Developing a Real-Time PCR Assay for Direct Identification and Quantification of *Pratylenchus penetrans* in Soil

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Abstract

The root-lesion nematode *Pratylenchus penetrans* is a major pathogen of potato worldwide. Yield losses may be exacerbated by interaction with the fungus *Verticillium dahliae* in the potato early dying disease complex. Accurate identification and quantification of *P. penetrans* prior to planting are essential for developing effective integrated pest control measures. However, distinction between *P. penetrans* and other *Pratylenchus* spp. based on morphology is a tedious task. A SYBR Green I-based qPCR assay was developed to discriminate, identify, and quantify *P. penetrans* in field soil. *P. penetrans*-specific qPCR primers were designed from the D2-D3 region of the 28S rDNA. The specificity of the assay was evaluated using eight isolates of *P. penetrans* populations and 31 isolates of other nematode species. A standard

curve relating threshold cycle and log values of nematode number was generated from artificially infested soils. There was a high correlation between the *P. penetrans* numbers artificially added to soil or estimated from naturally infested field soils by conventional methods, and the numbers quantified using the qPCR assay. Grinding the field soil prior to DNA extraction improved *P. penetrans* detection from soil. The qPCR assay will not only be useful for differentiating *P. penetrans* from mixed populations of *Pratylenchus* spp., but also for efficient detection and quantification of *P. penetrans* from field soil. The assay requires no expertise in nematode taxonomy and morphology, and may serve as a useful diagnostic tool in research, diagnostic labs, and extension services for pest management.

The root-lesion nematode, *Pratylenchus penetrans* is one of the most economically damaging species within the genus *Pratylenchus*. It has a worldwide distribution but is commonly found in temperate regions on a variety of crops. In Europe, *P. penetrans* is known to be damaging on field crops, vegetables, and ornamental plants (Coolen and D'Herde 1970; Pudasaini et al. 2007; Talavera et al. 2001; Westerdahl et al. 2003). The nematode is also recorded on different crops in Japan, Canada, Algeria and Morocco (Mokrini et al. 2012; Olthof and Potter 1973; Sato et al. 2007, 2010; Troccoli et al. 1992). In the U.S.A., the nematode is a major threat to many crops, especially potato growing in the northeastern part of the country (Francé et al. 1987; MacGuidwin and Rouse 1990; Martin et al. 1982; Rowe et al. 1987; Thies et al. 1995; Yan and Plaisance 2016). The nematode infects potato tubers and causes a scabby appearance with sunken lesions or dark, wart-like bumps that turn purple on tubers in storage. Yield losses may be exacerbated by interaction with the fungus *Verticillium dahliae* Kleb, causing a disease known as potato early dying disease complex (PED) (MacGuidwin and Rouse 1990; Martin et al. 1982; Rowe et al. 1987). This disease can lead to premature vine death and significant reduction in tuber size and total marketable yield and therefore can become a limiting factor in potato production. Reported yield reductions range from very slight to 30 to 50% (MacGuidwin and Rouse 1990; Martin et al. 1982; Rowe et al. 1985, 1987).

Seinhorst (1998) established that a significant correlation exists between the preplanting population numbers and the amount of damage caused by plant-parasitic nematodes. This makes accurate identification and quantification of *P. penetrans* prior to planting essential for developing effective integrated pest control measures and making yield-loss risk assessment. However, other *Pratylenchus* species, namely *P. crenatus*, *P. neglectus*, *P. scribneri*, and *P. thornei*, are commonly found coexisting with *P. penetrans* on potato in

North America (Brown et al. 1980; Kimpinski 1979; Wheeler et al. 1992). This coexistence makes it more challenging to reliably identify and quantify *P. penetrans* using the traditional microscopic enumeration methods based on morphological characters owing to the presence of only a small number of diagnostically valid characters between the *Pratylenchus* species (Luc 1987; Waeyenberge et al. 2009). Moreover, intraspecific variability has been demonstrated in *P. penetrans* (Tarte and Mai 1976). Molecular technologies provide a rapid and accurate alternative to the microscopic identification and quantification methods.

Quantitative PCR (qPCR) strategies using species-specific primers have been developed for plant-parasitic nematodes (Berry et al. 2008; Madani et al. 2005; Mokrini et al. 2012; Wishart et al. 2002; Ye 2012; Zijlstra et al. 2000). However, these methods require that the nematode is extracted from the soil prior to DNA extraction and amplification, which increases identification and/or quantification time, cost, and labor. Molecular assays for identification and quantification of specific plant-parasitic nematodes directly from soil DNA extracts have recently been developed (Goto et al. 2009; Li et al. 2014; Sato et al. 2010; Yan et al. 2008, 2012, 2013b). However, only few research describing methods for identification and quantification of *P. penetrans* have been reported (Koyama et al. 2016; Mokrini et al. 2013; Sato et al. 2007, 2010, 2011). However, the soil compactor used in the majority of these studies was a specialized machinery tailored to this end and is not readily available. A sensitive, rapid, specific, and reliable molecular diagnostic method is needed to identify and quantify *P. penetrans* directly in DNA extracts from soil for successful management. The goal of this study was therefore to develop a molecular assay to discriminate and quantify *P. penetrans* in commercial potato fields in Minnesota. The specific objectives were to i) develop a species-specific qPCR assay to identify *P. penetrans* and to discriminate the species from other closely related *Pratylenchus* species, namely *P. crenatus*, *P. neglectus*, *P. scribneri*, and *P. thornei* and other nematodes commonly found in MN potato fields; ii) develop a method to improve *P. penetrans* detection efficiency in field soils; and iii) develop a qPCR assay to detect and quantify *P. penetrans* directly from field soil DNA extracts.

