

Diagnostics of cyst nematodes: use of the polymerase chain reaction to determine species and estimate population levels

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Summary

The effective management of cyst nematodes requires accurate assessments of the species composition and size of field populations. Compared to traditional diagnostics the polymerase chain reaction offers high specificity, sensitivity and a more rapid means of identifying large numbers of cyst nematode samples. PCR-RFLP of the ITS1 ribosomal DNA region from *Heterodera* and *Globodera* species produced diagnostic profiles that allowed identification of six pest species. Quantitative PCR was assessed as a means of determining the population sizes of cyst nematode species. A positive correlation was demonstrated between numbers of viable juveniles in cysts and the amount of DNA that could be extracted from them. An internal control template was subsequently used in competitive PCR reactions with *G. rostochiensis* DNA and was shown to co-amplify with the nematode template. The resulting PCR product ratio allowed assessments of the levels of *G. rostochiensis* DNA extracted from cyst samples.

Key words: Cyst nematodes, *Heterodera schachtii*, *Globodera pallida*, *Globodera rostochiensis*, PCR, diagnostics

Introduction

Potato cyst nematodes, *Globodera rostochiensis* (Wollenweber) and *G. pallida* (Stone), and the beet cyst nematode, *Heterodera schachtii* (Schmidt) continue to represent a major threat to the production of potatoes and sugar beet throughout the world. Increasing restrictions on the application of nematicides and fumigants will necessitate the use of alternative methods of nematode control such as long crop rotations, catch crops and the use of resistant cultivars. A critical step in the use of all these control measures, is the accurate identification of target nematode species in the field and an assessment of the actual numbers of nematodes present. Periodic monitoring of the nematode species and their population levels in this way will

facilitate the long-term management of these pests. However, inaccurate diagnostics can result in inappropriate and costly management decisions.

Potato cyst and beet cyst nematode identification can be complicated by the presence of other cyst nematode species. Traditional methods of identifying and quantifying cyst nematodes in soil samples have relied on morphological examination and counts of cysts and juvenile worms. Isoelectric focusing of proteins has also been widely used for nematode diagnostics (Powers & Fleming, 1998). However, recent developments using immunological techniques (Davies, Curtis & Evans, 1996) and the polymerase chain reaction (PCR) offer the prospect of faster and more accurate nematode diagnostics (Szalansky, Sui, Harris & Powers, 1997).

DNA sequence variation in the ITS regions of the ribosomal DNA cistron can be used to identify many nematode taxa. This variation is detected by amplifying the ITS regions using conserved PCR primers which locate in the flanking 18S, 5.8S and 28S genes. Nematode taxa can then be distinguished on the basis of the size of the amplified PCR product or by cleaving the PCR products with sequence specific restriction enzymes and generating diagnostic PCR-restriction fragment length polymorphism (PCR-RFLP) profiles (Powers *et al.*, 1997). The use of PCR to quantify the amounts of pathogen DNA in a sample is becoming increasingly common in the diagnostics of plant pathogens (Robb & Nazar, 1996). Quantitative PCR can be achieved by adding a known amount of internal standard template DNA to the PCR reaction mixture. This template is competitively co-amplified with the target DNA and the relative amounts of target and internal standard PCR product generated can be used to determine the concentration of the unknown DNA. Here we describe the use of the polymerase chain reaction to identify and quantify levels of cyst nematodes extracted from UK field soils.

Materials and Methods

Source of nematode species and viability assessments of G. rostochiensis isolates

UK and Irish isolates of *H. schachtii*, the clover cyst nematode (*H. trifolii*), the carrot cyst nematode (*H. carotae*), the pea cyst nematode (*H. goettingiana*), *H. mani*, *G. rostochiensis* and *G. pallida* were extracted from field soil samples and field populations maintained at the Department of Agriculture for Northern Ireland (DANI) as laboratory cultures. *Globodera rostochiensis* cysts of varying ages, and used in the quantification experiments, were stored dry in the laboratory and viability assessments of eggs made prior to DNA extraction by performing hatching tests with potato root exudate on batches of 45 cysts (Shepherd, 1986).

DNA extraction from nematode cysts

Single or batches of nematode cysts were ground in liquid nitrogen and subjected to DNA extraction using the high pure PCR template preparation kit (Boehringer Mannheim) following the manufacturer's recommended protocol. DNA was quantified on agarose gels by comparison with known DNA standards (Sambrook, Fritsch & Maniatis, 1989).

