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ORIGINAL RESEARCH

Quantitative detection of the root-lesion nematode, *Pratylenchus penetrans*, using qPCR

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Abstract *Pratylenchus penetrans* is one of the most economically damaging plant-parasitic nematodes and is found on a wide variety of crops. Correct identification and quantification of this nematode are necessary for providing advice to farmers, but are not easily obtained with the traditional way of microscopic observation. We developed a qPCR assay to detect and quantify *P. penetrans* in a short but accurate manner. A qPCR primer set, including two primers and a TaqMan probe, was designed based on the sequence of the β-1,4-endoglucanase gene. The assay was optimized by using the primers in a qPCR assay with SYBR green I dye and setting the qPCR program to different annealing temperatures ranging from 60 °C to 64 °C. Based on the Ct-values, we retained the program with an annealing temperature of 63 °C. The assay with the probe was very sensitive as it was able to detect a single individual of *P. penetrans*, even when mixed with up to 80 individuals of *P. thornei*. The specificity of the reaction was confirmed by the lack of amplification of DNA from 28 populations of 18 other *Pratylenchus* species and from plant-parasitic nematodes from nine other genera. DNA from 21 different isolates from *P. penetrans* was amplified. DNA extraction from 80 individuals and quantification by qPCR was repeated four times; Ct-values showed consistent results (Ct=24.4±0.4). A dilution series from DNA of *P. penetrans* resulted in a standard curve showing a highly significant linearity between the Ct-values and the dilution rates (R^2=0.99; slope=−3.23; E=104 %). The tests showed a high correlation between the real numbers of nematodes and the numbers detected by the qPCR. The developed qPCR assay provides a sensitive means for the rapid detection and reliable quantification of individuals of this pest. This method 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 labs and extension services advising farmers for pest management.

Keywords β-1,4-endoglucanase gene · Diagnostics · Identification · qPCR · Quantification · Sequence

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Introduction

The root-lesion nematode, *Pratylenchus penetrans*, is one of the most economically damaging plant-parasitic nematodes. It is widely distributed and is found on a

wide variety of crops (Castillo and Vovlas 2007). In Europe, *P. penetrans* causes reductions in yield and/or quality of potato, vegetables, such as carrot, pea, bean, and ornamental plants, including rose, tulip and lily (Green and Verdejo 1985; Talavera et al. 2001; Pudasaini et al. 2007). The nematode also reduces productivity of potato in many production areas (Olthof and Potter 1973; Bernard and Laughlin 1976; Olthof 1986, 1989; Ball-Coelho et al. 2003; Belair et al. 2005; Holgado et al. 2009) In Québec, *P. penetrans* was reported as the dominant species in potato fields, and population densities above the damage threshold of 1,000 nematodes/kg soil were common (Olthof 1987). This nematode was also recorded in Algeria and Tunisia on several crops (Troccoli et al. 1992). In Morocco, *P. penetrans* is the most common pratylenchid in different wheat producing areas (Meskine and Abbad Andaloussi 1992; Mokrini et al. 2009, 2012).

It has been frequently demonstrated for diverse combinations of plants and nematodes, including *P. penetrans*, that a significant relationship exists between the pre-planting nematode density and the damage caused by the nematode on the host (Seinhorst 1998; Sato et al. 2009). Hence, a correct identification and quantification of *P. penetrans* is of major importance in nematode control strategies. However, identification of *Pratylenchus* spp. based on morphology and morphometric traits of adults is time-consuming, requires ample skill and training from the observer, and is frequently inconclusive because of the small number of diagnostically valid characters (Luc 1987; Loof 1991). Moreover, *Pratylenchus* spp. are frequently present in mixed populations, which makes their identification and quantification even more difficult. Waeyenberge et al. (2000), De Luca et al. (2004), and Subbotin et al. (2006) demonstrated that DNA-based methods provide efficient tools for a precise and rapid identification of *Pratylenchus* species. PCR using species-specific primers constitute a major step forward in the development of diagnostic technology, which has successfully been used for sensitive detection of *Pratylenchus* species. Species-specific primers to detect *P. penetrans* in a conventional PCR have been developed (Uehara et al. 1998; Al-Banna et al. 2004; Waeyenberge et al. 2009). However, these primers are not suitable to quantify the species. Recently, quantitative PCR (qPCR) strategies have been developed for a *P. zeae* (Berry et al. 2008) and *P. thornei* (Yan et al. 2012). qPCR allows continuous monitoring of the sample during PCR using hybridization probes. The log-linear region can be easily identified as the fluorescence data appear on the computer screen. Within this region, the number of cycles needed to obtain fluorescence above the background (Ct) is compared between samples and standards with known quantities of DNA. These data then can be used for quantification of the samples (Kingsnorth et al. 2003). The aim of the present study was to develop a rapid and precise method for the detection and quantification of *P. penetrans* a nematode suspension using qPCR and to evaluate its efficacy.