Materials and Methods

DNA extraction from pure culture and soil. Separate cultures of *Pratylenchus* spp., namely *P. penetrans*, *P. neglectus*, *P. thornei*, and *P. scribneri* were maintained on carrot discs at 22°C in an incubator

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*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary figures are available online.

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for 3 months (Pinochet et al. 1995) at the North Dakota State University Nematology Laboratory. The nematodes were extracted from the carrot discs for DNA extraction. A single nematode of each species was transferred separately into 10 µl of distilled water on a concave glass slide and crushed with a dental pick. The suspension (10 µl) containing nematode pieces was pipetted into a 0.5 ml sterile Eppendorf tube containing 10 µl of extraction buffer (2 µl of 10× PCR buffer, 2 µl of Proteinase K [600 µg/ml], and 6 µl of sterile water) and the tubes were incubated at –20°C for 20 min followed by 1 h incubation at 65°C and then 10 min incubation at 95°C (Subbotin et al. 2001). The DNA suspension was stored at –20°C and used as DNA template. The same procedure was followed to extract DNA from other vermiform nematode species commonly found in potato fields in Minnesota and North Dakota.

Total DNA was extracted from artificially infested soils and naturally infested field soils using the MoBio PowerSoil DNA Isolation Kit (MOBIO Laboratories Inc, Carlsbad, CA) according to the manufacturers' protocol and the MOBIO Vortex Adapter tube holder according to the manufacturer's recommendations. DNA extract from soil was stored at –20°C prior to qPCR.

Primer design, specificity, and sensitivity. The D2-D3 expansion region of the 28S rRNA gene of three populations of *P. penetrans* (PT-Edling_1, PT-DeChene_1, and PT-DeChene_2) originating from three different potato fields in MN was sequenced using the D2A/D3B universal primer set (Subbotin et al. 2008) (Supplementary Fig. S1) and deposited in GenBank with the following accession numbers: PT-Edling_1 = KY969631, PT-DeChene_1 = KY969632, PT-DeChene_2 = KY969633. The sequence information was compared with the published D2-D3 sequence information of *P. penetrans* and other *Pratylenchus* spp. in GenBank (BLAST option, <http://www.ncbi.nlm.nih.gov/>). All the D2-D3 sequences collected (Table 1) were aligned using the MegAlign Pro package within the DNASTAR software (Madison, WI) (Supplementary Fig. S2). To determine putative species-specific DNA fragments for *P. penetrans*, regions of the D2-D3 sequences conserved within *P. penetrans* but divergent from other *Pratylenchus* spp. for the aligned sequences were selected using the SeqBuilder module software package in DNA STAR. Preliminary evaluation of the primers was based on annealing temperature, GC content, and potential for self-duplex or primer dimer formation. The potential species-specific primers were further screened by looking for their presence in sequences stored in GenBank via BLAST. Putative primers were synthesized and salt-purified by Eurofins MWG Operon LLC (Huntsville, AL). Each primer pair was evaluated again on the basis of target specificity, amplification efficiency, endpoint fluorescence, and melting curve profile. Eventually, the forward primer Dechene-Pp-F (5'-GGTTTTCGGGCTCATATGGGTTC-3') and the reverse primer Dechene-Pp-R (5'-TTTACGCCGAGAGTGGGATTGTG-3') producing a 111 bp product (Supplementary Fig. S3) was chosen and used for all the experiments. This primer pair was evaluated for specificity to *P. penetrans* using DNA from eight isolates of *P. penetrans* from the U.S.A. and 12 isolates of other *Pratylenchus* spp., and 19 isolates of other plant-parasitic nematodes (Table 2). Nematode DNA extract from soil containing a nematode community including *Helicotylenchus* sp., *Hoplolaimus* sp., *Tylenchorhynchus* sp., *Heterodera* sp., and *Paratrichodorus* sp. commonly found on potato fields was used as a control, together with a soil DNA extract from potato field soil without detectable *P. penetrans*.

Detection sensitivity of the assay was performed using a fine textured sandy-loam soil, one of the field soil samples that did not have any detectable *P. penetrans* from De Chene. The soil, while moist, was autoclaved (121°C, 115 kPa) for 30 min for two consecutive days before use. Different numbers of *P. penetrans* (0.5, 1, 2, 3, and 5) were added to 0.5 g of autoclaved soil followed by soil DNA extraction and qPCR amplification for each level of infestation in triplicates. The sensitivity of the qPCR assay was determined by detection of quantification cycle (Cq) values at the minimum level of infestation.

Real-time PCR assay. The qPCR assay was performed using the Bio-Rad CFX96 Touch Real-time PCR Detection System (Bio-Rad

Laboratories, Inc., Hercules, CA, U.S.A.). The SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used with 1.0 µl DNA template and 0.5 µM primer concentration, according to the instructions. The cycling conditions were as follows: incubation at 95°C for 5 min; 35 cycles of 95°C for 10 s and 66°C for 20 s; and 72°C for 30 s followed by melting curve analysis using the default settings to evaluate the amplification specificity. Nontemplate control, using ddH₂O instead of DNA in the PCR, was run for each experiment. The data were analyzed using Bio-Rad CFX Manager Software V3.1. The Cq value was determined at the default settings. PCR product identity was confirmed by electrophoresis on a 2% agarose gel.

Development and validation of standard curves. The soil used for the generation of the standard curve was tested for the presence of PCR inhibitors. DNA was extracted from a total of 100 *P. penetrans* (mixed juveniles and adults) maintained in a carrot culture and serially diluted (1, 1/3, 1/9, 1/27, 1/81, 1/243) with PCR grade water. A similar dilution series was generated using an extract of the autoclaved De Chene field soil. Standard curves were constructed by plotting log value of DNA against Cq number. Presence or absence of PCR inhibitors in De Chene soil was verified by comparing amplification efficiency and the Cq values from serial dilutions of DNA in the soil extract to that from dilutions in water (Yan et al. 2012, 2013b).

