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Development and Application of a Multiplex qPCR Method for the Simultaneous Detection and Quantification of *Pratylenchus alleni* and *P. penetrans* in Quebec, Canada

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Abstract

Root lesion nematodes are very common plant-parasitic nematodes that affect a wide range of plants. More than one species can be found simultaneously in a field, and each has a different impact on crop yield. Unfortunately, identifying them using classical morphometric criteria is very difficult and time consuming. The species *Pratylenchus alleni* was recently observed for the first time in Canada, associated with severe damage in a soybean field in the province of Quebec. The major species, *P. penetrans*, is also known to be endemic in Quebec but no data exist on its distribution in field crops. This prompted the development of a multiplex quantitative polymerase chain reaction (PCR) assay for the simultaneous detection and quantification of *P. alleni* and *P. penetrans*. The method was found to be specific and sensitive, systematically detecting a single larva in a 100-cm^3 soil sample with no cross-amplification with other species, even when they outnumbered the target species. An exogenous internal positive control was included in the test to avoid false negatives due to the presence of PCR inhibitors. This assay was used to study the distribution of *P. alleni* and *P. penetrans* in 185 soybean fields in the major soybean-producing areas of Quebec during a 3-year survey. Overall, *P. penetrans* was found in 42% of the fields, *P. alleni* in 8%, and both species in 4%. The population density of *P. alleni* in positive fields was still very low, with only a few larvae detected. However, densities of *P. penetrans* were much higher: the provincial mean was 51.7 nematodes per 100 cm^3 of soil (in positive samples), and 8% of the fields (15 of 185) exceeded the theoretical economic threshold. The presence of *P. penetrans* was also strongly correlated with soil texture, with lighter soil being the most favorable.

Root lesion nematodes (*Pratylenchus* spp.) are polyphagous migratory endoparasites that cause severe damage to crops around the world. In a recent survey on the most scientifically and economically important nematodes, the genus *Pratylenchus* ranked third worldwide among all plant-parasitic nematodes (Jones et al. 2013). There are more than 70 described species of *Pratylenchus*, which attack a wide range of plant families (Castillo and Volvas 2007). In Canada and the United States, *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941, is the predominant species (Potter and McKeown 2003; Townshend et al. 1978; Yu 2008), with over 350 host plants, including weeds, woody plants, and herbaceous plants (Bélair et al. 2007; Singh et al. 2013; Thies et al. 1995). Many of its host plants are of major economic importance, such as corn and soybean.

In 2011, *P. alleni* Ferris, a new species for Canada, was detected for the first time in southwestern Quebec, in a soybean field (Bélair et al. 2013). This nematode was causing major yield reductions (38 to 54%) in heavily affected plants when compared with adjacent asymptomatic plants (Bélair et al. 2013). In the United States, *P. alleni* is present in some Midwestern states but is considered rare in the Northeast (Acosta and Malek 1981; Ferris 1961; Robbins et al. 1987, 1989; Townshend et al. 1978). Many studies have shown *P. alleni* to be a polyphagous species that reproduces well on soybean, tomato, corn, potato, wheat, oat, raspberry, and sunflower (Bernard and Keyserling 1985; Dickerson 1979; Hackney and Dickerson 1975; Singh et al. 2013; Wartman and Bernard 1985). Crop damage caused by *P. alleni* has rarely been studied. Ferris and Bernard (1962) noted that the weight of soybean roots was reduced by 25% in greenhouses tests with *P. alleni* but no effect on the growth of aerial parts or yield was observed.

Soybean and corn are major crops in Quebec, covering 317,000 and 380,000 ha, respectively (Statistics Canada 2016). The detection of this new pest, which has the potential to spread and damage major crops, has raised major concerns about the future management of *Pratylenchus* spp. Identification and quantification of *P. penetrans* and *P. alleni* based on morphology in mixed population samples remain major obstacles in research. Because of the small number of distinctive structures or characteristics and the intraspecific variability of some morphological characteristics (Loof 1978; Tarte and Mai 1976), new tools are needed. Many molecular diagnostic methods have been developed for *Pratylenchus* spp., including *P. neglectus* (Yan et al. 2013), *P. thornei* (Yan et al. 2012), *P. zeae* (Berry et al. 2008), and *P. penetrans* (Goto et al. 2011; Min et al. 2012; Mokrini et al. 2013; Sato et al. 2007, 2011; Waeyenberge et al. 2009) but none for *P. alleni*.

