PT436

Characterisation and detection of potato cyst nematode

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Know-how for Horticulture™

PT436

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1 EXECUTIVE SUMMARY

Potato cyst nematode (PCN) has been found in Western Australia and Victoria, Australia. Populations of PCN that we studied were all one species (*Globodera rostochiensis*). Pathotype studies confirmed that they were all the same pathotype, Rol.

Extensive testing of a range of resistant potato genotypes showed that the populations did not have any potential to develop virulence. This finding will enable Australian potato breeders to focus on a specific set of resistance genes.

A sensitive, specific, PCR-based test was developed to detect the presence of both species in a mixture of nematodes. The test is also capable of determining which species of PCN is present.

An overview of the current status of PCN research in Australia is presented.

2 INTRODUCTION

2.1 Background

Potato cyst nematode (PCN) was found in Western Australia in 1986 and subsequently in Victoria in 1991. The first discovery of PCN in Australia had a profound effect on the potato growing industry and quarantine departments. Dr John Marshall from the New Zealand Institute for Crop & Food Research Limited assisted both the Western Australian and Victorian quarantine service to develop their response to the finding. Legislation was immediately put in place to implement quarantine regulations.

2.2 What is potato cyst nematode?

Potato cyst nematode is a microscopic animal which lives in the soil and completes its life cycle inside the root of a potato plant. It belongs to the genus *Globodera*. There are two species, *Globodera rostochiensis* and *G. pallida*.

2.3 Why is potato cyst nematode a threat to farmers and a concern to quarantine authorities?

- As nematode numbers increase in the crop they damage the root system which reduces the vigour, yield and quality of the crop.

- PCN is microscopic—a handful of soil may contain thousands of the worms—and has very good survival mechanisms. The mature female (a round cyst of about 0.5 mm in diameter) can survive in the soil for up to 20 years. This characteristic makes it difficult to control. It is possible for the pest to be spread in soil carried by wind or water or in soil attached to potatoes, machinery and footwear.

- PCN is one of the most damaging pests in potato crops. In countries where it has been detected phyto-sanitary restrictions have been instituted on the movement of potatoes. Any country that cannot demonstrate an appropriate level of PCN control may not be able to export primary produce to the rest of the world.

3 DEVELOPMENT OF A NATIONAL STRATEGY

In 1993 the Horticultural Policy Council commissioned a review to develop a national strategy to deal with the threat posed by PCN. The first element of this strategy was:

- to set up a highly focused research programme to provide the information necessary for further development of the strategy and to provide the commercially acceptable resistant potato cultivars necessary to combat any outbreak of PCN.

In New Zealand we had undertaken extensive PCN research and had developed a clear understanding of the steps required by both farmers and the quarantine service to control this problem.

We identified for Australian authorities that research was needed to answer key questions about pest quarantine and management policy. In particular it was important to answer the questions:

- What is the distribution of PCN in Australia?
- Which species are present in Australia?

To answer these apparently simple questions required considerable effort.

3.1 Distribution in Australia

Surveys of each state showed that PCN was very restricted in its distribution. Given this information it was decided to continue to treat PCN as a closed quarantine pest.

3.2 Species present in Australia

It was important to know which species were present for two reasons:

- for quarantine purposes it is essential to accurately describe which pests are present in Australia, and
- if the Australian potato industry is to control PCN by using resistant potato crops growers must know which species is present in the field.

Resistant potato cultivars are the best long term solution to controlling PCN as they restrict PCN multiplication and allow the farmer to produce a high quality product.

Unfortunately, both species of PCN are not controlled by growing the same resistant potatoes. *Globodera rostochiensis* is completely controlled by the single major resistance gene, H1, but *G. pallida* reproduces on cultivars containing this gene.

An additional complication is that both species have developed a range of different virulences or pathogenicities to different resistance genes in potatoes.

3.3 Identifying the species and their population genetics

Samples of the microscopic organism found in diseased potatoes were sent to specialist taxonomists who confirmed that it was PCN. Samples from the major infected areas were also sent to Dr Ken Evans, a PCN nematologist at Rothamsted Research Station, UK. Dr Evans confirmed that all samples were *G. rostochiensis*, pathotype Ro1.

In addition to this Federal-funded research, the Horticultural Research Development Corporation (HRDC) funded a research project to look more closely at the species and the population structure of the Australian PCN infestations. HRDC contracted Dr J Hinch at the Victorian Department of Agriculture to determine the pathotype of all PCN populations found in Australia (PT 346, "Identification of potato cyst nematode pathotypes"). Dr Hinch used advanced electrophoretic techniques, known as iso-electric focusing, to verify the species, but more importantly he developed a novel system using capillary electrophoresis to demonstrate differences between the pathotypes.