The standard curve for the qPCR assay was then generated from a specified number of *P. penetrans* added to a sterilized De Chene soil. Quarter-gram samples of the soil were infested with vermiform *P. penetrans* from pure culture at five population densities (0.5, 1, 5, 25, 125, and 625 per 0.25 g of soil). Each infestation level and DNA extraction was done in triplicate and the qPCR amplification in duplicate. The soil standard curve was generated by plotting Cq versus log of the number of *P. penetrans* per gram of soil. Amplification efficiencies and the slopes of the plots were automatically

Table 1. *Pratylenchus* spp. and GenBank accession numbers of the 28S rRNA (D2/D3) sequences used in this study for designing *P. penetrans* specific primers

Species/isolate	Accession number	Origin
PT-Edling_1	KY969631	U.S.A.
PT-De Chene_1	KY969632	U.S.A.
PT-De Chene_2	KY969633	U.S.A.
<i>P. penetrans</i>	JX046990.1	Netherlands
<i>P. penetrans</i>	JX046986.1	Netherlands
<i>P. penetrans</i>	JX046970.1	Chile
<i>P. vulnus</i>	JQ003992.1	Japan
<i>P. vulnus</i>	JX047007.1	U.S.A.
<i>P. vulnus</i>	JQ003994.1	Japan
<i>P. vulnus</i>	JX261945.1	N/A ^a
<i>P. vulnus</i>	JX047010.1	U.S.A.
<i>P. pseudopratensis</i>	JX261965.1	N/A
<i>P. coffeae</i>	HQ688681.1	Cambodia
<i>P. coffeae</i>	AF170436.1	China
<i>P. scribneri</i>	JX047005.1	China
<i>P. scribneri</i>	JX047002.1	China
<i>P. scribneri</i>	EU130865.1	China
<i>P. speijeri</i>	KF974698.1	China
<i>P. speijeri</i>	KJ698686.1	China
<i>P. loosi</i>	KF430796.1	Japan
<i>P. loosi</i>	KF430797.1	Japan
<i>P. hexincisus</i>	DQ498832.1	N/A
<i>P. floridensis</i>	AF170437.1	N/A
<i>P. neglectus</i>	HM469438.1	China
<i>P. neglectus</i>	JX046968.1	China
<i>P. neglectus</i>	JX046969.1	China
<i>P. thornei</i>	JX261963.1	N/A
<i>P. thornei</i>	KT213559.1	N/A
<i>P. thornei</i>	EU130880.1	U.S.A.
<i>P. zaeae</i>	JN020930.1	U.S.A.
<i>P. zaeae</i>	JN020929.1	U.S.A.

^a N/A indicates information is not available.

generated from the slope of a plot of cycle threshold (Cq) (y-axis) and log of DNA (x-axis) ($E = 10^{(1/m)} - 1$; where m is the slope). The soil standard curve was validated using the autoclaved De Chene soil inoculated with lower numbers of vermiform *P. penetrans* at 1, 5, 10, 20, 40, and 80 per 0.25 g of soil. DNA was extracted from the infested soil and qPCR assay was used to quantify the number of nematodes in each level of infestation.

Assessing the effect of grinding field soil on detection efficiency. De Chene field soil without detectable *P. penetrans* was autoclaved as above and inoculated with *P. penetrans* 5 or 10 nematodes per 20 g of soil. Each population density was either ground into a fine texture or unground prior to DNA extraction. DNA was extracted in triplicate for each infestation level using 0.25 g of soil. Additionally, four field soil samples naturally infested with *P. penetrans* (Y2P1 and P25, Y2P25, and De Chene-1) were either ground or unground and DNA was extracted in triplicate for each treatment. A qPCR was carried out for each treatment using the DeChene-Pp-F/R primers. Detection efficiency was determined by the percentage of the total number of observations detectable by the assay.

***Pratylenchus penetrans* detection and quantification in field soils.** The qPCR assay was validated by comparing *P. penetrans* estimates determined by the qPCR assay and two conventional methods: Centrifugal sugar flotation (Jenkins 1964) and Whitehead tray method

(Whitehead and Hemming 1965). A total of 20 soil samples from potato, corn, and wheat fields naturally infested with different population densities of *P. penetrans* and other plant-parasitic and non-plant-parasitic nematodes were collected from MN. For each field soil sample, 600 g was collected for molecular detection and quantification and traditional extraction and quantification methods. The 600 g field soil was mixed thoroughly and divided into three subsamples of 200 g each. Two sets of subsamples were used for nematode identification and quantification using microscopy-based procedures and the third set for DNA extraction and qPCR. For the molecular assay, 200 g of the field soil was air-dried at room temperature in Petri dishes overnight (9 h) and ground in 70 × 90 mm porcelain mortar with pestle (VWR, Randor, PA, U.S.A.) for 4 min to obtain a smooth, homogenous texture; stones, roots debris, and other materials were removed. A subsample of 0.25 g was collected by at least 30 tiny scoops from different parts of the sample using a spatula. DNA was extracted from the 0.25 g subsample using the MoBio PowerSoil DNA Isolation Kit and amplified using the *P. penetrans* species-specific primer set DeChene-Pp-F/R. DNA extractions and qPCR amplifications were performed three times for each field sample under the optimum conditions as described above. A no-DNA template (water) was used as a negative control and DNA from pure *P. penetrans* suspension was used as a positive control.