An accurate identification and quantification method is extremely important for developing control strategies against the root lesion nematodes. The main objective of this study was to develop a multiplex quantitative polymerase chain reaction (qPCR) assay for the simultaneous detection and quantification of *P. alleni* and *P. penetrans*. This assay was then used to study the distribution of *P. alleni* and to update the presence of *P. penetrans* during a 3-year survey covering more than 180 soybean fields in the major soybean-producing areas of Quebec.

Materials and Methods

Root-lesion nematode culture. Root lesion nematodes of the species *P. alleni* (isolated on soybean from Saint-Anicet, Quebec, Canada) and *P. penetrans* (isolate from Quebec obtained from the Canadian National Collection of Insects, Arachnids, and Nematodes)

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were reared separately in Petri dishes on excised sweet corn roots (corn cultivar Extra Early Super Sweet; Stokes Seeds Ltd.) on Gamborg's B-5 medium with minimal organics (3.2 g/liter; Sigma-Aldrich) containing Phytagel (6 g/liter; Sigma Aldrich) and sucrose (20 g/liter). The Petri dishes were incubated in the dark at 30°C for *P. alleni* and 20°C for *P. penetrans* for 3 months. *Pratylenchus* larvae from each species were extracted from two Petri dishes by cutting the medium into 1-cm^2 pieces, followed by sieving with tap water through 75- and 20-μm sieves. The 20-μm sieve was backwashed, and nematodes were collected on a counting plate and manually picked for DNA extraction.

DNA extraction. All nematode suspensions obtained from in vitro cultures or soil extractions (see the "Field survey" section) were frozen at −80°C and freeze dried (Triad; Labconco) before DNA extraction. Genomic DNA was extracted with the DNeasy Blood and Tissue extraction kit from Qiagen according to the manufacturer's protocol, except for DNA elution. DNA was eluted twice with 50 μl of buffer AE for a total elution volume of 100 μl. DNA samples were stored at −20°C.

Primer and TaqMan probe design for real-time PCR. In silico alignments were performed with the sequences of the D2/D3 region of the large ribosomal subunit (28S) from 10 *Pratylenchus* spp. with the ClustalW function in BioEdit (version 7.2.5). The sequence of the *P. alleni* population found in Quebec (MF155653) was compared with sequences available in the National Center for Biotechnology Information (NCBI) GenBank from other *Pratylenchus* spp. (Supplementary Fig. S1). PrimerQuest (Integrated DNA Technologies) (http://www.idtdna.com/PrimerQuest/Home/Index) was used to develop specific primers and probes for *P. alleni* and *P. penetrans*. Primer and probe sequences were analyzed with OligoAnalyzer (Integrated DNA Technologies) (https://www.idtdna.com/calc/analyzer) in order to reduce the probability of homodimers, heterodimers, and hairpin loops. Sequences were also compared with the GenBank databases using the Basic Local Alignment Search Tool (BLAST; NCBI) in order to be sure that these sequences were unique to *P. alleni* and *P. penetrans*.

Primer specificity with SYBR green. Primer specificity was determined with the DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) in a final volume of 20 μl containing 10 μl of master mix (2×), 0.5 μl of each primer (10 μM) (Table 1), 7.6 μl of sterile distilled water, 0.4 μl of ROX dye (50×), and 1 μl of genomic DNA from 10 larvae of *P. alleni, P. coffeae, P. crenatus, P. penetrans, P. thornei*, and *P. neglectus* (Table 2). For each species, the test was replicated three times with two negative controls (sterile distilled water). Amplifications were performed under the following conditions: (i) 95°C for 5 min; (ii) 35 cycles of 95°C for 30 s and annealing or elongation at 62°C for 30 s; and (iii) dissociation curve from 55 to 95°C. All real-time PCR assays were run with the Mx3000P qPCR system from Stratagene (Agilent Technologies).