Species identification using PCR-RFLP

The ITS-1 regions of cyst nematodes were amplified using conserved primers located in the flanking 18S and 5.8S genes. The 18S primer, rDNA2 (5'-TTGATTACGTCCCTGCCCTTT-3'), was described by Vrain, Wakarchuk, Levesque & Hamilton, (1992). The 5.8S primer was

rDNA1.58S (5'- ACGAGCCGAGTGATCCACCG -3') (Szalansky *et al.*, 1997). Replicate (x3) PCR reactions were carried out in a reaction mixture containing 1µl (5ng/µl) DNA, 1µl (20mM) of each primer, 2.5µl 10x reaction buffer, 1.6µl (1.25mM) dNTP mix, 2.5 units of Taq DNA polymerase and double distilled water to 25µl. A PCR amplification profile of 94°C for 5 min, 40 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s and 72°C for 5 min was performed on a Perkin Elmer 2400 thermal cyclor.

PCR-RFLPs were generated using restriction enzymes *AluI* and *HinfI*. PCR products were digested for 4 hours at 37°C in restriction enzyme reaction mixtures comprising 5 units of restriction enzyme, 2µl 10x restriction enzyme buffer, 8µl PCR product and double distilled water to 20µl.

Estimation of G. rostochiensis population levels using quantitative PCR

Globodera rostochiensis DNA and internal standard DNA were amplified using the potato cyst nematode universal primer (5'-CTTCTAGCGATCGGAAGACG-3') and *G. rostochiensis* specific primer (5'-TGTTGTACGTGCCGTACCTT -3') of Mulholland *et al.* (1996). Internal standard DNA was a 748 base pair (bp) template comprising 708 bp of β-glucuronidase gene sequence flanked at each end by 20 bp of *G. rostochiensis* ribosomal DNA sequence matching the PCR primers.

Replicate (x3) PCR reactions were carried out in a reaction mixture containing various concentrations of internal standard DNA, various concentrations of target nematode DNA, 1µl (20mM) of each primer, 2.5µl 10x reaction buffer, 1.6µl (1.25mM) dNTP mix, 2.5 units of Taq DNA polymerase and double distilled water to 25µl. A PCR amplification profile of 94°C for 2 min, 37 cycles of 94°C for 30s, 50°C for 10s and 72°C for 30s and 72°C for 5 min was performed on a Perkin Elmer 2400 thermal cyclor.

Agarose gel electrophoresis

All PCR products and digests were visualised by agarose gel electrophoresis (Szalansky *et al.*, 1997). A DNA size marker (Gibco-BRL) comprising 2000,1200, 800, 400, 200 and 100bp bands was run-out on gels to enable PCR product sizing and DNA quantification. Gel images were recorded using Grab-It CCD image capture software (Ultra Violet Products Ltd.) and PCR products were subsequently analysed using the Phoretix 1D Advanced software package (Phoretix Ltd.).

Results

Species identification using PCR-RFLP

Amplification of *Heterodera* and *Globodera* DNA using the rDNA2 and rDNA1.58S primers generated single PCR products. Differences between the genera were evident with the *Heterodera* species producing products of 800 bp in size while *G. rostochiensis* and *G. pallida* both produced products of 750 bp (Fig. 1).

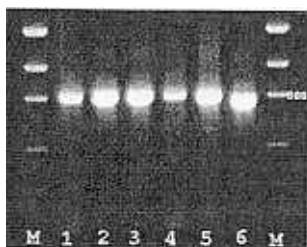


Fig. 1. ITS-1 PCR products for 5 cyst nematode species generated using primers rDNA2 and rDNA1.58s. M-DNA size marker with 800bp band; 1-*H. carotae*; 2,3-*H. goettingiana*; 4-*H. mani*; 5-*H. schachtii*; 6-*G. pallida*.

Restriction enzyme digestion of the ITS1 PCR products using *Alu* 1 and *Hinf* 1 revealed RFLP differences between species within both *Heterodera* and *Globodera* (Fig. 2). Complete digestion of the PCR products was demonstrated by the absence of any PCR product at the 800 and 750bp positions. *Alu* 1 and *Hinf* 1 PCR-RFLP profiles allowed all species to be discriminated.