Table 2. Plant-parasitic nematodes used to evaluate specificity of *Pratylenchus penetrans* primers used in this study for the qPCR assay

Code	Species	Cq	Host	Origin	Source	Reference
P.P 9C1	<i>P. penetrans</i>	27.23	Potato	MN, U.S.A.	G. Yan	Author's collection
P.P 154	<i>P. penetrans</i>	23.42	Potato	MN, U.S.A.	G. Yan	Author's collection
P16	<i>P. penetrans</i>	23.92	Potato	MN, U.S.A.	G. Yan	Author's collection
P.P. Edling	<i>P. penetrans</i>	25.39	Potato	MN, U.S.A.	G. Yan	Author's collection
De Chene-1	<i>P. penetrans</i>	24.15	Potato	MN, U.S.A.	G. Yan	Author's collection
P. P67	<i>P. penetrans</i>	24.00	Potato	MN, U.S.A.	G. Yan	Author's collection
P-59	<i>P. penetrans</i>	23.45	Potato	MN, U.S.A.	G. Yan	Author's collection
Pp-W	<i>P. penetrans</i>	23.55	Potato	WI, U.S.A.	A. Skantar	Yan et al. (2013a)
Pc	<i>P. crenatus</i>	N/A ^a	Grass	MD, U.S.A.	A. Skantar	Yan et al. (2013a)
P.t Oregon	<i>P. thornei</i>	N/A	Wheat	OR, U.S.A.	R. Smiley	Yan et al. (2013a, b)
Pt	<i>P. thornei</i>	N/A	Wheat	U.S.A.	R. Smiley	Yan et al. (2013a, b)
Pn-O	<i>P. neglectus</i>	N/A	Wheat	OR, U.S.A.	R. Smiley	Yan et al. (2013a)
Pn-N	<i>P. neglectus</i>	N/A	Wheat	ND, U.S.A.	G. Yan	Yan and Plaisance (2016)
Pz21	<i>P. zeae</i>	N/A	Spider lily	Singapore	A. Skantar	Yan et al. (2016b)
Pz20	<i>P. zeae</i>	N/A	Corn	NC, U.S.A.	A. Skantar	Yan et al. (2013a)
Ps	<i>P. scribneri</i>	N/A	Potato	ND, U.S.A.	G. Yan	Yan et al. (2016a)
Ps-c	<i>P. scribneri</i>	N/A	Corn	U.S.A.	A. Skantar	Yan et al. (2016a)
Pt	<i>P. thornei</i>	N/A	Wheat	OR, U.S.A.	R. Smiley	Yan et al. (2013a, b)
Pa	<i>P. agilis</i>	N/A	Corn	MD, U.S.A.	A. Skantar	Yan et al. (2013a)
MB-3	<i>P. allius</i>	N/A	Soybean	ND, U.S.A.	G. Yan	Yan et al. (2016c)
Hg13	<i>Hoplolaimus</i> sp.	N/A	Soybean	ND, U.S.A.	G. Yan	Yan et al. (2016c)
Mn36	<i>Mesocriconema</i> sp.	N/A	Soybean	ND, U.S.A.	G. Yan	Yan et al. (2016c)
Ha	<i>H. avenae</i>	N/A	Wheat	U.S.A.	R. Smiley	Yan et al. (2013a, b)
Hs	<i>H. schachtii</i>	N/A	Sugar beet	ND, U.S.A.	B. Nelson	Nelson et al. (2012)
Hg	<i>H. glycine</i>	N/A	Soybean	U.S.A.	G. Yan	Author's collection
Hc	<i>H. ciceri</i>	N/A	Chickpea	Syria	F. Toumi	Yan et al. (2013a)
Hsm	<i>Helicotylenchus</i> sp.	N/A	Sugarbeet	MN, U.S.A.	G. Yan	Author's collection
Pfp	<i>Paratylenchus</i> sp.	N/A	Field pea	MN, U.S.A.	G. Yan	Author's collection
Hsm	<i>Hoplolaimus</i> sp.	N/A	Soybean	MN, U.S.A.	G. Yan	Author's collection
Xsm	<i>Xiphinema</i> sp.	N/A	Soybean	MN, U.S.A.	G. Yan	Author's collection
Tsm	<i>Tylenchorhynchus</i> sp.	N/A	Soybean	MN, U.S.A.	G. Yan	Author's collection
Cw	<i>Cactodera weissii</i>	N/A	Potato	U.S.A.	A. Skantar	N/A
Ge	<i>Globodera ellingtonae</i>	N/A	Potato	U.S.A.	A. Skantar	N/A
Gp	<i>G. pallida</i>	N/A	Potato	IN, U.S.A.	A. Skantar	Skantar et al. (2007)
Gr56	<i>G. rostochiensis</i>	N/A	Potato	Canada	A. Skantar	N/A
Gr38	<i>G. rostochiensis</i>	N/A	Potato	NY, U.S.A.	A. Skantar	Skantar et al. (2007)
Gt	<i>G. tabacum tabacum</i>	N/A	Tobacco	U.S.A.	A. Skantar	Skantar et al. (2007)
Gtc	<i>G. tabacum tabacum</i>	N/A	Tobacco	U.S.A.	A. Skantar	Skantar et al. (2007)
Mn	<i>Meloidogyne naasi</i>	N/A	Poa/bent	U.S.A.	A. Skantar	N/A
NC	Nematode community	N/A	Potato	MN, U.S.A.	G. Yan	Author's collection
SDE ^b	Soil DNA extract	N/A	Potato	MN, U.S.A.	G. Yan	Author's collection

^a Not available.

^b Soil DNA extract from potato fields.

