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The *β-1,4-endoglucanase* gene is suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei*

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Summary – A real-time quantitative PCR assay was developed for the accurate detection and quantification of the root-lesion nematode, *Pratylenchus thornei*. A qPCR primer set, including two primers and a probe, was designed based on the sequence of the *β-1,4-endoglucanase* gene. The assay was optimised by using the primers with SYBR green I dye and setting the qPCR program to different annealing temperatures ranging from 62 to 69°C. Based on the Ct values, we retained the program with an annealing temperature of 69°C. The specificity of the qPCR assay including the probe was confirmed by the lack of amplification of DNA from 47 populations belonging to 15 other *Pratylenchus* species and nine isolates from *P. thornei*. The assay was very sensitive as it was able to detect a single individual of *P. thornei*, even when mixed with up to 80 individuals of *P. penetrans*. DNA was extracted from exactly 80 *P. thornei* individuals. A dilution series from this DNA resulted in a standard curve showing a highly significant linearity between the Ct values and the dilution rates ($R^2 = 0.98$; slope $= -3.38$; $E = 97.6\%$). The qPCR assay developed in this study proved to be specific and sensitive, thus providing a fast and accurate tool for detection and quantification of this pathogen during research, as well as for diagnostic labs.

Keywords – detection, lesion nematodes, plant-parasitic nematodes, Pratylenchidae, qPCR, sensitivity.

The migratory endoparasitic nematode *Pratylenchus thornei* is an important pathogen of wheat in Europe, Africa, North America, Asia, the Middle East and Australia (Greco *et al.*, 1992; Troccoli *et al.*, 1992; Smiley *et al.*, 2005). In the Mediterranean basin, the nematode also causes severe yield decline of crops such as chickpea, faba bean (Glazer & Orion, 1983; Greco *et al.*, 1984) and pulse crops (Di Vito *et al.*, 1992).

Identification of *Pratylenchus* spp. based on morphology and morphometrics is time-consuming, requires ample skill from the observer, and is frequently inconclusive because of the small number of diagnostically valid characters (Luc, 1987; Loof, 1991). Therefore, traditional nematode identification is more and more supplemented with molecular observations. Waeyenberge *et al.* (2000), De Luca *et al.* (2004) and Subbotin *et al.* (2006) demonstrated that DNA-based methods could be used for identification of *Pratylenchus* species. Species-specific primers were developed for distinguishing species of *Pratylenchus*, including *P. thornei* (Al-Banna *et al.*, 2004; Carrasco-Ballesteros *et al.*, 2007; Yan *et al.*, 2008). However, these methods are not suitable to quantify the species.

In view of a high throughput detection and quantification of *P. thornei*, our objective was to develop a rapid and precise method for the detection and quantification of *P. thornei* in a nematode suspension using qPCR. A similar strategy has been used for the detection and quantification of *P. neglectus*, *P. vulnus*, *P. thornei* and *P. zeae* in DNA extracts of soil using primers designed from the ITS region of rDNA (Qiu *et al.*, 2007; Berry *et al.*, 2008; Toyota *et al.*, 2008; Yan & Smiley, 2013). Mokrini *et al.* (2013), however, developed a real-time PCR assay using species-specific primers and a probe based on the *β-1,4-*

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endoglucanase gene to detect and to quantify *P. penetrans* in a nematode suspension. Here we report on the use of the same gene to develop a qPCR protocol for the rapid, specific, sensitive and quantitative detection of *P. thornei*.

Materials and methods

Nematode populations and DNA extraction

Forty-seven isolates of 15 *Pratylenchus* species originating from several countries and hosts (Table 1) were used in this study. For several species, especially *P. thornei*, more than one isolate was investigated to verify the specificity of the developed qPCR assay. DNA was extracted as described by Holterman *et al.* (2006). For many of the isolates DNA was available (all vermiform stages) as it had been used in another study (Waeyenberge *et al.*, 2009). The Moroccan populations, *viz.*, *P. penetrans*, *P. thornei* and *P. pseudocoffeae*, were identified on the basis of their morphology, morphometrics and D2D3 28S rRNA gene sequences. The DNA was used for testing the specificity of the selected primers and probe. For the sensitivity tests, the same DNA extraction method was used to obtain DNA from 1-80 individuals (see below).