4 CHARACTERISING AND DETECTING POTATO CYST NEMATODE

is known from overseas experience that PCN populations develop an increased virulence when repeatedly grown on the same, initially resistant cultivar. It was not known if the Australian populations had this ability. There was also a need to develop a rapid detection method that could identify both species and determine whether both species (*G. rostochiensis* and *G. pallida*) were present in a field. If both species are discovered in seed crops or in other Australian states authorities may be obliged to abandon the quarantine status accorded this pest. The status of PCN would then revert to that of a pest that had to be managed.

In 1994 Dr John Marshall, a nematologist from Crop & Food Research, was contracted by HRDC to undertake a research project entitled, "The characterisation and detection of potato cyst nematode" (PT 436). The project began in 1994 and will be completed in July 1997.

4.1 Characterising potato cyst nematode populations

Characterise available Australian PCN populations by determining the multiplication on selected host differentials and relative virulence to existing New Zealand populations.

We obtained a wide range of Australian PCN populations from both Victoria and Western Australia. Populations obtained were from Western Australia (Erceg and Jacovich) and from Victoria (Gembrook, Rose Bud, Wandin East and Sylvan South). We could not obtain populations for other infestations in Victoria.

All populations were bulked up for one reason on a non-selecting host. During this time all populations were confirmed to be *G. rostochiensis*, pathotype Ro1.

Virulence is defined as the ability of a nematode population to reproduce on a previously resistant potato cultivar as a result of repeated exposure to this cultivar. To identify any populations with potential virulence, nematodes were tested against potato plants containing a range of nematode resistance genes. If a population is virulent a few cysts are recovered at the end of the nematode's life cycle; if the population is avirulent, no cysts are found.

One hundred individual cysts from each population were inoculated onto individual tubers of Maris Anchor (a *G. rostochiensis* resistant cultivar containing the H1 gene) and

the plants were grown to maturity. If virulence potential was present the numbers of cysts present would have increased. If this proved to be the case we intended to challenge this progeny again against a range of resistant potato genotypes.

At the end of the first year no progeny were produced from the original inoculation so we could not reinoculate. The following year we repeated the original experiment and obtained the same result. From these results we concluded that the population we tested had no potential virulence and that the population tested was exclusively Ro1. The direction of our research, therefore, turned to the development of PCR primers.

Comparisons between New Zealand, international reference samples of *G. rostochiensis* and Australian populations showed that there were no discernible differences between the Australian *G. rostochiensis* populations and other *G. rostochiensis* pathotype Ro1 samples. Detailed examination of DNA sequences within the ribosomal internally transcribed spacer (ITS) region did not show any sequence differences. In conclusion, it is our opinion that the *G. rostochiensis* population in Australia is a very uniform pathotype with very little intra population variability.

4.2 Detecting PCN

We used advanced molecular methods to develop a set of primers for the polymerase chain reaction. It was intended that the primers would be capable of detecting and distinguishing PCN from other nematodes while determining which species of PCN was present.

The development of species-specific primers was a long process that involved the following steps:

- DNA was taken from both PCN species,
- a large region of the ITS from both species was amplified,
- this amplified DNA was sequenced so that we could read the order of the bases that make up the DNA,
- the two sequences were compared using a sophisticated computer package,
- from this comparison we identified sections of each sequence that were unique for each species. The sequences for *G. rostochiensis* and *G. pallida* were very similar but we did find a section that was sufficiently different to us to make specific primers that would only recognise a single species,

- the new primers were tested on nematodes of known species to make sure that they were specific to each species,

- each primer was found to be species-specific, and

- the final step was to identify the conditions under which both primers would work.

This last step was successfully achieved and the result was a single step test for the presence of PCN in a sample. The test is capable of distinguishing between *G. rostochiensis* and *G. pallida* and of determining if both are present in the sample.

A paper describing the development and application of molecular detection technology for PCN has been published (Appendix I). We are currently working on methods to allow us to test for PCN in crude processed soil samples.

From our tests of the virulence of Australian nematode populations it is clear that there is no need to develop virulence markers for these populations. New Zealand has a greater diversity of species and pathotypes and so were are continuing to identify potential virulence markers for *G. pallida* populations. We have produced a highly virulent Pa population from originally unselected populations. We will use AFLP methods to identify differences between selected and non selected populations. Consequently, we believe that we will be able to identify a marker for virulence in *G. pallida*.