Statistical analysis. The PROC REG in SAS (version 9.4, Statistical Analysis System; SAS Institute, Cary, NC) was used to establish the correlation relationship ($R^2 > 0.5$, $P < 0.05$) between nematode numbers based on the qPCR assay and traditional microscopic methods. The number of *P. penetrans* estimated by qPCR was regressed against the number of *P. penetrans* artificially added to autoclaved soil or quantified by the conventional methods. Comparison between *P. penetrans* estimates from the qPCR assay and microscopic methods was conducted by paired *t* test (PROC TTEST) ($P < 0.05$) where an acceptance of the null hypothesis ($P > 0.05$) indicates no significant difference between nematode counts obtained from the two procedures.

Results

DNA extraction from soil. Nematode DNA was extracted directly from soil using the PowerSoil DNA Isolation Kit (MoBio). To examine the presence of inhibitors that might interfere with fluorescence generated in the qPCR reaction, amplification of *P. penetrans* DNA serially diluted with water was compared with that of DNA diluted with extracts of sterilized and noninfested Dechane-1 sandy-loam soil. The amplification efficiency value for DNA in soil extracts was 103 whereas that of DNA in water was 104, indicating that the PowerSoil DNA Isolation Kit was able to remove inhibitors from extracts of sterilized and noninfested soil.

Primer specificity and sensitivity. The primer pair Dechene-Pp-F/R was designed based on the D2-D3 expansion region of the 28S rRNA sequence of *P. penetrans*. It produced the expected target amplicon of 111 bp only for *P. penetrans* but not from the other 23 plant-parasitic nematode spp. (31 isolates) used as control (Table 2). The Cq values of different numbers of *P. penetrans* isolates ranging from 23.42 to 27.23 produced a single melting peak at 85°C from the qPCR, indicating the presence of a single amplicon (Fig. 1).

Detection sensitivity of the assay was performed using a fine textured sandy-loam De Chene soil. The average Cq values obtained for the different numbers (0.5, 1, 2, 3, and 5) of *P. penetrans* added to 0.5 g of autoclaved soil were 31.83 ± 0.4 , 30.84 ± 0.2 , 30.39 ± 1.0 , 29.04 ± 0.65 , and 28.02 ± 0.7 , respectively, suggesting that lower proportions of the nematode could have been detected.

Generation of a standard curve from soil. The standard curve was generated from the artificially infested soils at different densities (0.5, 1, 5, 25, 125, and 625 *P. penetrans* per 0.25 g of soil). The equation obtained by plotting the Cq values versus the log of the starting materials was described as $y = -3.508x + 30.731$ (Fig. 2). The amplification efficiency (E) was 92.8% and the Cq values ranged from 32.88 to 21.52, and the relationship between the Cq values and the density of *P. penetrans* showed highly significant linearity ($R^2 = 0.983$, $P < 0.001$). No amplification was observed with control soils that were not infested with *P. penetrans*.

Validation of the standard curve. The equation of the above standard curve was used to estimate known numbers of *P. penetrans* in soil. Based on their Cq values, the average qPCR estimates for the infestation rates (1, 5, 10, 20, 40, or 80 per 0.25 g of soil) were 1.77, 11.09, 18.93, 43.96, 80.62, and 216.85 per 0.25 g of soil, respectively. Thus, the correlation between the numbers of *P. penetrans* added to soil and the numbers determined by qPCR was highly significant ($R^2 = 0.91$, $P < 0.001$, $n = 36$), described by the equation $y = 2.69x - 7.6$ (Fig. 3).

Effect of grinding field soil on detection efficiency. The impact of grinding field soil on qPCR detection and quantification of *P. penetrans* was investigated by comparing qPCR detections between ground and unground field soils. The results showed that 80% of the total number of ground samples were detected whereas only 47% of the unground soil samples were positive. The detection efficiency was density dependent and significantly higher ($P < 0.05$) in ground soil than unground soil (Fig. 4). At 50 *P. penetrans* per 200 g of soil, only one of the three replications of the ground soil samples was detected whereas none was detected for the unground soil. At 100 *P. penetrans* per 200 g of soil, two replications of the ground soil were detected whereas only one of the unground samples was detected. Although there was a difference in Cq values between ground and unground samples at certain densities, the gap was close at higher

densities > 196 nematodes per 200 g soil. At 196 *P. penetrans* per 200 g of soil, all the ground treatments were detected whereas only two of the unground treatments were detected. At 210 *P. penetrans* per 200 g of soil, all the replicates of both the ground and unground treatments were detected. Grinding of soil before DNA extraction was not necessary at population densities above 196 nematodes per 200 g of soil. The average Cq value obtained for all the ground treatments was 31.85, not significantly different from that of the unground treatments, 31.19 ($P = 0.82$, paired *t* test) (Table 3), indicating that grinding field soil may not have adverse effect on quantification accuracy.

Quantification of *P. penetrans* from field soil. Correlation analysis was conducted to determine the relationship between nematode numbers detected by the qPCR assay and the numbers determined by the two traditional microscopic methods for the 20 soil samples collected from infested potato fields in MN (Table 4). There was a strong and significant positive correlation ($R^2 = 0.82$; $P < 0.001$) between the numbers of *P. penetrans* quantified based on the qPCR assay and sugar centrifugal extraction method described by the equation $y = 8.66x - 393$ (Fig. 5). Whitehead tray method showed a relatively weaker but significant positive correlation ($R^2 = 0.61$; $P < 0.001$) described by the equation $y = 12.23x - 411$ (Fig. 6). Similarly, there was significant positive correlation ($y = 0.5305x + 22.731$; $R^2 = 0.75$; $P < 0.001$) between the sugar centrifugal extraction and quantification method, and the Whitehead tray method of extraction and quantification shown in Figure 7.

