Hydrolysis probe-based PCR for detection of Pratylenchus crenatus, P. neglectus and P. penetrans

Article *in* Nematology · November 2016

DOI: 10.1163/15685411-00003033

5 authors, including:

Claudio Marcelo G Oliveira
Instituto Biológico
106 PUBLICATIONS **1,075** CITATIONS
SEE PROFILE

Vivian Carol Blok
James Hutton Institute
185 PUBLICATIONS **6,738** CITATIONS
SEE PROFILE

Roy Neilson
James Hutton Institute
197 PUBLICATIONS **5,103** CITATIONS
SEE PROFILE

Tomasz L. Mróz
Warsaw University of Life Sciences - SGGW
13 PUBLICATIONS **36** CITATIONS
SEE PROFILE

BRILL

Nematology 19 (2017) 81-91

Nematology
brill.com/nemy

Hydrolysis probe-based PCR for detection of *Pratylenchus crenatus*, *P. neglectus* and *P. penetrans*

Claudio M.G. OLIVEIRA [1,*], Vivian BLOK [2], Roy NEILSON [2], Tomasz MRÓZ [3] and David ROBERTS [2]

[1] *Biological Institute, Campinas, SP, CEP 13 001 970, Brazil*
[2] *The James Hutton Institute, Dundee DD2 5DA, UK*
[3] *Warsaw University of Life Sciences WULS – SGGW, Warsaw 02-787, Poland*

Received: 13 June 2016; revised: 10 October 2016
Accepted for publication: 10 October 2016; available online: 11 November 2016

Summary – Molecular detection of pest and pathogens relies on rapid and dependable methods for their identification as well as an assessment of their abundance. This study describes the development and evaluation of a diagnostic method for detection of *Pratylenchus crenatus*, *P. penetrans* and *P. neglectus*, based on a hydrolysis probe qPCR assay. Primer/probe sets were designed targeting the ITS-1 rDNA. In order to assess the specificity, primer/probe sets were tested with samples of non-target *Pratylenchus* species and *Radopholus similis*. Experiments using dilutions of purified plasmid standards tested the sensitivity of the hydrolysis assay against detection of DNA extracted from individual nematodes. Target DNA was detected in soil samples collected from potato fields and this indicated that *P. crenatus*, *P. neglectus* and *P. penetrans* are widely distributed in Scotland, frequently co-existing in mixed populations, with *P. crenatus* more prevalent than either *P. neglectus* or *P. penetrans*.

Keywords – diagnosis, intraspecific variability, ITS-1, root-lesion nematodes.

Root-lesion nematodes belonging to the genus *Pratylenchus* are known to parasitise a range of crops, including potato, carrot, coffee and banana (Castillo & Vovlas, 2007), and are considered one of the primary global nematode pathogens (Jones *et al*., 2013). Root-lesion nematodes cause damage by directly feeding on roots or tubers and moving through cortical tissues, and they are often found associated with bacterial and fungal infections. For example, the combined infection of *Verticillium* spp. and *Pratylenchus* spp., known as potato early dying disease, has long been recognised as a problem in potato production areas of the USA, often resulting in yield reductions of 30-50% (MacGuidwin & Rouse, 1990; Powelson & Rowe, 1993). In Brazil, nine species of *Pratylenchus* (*P. brachyurus*, *P. coffeae*, *P. jaehni*, *P. penetrans*, *P. vulnus*, *P. zeae*, *P. pseudopratensis*, *P. jordaniensis* and *P. pseudofallax*) have been recorded (Gonzaga *et al*., 2016) associated with a range of crops. The three *Pratylenchus* species of focus in this paper (*P. crenatus*, *P. penetrans* and *P. neglectus*) are known pests of numerous global crops. In a glasshouse experiment, Holgado *et al*. (2009) postulated that *P. penetrans* induced tuber cross-lesions similar to those associated with common scab (*Streptomyces scabies*), and that the combined inoculation of the bacterium and the nematode enhanced symptom expression.

Pratylenchus crenatus is listed as a regulated pest in Brazil (Singh *et al*., 2013). This species has recently been detected from intercepted ornamental bulbs during quarantine inspection in Brazil and Korea (Oliveira *et al*., 2012; Kim & Chun, 2014) and in common bean fields in Paraná state, southern Brazil (Bonfim Junior *et al*., 2016); it is known to damage cv. Kuroda carrot resulting in seedling death in Australia (Hay & Pethybridge, 2005). *Pratylenchus neglectus* is one of the most widespread and economically important nematodes that invades plant roots and restricts cereal productivity in Australia and North America (Yan *et al*., 2013; Sheedy *et al*., 2015) but it has not been reported in Brazil. *Pratylenchus penetrans* has been recorded on over 350 hosts mainly in temperate areas of the world. In Brazil, this species has a restricted geographic distribution and has been reported on chrysanthemum, Peruvian carrot, artichoke and potato (Castillo & Vovlas, 2007).

* Corresponding author, e-mail: marcelo@biologico.sp.gov.br

DOI 10.1163/15685411-00003033

Generally, plants parasitised by root-lesion nematodes are stunted and exhibit pronounced leaf chlorosis and root shedding. *Pratylenchus* spp. cause extensive root cortical lesions leading to cortex destruction. Initially, reddish-brown lesions are caused on the roots but these later turn black. On potato tubers, root-lesion nematodes can penetrate through lenticels, parasitising the adjacent tissue and producing lesions of variable size, depending on the population level. Small lesions may be confused with lenticels, but large ones, although superficial, can affect tuber quality. Infected tubers may have pimples, pustules or wart-like protuberances affecting their marketability (Palomares-Rius *et al*., 2014). In Norway, Holgado *et al*. (2009) reported that the yield of potato cv. Saturna was reduced by 50% in affected areas with large populations of *P. penetrans*. In field-transect sampling, plant growth was negatively correlated with densities of *P. penetrans* and indicated a damage threshold for potato in soil of 100 individuals (250 g soil)$^{-1}$.

