region within Northern Ireland (Zahee *et al.,* 1993) though it is believed to exist at low levels over a wide area in eastern Britain (Stone *et al.,* 1986). The use of resistant and partially resistant potato cultivars may lead to an increase in the frequency of this and other *G. pallida* pathotypes thoughout the UK and Europe, thus diagnostic tests for Pa1 may be of increasing importance in the future (Turner, 1990; Whitehead, 1991). Since UK field isolates may often contain mixtures of Pa1 and Pa3, it is important to be able to characterise individual cysts. In the present study, the majority of Pa1 isolates examined at the level of the single cyst were distinguishable from *G. pallida* Pa3 by the presence of a single major PCR amplifiable band which corresponds in size to the smaller of the two Pa3 bands. The higher molecular weight 5S-SL variant, detectable in certain Pa1 isolates such as Dunminning Pa1, may have a number of possible origins. Firstly, *G. pallida* Pa1 could also contain the higher molecular weight version but for some reason (e.g. low copy number, divergence of primer sequences) this fails to amplify efficiently in most Pa1 isolates. Alternatively, pure *G. pallida* Pa1 may possess only the shorter of the two size variants indicating that the presence of the single small 5S-SL product is a true Pa1 diagnostic character. Since a PCN female can mate with several males and *G. pallida* Pa3 and *G. pallida* Pa1 are sexually compatible, it is possible that cysts which are predominantly *G. pallida* Pa1 (as assessed by their growth on differential clones) could also contain variable proportions of larvae with Pa3 5S-SL length variants. Introgression of Pa1 and Pa3 gene pools could account for results such as that obtained with Dunminning Pa1. The data obtained by probing PCN genomic DNA with the cloned 5S-SL repeat suggested that both size variants are in fact present in *G. pallida* Pa1 and that a reduction in copy number or sequence variation may account for the poor amplification of the larger size variant in Pa1 isolates. Regardless of the genetic basis of the observed divergence between Pa1 and Pa3, the ability to differentiate isolates of these pathotypes may be valuable in the management of UK *G. pallida* and in developing experimental studies on PCN population dynamics and gene flow.

The genomic locations of PCN avirulence/virulence genes are at present unknown, and it is uncertain if they are linked to loci containing the 5S and SL sequences; Consequently, it is probable that avirulence genes and loci containing 5S-SL can segregate independently resulting in the separation of " pathotype " (as determined by the alleles segregating at the avirulence gene) and the diagnostic 5S-SL DNA sequences. The minority of *G. pallida* Pal cysts which displayed both 5S-SL amplification products and also hybridised to the Pa1 specific probe pB 10-1 may or may not carry the Pa1 avirulence gene. Detailed testing of genetically defined nematode larvae on specific *Solanum* genotypes will be required to establish this point. Nevertheless, the generation time of potato cyst nematodes (usually one generation per year in the presence of a susceptible crop) coupled to a founder effect (where infestations are due to a single or few introductions) means that in the field, segregation of the Pa1 avirulence gene from an unlinked diagnostic DNA sequence would be relatively slow and for practical purposes the diagnostic sequences identified here are likely to be sufficient for distinguishing *G. pallida* Pa3 from most Pa1 isolates.

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