Discussion

An efficient and reliable diagnostic assay to quantify the presence of plant-parasitic nematodes in soil is critical for making management decisions. In this study, we report on the development of a qPCR assay for *P. penetrans* based on the D2-D3 expansion domain of the 28S rDNA gene. *P. penetrans* was quantified directly in DNA extracts from field soils using a species-specific SYBR Green I-based qPCR. The qPCR assay was evaluated based on primer specificity, sensitivity, efficiency, and the relationship between *P. penetrans* numbers estimated from field soils by conventional methods and the qPCR assay. The assay was highly specific and able to differentiate *P. penetrans* from other *Pratylenchus* spp. and non-*Pratylenchus* plant-parasitic nematodes commonly found in potato fields in MN. The qPCR assay was sensitive and detected genomic DNA of a single juvenile added to 1 g of sterilized soil. Artificially and naturally infested field soils with different *P. penetrans* population densities were used to validate the assay based on a standard curve generated by the assay. We also coupled mechanical grinding of field soil to qPCR amplification to improve *P. penetrans* detection efficiency in field soil.

The *P. penetrans*-specific primer set (Dechene-Pp-F/R) designed from the D2-D3 expansion region of the 28S rRNA showed high

Fig. 1. Melting curve profiles of *Pratylenchus penetrans*-specific amplicons with melting temperature at 85°C. Control reactions without *P. penetrans* DNA template did not produce any amplification.

specificity to *P. penetrans* and consistently produced a single amplicon in melt curve analysis without specific amplification when DNA from nontarget nematodes were used. Only few *P. penetrans*-specific primers have been reported (Al-Banna et al. 2004; Mokrini et al. 2013; Mekete et al. 2011; Sato et al. 2007; Setterquist et al. 1996; Uehara et al. 1998; Waeyenberge et al. 2009). The new *P. penetrans* primers designed from the D2-D3 was also shown to be specific to our local *P. penetrans* populations. Previous studies (Al-Banna et al. 2004; Carta et al. 2001; Duncan et al. 1999; Waeyenberge et al. 2009) demonstrated that this region is stable in length within the genus *Pratylenchus*. On the contrary, other studies (Orui 1996; Uehara et al. 1998; Waeyenberge et al. 2000) have indicated that ITS sequences can vary in size between different *Pratylenchus* species but may show extensive polymorphism within a species or an individual, limiting its suitability for designing specific primers for a species with much sequence variation among different populations. Further testing of these primers with other *Pratylenchus* spp. and local species not included in this study may be recommended.

The sensitivity of the assay was evaluated by its ability to detect a minimum number of *P. penetrans* in 0.5 g of autoclaved soil. The assay could detect 0.5 juvenile in 0.5 g of soil, equivalent to 200

juveniles per 200 g of soil. The average Cq value for 0.5 juvenile per 0.5 g of soil was 31.83, suggesting that our assay had the capacity to detect lower proportions of the nematode in 0.5 g of soil. This level of sensitivity is comparable with previous reports: detection sensitivity of 1 *P. thornei* per g of soil and 1 *P. neglectus* per g of soil was reported (Yan et al. 2012, 2013b). Sato et al. (2007) detected a single second-stage juvenile of *P. penetrans* mixed with 800 free-living nematodes whereas Toyota et al. (2008) reported a qPCR assay that detected a single second-stage juvenile of *G. rostochiensis* in 1,000 free-living nematodes. The economic threshold for *P. penetrans* is variable, and is contingent on the crop type, geographic location, environmental factors, edaphic factors, crop value, potential for disease complexes, nematode extraction procedures, etc. (Castillo and Vovlas 2007); however, with the presence of *Verticillium dahliae* (2 to 13 cfu/cm³), the economic threshold for *P. penetrans* could be as low as 10 to 20 vermiforms per 100 cm³ of soil (Powelson 1993). We could detect as low as 23 *P. penetrans* per 200 g of soil, suggesting that the assay could be useful in establishing economic threshold limits for *P. penetrans* in potato fields.

The standard curve was generated from a range of nematode individuals added to soils and was used to quantify known numbers of

Fig. 2. Standard curve of the qPCR assay for *Pratylenchus penetrans*: quantification cycle number (Cq) plotted against the log of the number of *P. penetrans* (0.5, 1, 5, 25, 125, and 625) added to 0.25 g of sterilized soil. The DNA was extracted in duplicates and the qPCR were run in triplicate.

Fig. 3. Correlation between the number of *Pratylenchus penetrans* determined by quantitative PCR (qPCR) and the number of *P. penetrans* (1, 5, 10, 20, 40, and 80) added to 0.25 g of sterilized and uninfested soil. DNA was extracted in duplicate and the qPCR were run in triplicate. *** indicates significant at $P < 0.0001$.

nematodes in artificially infested soil. Not only were the Cq values of the standard curve significantly inversely correlated with the log values of nematode numbers ($R^2 = 0.98$, $P < 0.001$), but also the correlation between the numbers of *P. penetrans* added to soil and the numbers determined by qPCR was highly significant ($R^2 = 0.91$, $P < 0.0001$), showing the accuracy of the qPCR assay in estimating the numbers of *P. penetrans* in artificially infested soil samples. Sato et al. (2007) intimated that the accuracy of using standard curve to identify nematodes in a nematode community would be higher when

Fig. 4. Comparison of quantitative PCR (qPCR) detection efficiencies of *Pratylenchus penetrans* between ground and unground soils. Detection efficiency was determined by the percentage of the total number of observations detectable by the assay. ** indicates significant *t* test ($P < 0.05$).

generated from individual nematodes than using serial dilutions from a concentrated DNA due to higher variations in DNA samples obtained from different numbers of nematode individuals than in simply diluted DNA samples. Moreover, it was demonstrated that standard curves generated from different nematode numbers, rather than serial dilution, tend to be more representative of the variation that may exist in natural field samples (Berry et al. 2008; Yan et al. 2012).