Due to minute morphological and morphometric differences, for example, the number of head annules, *Pratylenchus* is particularly problematic to identify accurately to species using light microscopy (Min *et al*., 2012). This is conflated with a continuing decline in classical taxonomic expertise of many taxa including Nematoda (Coomans, 2002). However, several identification keys are available that are based on presence/absence of males and a few morphological/morphometric characteristics (Castillo & Vovlas, 2007). Molecular techniques have proved valuable for nematode diagnostics and several PCR-based methods have been developed for a range of plant-parasitic nematodes including *Pratylenchus* (Al-Banna *et al*., 2004; Hübschen *et al*., 2004a, b; Blok, 2005; Oliveira *et al*., 2005; Holeva *et al*., 2006; Adam *et al*., 2007; Riga *et al*., 2007; Oliveira *et al*., 2009). Specific and sensitive assays using real-time PCR (qPCR) have enabled the detection and relative quantification of many plant pathogens (reviewed by Schaad & Frederick, 2002; Oliveira *et al*., 2011). For plant-parasitic nematodes, qPCR assays have primarily focused on the most economically important genera such as *Bursaphelenchus*, *Meloidogyne* and *Globodera* (Berry *et al*., 2008; Huang *et al*., 2010; Reid *et al*., 2010).

More recently, several qPCR strategies have been developed for detection and quantification of *Pratylenchus* species using the intercalating fluorescent reporter dye SYBR Green, including *P. neglectus* (Yan *et al*., 2013), *P. penetrans* (Sato *et al*., 2007; Goto *et al*., 2011; Mokrini *et al*., 2013; Koyama *et al*., 2016), *P. thornei* (Yan *et al*., 2012; Mokrini *et al*., 2014) and *P. zeae* (Berry *et al*., 2008). Only two protocols for detection of *P. penetrans* and *P. thornei* (Mokrini *et al*., 2013, 2014) utilise probes with reporter dyes that increase the specificity of the assay. Missing to date is a similar hydrolysis probe qPCR-based diagnostic for *P. crenatus* and *P. neglectus*.

The aim of the present study was to develop, validate and standardise a rapid, pragmatic and precise method for first line detection of *P. crenatus*, *P. neglectus* and *P. penetrans* using hydrolysis probes (formerly known as TaqMan). Such a test is important in a phytosanitary context where detection is key to ensuring that regulated species such as *P. crenatus* are not inadvertently imported into Brazil through infected plant material or soil.

Materials and methods

Extraction of nematodes from roots and soil samples

Pratylenchus penetrans and *P. crenatus* specimens were extracted from roots of lily cvs Oriental Early Yellow, Muscadet, True Emotion, Cobra and JetSet from The Netherlands (Oliveira *et al*., 2012). These species and *P. neglectus* were also extracted from 200 g soil samples (Wiesel *et al*., 2015) collected from a range of agricultural land in Scotland (Table 1). Nematodes were extracted from roots as described by Coolen & D'Herde (1972) and from soil samples using Baermann funnel extraction over

Table 1. Pratylenchidae used in the present study to test specificity of diagnostic primer/probes sets for *Pratylenchus crenatus*, *P. penetrans* and *P. neglectus*.

Species	Host	Origin
P. crenatus	Lily (*Lilium* sp.)	The Netherlands
P. crenatus	Soil (*Solanum tuberosum*)	Angus, Scotland
P. penetrans	Lily (*Lilium* sp.)	The Netherlands
P. neglectus	Soil (*Solanum tuberosum*)	Borders, Scotland
P. brachyurus	Rubber tree (*Hevea brasiliensis*)	Herculândia, SP, Brazil
P. coffeae	Yam (*Dioscorea alata*)	Recife, PE, Brazil
P. zeae	Sugar cane (*Saccharum* sp.)	Jaú, SP, Brazil
P. jaehni	Citrus (*Citrus* sp.)	Mogi Guaçu, SP, Brazil
Radopholus similis	Banana (*Musa* sp.)	Registro, SP, Brazil

Table 2. PCR primers used to amplify D2-D3 expansion of 28S rRNA and ITS regions.

Target	Primer	Sequence ($5' \rightarrow 3'$)	Region	Reference
Universal	D2A	ACAAGTACCGTGAGGGAAAGTTG	28S	Subbotin *et al*. (2006)
Universal	D3B	TCGGAAGGAACCAGCTACTA	28S	Subbotin *et al*. (2006)
Universal	TW81	GTTTCCGTAGGTGAACCTGC	ITS	Tanha Maafi *et al*. (2003)
Universal	AB28	ATATGCTTAAGTTCAGCGGGT	ITS	Tanha Maafi *et al*. (2003)

Primers synthesised by Eurofins MWG Operon.

48 h (Brown & Boag, 1988). Individual *Pratylenchus* nematodes used for qPCR assays were identified and picked out using light microscopy and their species identified by amplification and sequence analysis of the D2-D3 expansion of the 28S rRNA gene (Table 2). Exemplar sequences of each of the three species were deposited in GenBank; *P. crenatus* (KX683378), *P. neglectus* (KX683377) and *P. penetrans* (KX683379). Sequences had 97-100% affinity with published sequences of the three species (Subbotin *et al*., 2008; Kumari, 2015).

Total genomic DNA extraction

DNA from individual females from several non-target *Pratylenchus* species, representing species of *Pratylenchus* previously recorded in Brazil, and populations of target *P. crenatus*, *P. penetrans* and *P. neglectus* (Table 1) was extracted using a simplification of an existing protocol (Adam *et al*., 2007). Briefly, adult nematodes were placed individually in 0.2 ml micro-centrifuge tubes with 18 μl of buffer (1$\times$ GoTaq buffer; Promega) and frozen for 60 min at -20°C. Next, four 1.0 mm glass beads and 2 μl proteinase K (100 mg ml^{-1}; Promega) were added and the samples shaken in a Retsch MM300 shaker for 20 s at maximum speed. Thereafter, samples were incubated at 60°C for 90 min before inactivating the enzyme at 95°C for 15 min. After incubation, tubes were centrifuged for 1 min at 18 407 *g* and stored at -20°C until required.

PCR amplification

One Ready-to-Go PCR bead (GE Healthcare Biosciences) was placed into a 0.2 ml micro-centrifuge tube with 22 μl distilled water, 1 μl template DNA from a representative population of the target species (*P. crenatus*, *P. penetrans* and *P. neglectus*) or non-target species (Table 1) and 1 μl of each 10 μM primer. PCR products were generated using primers TW81 and AB28 (Table 2) located, respectively, in the 18S and 28S rDNA (Tanha Maafi *et al*., 2003). PCR conditions were as follows: 94°C for 4 min, then 35 cycles of: 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min with a final extension at 72°C for 10 min. PCR products were separated on a 1% agarose gel and visualised by staining with SybrSafe (Life Technologies). PCR products of sufficiently high quality were purified for sequencing using the QIAquick PCR Purification Kit (Qiagen) according to the protocol given by the manufacturer.