5 CONCLUSIONS

HRDC has played a pivotal role in co-ordinating research to address the problems caused by the discovery of PCN in Australia identified in the Horticultural Policy Council's impact report.

In Project 346 Dr J Hinch has been able to develop methods to directly identify the pathotypes of PCN. In Project 436 we have demonstrated that there is no virulence potential within Australian *G. rostochiensis* populations. We have developed a detection method for PCN that is rapid, sensitive and unequivocal. Ongoing funding for Roger Kirkham's potato breeding programme projects (PT 634, PT 638 and PT 637) has enabled him to produce a new, resistant potato cultivar.

Finally, HRDC has supported the collaboration of nematologists and potato breeders on both sides of the Tasman. Australia has directly benefited from the resulting rapidly obtained and extensive understanding of PCN.

5.1 Benefits of the project

The project has had a number of beneficial outcomes for the Australian economy, particularly for Australian farmers. It has produced the benefits identified in the original proposal.

- The Australian quarantine service can now give categorical assurance to its trading partners that *G. rostochiensis* is the only PCN species present in Australia.

- Farmers can be reassurred that *G. pallida* has not been found in any PCN-infested areas. They will be able to rely on resistant potato cultivars for control and avoid having to use expensive and toxic nematicides ($1000/ha) .

- The Victorian Department of Agriculture's potato breeder, Roger Kirkham, now has this information and it has given him the confidence to focus his breeding programme on *G. rostochiensis* resistance rather than spreading his resources to produce crosses containing *G. pallida* resistance. He also knows that the genes for resistance used in his breeding programme will not be overcome by virulent PCN populations. This is a major saving for the industry.

Roger Kirkham has been supported by HRDC (PT 634, PT 638 and PT 637) to develop a range of new cultivars for the fresh, crisp and French fry market. Many of his crosses contain the H1 resistance gene.

- Further collaboration has been established with John Marshall's research group in New Zealand which screens Roger's advanced lines for *G. rostochiensis* resistance. This collaboration has resulted in significant cost savings for Australian growers. PCN is still a quarantine pest in Australia but no major quarantine facilities have had to be built. (They would probably cost in excess of AUS$250 000). A simple subcontract to Crop & Food Research has been a much cheaper option (annual cost AUS$600).

- Roger Kirkham has released two new PCN resistant cultivars (Wontscab and Dalmore) into the crisp market and has more advanced material for the fresh market which is also resistant to *G. rostochiensis*.

- The rapid PCR detection test will form the basis of a rapid laboratory-based test for farmers and quarantine staff to help them monitor and manage any future outbreaks of PCN.

5.2 Future R&D

The development of the next generation of phyto-sanitary certification based on unequivocal diagnosis is the main focus of future R&D directed towards the control of PCN.

Soil-based detection tests currently used in specialist laboratories must be optimised to allow this technology to be carried out in any diagnostic laboratory.

6 FURTHER INFORMATION

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7 APPENDIX

Appendix I Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR)

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Abstract Molecular examination of the ribosomal internal transcribed spacer (ITS) region in potato cyst nematodes (PCN) is described. The ITS was amplified and sequenced from a number of PCN collections. A low level of sequence variation was found between *Globodera rostochiensis*, *G. pallida*, and a Peruvian PCN collection, but no variation within Australasian collections of species was noted. Polymerase chain reaction (PCR) primers based upon the *G. rostochiensis* - *G. pallida* sequence differences were designed and successfully used to identify mixed PCN species in a single PCR reaction.

Keywords PCN; *Globodera rostochiensis*; *Globodera pallida*; ribosomal ITS; PCR; microheterogeneity

INTRODUCTION

Potato cyst nematodes (PCN) are pests of world-wide importance, causing serious damage to potato (*Solanum tuberosum* L.) crops. In New Zealand, the two PCN species, *Globodera pallida* and *G. rostochiensis*, have been present since 1972 (Dale 1972) and are now widely distributed (Marshall 1993). Effective control of PCN relies on accurate knowledge of the species and pathotype present in soil. Physical detection methods, such as fork sampling, are slow and often ineffective. Molecular biology tools have been applied to the separation of PCN species and pathotypes (e.g., Schnick et al. 1990; Stratford et al. 1992; Folkertsma et al. 1994). Marshall (1993) applied molecular dot-blot hybridisation to define the distribution of PCN species throughout New Zealand.