Materials and methods

Nematodes populations and DNA extraction

Forty-nine isolates of 20 *Pratylenchus* species originating from several countries and hosts (Table 1) were used in this study. For several species, especially *P. penetrans*, more than one isolate was investigated to verify the specificity of the developed qPCR assay. From many of the isolates, DNA had already been extracted (all stages confounded) and used in another study to develop a species-specific PCR for the detection of *P. penetrans* (Waeyenberge et al. 2009). This DNA was also used in our study. Other isolates were obtained during a survey conducted in different wheat growing areas in Morocco (Mokrini et al. 2012). The 21 Moroccan populations, comprising *P. penetrans*, *P. thornei* and *P. pseudocoffeae*, were identified on the basis of their morphology, morphometrics and D2-D3 28S rRNA gene sequences. From these latter populations, DNA was extracted as described by Holterman et al. (2006). For this purpose, one or five individuals (all stages confounded) were hand-picked and transferred to an Eppendorf tube containing 25 µl double distilled water (ddH2O) and 25 µl nematode lysis buffer (final concentration: 200 mM NaCl, 200 mM Tris–HCl (pH8), 1 % mercaptoethanol and 800 µg of Proteinase K). The tubes were incubated at 65 °C for 1.5 h and 99 °C for 5 min, consecutively. This DNA was used for testing the specificity of the selected primers and probe. For the sensitivity tests, the same DNA-extraction method was applied to obtain DNA from 1 to 100 individuals (see below).

Development of primers and probe

We collected all gene sequences of *Pratylenchus* spp. available in the GenBank database in search for a DNA-region with potential for use as a diagnostic tool. However, we avoided the sequences based on ribosomal DNA as it is

Table 1 Origin and codes of populations of *Pratylenchus* spp. and species from other nematode genera used in this study, together with the mean Ct value (0.05) and standard deviation obtained in a qPCR reaction