Cloning and sequencing

Purified plasmid DNA containing clones of the ITS1-5.8S-ITS2 regions of *P. crenatus*, *P. penetrans* and *P. neglectus* was prepared as follows. PCR products from each template species were obtained in 25 μl reactions as previously described using primers TW81 and AB28 (Table 2). Ligation into a plasmid vector (pGEM-T Easy Vector; Promega) was carried out overnight and the plasmid introduced into in-house *Escherichia coli* dH5α electrocompetent cells by electroporation using a micropulser electroporator (Bio-Rad). Colonies with recombinant plasmids were selected and DNA purified using GenJet plasmid minipreps kit (Thermo Scientific). Sequencing reactions were carried out using a Big Dye Sequencing kit (Applied Biosystems) and the sequence analysis was run on an ABI Prism3700 DNA Analyser (Applied Biosystems) by the JHI in-house sequencing service. The full ITS length varied between species: 685 bp (*P. neglectus*), 717 bp (*P. penetrans*) and 778 bp (*P. crenatus*). At least three clones of each population were sequenced (six clones of *P. crenatus*; four clones of *P. neglectus*; three clones of *P. penetrans*). Exemplar clone sequences were deposited in GenBank; *P. crenatus* (KX683384-KX683389), *P. neglectus* (KX683380-KX683383) and *P. penetrans* (KX683390-KX683392). At the species level, the majority of the clones had minimal variation (0.0-0.3%), although we did note outliers *P. crenatus* (11.1%, Pcc5), *P. neglectus* (7.1%, Pnc4) and *P. penetrans* (0.6%, Ppc3).

STANDARD CURVES

The molecular weight of each representative clone was calculated based on the number of bases of the pGEM-T vector and the inserts. Estimation of gene-copy number in each representative clone of *P. crenatus*, *P. penetrans* and *P. neglectus* was based on the molecular weight in accordance with http://www.thermoscientificbio.com/webtools/copynumber/ after linearisation with *NotI* (Promega), with copy number adjusted in a ten-fold dilution series used to generate standard curves for real-time experiments. The calibration curves were linear over eight orders of magnitude (10-10^7).

HYDROLYSIS PRIMER/PROBE SETS DESIGN

Three independent primer/probe sets targeting the ITS-1 region of the ribosomal DNA (rDNA) representing each of the three target species (*P. crenatus*, *P. penetrans* and *P. neglectus*) were designed (Table 3) and synthesised by PrimerDesign. All hydrolysis probes were labelled at the 5′-end with FAM reporter dye and the 3′-terminal quencher was TAMRA. Each lyophilised primer/probe set was re-suspended in 600 μl of RNAse/DNAse free water and stored at −20°C. Care was taken to identify ITS-regions with the least number of within-species polymorphisms to enable amplification from all known examples of each target species. Where within-species polymorphisms were unavoidable, A-T polymorphisms were preferentially chosen.

HYDROLYSIS ASSAY CONDITIONS

Real-time PCR was performed using the StepOnePlus Real-Time PCR System, with MicroAmp optical 96-well plates and MicroAmp optical adhesive film (all Applied Biosystems).

For each sample, 2 μl of DNA template for *P. crenatus*, *P. penetrans* and *P. neglectus* were used from the final DNA suspension volume and transferred into a mix containing 10 μl SensiFast Probe Hi-Rox Mix (2×) (Bioline Reagents), 1 μl of primer (6 pg μl^{-1}) per probe (3 pg μl^{-1}) master mix. The final volume was 20 μl. Negative control samples were also included in each experiment using distilled water instead of DNA. The thermo-cycling profile for all PCR reactions was the default cycling profile suggested by the manufacturer using a fast ramp speed, consisting of one cycle at 95°C for 3 min and 40 cycles at 95°C for 10 s and 60°C for 20 s. Thermal cycling was completed in less than 40 min and all samples were run in duplicate.

PCR product and both negative (no DNA template) and positive (genomic DNA from individual nematodes) controls for *P. crenatus* (n = 2), *P. penetrans* (n = 2) and *P. neglectus* (n = 2), were included in each assay. The specificity of the primer/probe sets was tested using genomic DNA extracted from individual nematodes of four non-target *Pratylenchus* species, *P. brachyurus*, *P. zeae*, *P. coffeae* and *P. jaehni* and *Radopholus similis* (Table 1). Standards in ten-fold dilutions were run in duplicate (Fig. 1) for quantification experiments. By increasing the amount of plasmid standard DNA, the respective threshold cycle

Table 3. Diagnostic primer/probe sets developed for detection of *Pratylenchus crenatus*, *P. penetrans* and *P. neglectus*.

Target (GenBank accession No.)	Primer/probe[1]	Sequence (5′ → 3′)	Position	T_m (°C)	GC%	Product length (bp)
P. crenatus (FJ712916)	PcreF[2]	TTCTTGACAAGTTCATTGCTTC	50	54.0	36.4	116
	PcreR[2]	CACTCACGATGTGCTTCTG	165	54.2	52.6	
	PcreP[2]	GCATGATTGAGCGAGAATGGAACTGTAAAT				
P. penetrans (FJ712987)	PpenF	AATGTGTCTCGCCCTGAGG	109	57.1	57.9	80
	PpenR	GCAACCACGGACGGAATAC	188	56.7	57.9	
	PpenP	ACACAGACGCCAGCAGAAGCCG				
P. neglectus (FJ717818)	PneglF	ACTGTGCGAAGTGTCCG	221	54.3	58.8	121
	PneglR	GATCCACCGATAAGGCTAGA	341	53.9	50	
	PneglP	ASGGGACGCCAGCAACCAATGTTTT				

[1] Primer/probe sets were designed and synthesised by PrimerDesign.
[2] F: sense primer; R: reverse primer; P: hydrolysis probe. All hydrolysis probes were labelled at the 5′-end with FAM reporter dye and the 3′-terminal quencher was TAMRA.