Recently, Mulholland et al. (1996) used ribosomal internal transcribed spacers (ITS) sequence data to distinguish European PCN populations. The ribosomal ITS is a variable region of DNA that has been widely examined in population studies. In this study, we set out to examine molecular differences in Australasian PCN populations, and to develop a simple means to differentiate *G. pallida* from *G. rostochiensis*, by sequencing the ribosomal ITS.

MATERIALS AND METHODS

Nematode collections

All PCN collections were maintained at the Lincoln S4 quarantine station. New Zealand *G. pallida* collections were taken from field grown potato plants, and multiplied over 15 generations on the *G. rostochiesis* resistant potato cultivar 'Maris Anchor' using a standardised method (Marshall et al. 1986). *G. rostochiensis* collections were maintained on 'Ilam Hardy'. All collections were regenerated twice from single cyst culture. The collections Ro1 7IH and 12IH, and Pa2/3 8IH and 36IH are Canterbury, New Zealand, PCN collections which were previously assessed by dot-blot hybridisation (Marshall 1993). An unselected Kakanui, New Zealand, collection was obtained in 1990 and maintained on 'Ilam Hardy'.

Australian PCN collections, Ro1 Victoria and Ro Western Australia, were collected in 1989 and 1986, respectively, and maintained on 'Ilam Hardy'.

The European PCN collections, Ro2 Obersteinbach (Germany), Ro3 Wageningen (the Netherlands), and Pa1 Dunminning (Northern Ireland), and the South American P6A Curgos (Peru) collection, were obtained during the 1980s and maintained at Lincoln on 'Ilam Hardy'.

DNA preparation

Twenty nematode cysts were ground in Eppendorf tubes, using plastic micro-pestles (Treff), with 200 μl of solution containing 5*M* guanidine isothiocyanate, 10 m*M* EDTA, 50 m*M* Tris-HCl (pH 7.5), and 8% mercaptoethanol. After room temperature incubation for up to 1 h, the DNA-containing solution was extracted once with equal volumes of phenol and chloroform-isoamyl alchohol (24:1) and once with chloroform-isoamyl alcohol, then precipitated with 0.3*M* sodium acetate and two volumes ethanol. DNA was resuspended in 100 μl of H_2O.

DNA was extracted from single nematode cysts by the same method, starting with 50 μl extraction buffer and using 10 μg glycogen carrier during precipitation. DNA was resuspended in 20 μl of H_2O.

Polymerase chain reaction and PCR primer design

The ribosomal gene spacer region was amplified using primers ITS5 (White et al. 1990) and ITS26 (AB28, Howlett et al. 1992). One μl of DNA was used as the template in a final PCR reaction volume of 25 μl. PCR conditions were 20 m*M* Tris-HCl (pH 8.3),

50 m*M* KCl, 2.0 m*M* MgCl2, 160 μM each dNTP, 250 μM each primer, and 0.6 U Taq DNA polymerase (Life Technologies). Reactions were run for 35 cycles of 94°C (30 s), 55°C (30 s) and 72°C (2 min), with an initial denaturation of 94°C for 2 min.

PCR reactions to distinguish between *G. rostochiensis* and *G. pallida* were carried out using primers PITSr3 (5'-AGCGCAGACATGCCGCAA-3') and PITSp4 (5'-ACAACAGCAATCGTCGAG-3') in combination with primer ITS5 (White et al. 1990). PCR reaction components were as above; reactions were cycled 35 times at 94°C (30 s), 60°C (30 s) and 72°C (30 s) with an initial denaturation of 94°C for 2 min.

All PCR reactions included a no DNA control. Reactions showing amplification in the negative control were discarded.

DNA sequencing

PCR products from two 25 μl reactions were pooled, then purified by chloroform-isoamyl alchohol extraction and precipitation with 2*M* ammonium acetate and one volume isopropanol. DNA was resuspended in H_2O and the concentration estimated spectrophotometrically. DNA products were cycle sequenced using Δ-Taq DNA polymerase (Amersham Life Science) or Thermo-Sequenase (Amersham Life Science), according to manufacturers' instructions.