Standard curves. In order to develop a calibration curve that would relate quantification cycle (Cq) values to a known amount of DNA from *P. alleni* and *P. penetrans*, we elaborated two types of curves. The first standard curve was done using genomic DNA from 5, 10, 25, 50, and 100 larvae from all stages except eggs for both species. Three biological replicates (independent DNA extractions) were done for each species. The second curve used synthetic double-stranded (ds)DNA (gBlock) containing the DNA sequence of the D2/D3 region of the large ribosomal subunit (28S) of *P. alleni* and *P. penetrans*. These gBlocks were synthesized by Integrated DNA Technologies. This DNA fragment was suspended in sterile Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0), and the concentration was determined by the Qubit Fluorometer (Life Technologies). The amount of gBlock solution was converted to the number of copies using a converter (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr). Sets of serial dilutions (1/10) were prepared from 1×10^6 to 100 copies/μl and used as standard curves. The sensitivity of the qPCR assay was also determined by evaluating the minimum number of copies in a sample that could be measured accurately.

Multiplex real-time PCR and exogenous internal positive control. An exogenous internal positive control (EIPC) was included in the assay to detect the presence of inhibitors and avoid this type of false-negative result. The EIPC used was a 500-bp synthetic fragment of dsDNA with a known sequence (Supplementary Table S1) (Fall et al. 2015). It was added directly to the master mix in order to obtain 2,000 copies/reaction. Specific primers and probes (Table 1) were also added. A difference (delay) of one or more Cq between a sample and the negative control (sterile distilled water) was indicative of the presence of inhibitors. In such cases, samples were diluted 1/10 and retested.

Multiplex qPCR was conducted on a total volume of 20 μl, including 10 μl of QuantiFast Multiplex PCR buffer (Qiagen) (2×), 0.4 μl of ROX reference dye (6-carboxyl-X-rhodamine; 50×), 0.25 μl of bovine serum albumin at 10 mg/μl, 250 nM primers (4 × 0.5 μl) and 250 nM TaqMan hydrolysis probes (2 × 1 μl) for each nematode species, 300 nM EIPC primers (2 × 0.6 μl), 375 nM EIPC hydrolysis probe (1.5 μl), 2,000 copies of EIPC gBlock (0.04 μl of 5×10^4 copies), sterile distilled water (1.61 μl), and 1 μl of DNA sample. The qPCR dyes used were CY5 for *P. alleni*, FAM for *P. penetrans*, and HEX for EIPC. Negative controls (sterile distilled water instead of DNA) were included in each assay. Amplifications were performed under the following conditions: (i) 95°C for 5 min and (ii) 35 cycles of 95°C for 30 s and annealing at 62°C for 30 s. Every sample, standard, and control was made in triplicate.

DNA extracts obtained from 50 larvae of *P. alleni* or *P. penetrans* were tested alone or in combination using this multiplex master mix. Also, different ratios of gBlocks from the two organisms were tested to see if there was template competition between them. We tested two dilutions of each species gBlock (10^5 and 10^7 copies) alone or with the other species in all possible combinations. DNA from 10 larvae of *P. coffeae, P. crenatus, P. thornei*, and *P. neglectus* were also tested to confirm probe specificity.

Impact of the developmental stages on the Cq values. To evaluate the impact of the developmental stages on the quantification of nematodes, we quantified DNA from 10 second-stage juveniles (J2),

Table 2. Source and origin of the *Pratylenchus* spp. used as control species

Species	Source	Origin
Pratylenchus coffeae	S. Subbotin	Japan
P. crenatus	S. Subbotin	United Kingdom
P. neglectus	E. Grenier	France
P. thornei	E. Grenier	France

Table 1. Primers and probes developed for *Pratylenchus alleni* and *P. penetrans*

Primer or probe	*P. alleni*	*P. penetrans*	EIPC[y]
Forward (5′–3′)	GGATTTCTGTTCGTTCTAAGTGTTT	GAGACTTTCGAGAAGGCGATATG	CTGAGTAGCCACGTTATTATC
Reverse (5′–3′)	GTAGAGCTCGGCCAGGA	AGGACCGAATTGGCAGAAG	GCGAACAACTAACTTGATTG
Probe (5′–3′)	CGACACGTACTGTGCCACCAGTTC	CCCGGATTGGAGGAATGTTGTTCGT	CGCACTTGTCCTACACCCTTCATAC
Fluorochrome[z]	Cy5	FAM	HEX
Amplicon size (bp)	133	176	87

[y] Exogenous internal positive control.
[z] Fluorochrome transmitters FAM (5-carboxyfluorescein), Cy5 (cyanine), and HEX (6-carboxy-1,4-dichloro-2′,4′,5′,7′-tetrachlorofluorescein) are located at the 5′ end of the probe.

10 females, 10 males, and 10 mixed stages for both species. Three biological replicates and three technical replicates were analyzed in a qPCR assay using the same parameters as described above.