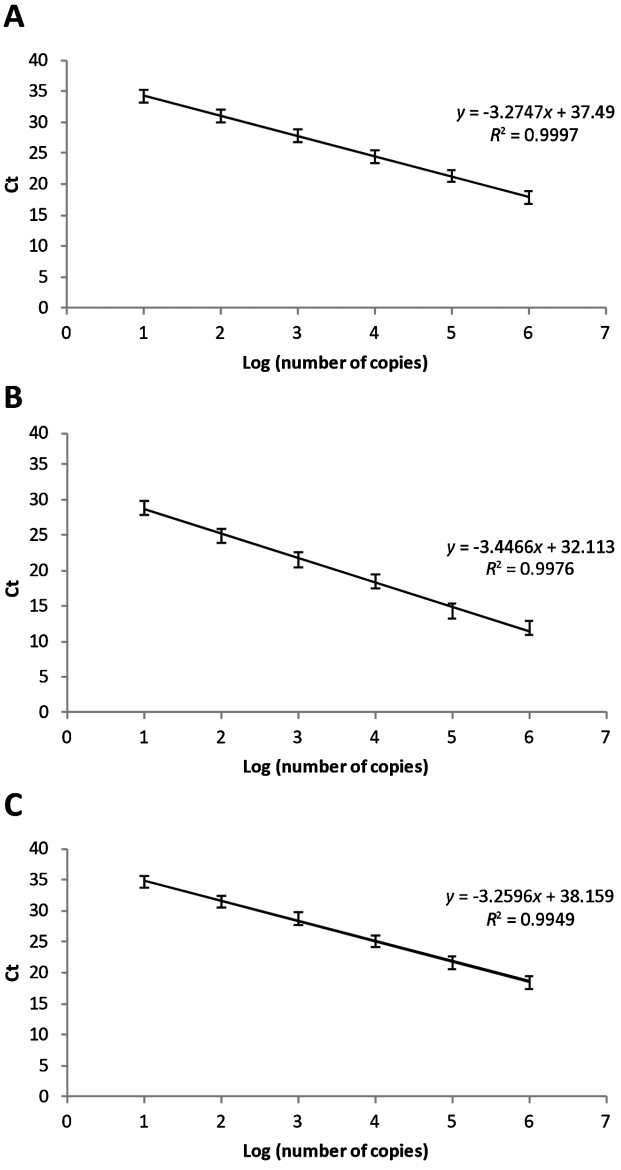

Fig. 1. Standard linear curves of cycle threshold (C_t) values plotted against log-transformed plasmid DNA standards of known concentrations of A: *Pratylenchus crenatus*; B: *P. penetrans*; C: *P. neglectus*. The standard curves were run in duplicate for each *Pratylenchus* species and the standard error bars represent the differences between different runs.

(C_t) was calculated and the relationship between these values and the logarithm of initial template amounts determined by regression analysis. Quantification of target DNA present in unknown samples was performed from measured C_t values by interpolation using the derived regression equation.

Detection of *Pratylenchus* nematodes in field samples

In order to verify that the qPCR diagnostic was able to detect and quantify target *Pratylenchus* spp., soil samples were obtained from 235 potato fields across Scotland. Nematodes were extracted from 200 g soil using a modified Baermann funnel technique (Brown & Boag, 1988). The collected eluent was reduced to *ca* 1.5 ml and freeze-dried in preparation for DNA extraction. DNA was extracted using Purelink DNA (Invitrogen) extraction kits, according to the manufacturer's instructions. Elution was in a total volume of 100 μl of which 2 μl was used in the qPCR assay for each target species as described above.

Comparison between the number of *Pratylenchus* estimated by qPCR and microscopy

As a comparator, qPCR data were compared with *Pratylenchus* spp. abundance determined manually under microscopy for a subset of 50 of the 235 tested soil samples. For those samples, total nematode abundance was determined using a microscope and then subsequently used for total DNA extraction and processed as described above. Data were subjected to an analysis of variance (ANOVA), using the statistical package SISVAR.

Results

DNA extracted from individual female *P. crenatus*, *P. penetrans* and *P. neglectus* nematodes was tested against primer/probe sets for all target species. No cross-reactions were observed amongst the three target *Pratylenchus* species when tested against each other. Similarly, neither of the target primer/probe sets amplified DNA extracted from the four non-target *Pratylenchus* species (*P. brachyurus*, *P. zeae*, *P. coffeae* and *P. jaehni*) or *R. similis*. In all experiments, no increase in fluorescence was detected from the non-template control reactions containing sterile distilled water instead of DNA, confirming that all reagents were free of contamination.

Sensitivity of the qPCR assays was measured with a ten-fold dilution series of purified plasmids. The standard curves were run in duplicate for each *Pratylenchus* species and the samples were almost identical as represented by the standard error values on the different runs (Fig. 1). Each of the three qPCR assays developed in this study reliably detected 10 copies μl^{-1} of template DNA in each

reaction. The standard curve used to calculate the starting concentration of *P. crenatus* template DNA had a high correlation coefficient ($R^2 = 0.99$) indicating a reproducible linear relationship with increasing DNA concentration. The assay efficiency (E) was 100%, calculated from the slope (−3.27) of the standard curve using the regression equation $E = ((10^{(-1/\text{slope})}) - 10) \times 100$. Similarly, there was a negative correlation between C_t values and the $\log_{10}$ of the initial known DNA quantities ($R^2 = 0.99$ for both *P. penetrans* and *P. neglectus*) over a range of 10^7 to 10 copies μl^{-1}, illustrating the sensitivity and linearity of the technique (Fig. 1).

Quantification of target DNA from individual nematodes was performed by comparison of the fluorescence signals to that obtained from standard plasmid dilutions. Based on genomic DNA extracted from single female nematodes the estimated number of ITS-1 copies per nematode was 6981 ± 1343 (*P. penetrans*), 741 ± 163 (*P. neglectus*) and 6168 ± 625 (*P. crenatus*) (Table 4).

Relative quantification of target DNA present in unknown samples (ITS-1 copies in 100 μl DNA solution from nematodes extracted from field soil samples) was performed by comparison of the fluorescence signals of the samples with those obtained from plasmid standard dilutions. Using the primer/probe combinations for *P. crenatus*, *P. penetrans* and *P. neglectus*, qPCR analysis yielded detectable amplification from all tested soil samples obtained from potato fields across Scotland. Based on these data (n = 235), estimated prevalence of the three target species varied with *P. crenatus* present in 94% of the tested soil samples, *P. penetrans* 75% and *P. neglectus* 64%.