Code	Species	Host/soil	Origin	Ct
Pl1	*P. loosi*	native plants	USA	N/A
Pl2	*P. loosi*	Tea	Gilan, Iran	N/A
Pj	*P. jaehni*	Citrus	Sao Paulo, Brazil	N/A
Ph	*P. hippeastri*	Amaryllis	Florida, USA	N/A
Pgu1	*P. gutierrezi*	Maize	Kwazulu Natal, South Africa	N/A
Pgu2	*P. gutierrezi*	Coffee	Guatemala	N/A
Pg	*P. goodeyi*	Banana	Tenerife, Canary Islands	N/A
Pf1	*P. fallax*	Soil	Merelbeke, Belgium	N/A
Pf2	*P. fallax*	Soil	Redu, Belgium	N/A
Pcr1	*P. crenatus*	Soil	Gottem, Belgium	N/A
Pcr2	*P. crenatus*	Soil	Laukaa, Finland	N/A
Pcon	*P. convallariae*	Convallaria	Sassenheim, The Netherlands	N/A
Pcf	*P. coffeae*	Coffee	Vietnam	N/A
Pbr	*P. brachyurus*	Aster	Florida, USA	N/A
Pbo	*P. bolivianus*	Alstroemeria	West Sussex, UK	N/A
Pa	*P. agilis*	Maize	Maryland, USA	N/A
Pme	*P. mediterraneus*	Wheat	Saad, Isreal	N/A
Pne1	*P. neglectus*	Faba bean	Cerignola, Italy	N/A
Pne2	*P. neglectus*		Turkey	N/A
Ppi	*P. pinguicaudatus*	Faba bean	Beja, Tunisia	N/A
Pz	*P. zeae*	Grassland	Florida, USA	N/A
Pth1	*P. thornei*	Wheat	Ain Auda, Morocco	N/A
Pth2	*P. thornei*	Wheat	Marchouch, Morocco	N/A
Pth3	*P. thornei*	Wheat	Sidi Bettach, Morocco	N/A
Pth4	*P. thornei*	Wheat	Marchouch, Morocco	N/A
Pth5	*P. thornei*		Turkey	N/A
Pps1	*P. pseudocoffeae*	Wheat	Settat, Morocco	N/A
Pps2	*P. pseudocoffeae*		Iran	N/A
Ppe1	*P. penetrans*	Wheat	Turkey	28.9±0.5
Ppe2	*P. penetrans*	Soil	Belgium	28.7±0.1
Ppe3	*P. penetrans*	Soil	Kinrooi, Belgium	31.6±0.3
Ppe4	*P. penetrans*	soil	The Netherlands	28.8±1.3
Ppe5	*P. penetrans*	Soil	Kerkom, Belgium	29.3±0.7
Ppe6	*P. penetrans*	Wheat	Ain auda, Morocco	28.5±0.7
Ppe7	*P. penetrans*	Wheat	Marchouch, Morocco	28.4±0.2
Ppe8	*P. penetrans*	Wheat	Ain auda, Morocco	28.2±0.5
Ppe9	*P. penetrans*	Wheat	Ouled said, Morocco	29.9±0.3
Ppe10	*P. penetrans*	Wheat	Sidi Bettach, Morocco	29.7±0.5
Ppe11	*P. penetrans*	Wheat	Berchid, Morocco	27.2±0.2
Ppe12	*P. penetrans*	Wheat	Mediona, Morocco	27.5±0.4
Ppe13	*P. penetrans*	Wheat	Settat, Morocco	29.1±0.7
Ppe14	*P. penetrans*	Wheat	Sidi slimane, Morocco	28.9±0.1
Ppe15	*P. penetrans*	Wheat	Mhaya Morocco	29.4±0.5
Ppe16	*P. penetrans*	Wheat	Ain Taoujdate, Morocco	27.1±0.4

Table 1 (continued)

Code	Species	Host/soil	Origin	Ct
Ppe17	*P. penetrans*	Wheat	Sebaa ayoune, Morocco	29.4±1.5
Ppe18	*P. penetrans*	Wheat	Meknes, Morocco	29±0.1
Ppen19	*P. penetrans*	Wheat	Kenitra, Morocco	29.5±0.3
Ppen20	*P. penetrans*	Wheat	Ait Malk, Morocco	28.7±0.6
Ppen21	*P. penetrans*	Wheat	Taso, Morocco	29.3±0.5
Glr	*Globodera rostochiensis*	Potato	Bioska, Serbia	N/A
Melh	*Meloidogyne hapla*	Culture	Belgium	N/A
Rad	*Radopholus duriophilus*	Coffee	Vietnam	N/A
Tys	*Tylenchulus semipenetrans*	Citrus	Gharb, Morocco	N/A
Xid	*Xiphinema diversicaudatum*	Citrus	Gharb, Morocco	N/A
Ha	*Heterodera avenae*	Wheat	Zaers, Morocco	N/A
Hl	*H. latipons*	Wheat	Sais, Morocco	N/A
Para	*Paratylenchus* sp.	Lettuce	Belgium	N/A
Scu	*Scutellonema* sp.	Yam	Ghana	N/A

N/A not applicable

known that the LSU, SSU and D2D3 regions in this gene are not very suitable to distinguish *P. penetrans* from other closely related *Pratylenchus* spp. (Orui 1996; Waeyenberge et al. 2000, 2009). Because most sequence information for several *Pratylenchus* species was found for the β-1,4-endoglucanase gene, we decided to retain this gene for further study. All retrieved β-1,4-endoglucanase gene sequences (Table 2) were aligned for the selection and design of primers and probes using the software package AlleleID 7.75. The sequences selected for the forward primer, reverse primer and probe were PpenMFor 3′-CCA ACC TCT GCT ACA CTA-5′, PpenMRev 3′-CAG TGC CGT ATT CAG TGA-5′ and PpMPb 3′-CAC TAT TAT GCC GC-5′, respectively. The MGB-probe was labelled with 6-FAM (Life Technologies Europe).