Development of primers and probe

We used the same sequence information of the *β-1,4-endoglucanase* gene of six *Pratylenchus* spp. available from GenBank as in Mokrini *et al.* (2013). The sequences were aligned using the software package AlleleID 7.75. On the basis of this alignment we selected the following sequences for the forward primer, reverse primer and probe: PthMFor 3′-GGA TGC GGT CAT CAA GGC-5′, PthMRev 3′-TTG GCT CTG GTG GTT CTG-5′ and PthMPb 3′-CGA CTG GCA CGA CCA CAA CG-5′, respectively. The MGB-probe was labelled with 6-VIC (Life Technologies Europe).

Real-time PCR assay

Optimisation of the annealing temperature

All qPCR kits (SensiFAST Probe Hi-ROX kit and SensiFAST SYBR Hi-ROX) were validated by the producer (Bioline Reagents) on all commonly used real-time instruments and did not need further optimisation regarding their composition. qPCR using SYBR Green I dye was done under annealing temperatures ranging from 62 to 69°C in a final volume of 20 µl reaction mixture containing 10 µl of SensiFAST SYBR Hi-ROX (2×), 400 nM of each primer, and 3 µl of template DNA extracted from a single individual of *P. thornei* PthZ1 and five individuals of PthZ2 (Table 2). Each sample was run in duplicate using an automated ABI PRISM 7900 HT sequence detection system (Applied Biosystems). The amplification program consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62-69°C for 30 s and 72°C for 1 min. Sequence Detection Software (SDS) 2.4 was used to generate the amplification curves for each reaction. The threshold cycle number (Ct) was determined at a threshold set on 0.2. To differentiate species amplicons from non-specific products, a dissociation curve was generated after each reaction. Control samples without DNA template (NTC) were included in each experiment in duplicates.

Testing specificity of primers and probe

To determine whether the primers and probe were specific for amplification and detection of *P. thornei*, DNA from 47 populations comprising 15 different *Pratylenchus* species (Table 1) was used. Each sample was loaded in triplicate. A negative control sample was also prepared in two replicates using distilled water instead of a DNA template. All runs were done in a final volume of 20 µl containing 10 µl of a SensiFAST Probe Hi-ROX (2×), 400 nM of each primer, 200 nM of the probe and 3 µl of template DNA. The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 69°C for 30 s and 72°C for 1 min.

Testing sensitivity of primers and probe

The sensitivity of the primers and probe was determined in two different experiments run with SensiFAST Probe Hi-ROX (2×). The relationship between DNA concentration and Ct values was estimated in a first experiment in which DNA was extracted from three series of 1, 5, 10, 20, 40 and 80 individuals (all vermiform stages) of *P. thornei* (Holterman *et al.*, 2006). All DNA extracts were run in triplicate. A negative control was prepared in two replicates using distilled water instead of a DNA template. The second experiment examined the detection limit of one individual of *P. thornei* in the presence of an increasing number of individuals (all vermiform stages) of *P. penetrans*. One, 5, 10, 20, 40 and 80 individuals (all vermiform stages) of *P. penetrans* were hand-picked and transferred to an Eppendorf tube containing 25 µl water along with a single *P. thornei*. DNA was extracted (Holterman *et al.*, 2006). Three qPCR were run for each DNA extract. A negative control was also prepared in two replications using distilled water instead of a DNA template.

Table 1. Origin and codes of populations of *Pratylenchus* spp. used in this study, together with the mean Ct value (0.03) and standard deviation, where determined, obtained in a qPCR reaction.