In addition to the ITS5 and ITS26 primers, primers ITS3 (White et al. 1990), PI1 (5'-TGCGTCGTTGAGCGGTTGTT-3'), PI2 (5'-AACACGTCGTCTCATCACGG-3'), and PI4 (5'-CGTGCCTAAATTACGGTCTG-3') were used to complete sequencing of the products in both directions. ITS products cloned into the pGEM-T vector (Promega) were sequenced with the SP6 and T7 primers to confirm sequences near to the ITS5 and ITS26 primers. ITS microheterogeneity was examined by sequencing 12 and 11 clones, respectively, from single Ro1 Lincoln and Pa2/3 Lincoln cysts, using primers ITS26 and ITS5.

Sequence manipulation and primer design

DNA sequences were recorded using the Seqaid II (Rhoads & Roufa 1989) program. Sequences were visually aligned. Boundaries for the 18S, 28S, and 5.8S genes were estimated by comparison with the sequences of *Caenorhabditis elegans* (Ellis et al. 1986) and *Meloidogyne arenaria* (Genbank X57216).

PCR primers were designed manually or by using the OSP program (Hillier & Green 1991).

RESULTS

G. rostochiensis - *G. pallida* ITS sequence

A 1 kb ribosomal ITS product spanning ITS 1, ITS 2, and the 5.8S gene was amplified from New Zealand PCN DNA. The product was directly sequenced from *G. pallida* Pa2/3 Lincoln and *G. rostochiensis* Ro1 Lincoln. Initial difficulties ascertaining the sequence of Ro1 Lincoln ITS 2 were overcome by sequencing cloned ITS products. This revealed a number of clones with deletions at nucleotide 775 (nucleotides numbered from the 18S gene end). The aligned ITS sequences from *G. pallida* Pa2/3 Lincoln and *G. rostochiensis* Ro1 Lincoln are shown in Fig. 1.

ITS products from Ro1 Victoria, Ro2 Obersteinbach, Ro3 Wageningen, Pa1 Dunminning, Pa2/3 Pukekoke, and P6A Curgos were also directly sequenced. No unambiguous within-species sequence differences were observed in these collections, with the exception of P6A Curgos which exhibited 12 nucleotide differences in comparison with the Australasian *G. pallida* sequence (data not shown). Overall, 29 bp differences in 743 bp aligned nucleotides of ITS 1 and ITS 2 were found between *G. pallida* (excluding P6A Curgos) and *G. rostochiensis*.

ITS microheterogeneity

Direct sequencing of the Ro1 Lincoln ITS revealed several ambiguous or heterogeneous nucleotide positions. This microheterogeneity was evident at the same nucleotide positions, at varying intensities, in all five *G. rostochiensis* collections examined. The stronger banding of nucleotides at the heterogeneous positions has been shown in lower case lettering in Fig. 1.

To examine PCN ITS microheterogeneity more closely, ITS products, amplified from single Ro1 Lincoln and Pa2/3 Lincoln cysts, were cloned and then sequenced with the ITS5 and ITS26 primers. This allowed sequence differences between clones to be identified over the majority of ITS 1 (nucleotides 70-420) and the 5.8S gene/ITS 2 (nucleotides 537-940) (Table 1). The Ro1 Lincoln clones showed greater variability, with most nucleotide differences occurring at the previously identified heterogeneous positions. In addition, three nucleotide positions in the Ro1 Lincoln clones and a single position in the Pa2/3 Lincoln clones were identified where more than one clone shared sequence differences. Five nucleotide positions at which a single clone varied were noted in both collections. One nucleotide position from each collection showed single base-pair deletions.

All variable sites detected in the PCN ITS products occurred in ITS 1 and ITS 2, with the exception of nucleotide 726 in the *G. rostochiensis* 5.8S gene.

Species-specific PCR

Species-specific PCR primers differing at the 3' end were designed from the Australasian *G. rostochiensis* and *G. pallida* ITS 1 sequences. In combination with primer ITS5 (White et al. 1990), primers PIr3 and PIp4 (Fig. 1) accurately distinguished Ro1 Lincoln and Pa2/3 Lincoln (Fig. 2). Primer PIr3 gave a PCR product of 434 bp while primer PIp4 gave a product of 265 bp.

Reactions with ITS5, PIr3, and PIp4, carried out against dilutions of Ro1 Lincoln DNA with Pa2/3 Lincoln DNA, showed that at an approximately 1:20 dilution the *G. pallida*-specific band became much fainter but was still visible at a 1:100 dilution (Fig. 2A). PCR reactions upon these dilutions of DNA, using ITS5 and PITSp4 alone, gave strong amplification at the 1:100 dilution (Fig. 2B).