Table 2 List with GenBank accession numbers of the β-1,4-endoglucanase sequences of *Pratylenchus* species used in this study for designing the primers and probe

Pratylenchus species	Accession numbers
P. penetrans	AB045781
P. penetrans	AB045780
P. penetrans	JN052038
P. penetrans	JN052037
P. penetrans	JN052036
P. penetrans	JN052035
P. vulnus	JN052050
P. vulnus	JN052051
P. thornei	JN052046
P. pratensis	JN052043
P. pratensis	JN052042
P. pratensis	JN052044
P. neglectus	JN052029
P. neglectus	JN052030
P. neglectus	JN052031
P. neglectus	JN052032
P. neglectus	JN052033
P. neglectus	JN052034
P. convallariae	JN052028

Real time PCR assay

All qPCR kits that were used (SensiFAST Probe Hi-ROX kit (2×) and SensiFAST SYBR Hi-ROX), were validated by the producer (Bioline Reagents Company, London, UK) on all commonly used real-time instruments and did not need further optimization regarding their composition. The SensiFAST SYBR Hi-ROX kit was only used to optimise the annealing temperature (by melting curve analysis). All other tests (specificity, sensitivity, construction of standard curve) were done with the SensiFAST

Probe Hi-ROX (2×) kit. The finally retained *P. penetrans* species-specific qPCR assay is a TaqMan based assay.

1. Optimisation of the annealing temperature

We optimized the efficiency of the primers for different annealing temperatures with two Moroccan populations of *P. penetrans* (Ppe11, Ppe12) and one population of *P. thornei* (Pth2). The qPCR was performed for different annealing temperatures ranging from 60 °C to 64 °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 Ppe11, Ppe12 or Pth2 (Table 3). Each sample was run in triplicate 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, 60 °C to 64 °C for 30 s and 72 °C for 1 min. The 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.

2. Testing specificity of primers and probe

To determine whether the primers and probe were specific for amplification and detection of *P. penetrans*, DNA from 49 populations comprising 19 different *Pratylenchus* species was used, as well as DNA from plant-parasitic nematodes from 9 other genera (Table 1). 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, 63 °C for 30 s and 72 °C for 1 min.

3. Testing sensitivity of primers and probe

To determine the sensitivity of the primers and probe for *P. penetrans*, two different experiments were run with SensiFAST Probe Hi-ROX (2×). The relationship between DNA concentration and Ct values was estimated in the first experiment as follows. DNA was extracted from three series of 1, 5, 10, 20, 40 and 80 individuals (all stages confounded) of *P. penetrans* (Holterman et al. 2006). All DNA-extracts were run in triplicates, one for each series. A negative control was also prepared in two replications using distilled water instead of a DNA template. The second experiment examined the detection limit of one individual of *P. penetrans* in the presence of an increasing number of individuals (all stages confounded) of *P. thornei*. Therefore, 1, 5, 10, 50 and 100 individuals (all stages confounded) of *P. thornei* were hand-picked and transferred to an Eppendorf tube containing 25 µl water along with a single *P. penetrans*. DNA was extracted (Holterman et al. 2006) and two samples were taken from the extract. 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.

4. Construction of standard curve

DNA was extracted from four series of 80 individuals (all stages confounded) of *P. penetrans* (Ppe14). A single qPCR was run for each DNA-extract and Ct-values were compared. Subsequently, all four tubes of

Table 3 Mean and standard deviation of Ct values obtained at different melting temperatures of DNA extracted from single individuals of two *Pratylenchus penetrans* and one *P. thornei* population (n=3)

Sample	60 °C		62 °C		63 °C		64 °C	
	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
P. penetrans (1)	26.2	0.1	30.4	0.4	31.3	0.4	34.5	0.4
P. penetrans (1)	27.5	0.2	30.1	0.7	31.5	1.1	35.2	1.6
P. thornei (1)	N/A	–	39.2	0.5	N/A	–	N/A	–
Negative control	39.5	0.4	N/A	–	N/A	–	N/A	–