Code	Species	Host/Soil	Origin	Ct
Pa	*P. agilis*	Maize	USA	Und
Pbr2	*P. brachyurus*	Aster	Florida, USA	Und
Pcre	*P. crenatus*	Soil	Belgium	Und
Pc	*P. coffeae*	Coffee	Vietnam	Und
Pfa1	*P. fallax*	Soil	Merelbeke, Belgium	Und
Pfa2	*P. fallax*	Soil	Redu, Belgium	Und
Pg	*P. goodeyi*	Banana	Tenerife, Canary Islands	Und
Pgu	*P. gutierrezi*	Maize	South Africa	Und
Pme	*P. mediterraneus*	–	Spain	Und
Pneg	*P. neglectus*	–	Turkey	Und
Pping1	*P. pinguicaudatus*	–	Italy	Und
Pping2	*P. pinguicaudatus*	–	Morocco	Und
Pps1	*P. pseudocoffeae*	Wheat	Settat, Morocco	Und
Pps2	*P. pseudocoffeae*	–	Iran	Und
Pter	*P. teres*	–	South Africa	Und
Pz	*P. zeae*	–	Australia	Und
Ppe1	*P. penetrans*	Wheat	Turkey	Und
Ppe2	*P. penetrans*	Soil	Belgium	Und
Ppe3	*P. penetrans*	Soil	Kinrooi, Belgium	Und
Ppe4	*P. penetrans*	Soil	The Netherlands	Und
Ppe5	*P. penetrans*	Wheat	Ain Aouda, Morocco	Und
Ppe6	*P. penetrans*	Wheat	Marchouch, Morocco	Und
Ppe7	*P. penetrans*	Wheat	Mediona, Morocco	Und
Ppe8	*P. penetrans*	Wheat	Settat, Morocco	Und
Ppe9	*P. penetrans*	Wheat	Ain Taoujdate, Morocco	Und
Ppe10	*P. penetrans*	Wheat	Mhaya, Morocco	Und
Ppe11	*P. penetrans*	Wheat	SidiSlimane, Morocco	Und
Ppe12	*P. penetrans*	Wheat	Berchid, Morocco	Und
Pp	*P. penetrans*	–	Belgium	Und
PthN1	*P. thornei*		New Zealand	27.7 ± 0.6
PthN2	*P. thornei*	–	New Zealand	19.3 ± 0.6
PthMo	*P. thornei*	Almond	Souk El Gour, Morocco	22.6 ± 0.3
PthAus	*P. thornei*	–	Australia	29 ± 0.5
PthZ1	*P. thornei*	Wheat	Ain Aouda, Morocco	28.9 ± 0.4
PthZ2	*P. thornei*	Wheat	Ain Aouda, Morocco	28.5 ± 0.2
PthZ3	*P. thornei*	Wheat	Marchouch, Morocco	28.2 ± 0.5
PthZ4	*P. thornei*	Wheat	Marchouch, Morocco	30.1 ± 0.4
PthTu	*P. thornei*	Wheat	Turkey	27.9
Tys	*Tylenchulus semipenetrans*	Citrus	Gharb, Morocco	Und
Xid	*Xiphinema diversicaudatum*	Citrus	Gharb, Morocco	Und
Ha	*Heterodera avenae*	Wheat	Zaers, Morocco	Und
Hl	*Heterodera latipons*	Wheat	Sais, Morocco	Und
Gro	*Globodera rostochiensis*	Potato	Bioska, Serbia	Und
Par	*Paratylenchus* sp.	Lettuce	Belgium	Und
Mh	*Meloidogyne hapla*	–	Serbia	Und
Rd	*Radopholus duriophilus*	Coffee	Vietnam	Und
Scu	*Scutellonema* sp.	Yam	Ghana	Und

Und = undetermined; – = unknown.

Table 2. Mean and standard deviation of Ct values obtained at different melting temperatures of DNA extracted from single individuals (PthZ1) and five individuals (PthZ2) of *Pratylenchus thornei* ($n = 2$).