It is worthwhile to note that variation exists between the two methods of estimating *P. penetrans*. The variation between the qPCR estimates and the numbers of *P. penetrans* actually added to the soil was lower at lower densities (1, 5, 10 nematodes per 0.25 g soil) and highest at 80 nematodes per 0.25 g soil. Variations at this high population density are likely due to reduction in DNA extraction efficiency and the variation in the nematode DNA at different developmental stages. Sato et al. (2007) found that the Ct values of a male *P. penetrans* (22.0 ± 0.7) and a female (23.0 ± 0.3) with larger body sizes were significantly lower ($P < 0.05$) than those from small juveniles (25.1 ± 0.4). However, under field conditions, densities <8,000 nematodes per 200 g soil may be more realistic than 64,000 nematodes per 200 g soil, which further suggests that the assay can reliably be used to estimate *P. penetrans* numbers under normal field conditions.

In our previous study, a combination of soil grinding and conventional PCR was developed to sensitively detect soybean cyst nematode *Heterodera glycines* (Baidoo et al. 2017). The present study demonstrates that this method is applicable to the root-lesion nematode *P. penetrans*. The effect of grinding was parallel to compaction

Table 3. Effect of grinding field soil on *Pratylenchus penetrans* detection and quantification efficiency^a

Sample ID	Replication	Nematodes/200 g soil	Detected/not detected	Detection efficiency (%)	Cq	Mean Cq
5 nematodes ground	Rep 1	50	n.d.	33.3	N/A	31.99
	Rep 2	50	+		31.99	
	Rep 3	50	n.d.		N/A	
5 nematodes unground	Rep 1	50	n.d.	0	N/A	n.d.
	Rep 2	50	n.d.		N/A	
	Rep 3	50	n.d.		N/A	
10 nematodes ground	Rep 1	100	n.d.	66.6	N/A	34.6
	Rep 2	100	+		34.9	
	Rep 3	100	+		33.62	
10 nematodes unground	Rep 1	100	n.d.	33.3	N/A	31.73
	Rep 2	100	n.d.		N/A	
	Rep 3	100	+		31.73	
Y2-P25 ground	Rep1	23	+	100	34.76	33.82
	Rep 2	23	+		34.94	
	Rep 3	23	+		31.78	
Y2-P25 unground	Rep 1	23	+	33.3	34.24	34.24
	Rep 2	23	n.d.		N/A	
	Rep 3	23	n.d.		N/A	
Y2p.1 ground	Rep 1	210	+	100	31.69	31.81
	Rep 2	210	+		32.72	
	Rep 3	210	+		31.04	
Y2p.1 unground	Rep 1	210	+	100	30.28	31.72
	Rep 2	210	+		33.46	
	Rep 3	210	+		31.43	
P25 ground	Rep 1	196	+	100	28.71	28.26
	Rep 2	196	+		28.00	
	Rep 3	196	+		28.08	
P25 unground	Rep 1	196	n.d.	66.6	N/A	28.56
	Rep 2	196	+		28.55	
	Rep 3	196	+		28.64	
De Chene 1 ground	Rep 1	0	n.d.	-	N/A	n.d.
	Rep 2	0	n.d.		N/A	
	Rep 3	0	n.d.		N/A	
De Chene 1 unground	Rep 1	0	n.d.	-	N/A	n.d.
	Rep 2	0	n.d.		N/A	
	Rep 3	0	n.d.		N/A	

^a n.d = not detected; N/A = not applicable; + = detected.

reported by Goto et al. (2009). We determined that grinding of the field soil before DNA extraction could increase *P. penetrans* detection efficiency from 47 to 80%. We found that grinding did not significantly affect the amount of DNA recovered from the soil at higher population densities (≥ 196 nematodes per 200 g of soil) evident in similarity in Cq values between the ground and the unground samples. On the contrary, there was a difference in Cq between ground (34.6) and unground (31.73) at 100 nematodes per 200 g soil. The most reasonable explanation to this is that, in the unground sample, the nematodes are picked intact in the 0.25 g sample used for DNA extraction and the amount of DNA recovered depends on the number

of intact nematodes per 0.25 g of soil. Thus, at lower densities, the probability of picking an intact nematode is low, resulting in low detection efficiency, but once detected, the Cq value is expected to be equivalent to that of at least 1 nematode per 0.25 g soil. This suggests that at lower densities (≤ 100 nematodes per 200 g soil), not grinding the field soil before DNA extraction may not only lead to lower detection efficiency (false negative results), but also could lead to overestimation of *P. penetrans* population densities. However, at lower density (≤ 100 nematodes per 200 g soil), the Cq value may be high but the possibility of false negative is reduced. Thus, grinding the soil helps break the nematode body and homogenizes its content in the sample, which appears to be critical for detection. Mixing is important because of the patchy spatial distribution of nematodes in soil plus only a small amount of soil is used for detection. In agreement with our observation, Tsai and Olson (1992) used a rapid freeze-and-thaw method to detect less than three *Escherichia coli* cells per g of infested soils, similar to that of Volossiouk et al. (1995), who reported that disruption of *Verticillium dahliae* by grinding in liquid nitrogen with natural abrasives enabled its direct detection from the soil. Nevertheless, grinding field soil can be difficult for heavy and moist soils, but air drying the field soil for at least 8 h facilitates grinding.