N/A not applicable

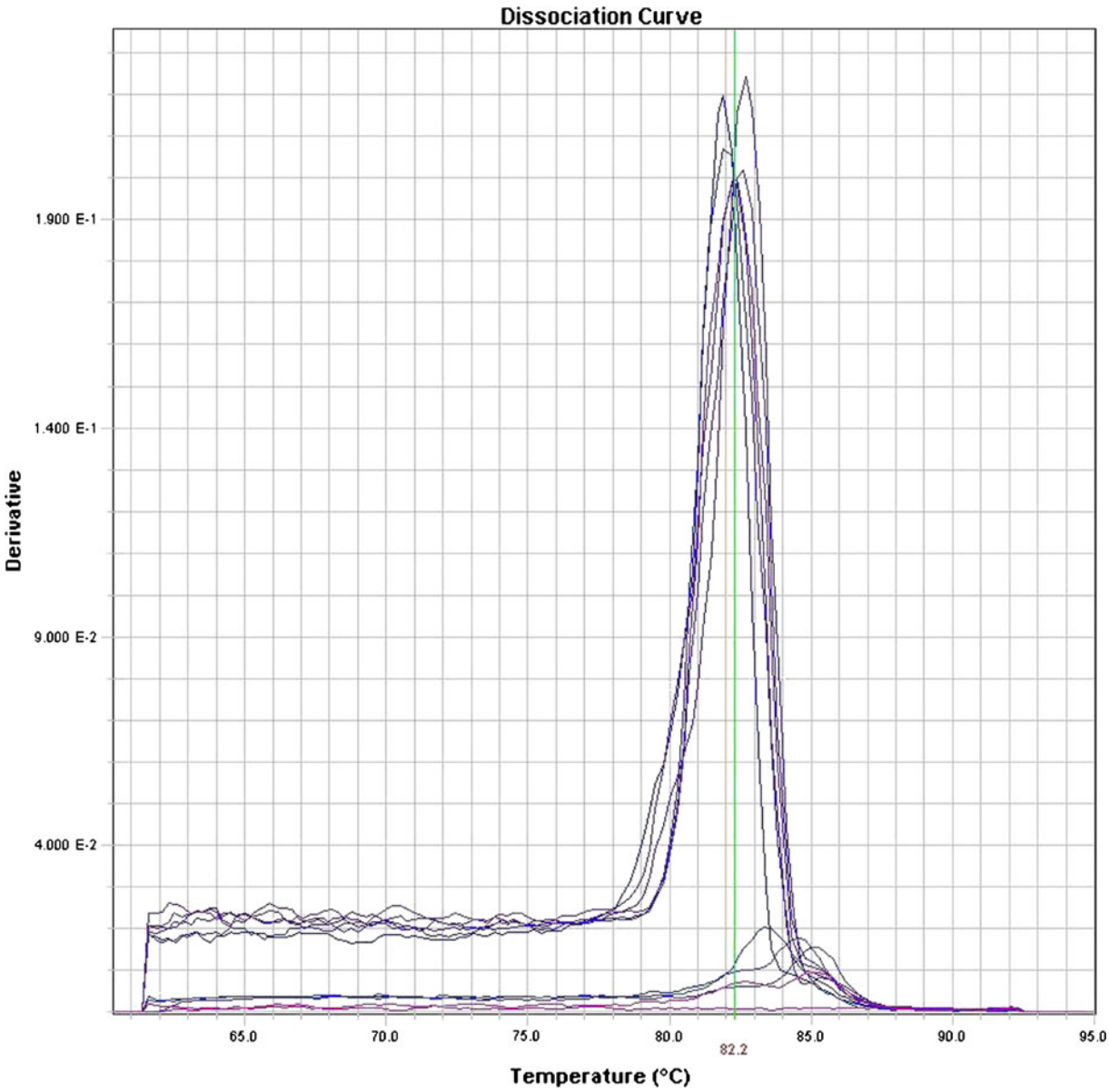

Fig. 1 Dissociation curve of the qPCR test (SensiFAST SYBR Hi-ROX) with annealing temperature set at 62 °C showing high peaks at ±82.2 °C of two *P. penetrans* populations (*n*=3) and minor peaks for a population of *P. thornei* (*n*=3) and NTC (*n*=2)

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 and 1/80 of the original concentration (representing 80 individuals of *P. penetrans)*. These concentrations were used as templates in a qPCR. Plotting logarithmic values of DNA concentration versus Ct-values generated a standard curve.