Sample	62°C		63°C		65°C		68°C		69°C	
	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
PthZ1	28.1	0.6	28.9	1.2	29	0.2	30.2	0.5	31.9	0.2
PthZ2	27.6	0.2	27.9	0.4	28.4	0.1	29.2	0.5	29.4	1
Negative control	Und	–	>35	–	Und	–	Und	–	Und	–

Und = undetermined.

Construction of standard curve

DNA was extracted (Holterman *et al.*, 2006) from three times 80 individuals (all vermiform stages) of *P. thornei* (PthZ1). A single qPCR was run for each DNA extract and Ct values were compared. Subsequently, all three tubes of DNA were mixed and a dilution series was prepared. The mixed DNA sample was serially diluted to 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 of the original concentration representing 80 individuals of *P. thornei*. These concentrations were used as templates in a qPCR. Plotting logarithmic values of DNA concentration *vs* Ct values generated a standard curve.

Robustness of the method

To verify if the qPCR method is able to detect and quantify nematodes extracted from a soil sample, eight soil samples from a running pot test with cereals infected with *P. thornei* were analysed. Nematodes were extracted from soil and roots with zonal centrifugation (Hendrickx, 1995). The whole nematode suspension was first counted using a microscope and then used for DNA extraction, followed by the developed qPCR assay.

Results

Optimisation of the annealing temperature

Based on the Ct values, 69°C was selected as annealing temperature. Increasing temperatures resulted in higher Ct values. At 62°C, 63°C, 65°C and 68°C, the dissociation curve of the qPCR test showed multiple peaks (Fig. 1). At 69°C, the assay was able to detect a single individual of *P. thornei* whereas no signals were observed in the NTC samples (Table 2). Non-specific fluorescence due to amplification of primer-dimers or other non-specific amplification product was not observed (Fig. 2).

Specificity of primer and probe set

The target fragment (88 bp) of all *P. thornei* isolates was amplified running the qPCR protocol with the primer pair PthMFor and PthMRev, and the probe PthMPb. DNA from other *Pratylenchus* species, or from species of other nematode genera tested, was not amplified (Table 1). In addition, DNA was not amplified or detected in any of the controls that contained water instead of DNA. The Ct values for DNA derived from 1-5 *P. thornei* individuals from different populations varied between 27.7 ± 0.6 and 30.1 ± 0.4 with the exception of two isolates. These isolates (PthN2 and PthMo) were cultured, and pure genomic DNA from thousands of individuals was obtained using a DNA extraction kit *ca* 7 years ago (High pure PCR template preparation kit, Roche Diagnostics). The Ct values of the latter were lower (19.38 and 22.6) because of the more concentrated DNA (Table 1).

Sensitivity of primers and probe

The qPCR (first experiment) successfully amplified DNA extracted from all *P. thornei* quantities (1, 5, 10, 20, 40, or 80 individuals). The corresponding Ct values were 28.7 ± 0.5, 26.9 ± 0.2, 26.1 ± 0.1, 24.8 ± 0.2, 23.9 ± 0.2, and 23.1 ± 0.2. The Ct value of the negative control was always undetermined. The Ct values obtained after qPCR with DNA from a single *P. thornei* in the presence of increasing individuals of *P. penetrans* (second experiment) were almost constant: 28.8 ± 0.1, 28.6 ± 0.4, 28.7 ± 0.1, 28.9 ± 0.3, 28.8 ± 0.5 and 28.8 ± 1, respectively. The negative control was always undetermined. The Ct values were very stable.