Field survey. A 3-year survey was conducted in 185 soybean fields in different regions of Quebec in autumn 2013 (n = 45), 2014 (n = 54), and 2015 (n = 86). In each field, 25 on-row soil cores were taken near the soybean root zone with a soil auger from the 0- to 20-cm depth in a zigzag pattern to cover a 2-ha area. One liter of soil was transferred to a plastic bag labeled with the address, GPS coordinates, and sampling date. The type of soil was determined by the hydrometer method (Bouyoucos 1962) and recorded for the 2014 and 2015 samples. For each field, a 100-cm^3 soil subsample was submitted for nematode extraction according to Baermann's pan method (Townshend 1963). Species were quantified using the DNA extraction and multiplex qPCR assay described above. Based on morphological characteristics, the number of nematodes similar to the *Pratylenchus* genus was evaluated in 70 samples. The nucleotide sequences of six random positive field samples were also determined by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using a capillary-array automated DNA sequencer (ABI3730xl DNA Analyzer; Applied Biosystems) at McGill University and Génome Québec Innovation Centre to confirm species identity.

Statistical analysis. Linear regression analyses were carried out using the MxPRO 4.10 qPCR software for Mx3000P. Student's t tests (P = 0.05) were calculated to compare Cq between singleplex and multiplex assays, and χ^2 tests were used to determine the influence of the type of soil on the distribution of *P. penetrans* using XLSTAT 2015 (Addinsoft).

Results

Development of a multiplex qPCR. The multiple alignments of the D2/D3 sequence of the large ribosomal subunit (28S) of 10 *Pratylenchus* spp. made it possible to identify specific regions and develop primers for *P. alleni* and *P. penetrans* (Table 1). SYBR green validation of these primers showed good amplification of the target species and no amplification when DNA from *P. coffeae*, *P. crenatus*, *P. thornei*, *P. neglectus*, or the other species studied was used as a template. The dissociation curves were unimodal in assays that included one or both targets, confirming that the primers were specific to each species and did not interact with one another. The dissociation temperature was 88.1°C for *P. alleni* and 83.6°C for *P. penetrans* (Fig. 1).

The PCR efficiency of the standard curves ranged from 94.9 to 101.1%, with high coefficients of variation between 0.936 and 0.999 (Fig. 2). The lowest concentration of gBlock that consistently amplified was 100 copies/µl for *P. alleni* and *P. penetrans*. The slopes of the linear regressions using gBlock were similar to those obtained from larvae. Using the equations resulting from these regressions, it was possible to establish a theoretical number of copies of gBlock corresponding to a single larva for each species. This number was 238 for *P. alleni* and 133 for *P. penetrans*. To facilitate the preparation of standard curves and ensure reproducibility between runs, gBlocks were always used as standards for the subsequent tests. The number of *Pratylenchus* larvae was estimated by converting the number of gBlock copies derived from the Cq using the correspondence described above.

The detection of *P. alleni* and *P. penetrans* in multiplex assays using 50 larvae was not influenced by the presence of the other species, and when only one species was present, no signal for the other species was recorded (Table 3). These interactions between species were also tested using gBlocks at higher concentrations (equivalent to 1,000 and 100,000 larvae). No false positives were detected. Moreover, the Cq value was not influenced when the DNA of a species was underrepresented (1:100) or overrepresented (100:1) in the reaction mixture (Table 3). No amplification was observed for *P. coffeae, P. crenatus, P. thornei*, and *P. neglectus.* The amplicons from six positive field samples (see below) were also sequenced and confirmed the identity of the target species.

Impact of the developmental stages on the Cq values. Quantification was not significantly influenced by life stage for *P. penetrans* (DF = 3, F = 1.492; Prob F = 0.289) (Table 4). However, a significant difference was noticed between the Cq observed with juveniles (J2) (28.42 ± 0.85) versus adult males (26.83 ± 0.55) for *P. alleni* (DF = 3; F = 4.418; Prob F = 0.041) (Table 4).

Field survey. During this survey, 185 fields were sampled. Molecular identification of these specimens showed that *P. penetrans* was present in 78 fields (42%), whereas *P. alleni* was detected in only 14 fields (8%) (Table 5). Eight fields (4%) were infested by both species. The region with the highest incidence of *P. penetrans* was Estrie, where 85% (23 of 27) of fields were infested. For *P. alleni*, the region with the highest incidence was Montérégie-Ouest, where 20% (6 of 30) of the fields were positive (Table 5).