Calibrating against gene copy number, C_t values were converted into approximate nematode abundance and compared with the manual estimates of *Pratylenchus* spp. abundances from 50 soil field samples (Fig. 2). In Figure 2A, the nematode abundances of each sample calculated through both methods is shown, whilst Figure 2B represents the estimated abundance of each *Pratylenchus* species by qPCR. There was also a positive but low correlation ($R^2 = 0.188$) between qPCR values and the *Pratylenchus* spp. numbers estimated by microscopy. Manual counts averaged 36 ± 16 individuals, ranging from 3 to 83 nematodes (200 g soil)$^{-1}$. Conversely, the average nematode abundance based on qPCR data was higher (85 ± 46) for the same samples. For each target species, the qPCR tests provided the following mean ± standard error and range: *P. crenatus* (59 ± 40; 6-176), *P. penetrans* (16 ± 22; 0-96) and *P. neglectus* (9 ± 13; 0-57) for the 50 samples tested.

Table 4. Quantification of target DNA (ITS-1 copy number) from individual nematodes of *Pratylenchus penetrans*, *P. crenatus* and *P. neglectus* (Scottish populations) performed by comparison of the fluorescence signals of the samples to those obtained from standard plasmid dilutions.

Species	C_t (mean ± SE)	ITS-1 copy number (mean ± SE)
P. penetrans (n = 5)	25 ± 0.3	6981 ± 1343
P. crenatus (n = 14)	29 ± 0.2	6168 ± 625
P. neglectus (n = 9)	28 ± 0.3	741 ± 163

Discussion

In recent years, PCR-based molecular diagnostics have demonstrated that they offer sensitivity, accuracy and confidence when used to complement conventional descriptive information. Indeed, molecular diagnostics are evolving and recently qPCR assays have been designed for several plant-parasitic nematodes, including *P. neglectus*, *P. penetrans*, *P. thornei* and *P. zeae* (Sato *et al*., 2007; Berry *et al*., 2008; Yan *et al*., 2012, 2013; Mokrini *et al*., 2013, 2014). However, there are technical differences between these qPCR assays; some use the intercalating fluorescent reporter dye SYBR Green whereas others utilise hydrolysis probes (formerly known as TaqMan). The advantage of hydrolysis probes over SYBR Green is that specific hybridisation between probe and target DNA sequence is required to generate a fluorescent signal (Schena *et al*., 2013). The hydrolysis probe method provides an additional level of assay specificity as all three oligonucleotides (forward and reverse primers and probe) hybridise to the target sequence.

In the present study, a standardised molecular diagnostic protocol using specific primers and hydrolysis probes for *P. crenatus*, *P. neglectus* and *P. penetrans* was developed based on the ITS-1 region. The applicability of these primer/probe assays was demonstrated with a reliable pre-plant soil test that determined the presence of these species. The assays were developed for use in routine sample screening as a quick method to detect and quantify each one of these three economically important *Pratylenchus* species. These qPCR assays can be used for identification and assessments of abundance of each of the species and provide new possibilities to deal with large numbers of samples such as from statutory surveys through automation of extraction and molecular proce-