Construction of a standard curve

The primers PthMFor, PthMRev and PthMPb generated a PCR product. No fluorescent signal was recorded from the negative control. The threshold was set on 0.03,

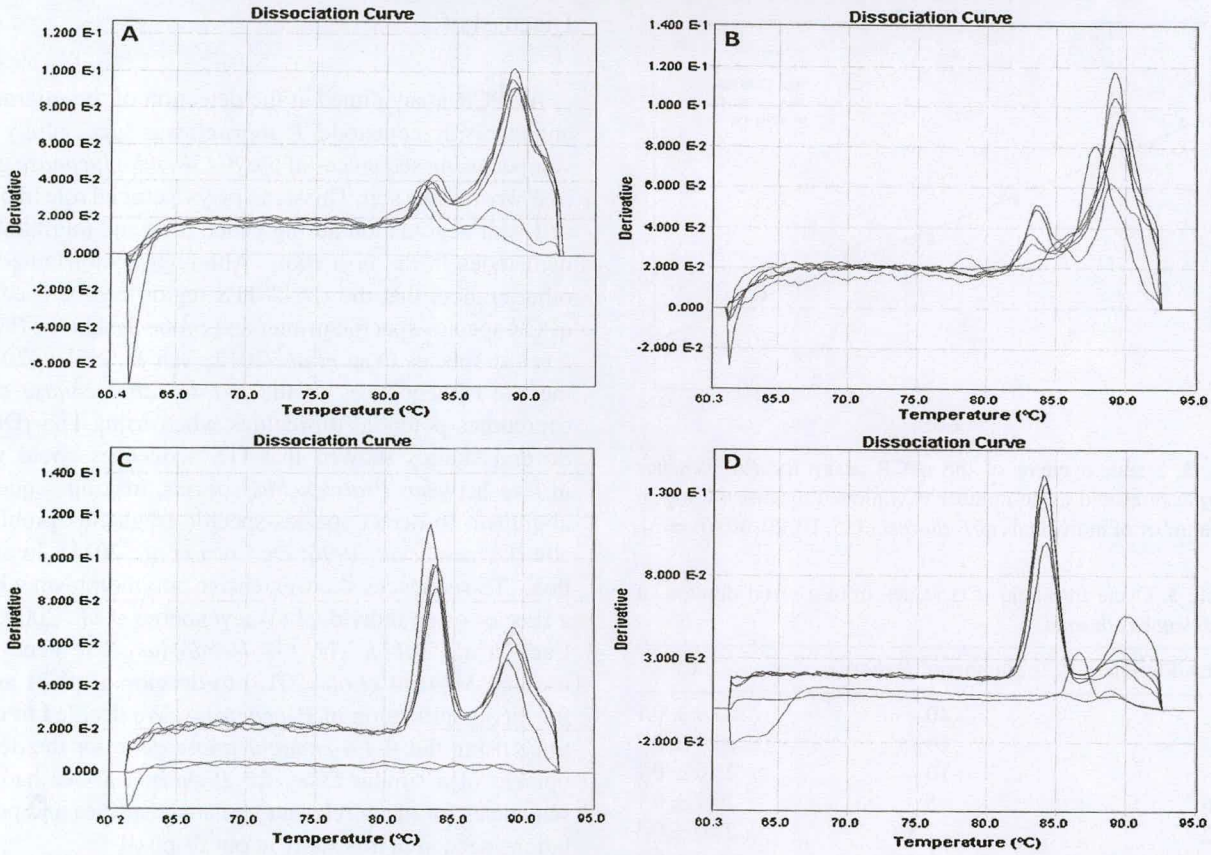

Fig. 1. A, B: Dissociation curve of the qPCR test (SensiFast SYBR Hi-ROX) with annealing temperatures set at 62°C and 63°C showing multiple peaks of two *Pratylenchus thornei* populations ($n = 3$) and NTC ($n = 2$); C, D: Dissociation curve of the qPCR test (SensiFast SYBR Hi-ROX) with annealing temperatures set at 65°C and 68°C showing multiple peaks of two *P. thornei* populations ($n = 3$) and NTC ($n = 2$).