The number of *P. alleni* recovered from positive fields (3 years inclusive) was very low, with a provincial mean of 3.5 nematodes per 100 cm^3 of soil (1 to 27). For *P. penetrans*, 51.7 nematodes per 100 cm^3 of soil (1 to 274) were detected (Table 5). Soil type significantly influenced the occurrence of *Pratylenchus* (F = 251.67; DF = 5; P = 0.048). The presence of *P. penetrans* was observed in all types of soil except organic (n = 1) and was strongly correlated with soil texture, with lighter soil being the most favorable (χ^2 = 26.27, df = 5, P value = 0.0001) (Fig. 3). The soil type with the highest percentage of positive sites for *P. penetrans* was sand at 80% (12 of 15), whereas the nematode was found in only 13% (2 of 16) of clay sites.

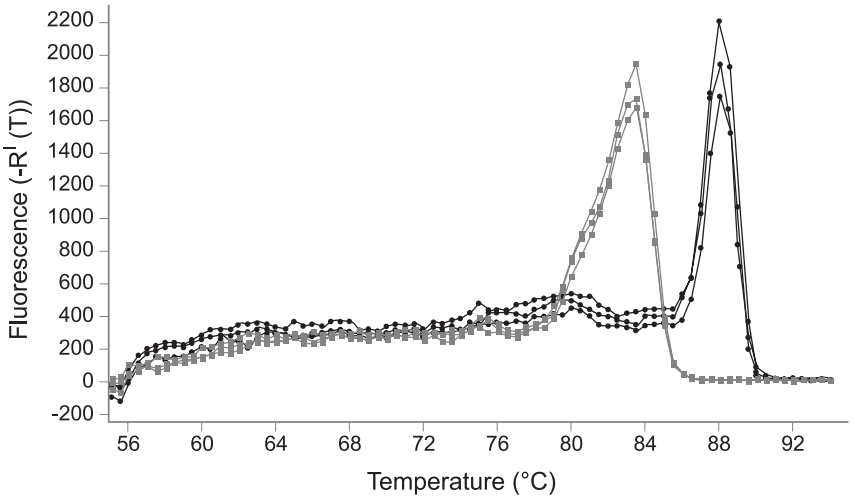

Fig. 1. DNA dissociation curves analysis for *Pratylenchus alleni* (black circles) and *P. penetrans* (gray squares).

The low number of positive sites for *P. alleni* did not allow for a proper comparison of the impact of soil type. However, the species was found in 24% of silt loam fields, 7% of sandy soils, and 5% of clay loams.

The comparison of the number of nematodes morphologically similar to the *Pratylenchus* genus (any species) by visual examination with the sum of the estimation of *P. penetrans* and *P. alleni* abundance by qPCR indicated a good correlation, with an R^2 of 0.77 (Fig. 4).

Discussion

Root lesion nematodes, which belong to the genus *Pratylenchus*, cause substantial yield losses in many crops in North America. The detection in 2011 of a species new to Quebec (*P. alleni*, which could pose a threat to soybean and corn production) emphasizes the importance of prompt and adequate identification of *Pratylenchus* spp. Identification at the species level using morphological criteria only is complex and extremely time consuming, if not impossible (Loof 1978; Tarte and Mai 1976). Given that the two species of *Pratylenchus* in this study are sympatric, the development of a multiplex molecular identification tool will be very useful for identification and quantification. The proposed qPCR assay has the advantage of being faster and more efficient than previous methods that used restriction enzymes and electrophoresis. The use of the D2/D3 region (28S) instead of the internal transcribed spacer (ITS) allowed specific primers and probes to be developed to distinguish between *P. alleni* and *P. penetrans* for simultaneous detection. The ITS region is known to contain intraspecific variation in *Pratylenchus* spp. (De Luca et al. 2011; Waeyenberge et al. 2009), whereas the D2/D3