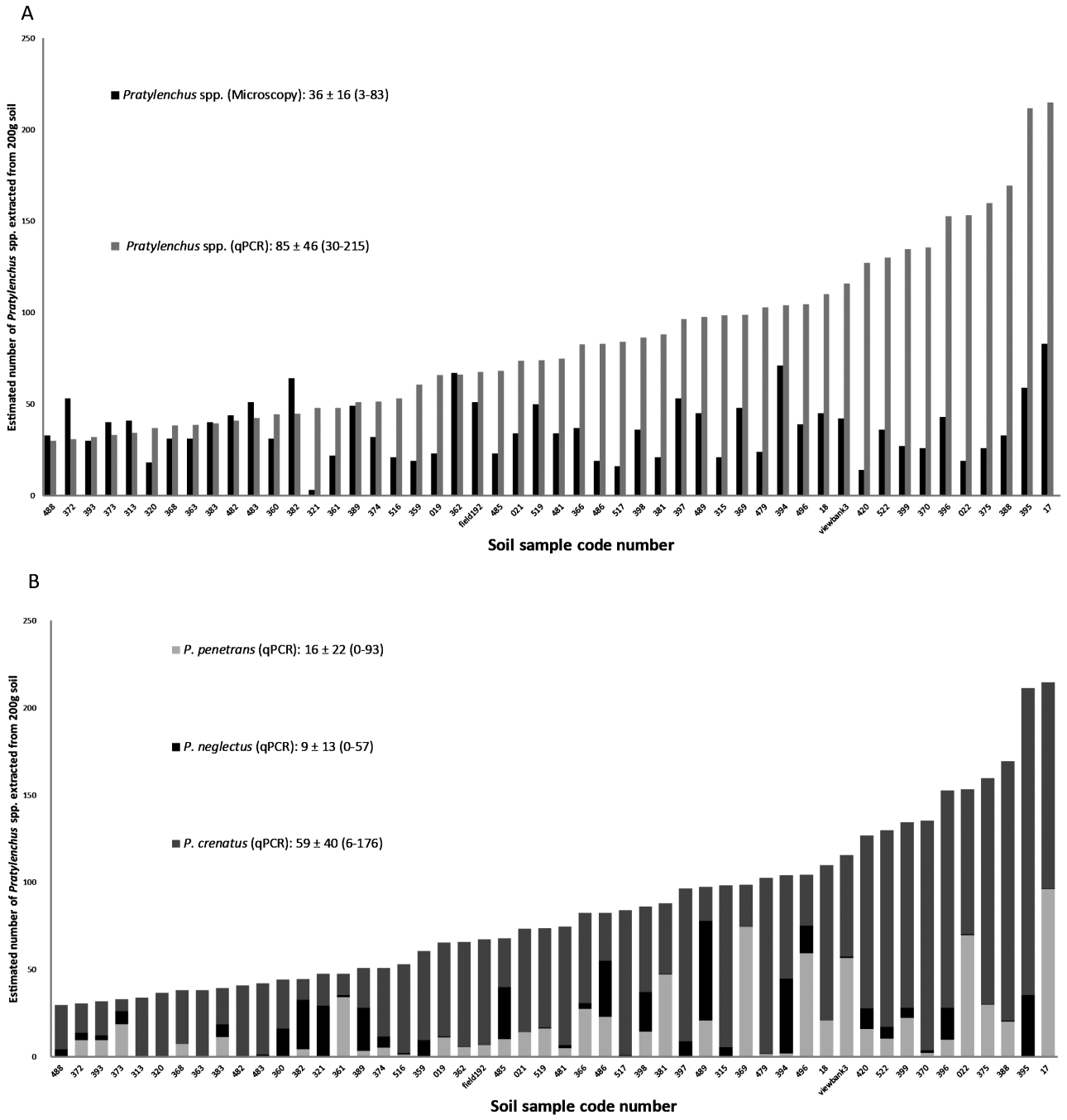

Fig. 2. Estimated *Pratylenchus* spp. populations from 50 Scottish potato fields using microscopy and qPCR (average, standard deviation, minimum and maximum values). A: Nematode abundances of each sample calculated through both methods ($P < 0.01$); B: Number of each *Pratylenchus* species estimated by qPCR.

dures that increase sample throughput and accuracy (Reid *et al*., 2010). Additionally, these standardised diagnostic protocols can be useful for plant protection agencies to limit the movement of regulated species, for example, *P. crenatus*, considered an A1 quarantine pest to Brazil (Singh *et al*., 2013).

Sensitivity of these assays enabled the specific detection of genomic DNA extracted from each *P. crenatus*, *P. neglectus* or *P. penetrans* individual with no cross-reaction between these three species. For the selected non-target *Pratylenchus* species, specificity was demonstrated by the absence of cross-reactions with any of the three assays.

Standard curve calibration experiments with known amounts of plasmid DNA demonstrated that starting quantities differing in over eight orders of magnitude were detected within the linear dynamic range of the qPCR system. A dilution series using DNA of each studied target species resulted in a standard curve showing highly significant linearity between the C_t values and the dilution rates.

Sequence variability in the ITS regions has previously been utilised for designing qPCR diagnostics of several species of plant-parasitic nematodes, including *P. neglectus* (Yan *et al*., 2013), *P. thornei* (Yan *et al*., 2012) and *P. zeae* (Berry *et al*., 2008). Whilst intra-individual variability in the ITS regions can be sufficiently large to confound interpretation for phylogenetic analysis of some species, such as *P. vulnus* and *P. neglectus* (De Luca *et al*., 2011), it remains a useful region for the development of qPCR diagnostics. The ribosomal genes and interspecific regions (rDNA) have been widely used for both diagnostic and phylogenetic studies and the considerable amount of sequence data available for Nematoda provides a useful framework for *in silico* analyses and comparison with non-target species. Conserved sequences in the rDNA also make it very accessible for PCR amplification and subsequent sequence analyses. The multi-copy nature of rDNA is also of considerable utility for enhancing the detection potential, which is particularly important with microscopic organisms that are of phytosanitary risk and that contain restricted quantities of DNA. We did observe some intraspecific sequence variability in the ITS rDNA of the different *Pratylenchus* species; however, this did not preclude the designing of primers and probes that differentiated the three target species. The primers and probes designed detected all individuals in their target species and did not detect those in the other species that are of concern for the import of seed potatoes into Brazil.

The proposed primer/probes presented here cover regions where polymorphisms exist within species. These polymorphisms are mainly single nucleotide mismatches, predominantly A-T mismatches. It is therefore accepted that whilst the presence of intra-species variation within the ITS regions utilised in this study will result in the amplification of some variants of the ITS regions (on a species-by-species basis) with lowered efficiency, each diagnostic tool amplified all individuals of each target species tested. As with all diagnostics, the suitability of these primer/probes for use on targets from outside the geographic regions tested during the design phase would need to be confirmed.

The estimated number of ITS-1 copies per nematode was lower in *P. neglectus* (741) than in *P. crenatus* (6168) and *P. penetrans* (6981). One possible explanation for the lower number of ITS-1 copies in *P. neglectus* may be related to the intra-individual variability in ITS regions (Pecson *et al*., 2006; De Luca *et al*., 2011). If ITS-1 from *P. neglectus* has polymorphisms at the primer or probe binding sites, or if target ITS-1 varied in copy number, it would result in an underestimation of copy number of the ribosomal array. Thus, qPCR efficiency and sensitivity among populations may also vary as fluorescence and copy number in template DNA are linearly related. Also, if different ITS types were present at different relative proportions in different individuals/populations then the signal strength cannot be used to determine accurately the number of individuals present. Alternatively, the putative copy number variability between the three species could be the result of variation in the numbers of copies of the rDNA cistrons between species or the DNA extraction efficiency was not the same for the three *Pratylenchus* species (Holeva *et al*., 2006). It has been reported that rDNA copy number can vary among different nematode species including plant-parasitic or animal-parasitic nematodes. For example, Holeva *et al*. (2006) reported a lower relative quantity of amplifiable target DNA from *Trichodorus similis* than in *Paratrichodorus pachydermus*. The number of ITS-1 copies ranged from 8.4×10^3 to 5.3×10^4 copies per viable egg of *Ascaris lumbricoides* (Pecson *et al*., 2006). Another possible contributory factor to the observed variation was the use of different developmental stages; however, Yan *et al*. (2013), using SYBR Green, reported no significant difference between different life stages of *P. neglectus*, suggesting that the amount of DNA is similar in different stages of development of this species. By contrast, using more sensitive hydrolysis probes, Ghelder *et al*. (2015) observed differences be-

tween samples of equal numbers of juveniles and adult *Xiphinema index*.

Our data confirmed that quantitative differences in the number of each *Pratylenchus* species can be detected from a bulk nematode DNA extraction from soil samples. Additionally these results indicate that *P. crenatus*, *P. neglectus* and *P. penetrans* are widely distributed in Scottish potato fields, frequently co-existing in mixed populations, but with *P. crenatus* more prevalent than *P. neglectus* and *P. penetrans*.

Comparison between the number of *Pratylenchus* estimated by qPCR and microscopy showed that most of those analysed by qPCR yielded higher numbers. Potential reasons for this include: *i*) eggs carried by females would also be detected by qPCR but not by classical microscopy; *ii*) gravid females would generate a stronger signal than non-gravid females; and *iii*) males containing sperm will generate a higher signal than non-gravid females or males without sperm, although this is only valid for *P. penetrans*. Thus, a signal generated by qPCR could be greater than nematode abundance estimated by microscopy.