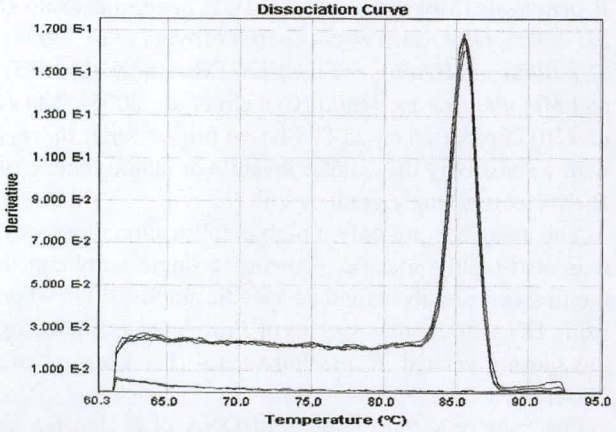

Fig. 2. Dissociation curve of the qPCR test (SensiFast SYBR Hi-ROX) with annealing temperatures set at 69°C showing high peaks at ±85.5 of two *Pratylenchus thornei* populations ($n = 3$) and NTC ($n = 2$).

measuring amplification during the logarithmic phase of the qPCR (Fig. 3). The qPCR was run three times using DNA extracted from 80 individuals of *P. thornei*. A standard curve for *P. thornei* was generated using a serial dilution of *P. thornei* DNA (Fig. 3; Table 3). It showed a highly significant relationship between the Ct value and number of nematodes over the range studied ($R^2 = 0.98$; slope $= -3.38$; $E = 97.6\%$). Based on three sample replications, the ABI PRISM fluorescence detection system automatically calculated the starting number of *P. thornei* by comparison of the Ct values from the unknown samples with the values of the standard curve.

Robustness of the method

The eight soil samples contained between 26 and 228 vermiform stages of *P. thornei* (no eggs) and some sapro-

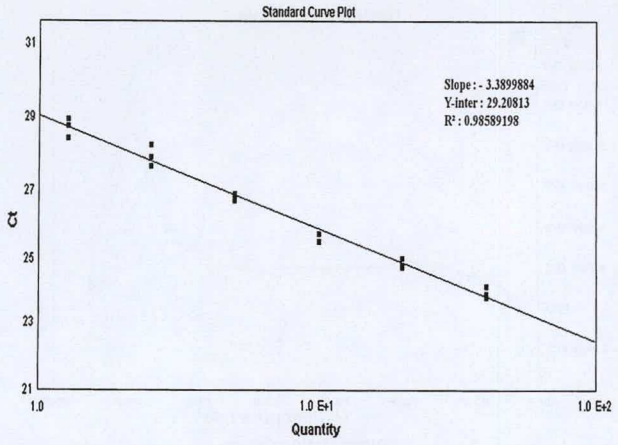

Fig. 3. Standard curve of the qPCR assay for *Pratylenchus thornei* threshold cycle number (Ct) plotted against the log of the number of individuals of *P. thornei* (1, 5, 10, 20, 40) ($n = 3$).

Table 3. Cycle threshold (Ct) values from a serial dilution of *Pratylenchus thornei*.

Serial dilution	Number of *P. thornei*	Ct
1:2	40	23.9 ± 0.1
1:4	20	24.7 ± 0.7
1:8	10	25.6 ± 0.1
1:16	5	26.7 ± 0.1
1:32	2.5	28.0 ± 0.3
1:64	1.25	28.8 ± 0.3

Table 4. Cycle threshold (Ct) values of nematodes from soil and root samples with *Pratylenchus thornei*.

Sample	Ct value	Number of *P. thornei*	
		Calculated	Counted
1	27.46	228.0	233
2	30.80	38.4	40
3	29.41	81.0	82
4	30.25	102.8	107
5	29.68	70.0	73
6	29.95	60.4	65
7	32.79	26.0	29
8	26.39	103.2	106

The total number of *P. thornei* in the nematode suspension was counted, then a subsample of the DNA extract of the nematodes was used for qPCR. The calculated number of *P. thornei* is the number of individuals for the whole sample.

phytic nematodes. About the same, or very similar, numbers of *P. thornei* were counted using visual assessment as with the qPCR method (Table 4).