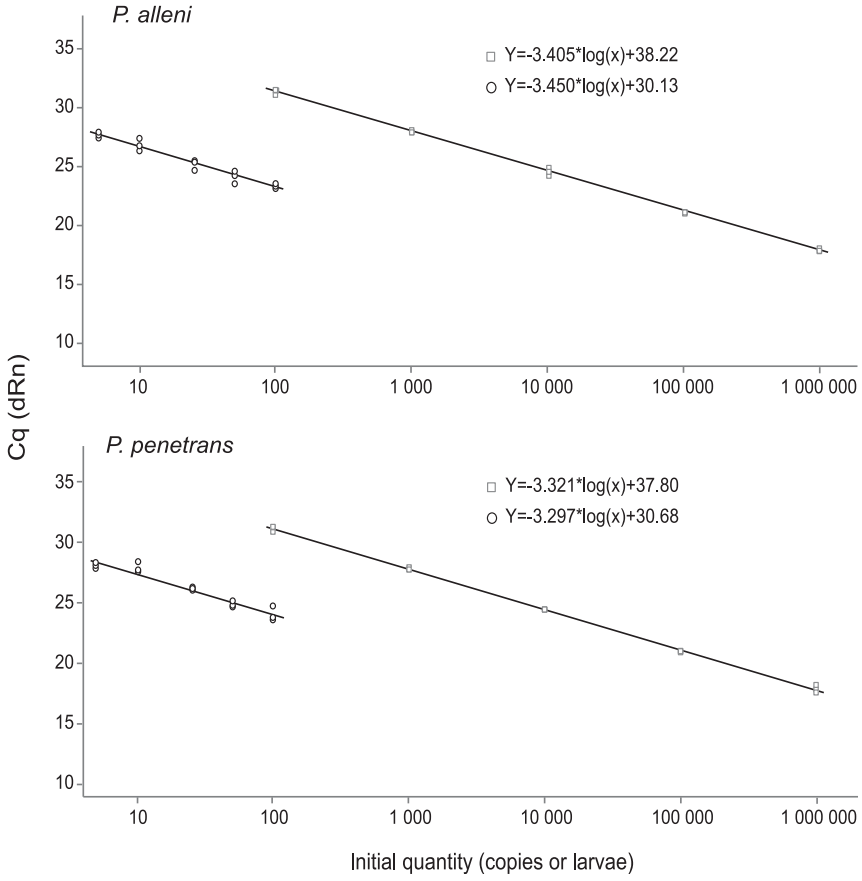

Fig. 2. Comparison of standard curves with genomic DNA from known numbers of larvae of *Pratylenchus alleni* and *P. penetrans* (circles) and their corresponding synthetic DNA gBlock (squares).

Table 3. Influence of the presence of 50 individuals of the other species on the quantification cycle (Cq) of 50 individuals of *Pratylenchus penetrans* or *P. alleni* and impact of different ratios of synthetic DNA on the Cq for each species[z]

DNA template		qPCR dye	
Source	**Species**	**FAM (*P. penetrans*)**	**CY5 (*P. alleni*)**
Larvae	*P. alleni*	No Cq	26.00 ± 0.07
	P. penetrans	24.99 ± 0.10	No Cq
	P. alleni + *P. penetrans*	25.00 ± 0.07	26.00 ± 0.08
Synthetic DNA	*P. alleni* 10^5	No Cq	20.89 ± 0.19
	P. alleni 10^7	No Cq	14.37 ± 0.35
	P. penetrans 10^5	20.97 ± 0.10	No Cq
	P. penetrans 10^7	13.80 ± 0.11	No Cq
	P. alleni 10^5 + *P. penetrans* 10^7	13.89 ± 0.07	20.30 ± 0.29
	P. alleni 10^7 + *P. penetrans* 10^5	20.75 ± 0.27	13.91 ± 0.49
	NTC	No Cq	No Cq

[z] Abbreviations: qPCR = quantitative polymerase chain reaction and NTC = no-template control.

region has been used for the development of specific primers for several nematode species and has proven to be reliable (Kumari and Subbotin 2012; Ye and Giblin-Davis 2013). The assay was found to be very sensitive, systematically detecting the lowest tested quantity of the target sequences (100 copies of the gene) or the equivalent of less than one larva for *P. alleni* and *P. penetrans*. This result is similar to other studies such as those of Sato et al. (2007) and Yan et al. (2012), in which single individuals were also detected. For these reasons, we think that a nematode enrichment through Baermann's pan is a good strategy compared with the analysis of DNA directly extracted from soil. Most of the commercial kits available still work with a very low input, such as 0.5 g of soil, a quantity that is hardly representative of a field status for nematodes, even if it comes from composite samples. Also, different soil types could greatly affect the efficiency of DNA extraction because of the presence of chemical components interacting with the matrix of the kit or soil physical properties. Although slightly more time consuming, a concentration step overcomes these problems on top of lowering the detection limit.