Species-distinguishing PCR reactions were then carried out on several Australasian PCN collections in addition to Pa1 Dunminning Ireland and P6A Curgos (Fig. 3). In all instances designation of these collections as either *G. pallida* or *G. rostochiensis*, or a mixture of the two, was in accordance with results obtained from field-based testing.

DISCUSSION

Evolutionary implications

The sequence of the ribosomal ITS in Australasian *G. pallida* and G. *rostochiensis* collections did not differ from that found in a number of European PCN collections examined in this study. This included a Pa1 (Dunminning) collection which previously appeared genetically distant from other European *G. pallida* collections (e.g., Schnick et al. 1990). Although a relatively low level of intra-specific variation was evident in the ITS of European PCN populations, more significant differences have been found in South American PCN collections (P6A Curgos, Peru; Szalanski et al. 1995; C. Fleming pers. comm.). Together, these points suggest that Australasian PCN populations originated in Europe, consistent with observations of another potato pathogen, *Spongospora subterranea* (Bulman & Marshall unpubl. data).

Lacking sequence information from other *Globodera* spp. ITS products, we are unable to draw any evolutionary conclusions with regard to hybridisation events leading to the microheterogeneity in *G. rostochiensis* collections.

Species diagnostics

During construction of diagnostic primers based upon 1-2 bp differences in the ITS, we found that several putatively species-specific primers amplified DNA from both

species of PCN. We are unable to attribute this amplification to difficulties in performing "allele-specific" PCR or to heterogeneity in the sequence of the PCN ITS. However, in contrast to Mulholland et al. (1996), we have not found the use of an exonuclease negative polymerase to be necessary for species-specific PCN amplification with similarly positioned primers.

Aside from the heterogeneous sites noted during direct sequencing of all four *G. rostochiensis* collections, several points suggest that the sequence variability in both Ro1 Lincoln and Pa2/3 Lincoln was not caused solely by Taq amplification error: (1) cloned ITS products from *S. subterranea* (Bulman & Marshall unpubl. data), generated using essentially identical PCR parameters, did not display similar sequence variability; (2) all but one varying nucleotide position occurred in the non-coding ITS 1 and ITS 2, not the 5.8S gene; (3) the Ro1 Lincoln nucleotide 726 deletions, apparent during direct sequencing, demonstrate that a physically well characterised PCN collection can carry a unique sequence polymorphism at high levels; and (4) ITS sequence microheterogeneity has previously been recorded, e.g., Vogler & DeSalle (1994) found 42 out of a total of 50 ITS 1 clones with sequence differences from the tiger beetle, *Cicindela dorsalis*.

A number of factors should be considered in assessing the overall applicability of this test. Most previous PCN studies have used techniques that are not ideally suited to rapid species identification. In this study, the design of species-specific PCR primers enabled us to quickly identify clean cysts using a single PCR reaction. Although the ITS5 primer used here is not specific to PCN, we observed no amplification from fungal contaminants of cysts, despite some amplification of this type when using both ITS5 and ITS26 primers in combination.

Low levels of *G. pallida* (for which resistance is unavailable) in a mixed PCN population can be confirmed using the PITSp4/ITS5 primer pair alone or more laboriously, DNA can be tested from individual cysts. Because PCN cysts can easily be separated from soil by physical means we have routinely used this method to distinguish *G. pallida* from *G. rostochiensis* cysts in field situations.

The effect of ITS microheterogeneity on PCN species diagnostics is unclear but does raise the possibility of incorrect species identification. Wider comparison of PCR-based PCN diagnoses with field-based characterisation may alleviate such concerns.