Intraspecific variability in the ITS rDNA does has implications for accurate quantification of the three *Pratylenchus* species in this study. However, the primary objective was to have a sensitive method to detect these species due to their quarantine importance. Results presented here describe a hydrolysis qPCR assay for the detection and assessment of the abundance of three species of *Pratylenchus*. The developed diagnostics represent a simple, rapid and pragmatic assessment tool for evaluating the risks associated with large numbers of samples as the first line of defence in a phytosanitary context to minimise importation of infected material into Brazil.

Acknowledgements

This research was funded by FAPESP (Sao Paulo State Foundation, Brazil), Project number 2012/19101-9. The James Hutton Institute receives financial support from the Scottish Government, Rural and Environment Science and Analytical Services Division.

References

Adam, M.A.M., Phillips, M.S. & Blok, V.C. (2007). Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology* 56, 190-197.

Al-Banna, L., Ploeg, A.T., Williamson, V.M. & Kaloshian, I. (2004). Discrimination of six *Pratylenchus* species using PCR and species-specific primers. *Journal of Nematology* 36, 142-146.

Berry, S.D., Fargette, M., Spaull, V.W., Morand, S. & Cadet, P. (2008). Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes* 22, 168-176.

Blok, V.C. (2005). Achievements in and future prospects for molecular diagnostics of plant-parasitic nematodes. *Canadian Journal of Plant Pathology* 27, 176-185.

Bonfim Junior, M.F., Consoli, E.A., Inomoto, M.M. & Oliveira, C.M.G. (2016). First report of *Pratylenchus crenatus* in Brazil. *New Disease Reports* 34, 7. DOI:10.5197/j.2044-0588.2016.034.007.

Brown, D.J.F. & Boag, B. (1988). An examination of methods used to extract virus-vector nematodes (Nematoda: Longidoridae and Trichodoridae) from soil samples. *Nematologia Mediterranea* 16, 93-99.

Castillo, P. & Vovlas, N. (2007). Pratylenchus *(Nematoda: Pratylenchidae): diagnosis, biology, pathogenicity and management*. *Nematology Monographs and Perspectives 6* (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill.

Coolen, W.A. & D'Herde, C.J. (1972). *A method for quantitative extraction of nematodes from plant tissue*. Merelbeke, Belgium, State Nematology and Entomology Research Station.

Coomans, A. (2002). Present status and future of nematode systematics. *Nematology* 4, 573-582.

De Luca, F., Reyes, A., Troccoli, A. & Castillo, P. (2011). Molecular variability and phylogenetic relationships among different species and populations of *Pratylenchus* (Nematoda: Pratylenchidae) as inferred from the analysis of the ITS rDNA. *European Journal of Plant Pathology* 130, 415-426.

Ghelder, C.V., Reid, A., Kenyon, D. & Esmenjaud, D. (2015). Development of a real-time PCR method for the detection of the dagger nematodes *Xiphinema index*, *X. diversicaudatum*, *X. vuittenezi* and *X. italiae*, and for the quantification of *X. index* numbers. *Plant Pathology* 64, 489-500.

Gonzaga, V., Santos, J.M., Mendonça, R.S. & Santos, M.A. (2016). Gênero *Pratylenchus*. In: Oliveira, C.M.G., Santos, M.A. & Castro, L.H.S. (Eds). *Diagnose de Fitonematoides*. Campinas, SP, Brazil, Millennium Editora, pp. 71-98.

Goto, K., Min, Y.Y., Sato, E. & Toyota, K. (2011). A multiplex real-time PCR assay for the simultaneous quantification of the major plant-parasitic nematodes in Japan. *Nematology* 13, 713-720.

Hay, F.S. & Pethybridge, S.J. (2005). Nematodes associated with carrot production in Tasmania, Australia, and the effect of *Pratylenchus crenatus* on yield and quality of Kuroda-type carrot. *Plant Disease* 89, 1175-1180.

Holeva, R., Phillips, M.S., Neilson, R., Brown, D.J.F., Young, V., Boutsika, K. & Blok, V.C. (2006). Real-time PCR detection and quantification of vector trichodorid nematodes and tobacco rattle virus. *Molecular and Cellular Probes* 20, 203-211.

Holgado, R., Oppen Skau, K.A. & Magnusson, C. (2009). Field damage in potato by lesion nematode *Pratylenchus penetrans*, its association with tuber symptoms and its survival in storage. *Nematologia Mediterranea* 37, 25-29.

Huang, L., Ye, J.R., Wu, X.Q., Xu, X.L., Sheng, J.M. & Zhou, Q.X. (2010). Detection of the pine wood nematode using a real-time PCR assay to target the DNA topoisomerase I gene. *European Journal of Plant Pathology* 127, 89-98.

Hübschen, J., Kling, L., Ipach, U., Zinkernagel, V., Bosselut, N., Esmenjaud, D., Brown, D.J.F. & Neilson, R. (2004a). Validation of the specificity and sensitivity of species-specific primers that provide a reliable molecular diagnostic for *Xiphinema diversicaudatum*, *X. index* and *X. vuittenezi*. *European Journal of Plant Pathology* 110, 779-788.

Hübschen, J., Kling, L., Ipach, U., Zinkernagel, V., Brown, D.J.F. & Neilson, R. (2004b). Development and validation of species-specific primers that provide a molecular diagnostic for virus-vector longidorid nematodes and related species in German viticulture. *European Journal of Plant Pathology* 110, 883-891.

Jones, J.T., Haegeman, A., Danchin, E.G., Gaur, H.S., Helder, J., Jones, M.G., Kikuchi, T., Manzanilla-López, R., Palomares-Rius, J.E., Wesemael, W.M. *et al.* (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology* 14, 946-961.

Kim, D.W. & Chun, J.Y. (2014). Identification of root-lesion nematode (Pratylenchidae: *Pratylenchus*) intercepted on imported plants. *Korean Journal of Applied Entomology* 53, 51-57.

Koyama, Y., Toyota, K., Miyamaru, N., Yoshida, K. & Uesugi, K. (2016). Development of a quantification method with real-time PCR for three *Pratylenchus* species causing damage to chrysanthemum in Japan. *Nematology* 18, 687-695. DOI:10.1163/15685411-00002984.