Discussion

A qPCR assay aimed at the detection of the migratory endoparasitic nematode, *P. thornei*, was successfully developed using sequences of the *β-1,4-endoglucanase* gene of *Pratylenchus* spp. This gene plays a crucial role in plant cell-wall degradation during penetration and migration of nematodes in the host roots. Although demonstrated by other authors that the rDNA-ITS region can be used for qPCR species-specific primer and probe design for *Pratylenchus* species (Yan *et al.*, 2012; Yan & Smiley, 2013), the use of sequences of the *β-1,4-endoglucanase* gene overcomes potential difficulties when using ITS-rDNA. Several studies showed that ITS sequences could vary in size between *Pratylenchus* species, making sequence alignment to detect species-specific fragments problematic (Uehara *et al.*, 1998; De Luca *et al.*, 2011). In addition, ITS sequences show extensive polymorphism within a species or an individual (Waeyenberge *et al.*, 2009; De Luca *et al.*, 2011). The *β-1,4-endoglucanase* gene was used by Mokrini *et al.* (2013) to develop a qPCR assay for the identification of *P. penetrans*. We decided to continue using the *β-1,4-endoglucanase* gene for the development of a similar assay for *P. thornei* as we had the sequences for many relevant nematode species and populations needed in this study at our disposal.

The assay allowed accurate and consistent detection of the DNA of single individuals of *P. thornei* when mixed with DNA from 80 individuals of *P. penetrans*. This sensitivity compares well with findings reported for other nematode species, *viz.*, *P. neglectus* (Yan & Smiley, 2013), *P. penetrans* (Mokrini *et al.*, 2013), *P. penetrans* (Sato *et al.*, 2007), *Globodera rostochiensis* (Toyota *et al.*, 2008), *G. pallida* and *Heterodera schachtii* (Madani *et al.*, 2005), and *Meloidogyne incognita* (Ciancio *et al.*, 2005). Yan *et al.* (2012) reported on an ITS-based primer for *P. thornei* with a sensitivity of a single juvenile or single female of *P. thornei* in a single gram of soil.

The assay has not only a high amplification efficiency, it is also highly specific, showing a single amplicon in melting curve analyses and no specific amplification when using DNA from other species of *Pratylenchus*, including the closely related *P. mediterraneus* (De Luca *et al.*, 2004).

Our runs of serial dilutions of DNA of *P. thornei*, as well as runs with DNA extracted from increasing amounts of nematodes showed a highly significant linear relationship between the Ct value and number of *P. thornei*. These results are similar to those obtained with the qPCR test

developed for *P. penetrans* by Mokrini *et al.* (2013). Yan & Smiley (2013) reported a significant positive relationship between the numbers of *P. neglectus* added to soil and the numbers quantified using their soil standard curve and qPCR. They found much variation between the qPCR tests of replicate samples of *P. neglectus* added at 20 and 40 nematodes per g of soil. The authors mention this is probably due to the commercial kits used for extraction of DNA directly from soil causing variable number of nematodes to be disrupted and releasing their DNA during vortexing steps. Our tests were performed with nematode suspensions in water, requiring extraction of nematodes from soil prior to performing the qPCR test, but increasing accuracy in quantification. We demonstrated that the test also performs very well with nematodes extracted from soil using centrifugation, thus indicating its robustness.

This qPCR assay has the capacity of simultaneously detecting and quantifying *P. thornei* in mixed populations of *Pratylenchus* spp. where visual identification of individual nematodes at species level is extremely difficult. This technique does not require expertise in nematode taxonomy and morphology, and can be used as a rapid diagnostic tool in research, as well as in diagnostic laboratories to avoid the time-consuming steps of traditional nematode extraction, microscopic identification, and quantification.

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