Considering that the DNA extract comes from 100 cm^3 of soil, the limit of detection is well below (approximately 100×, depending on soil density) the economic threshold for *Pratylenchus* spp., which is established at roughly 100 nematodes per 100 cm^3 of soil. Conversely, quantification was linear up to the highest tested concentration (1×10^6 copies), which corresponds to approximately 5,000 nematodes per 100 cm^3 of soil, depending on species and soil density. Thus, the range of this assay is sufficient to cover most agronomic

Table 4. Influence of the developmental stage of *Pratylenchus* spp. on the quantification cycle (Cq) of 10 individuals of *P. penetrans* and *P. alleni* by quantitative polymerase chain reaction[z]

Stage	*Pratylenchus penetrans* Cq (mean ± SD)	*P. alleni* Cq (mean ± SD)
Juvenile (J2)	28.07 ± 0.27 a	28.42 ± 0.85 a
Adult female	27.37 ± 0.27 a	27.43 ± 0.17 ab
Adult male	27.91 ± 0.79 a	26.83 ± 0.55 b
Mixed stages	27.53 ± 0.28 a	27.53 ± 0.34 ab

[z] Results with the same letter were not significantly different; SD = standard deviation.

Table 5. Number of fields that tested positive for the presence of *Pratylenchus alleni* and *P. penetrans* by region of the province of Quebec

Region	Number of fields tested	Number of positive fields[z]		
		P. alleni	*P. penetrans*	Both species
Abitibi-Témiscamingue	2	–	1 (1)	–
Centre-du-Québec	13	–	8 (1–172)	–
Chaudière-Appalaches	6	2 (1)	–	–
Estrie	27	3 (1)	23 (2–254)	3
Laurentides	3	–	1 (3)	–
Mauricie	8	–	5 (4–138)	–
Montérégie-Est	50	1 (1)	18 (1–91)	1
Montérégie-Ouest	30	6 (1–27)	8 (1–201)	4
Montréal-Laval-Lanaudière	37	2 (3)	9 (1–27)	–
Outaouais	9	–	5 (5–274)	–
Total	185	14 [3.95]	78 [51.71]	8

[z] Numbers in parentheses are the range of the number of nematodes found per 100 cm^3 of soil in positive samples and numbers in brackets are the mean number of nematodes per 100 cm^3 of soil in positive samples by species.

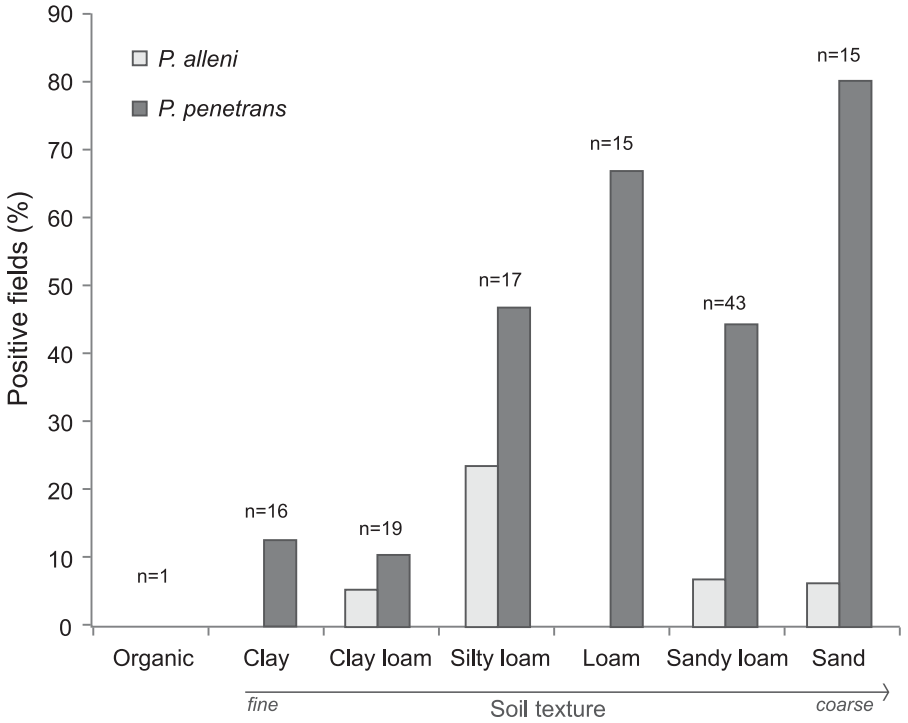

Fig. 3. Percentage of fields that tested positive for *Pratylenchus alleni* and *P. penetrans* by type of soil.