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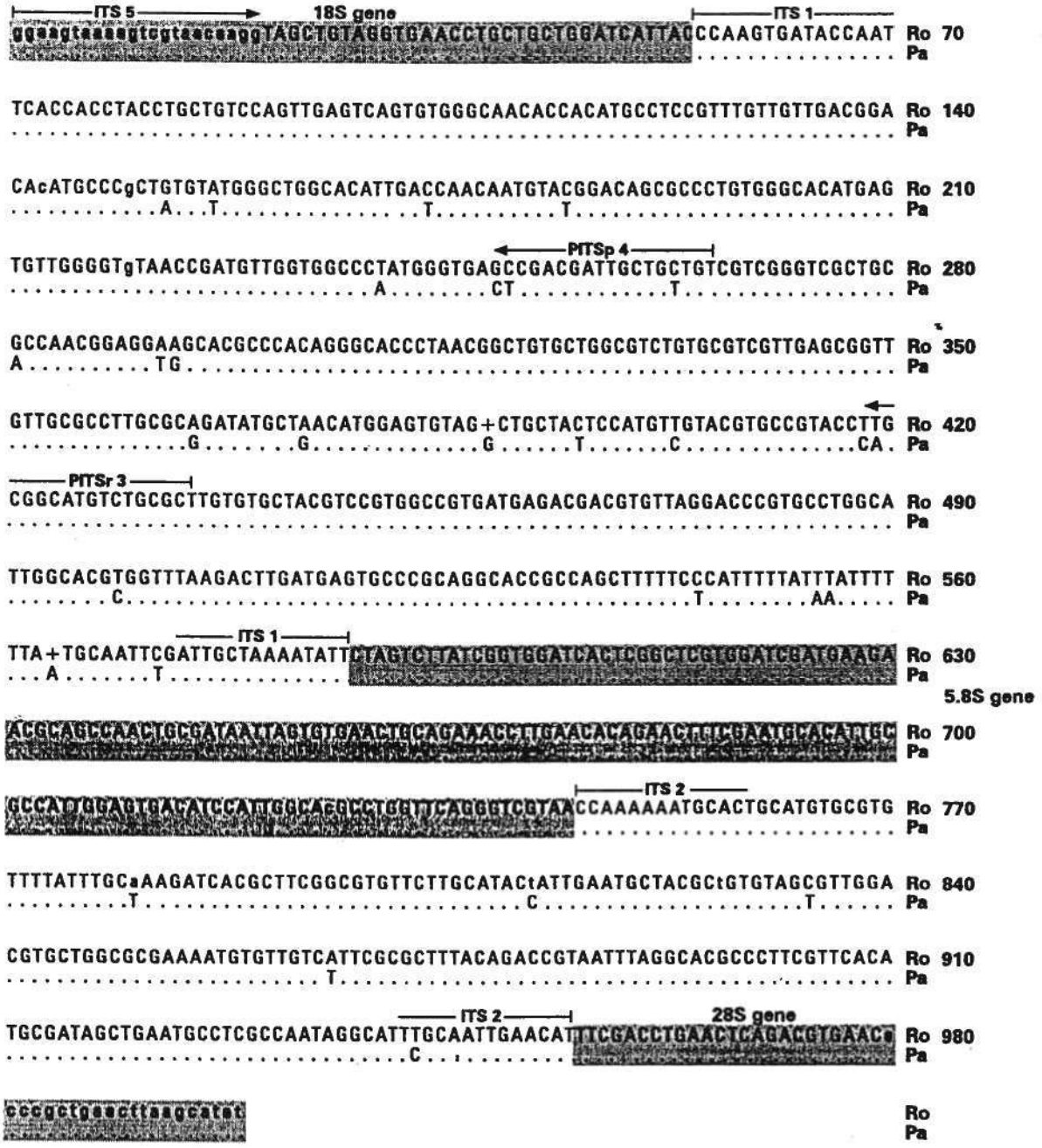

Fig. 1 Alignment of the ribosomal internal transcribed spacer (ITS) sequence from *Globodera rostochiensis* Ro1 Lincoln (top) and *G. pallida* Pa2/3 Lincoln (bottom). Sequence differences between the two species are shown, (.) represents identical bases. Estimated positions of the 18S, 28S, and 5.8S genes, and ITS 1 and ITS 2 are indicated. Heterogeneous nucleotide positions in Ro1 Lincoln ITS 1 and ITS 2 are represented with lower case lettering. The polymerase chain reaction (PCR) primers, ITS5 and ITS26, are also shown in lower case lettering at the beginning and end of the sequence. Positions of the species diagnostic primers PIp4 and PIr3, along with ITS5, are indicated with arrows.