Results

Optimisation of the annealing temperature

Based on the Ct-values, the program with an annealing temperature of 63 °C was selected (T). Increasing annealing temperatures resulted in higher Ct-values for *P. penetrans*

(Table 3), whereas lower temperatures did not completely avoid the formation of non-specific products, as shown by the dissociation curves (Figs. 1 and 2). At 62 °C, the dissociation curve of the qPCR test showed minor peaks for *P. thornei* (Fig. 1) and a Ct value of 39 (Table 3) for this non-target species. At 63 °C, the assay was able to detect a single individual of *P. penetrans* whereas no signals were observed in the NTC samples nor in the sample with *P. thornei* (Table 3). At this temperature, peaks for *P. thornei* were hardly noticeable in the dissociation curve while they were high for *P. penetrans* (Fig. 2),

Specificity of primer and probe set

The targeted fragment of all isolates of *P. penetrans* was amplified by utilizing the qPCR protocol with the primer pair PpenMFor/PpenMRev along with the probe PpMPb. The qPCR assay did not show any amplification of DNA

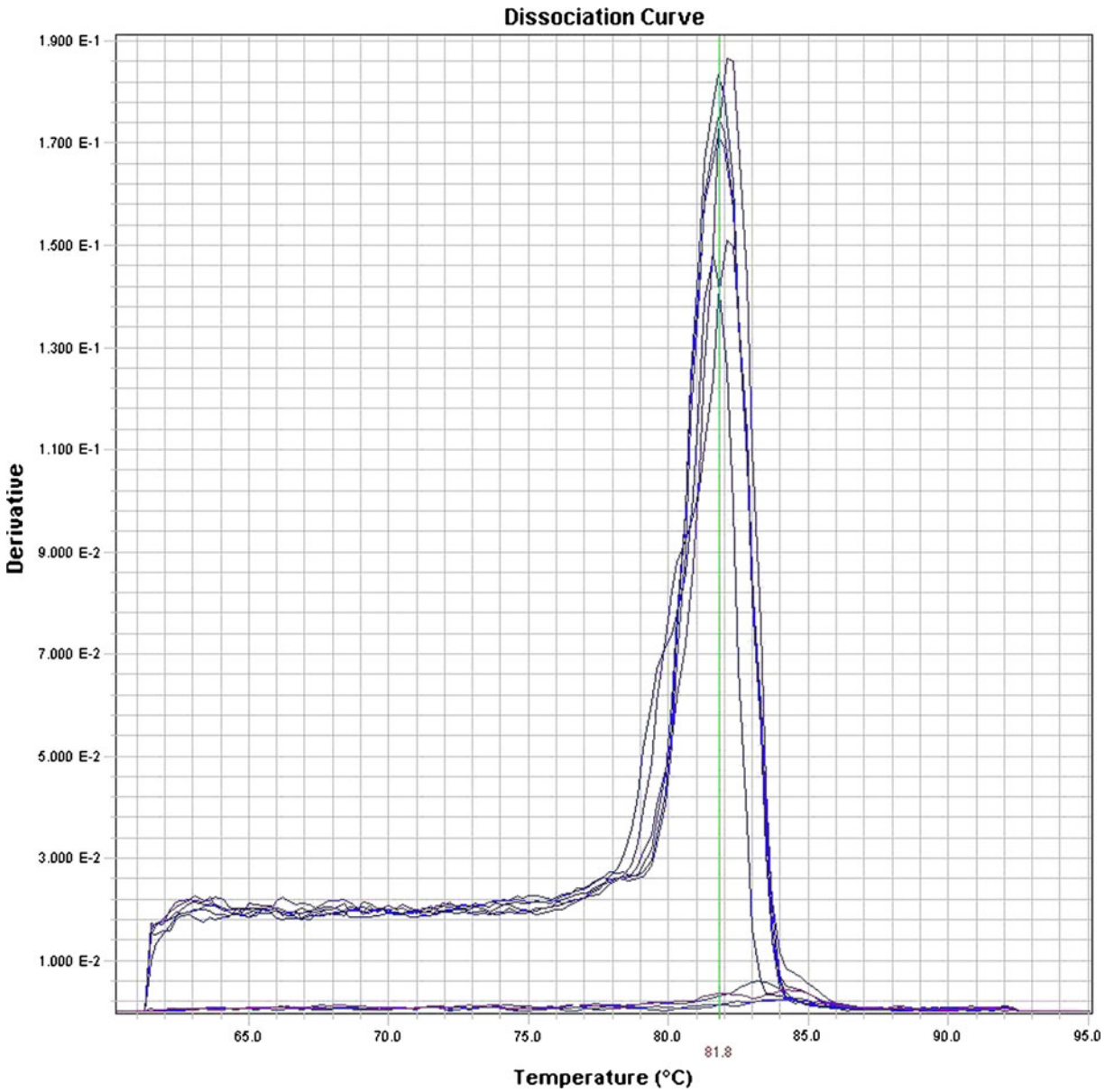

Fig. 2 Dissociation curve of the qPCR test (SensiFAST SYBR Hi-ROX) with annealing temperature set at 63 °C showing high peaks at ±81.8 °C of two *P. penetrans* populations (n=3) and very low peaks for a population of *P. thornei* (n =3) and NTC (n=2)

from other *Pratylenchus* species, neither of DNA from species of other nematode genera (Table 1). In addition, DNA was not amplified or detected in any of the non-template controls that contained water instead of DNA. The Ct-values for DNA derived from 1–5 *P. penetrans* individuals from different populations from Morocco varied between 27.1±0.4 and 29.9±0.3 (Table 1).

Sensitivity of primers and probe