Kumari, S. (2015). Characterization of *Pratylenchus crenatus* and *P. neglectus* (Nematoda: Pratylenchidae) associated with wheat crop. *Helminthologia* 52, 280-286.

MacGuidwin, A.E. & Rouse, D.I. (1990). Role of *Pratylenchus penetrans* in the potato early dying disease of Russet Burbank potato. *Phytopathology* 80, 1077-1082.

Min, Y.Y., Toyota, K. & Sato, E. (2012). A novel nematode diagnostic method using the direct quantification of major plant-parasitic nematodes in soil by real-time PCR. *Nematology* 14, 265-276.

Mokrini, F., Waeyenberge, L., Viaene, N., Abbad Andaloussi, F. & Moens, M. (2013). Quantitative detection of the root-lesion nematode, *Pratylenchus penetrans*, using qPCR. *European Journal of Plant Pathology* 137, 403-413.

Mokrini, F., Waeyenberge, L., Abbad Andaloussi, F., Viaene, N. & Moens, M. (2014). The β-1,4-endoglucanase gene is suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei*. *Nematology* 16, 1-8.

Oliveira, C.M.G., Fenton, B., Malloch, G., Brown, D.J.F. & Neilson, R. (2005). Development of species-specific primers for the ectoparasitic nematode species *Xiphinema brevicolle*, *X. diffusum*, *X. elongatum*, *X. ifacolum* and *X. longicaudatum* (Nematoda: Longidoridae) based on ribosomal DNA sequences. *Annals of Applied Biology* 146, 281-288.

Oliveira, C.M.G., Machado, A.C.Z., Kubo, R.K. & Harakava, R. (2009). Diagnose de *Aphelenchoides fragariae* e *Pratylenchus* spp. pela aplicação da tecnologia do código de barras do DNA. *Nematologia Brasileira* 33, 218-225.

Oliveira, C.M.G., Monteiro, A.R. & Blok, V.C. (2011). Morphological and molecular diagnostics for plant-parasitic nematodes: working together to get the identification done. *Tropical Plant Pathology* 36, 65-73.

Oliveira, S.A., Eulalio, J., Barozzi, I.C.C., Giora, F.C., Kubo, R.K. & Oliveira, C.M.G. (2012). Identification of plant-parasitic nematodes of ornamental bulbs and use of hidrotermotherapy to control *Pratylenchus crenatus* on lily. *Revista Brasileira de Horticultura Ornamental* 18, 155-162.

Palomares-Rius, J.E., Oliveira, C.M.G. & Blok, V.C. (2014). Plant parasitic nematodes of potato. In: Navarre, R. & Pavek, M. (Eds). *The potato: botany, production and uses*. Wallingford, UK, CAB International, pp. 148-166.

Pecson, B.M., Barrios, J.A., Johnson, D.R. & Nelson, K.L. (2006). A real-time PCR method for quantifying viable *Ascaris* eggs using the first internally transcribed spacer region of ribosomal DNA. *Applied and Environmental Microbiology* 72, 7864-7872.

Powelson, M.L. & Rowe, R.C. (1993). Biology and management of early dying of potatoes. *Annual Review of Phytopathology* 31, 111-126.

Reid, A., Kenyon, D.M., Evans, F.F., Mulholland, V., Pickup, J., Blok, V.C., Paterson, A. & Phillips, M.S. (2010). Development of a high-throughput method for the detection and species determination of PCN. In: Haydock, P.P. (Ed.). *Proceedings of the 3rd symposium on potato cyst nematodes*. Harper Adams University, Newport, UK, Association of Applied Biology, pp. 11-14.

Riga, E., Karanastasi, E., Oliveira, C.M.G. & Neilson, R. (2007). Molecular identification of two stubby root nematode species. *American Journal of Potato Research* 84, 161-167.

Sato, E., Min, Y.Y., Shirakashi, T., Wada, S. & Toyota, K. (2007). Detection of the root-lesion nematode, *Pratylenchus penetrans* (Cobb), in a nematode community using real-time PCR. *Japanese Journal of Nematology* 37, 87-92.

Schaad, N.W. & Frederick, R.D. (2002). Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology* 24, 250-258.

Schena, L., Li Destri Nicosia, M.G., Sanzani, S.M., Faedda, R., Ippolito, A. & Cacciola, S.O. (2013). Development of

quantitative PCR detection methods for phytopathogenic fungi and oomycetes. *Journal of Plant Pathology* 95, 7-24.

Sheedy, J.G., McKay, A.C., Lewis, J., Vanstone, V.A., Fletcher, S., Kelly, A. & Thompson, J.P. (2015). Cereal cultivars can be ranked consistently for resistance to root-lesion nematodes (*Pratylenchus thornei* & *P. neglectus*) using diverse procedures. *Australasian Plant Pathology* 44, 175-182.

Singh, S.K., Hodda, M. & Ash, G.J. (2013). Plant-parasitic nematodes of potential phytosanitary importance, their main hosts and reported yield losses. *EPPO Bulletin*, 334-374.

Subbotin, S.A., Sturhan, D., Chizhov, V.N., Vovlas, N. & Baldwin, J.G. (2006). Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences. *Nematology* 8, 455-474.

Subbotin, S.A., Ragsdale, E.J., Mullens, T., Roberts, P.A., Mundo-Ocampo, M. & Baldwin, J.G. (2008). A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): evidence from 18S and D2-D3 expansion segments of 28S ribosomal RNA genes and morphological characters. *Molecular Phylogenetic Evolution* 48, 491-505.

Tanha Maafi, Z., Subbotin, S.A. & Moens, M. (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences. *Nematology* 5, 99-111.

Wiesel, L., Daniell, T.J., King, D. & Neilson, R. (2015). Determination of the optimal soil sample size to accurately characterise nematode communities in soil. *Soil Biology & Biochemistry* 80, 89-91.

Yan, G.P., Smiley, R.W. & Okubara, P.A. (2012). Detection and quantification of *Pratylenchus thornei* in DNA extracted from soil using real-time PCR. *Phytopathology* 102, 14-22.

Yan, G.P., Smiley, R.W., Okubara, P.A., Skantar, A.M. & Reardon, C.L. (2013). Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. *Plant Disease* 97, 757-764.