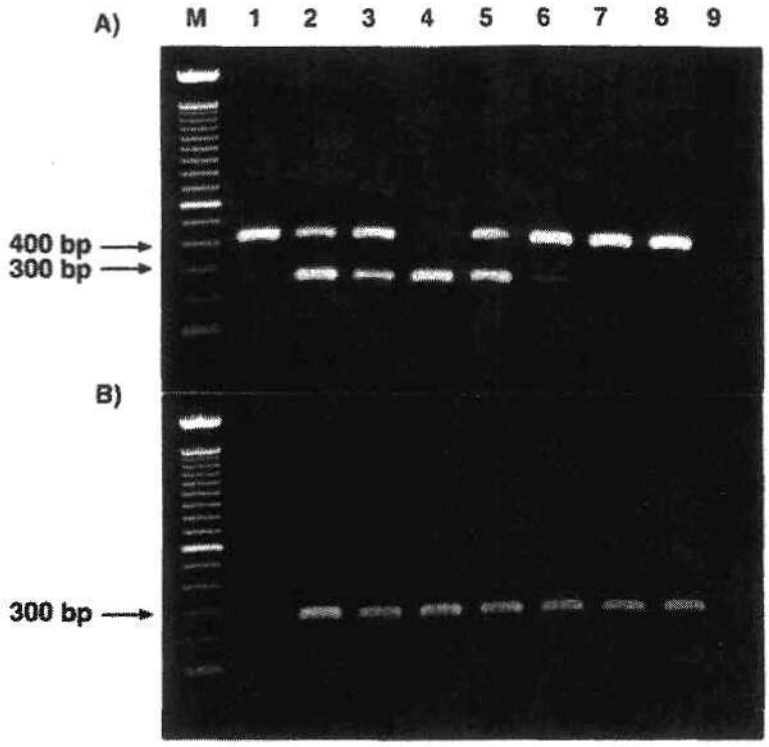

Fig. 2 Polymerase chain reaction (PCR) differentiation of the potato cyst nematode (PCN) species, *Globodera rostochiensis* and *G. pallida*, with varying concentrations of DNA. **A,** multiplex PCR with primers PIp4, PIr3, and ITS5 upon DNA from Ro1 Lincoln and Pa2/3 Lincoln. Lane 1, Ro1 1:20 H2O; Lane 2, Ro1 1:20 Pa2/3; Lane 3, Ro1 1:1 Pa2/3 (1:20 H2O each); Lane 4, Pa2/3 1:20 H2O; Lane 5, Ro1 10:1 Pa2/3; Lane 6, Ro1 20:1 Pa2/3; Lane 7, Ro1 50:1 Pa2/3; Lane 8, Ro1 100:1 Pa2/3; Lane 9, no DNA control. **B,** PCR with primers ITS5 and PIp4 alone, performed upon the same DNA samples as in A. Molecular weight markers (M) = 100 bp DNA ladder (Life Technologies).

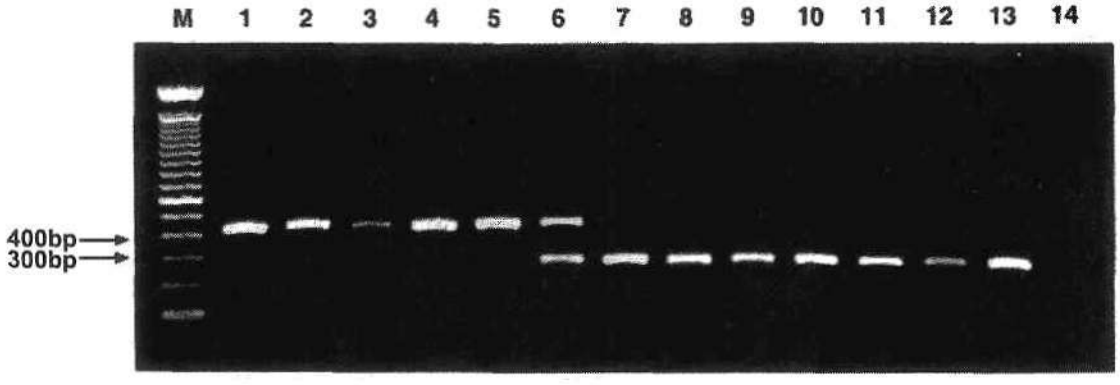

Fig. 3 Molecular differentiation of Australasian potato cyst nematode (PCN) (*Globodera rostochiensis* and *G. pallida*) populations. Multiplex polymerase chain reaction (PCR) using primers PIp4, PIr3, and ITS 5 upon DNA from lane 1, Ro1 Lincoln; Lane 2, Ro1 Victoria; Lane 3, Ro Western Australia; Lane 4, Ro1 7IH; Lane 5, Ro1 12IH; Lane 6, Kakanui unselected; Lane 7, Pa2/3 8IH; Lane 8, Pa2/3 36IH; Lane 9, Pa1 Dunminning, Northern Ireland; Lane 10, P6A Curgos, Peru; Lane 11, Pa2/3 Pukekohe; Lane 12, Pa2/3 Lincoln; Lane 13, DNA from single cyst Pa2/3 Lincoln; Lane 14, no DNA control. Molecular weight markers (M) = 100 bp DNA ladder (Life Technologies).