The qPCR assay (first experiment) successfully amplified DNA extracted from a nematode suspension containing 1, 5, 10, 20, 40 or 80 individuals of *P. penetrans*. The corresponding decreasing Ct-values were 32.5±0.3, 30.1±0.2, 29.9±0.09, 28.6±1.6, 27.1±0.6, 26.5±0.3, respectively. The Ct-value of the negative control was always undetermined.

The Ct-values obtained after qPCR, with DNA from a single *P. penetrans* in the presence of 1, 5, 10, 20, 40 and 80 individuals of *P. thornei* (second experiment) were almost the same: 32.3±0.4, 32.1±0.2, 31.9±0.1, 32.2±0.2, 31.9±0.4 and 32.1±0.3, respectively. The negative control was always undetermined. The Ct-values were significantly stable.

Construction of a standard curve

qPCR was run 4 times using DNA extracted from exactly 80 individuals of *P. penetrans*. Again, Ct-values showed a consistent result (Ct=24.4±0.4). The standard curve (Fig. 3) generated from the data obtained with the qPCR of the serial dilution (Table 4) showed a highly significant relationship between the Ct-value and number of nematodes over the range studied (R^2=0.99; slope=−3.23; E=104 %). Based on three sample replications, the ABI PRISM fluorescence detection system automatically calculated the starting number of *P. penetrans* by comparison of the Ct-values from the unknown samples with the values of the standard curve.

Table 4 Cycle threshold (Ct) values from a serial dilution of *Pratylenchus penetrans*

Serial dilution	Number of *P. penetrans*	Ct
1 :1	80	26.4±0.21
1 :2	40	27.4±0.14
1 :4	20	28.8±0.06
1 :8	10	29.9±0.2
1 :16	5	30.5±0.08
1 :80	1	32.5±0.25

Discussion

A rapid and reliable diagnostic test to quantify the presence of *P. penetrans* in samples is an essential step in the management of this economically very important plant-parasitic nematode. In this paper we report on the development of a qPCR assay for *P. penetrans* based on the β-1,4-endoglucanase gene. This gene may play a crucial role in plant cell wall-degradation during penetration and migration of nematodes in the host roots.