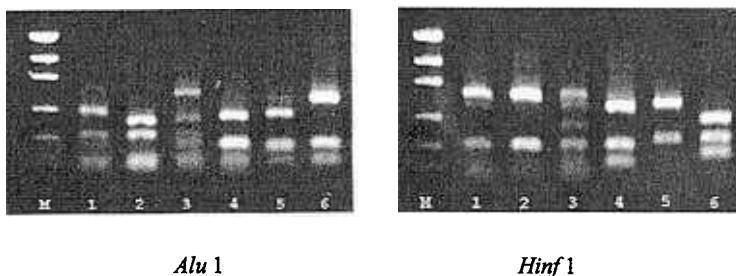


Fig. 2. ITS1 PCR-RFLP profiles for 6 cyst nematode species. M-DNA size marker; 1-*H. carotae*; 2-*H. goettingiana*; 3-*H. mani*; 4-*H. schachtii*; 5-*G. rostochiensis*; 6-*G. pallida*.

Estimation of PCN population levels using quantitative PCR

The relationship between G. rostochiensis viability and extracted DNA

Fundamental to the process of nematode quantification using DNA measurements is the establishment of a correlation between cyst DNA content and the numbers of viable nematode eggs and juveniles present. This relationship was examined by extracting DNA from a series of *G. rostochiensis* populations up to 15 years old. The DNA yield from *G. rostochiensis* cysts was positively correlated with the numbers of viable juvenile nematodes which hatched after treatment with potato root exudate for 6 weeks (Fig. 3; $\text{Log hatched juvenile numbers} = 1.53 + 0.00112 \times \text{DNA extracted}$; $r = 0.885$; $p < 0.001$).

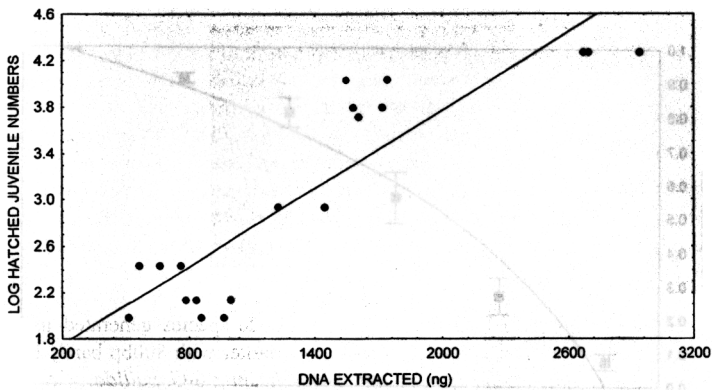


Fig 3. Correlation between DNA yield and numbers of viable juvenile *G. rostochiensis* hatched from batches of cysts.

PCR amplification of internal standard template DNA yielded the predicted 748bp product, while *G. rostochiensis* DNA yielded the expected 238bp product of Mulholland *et al.* (1996). In order to determine if the internal standard template and nematode DNA would co-amplify during PCR and whether changing the amount of nematode DNA would affect the yield of internal standard template product, PCR reactions were run containing 10 pg internal control template and varying amounts of *G. rostochiensis* DNA.

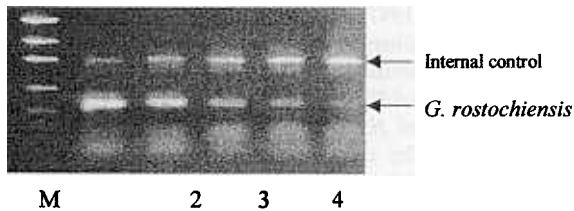


Fig. 4. PCR products generated using 10pg internal control template and varying quantities of *G. rostochiensis* DNA (1- 50ng; 2- 5ng; 3- 0.5ng; 4- 0.05ng; 5- 0.005ng; M- DNA size marker).

Fig. 4 shows the results of a competitive PCR experiment and the successful co-amplification of internal standard and *G. rostochiensis* PCR products. The relationship between the amount of *G. rostochiensis* DNA and the resulting PCR product ratio can be seen graphically in Fig. 5. Other experiments (unpublished) have indicated that reactions containing equal amounts of internal control template and *G. rostochiensis* DNA result in the generation of equal amounts of the two PCR products.

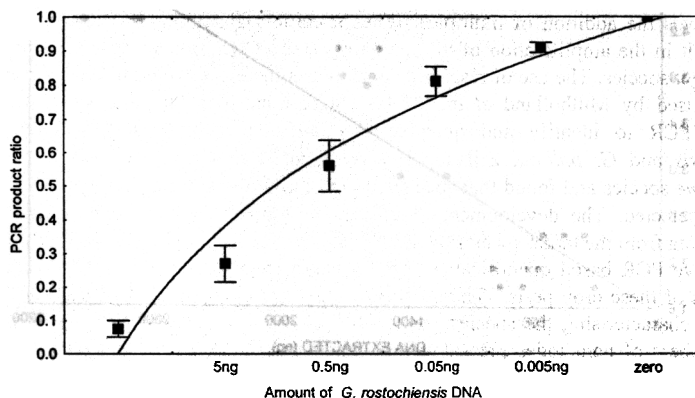


Fig. 5. Relationship between the amount of *G. rostochiensis* DNA and the resulting PCR product ratio (internal control product as a proportion of the total PCR product) after competitive PCR with 10pg of internal control template. Data points are means with SE bars.