The qPCR assay was used to quantify *P. penetrans* in 20 field soil samples. There was a strong, significant positive correlation between the *P. penetrans* numbers estimated by conventional nematode extraction and quantification methods (centrifugal sugar flotation method and Whitehead tray method) and the qPCR method, suggesting that the assay can be used to estimate *P. penetrans* population density in field soils. However, the strength of the correlation was lower in field soil samples ($R^2 = 0.82$ or $R^2 = 0.61$; $P < 0.001$) compared with the artificially infested soil ($R^2 = 0.91$; $P < 0.001$). Such discrepancies may be attributed to the lack of uniformity in nematode distribution within a given soil sample: giving the same extraction method, different laboratories produced different numbers of *P. neglectus* using the same sample reflecting the difficulty in *Pratylenchus* sp. quantification using traditional microscopic methods (Yan et al. 2013b). In spite of these variations, the correlations obtained in this study between the two methods and the qPCR was comparable to previous reports (Ophel-Keller et al. 2008; Yan et al. 2012, 2013b).

This developed qPCR assay tended to overestimate the numbers of nematodes in both artificially infested and the field soils in

Table 4. Comparison of *Pratylenchus penetrans* estimations by different methods in 200 g of soil

Field sample ID	Sugar centrifugation method	Whitehead tray method	qPCR assay
P25-Nat-Rep 10	663	354	3,068
P25-Rep 3	224	98	925
P90	213	126	937
P25	260	231	878
P15	120	176	697
P51	143	80	592
P86	126	120	547
P59	201	56	463
P89	64	173	289
H-11	42	25	284
Y2P1	133	115	234
P54	140	125	188
P.13	75	40	127
Hayes plant centre	12	22	86
P14	42	18	77
Cambel East 1	13	0	0
P18	13	0	0
Y2P3 east	0	40	0
Dechane 1	0	0	0
Y2P16	0	0	0

Fig. 5. Correlation between the numbers of *Pratylenchus penetrans* determined by the quantitative PCR (qPCR) and by the centrifugal sugar flotation extraction and microscopic counting method (SugarM) from 20 different field soil samples. *** indicates significant at $P < 0.001$. DNA was extracted from each field sample in triplicate.

comparison with the conventional methods similar to previous reports (Goto et al. 2009; Ophel-Keller et al. 2008; Toyota et al. 2008; Yan et al. 2012). These differences could be attributed to a number of factors. Firstly, conventional methods estimate the number of *P. penetrans* based on the number of adults and juveniles with visual diagnostic features without consideration of the eggs in the sample. Also, ergonomic errors associated with nematode extraction, sampling from nematode suspensions, and nematode counting may undermine the accuracy of the conventional estimates. Bellvert et al. (2008) observed that nematode extraction efficiency was dependent on the initial soil volume used, soil type, residual moisture in

samples prior to extraction, and the extraction method with efficiencies ranging from 33 to 84%, suggesting a high risk of underestimating numbers with the traditional detection methods. The patchy spatial and uneven distribution of nematodes in field soils may affect their quantification by different methods (Ophel-Keller et al. 2008; Waeyenberge et al. 2000; Whitehead and Hemming 1965). In comparison, a small amount of soil (0.25 g) was used for DNA extraction using the PowerSoil DNA Isolation Kit whereas approximately 200 g of soil was processed using the conventional methods. These discrepancies may help explain why sample Y2P1, with 210 nematodes estimated by the centrifugal sugar flotation method, produced a higher

Fig. 6. Correlation between the numbers of *Pratylenchus penetrans* determined by the quantitative PCR (qPCR) and by the Whitehead tray extraction and microscopic counting method (Whitehead) from 20 different field soil samples. *** indicates significant at $P < 0.001$. DNA was extracted from each field sample in triplicate.

Fig. 7. Correlation between the numbers of *Pratylenchus penetrans* determined by the centrifugal sugar flotation extraction and microscopic counting method (SugarM), and by the Whitehead tray extraction and microscopic counting method (WhiteheadM) from 20 different field soil samples. *** indicates significant at $P < 0.001$. DNA was extracted from each field sample in triplicate.

Cq value of 31.8 (meaning a lower population density) than P25 (28.4) with 196 nematodes. Although the qPCR method seems to overestimate nematode numbers, it is important to note that the assay establishes equations relating qPCR numbers to the traditional quantification numbers, which means that the qPCR estimates can be used to obtain the traditional estimates mathematically.

Identification and quantification of plant-parasitic nematodes directly from soil DNA extract (Li et al. 2014; Sato et al. 2010; Yan et al. 2008, 2012, 2013b) is an extra step in technological advancement compared with quantification using individual nematodes from nematode suspension or communities (Berry et al. 2008, Koyama et al. 2016; Madani et al. 2005, Sato et al. 2007; Toyota et al. 2008). This paper presents another protocol using a SYBR Green I dye species-specific qPCR assay to detect and quantify *P. penetrans* directly from infested soils from potato fields. The qPCR assay will be useful for differentiating *P. penetrans* from mixed populations of either *P. crenatus*, *P. neglectus*, *P. scribneri*, or *P. thornei* on potato in North America. We coupled manual grinding of field soil to qPCR quantification for improved direct detection and quantification of *P. penetrans* from a large number of field soil samples. The assay obviates the time-consuming steps of conventional nematode extraction, microscopic identification, and counting, requires no expertise in nematode taxonomy and morphology, and can serve as a useful diagnostic tool not only in research, but also in diagnostic labs and extension services for pest management. Although the soil processing and DNA extraction methods presented in this study did not seem to affect PCR amplification efficiency for the different field soils used, it is important to note that this may not accurately reflect all other soil types and conditions. Our understanding of how different soil types and conditions affect direct DNA amplification from soil is limited and needs to be elucidated in future research.

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