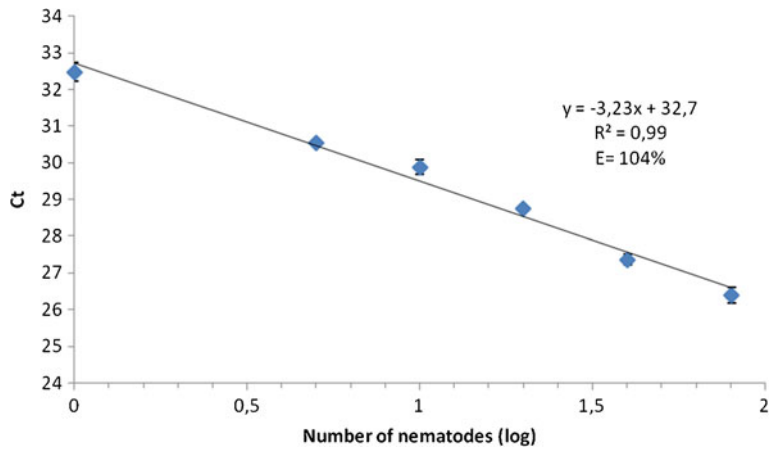

Fig. 3 Standard curve of the qPCR assay (SensiFAST Probe Hi-ROX) for *P. penetrans*: threshold cycle number (Ct) plotted against the log of the number of individuals of *P. penetrans* (1, 5, 10, 20, 40, 80) (*n*=3)

ITS-sequences of the ribosomal gene are frequently used for the development of molecular tools to identify plant-parasitic nematodes (Subbotin and Moens 2006). However, Waeyenberge et al. (2000) and Uehara et al. (1998, 1999) demonstrated that ITS-sequences can vary in size between different *Pratylenchus* species, which makes sequence alignment to detect species-specific fragments problematic. In addition, ITS sequences show extensive polymorphism within a species or an individual (Orui 1996; Waeyenberge et al. 2000). This drastically limits the number of potential DNA fragments suitable for the design of species-specific primers. Because of the limited availability of comparable sequences of *Pratylenchus* spp. in GenBank we decided to use the β-1,4-endoglucanase gene. To our knowledge, this gene has never been used to identify plant-parasitic nematodes.

The DNA-extraction method that we used appeared to be stable and capable of extracting DNA from up to 80 individuals. Five individuals, however, were suggested to be the maximum number of nematodes to be used in the DNA-extraction method described by Holterman et al. (2006). Our results showed the presence of an increasing amount of DNA extracted from a proportional increasing amount of nematodes.

The qPCR assay is very sensitive, reliably detecting the DNA of a single individual of *P. penetrans* when mixed with DNA from 80 individuals of *P. thornei*. This sensitivity compares well with findings reported for other nematode species. Madani et al. (2005) could detect a single second-stage juvenile of the cyst-forming nematodes *Globodera pallida* and *Heterodera schachtii*. Toyota et al. (2008) reported that real-time PCR sensitively detected a single second-stage of the potato cyst nematode *Globodera rostochiensis* from 1000 free-living nematodes.

Repeatability of the test was demonstrated by the similar Ct values (24,4±0.4) obtained from four tests performed with 80 individuals of *P. penetrans,* and the almost equal Ct values of about 32 obtained in the 18 runs with 1 individual of *P. penetrans* mixed with variable numbers of *P. thornei*.

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*. The specificity was tested on several isolates of 19 different *Pratylenchus* species. Different populations of the morphologically and phylogenetically closely related species *P. fallax, P. convallariae* and *P. penetrans* (Subbotin et al. 2008) were also tested. The specific primers and probe did not produce any amplification for the three populations of *P. fallax* and *P. convallariae*, but were capable of detecting all 21 *P. penetrans* isolates tested, originating from all over the world. Neither was there amplification for the plant-parasitic species of nine other genera. Although the chosen target (the β-1,4-endoglucanase gene) is common for many nematode species, the developed primers and probe were only compatible with *P. penetrans*.

The results of runs of the serial dilutions of DNA samples of *P. penetrans* as well as the runs conducted with DNA from increasing amounts of nematodes showed a highly significant linearity (R^2=0.99). The particular strength of the qPCR assay is that it is useful for quantifying *P. penetrans* in mixed populations of *Pratylenchus* spp., where visual identification of individual nematodes at species level is extremely difficult. Particularly, the juvenile stages are difficult to identify at species level because their species-specific features are not defined. Within the different developmental stages of pratylenchids, differences in size exist; second-stage juveniles of *Pratylenchus* spp. are small compared to adults of the same species. We believe that despite this difference, qPCR is able to quantify numbers of *P. penetrans* even when different developmental stages are mixed. The DNA signal can be converted into an accurate estimation of the number of individuals involved. Although the number of cells in an individual nematode increases during growth (Cunha et al. 1999), this given does not have a large influence on the estimation of the numbers of nematodes in a sample as the degree of accuracy required in agronomic and ecological studies will be much more determined by factors such as sampling and extraction efficiency.

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