Discussion

The ITS1 region of ribosomal DNA has been used successfully to discriminate a range of plant parasitic nematode species (Vrain, Wakarchuk, Levesque & Hamilton, 1992; Ferris, Ferris & Faghihi, 1993; Ferris, Ferris, Faghihi & Ireholm, 1994; Thiery & Mugniery, 1996; Zijlstra, Uenk & Van Silfhout, 1997). This is particularly relevant to the diagnostics of the cyst forming nematodes which often occur in mixed populations in fields and where correct identification is critical to the provision of accurate pest management advice. Szalansky *et al.* (1997) demonstrated the power of PCR-RFLP in distinguishing cyst nematode genera and *N. American* and Asian isolates of *H. schachtii*, *H. trifolii*, *H. glycines*, *H. cruciferae*, *H. goettingiana* and *H. zaeae*. The current study revealed similar PCR-RFLP profiles in European isolates of *H. schachtii* and *H. goettingiana* confirming the value of the PCR approach for quarantine and advisory work. In addition, European isolates of *H. mani* and *H. carotae* were shown to exhibit species specific PCR-RFLP profiles. Traditionally, cyst nematode identification has required the work of highly skilled taxonomists and was characterised by relatively slow turnaround time of samples. Protein isoelectric focusing enables nematode identification to be performed by staff with less formal taxonomic training and has proved particularly suited to laboratories that deal with large numbers of nematode samples. Experience with PCN has shown that, while individual cysts can be identified using protein profiles, they generally need to contain large numbers of viable eggs. Low viability cysts often contain insufficient levels of protein to enable effective diagnostics. Analysis of nematode DNA using PCR can also be carried out by most laboratory staff and without the need for specialised taxonomic training. The procedures also enable large numbers of samples to be screened quickly and accurately. Most significantly, the sensitivity of PCR is much greater than isoelectric focusing, with the DNA from single eggs or juveniles sufficient to enable identification. The presence of DNA sequence differences in *Heterodera* species, as exemplified by PCR-RFLP in this and other studies, offers the prospect of a further

advance through the development of multiplex PCR tests for species identification. Multiplex PCR involves the addition of multiple species specific PCR primers to reaction mixtures which result in the amplification of differentially sized PCR products which are specific to certain target species. The use of restriction enzymes is therefore not required. This approach has been used by Mulholland *et al.* (1996) and Bulman & Marshall (1997) who used multiplex PCR to identify and quantify pure and mixed species populations of *G. rostochiensis* and *G. pallida*. Zijlstra (1997) also used multiplex PCR to identify four *Meloidogyne* species and found that species present in mixtures in proportions as low as 2% could be detected. The development of effective multiplex PCR primers requires DNA sequence data from the target species, work that is currently ongoing at DANI.

An effective PCR based quantification test for cyst nematodes would greatly enhance the diagnostics of these crop pests. The sensitivity and specificity of PCR make it an obvious choice for characterising the species composition of field populations. However assessing actual numbers of nematodes present in the soil is crucial for determining an appropriate management strategy. In the past, egg and juvenile counts and hatching tests have been the major methods for determining the levels of cyst nematode infestation. These approaches are time consuming, and in the case of egg and juvenile counts, also require viability assessments using for example, vital stains (Shepherd, 1986). The current study has demonstrated a correlation between the numbers of hatching juvenile *G. rostochiensis* and the amount of DNA that can be extracted from cysts. This relationship indicates that quantitative PCR may allow population levels of cyst nematodes to be measured along with species composition. The artificial internal control template was found to competitively co-amplify with *G. rostochiensis* DNA. The dynamics of the competitive PCR reaction showed that this control template could be used to assess the quantity of target nematode DNA in a sample and thus determine the viability and pest potential of the population. Comparative tests using traditional methods and quantitative PCR are currently being carried out on populations of *G. rostochiensis* and on *G. pallida* using a *G. pallida* specific internal control template. Initial assessments suggest that the technique can provide good assessments of PCN population levels. The construction of *Heterodera* internal control templates will be required before this approach can be used to quantify populations of *H. schachtii*.

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