situations. The method was also specific, given that no cross-amplification with other species was observed. Furthermore, the inclusion of an internal control improved the robustness of the method by preventing false-negative results due to the presence of PCR inhibitors. The quantification of a given species was not affected by the presence of DNA from the other species, even when the other species was a hundred times more abundant. This feature is important because these two closely related species are found simultaneously in some fields. Accuracy was also very good for *P. penetrans* but was found to be slightly affected by the presence of different life stages for *P. alleni.*

Contradictory results have been reported about the influence of the developmental stages and the Cq variation of *Pratylenchus* spp. Our results are in accordance with those of Kawanobe et al. (2015), who found no significant differences in Cq for juveniles or adults of *P. penetrans*. The same result has been obtained for other species such as *P. neglectus* (Yan et al. 2013). However, the difference observed between juveniles and males of *P. alleni* suggests that this phenomenon is probably not universal within the genus. From a practical point of view, this situation is not a serious concern, because the difference between each stage was minor. Mixed stages were used for the development of the assay, and this pool was not significantly different from any of the life stages tested alone. In the field, populations of *P. alleni* are not expected to be found at a single life stage at a given moment during the growing season.

Using the proposed method, *P. alleni* was found in 14 soybean fields scattered across five regions of Quebec. Populations of *P. alleni* were very low, with the number of larvae per sample ranging from 1 to 27 (Table 5). Thus, population densities were at the limit of detection for most of these positive fields. The only region with samples containing higher densities was Montérégie-Ouest, which is also where this species was initially discovered in 2011 (Bélair et al. 2013) and the warmest area in the province.

Of the 185 fields tested, 78 were found to be infested by *P. penetrans*, including 8 where *P. alleni* was also found. These fields were located in nine different regions. There have already been numerous reports of *P. penetrans* in different areas of Quebec (Santerre and Lévesque 1982) but this was the first time that root lesion nematodes were surveyed in field crops. The high number of positive fields is not surprising because the species is reported to be endemic in this part of the world. However, many of the fields exhibited high population densities, and 8% (15 of 185) of the fields tested were above the theoretical economic threshold. Unfortunately, this problem is overlooked most of the time, and no data currently exist to correlate these numbers on soybean or corn with actual yield losses under the bioclimatic conditions of Quebec.

Soil type was observed to have a significant impact on the presence of *P. penetrans*. Other authors, such as Zasada et al. (2015) and Townshend and Webber (1971), have demonstrated that soil type, especially soil texture, appears to be one of the most important factors affecting the damage potential of *P. penetrans*. Soil texture influences the damage potential of *P. penetrans* through a combination of nematode factors (such as root movement and penetration, fecundity, and survival) and plant factors (such as water and nutrient uptake and general stress). It has been reported that, in general, *P. penetrans* causes more severe damage in sandy soils than in finer-textured soils (Endo 1959; Schmitt and Barker 1981; Townshend 1972). The number of fields that tested positive for *P. alleni* was too low to establish a good correlation with soil type. However, a greenhouse assay using the population from Quebec surprisingly showed that the population increases more in heavier-textured soils (Vandal 2017). This was also demonstrated for *P. thornei* and other species (Thompson et al. 2010). If this were to be confirmed in the field, the impact could be considerable, because most field crops are cultivated in heavy-textured soil in Quebec. These crops have been relatively spared from *P. penetrans* but could be a good niche for *P. alleni*.

The comparison of the number of *Pratylenchus* spp. detected in these fields by visual enumeration with the estimations by qPCR showed a good correlation. Still, the molecular method targeting two specific species often yielded lower populations than actual counting. It is important to remember that other species of *Pratylenchus* that are morphologically similar to the target species are also present in some of these fields. Most of these species are not problematic in corn and soybean but will be counted by a diagnostic lab using classical techniques. This overestimates the pest presence and could lead to unnecessary nematicide or fumigation treatments. Therefore, the utilization of this molecular technique could reduce the impact of pest management on the environment.

Canada's nematological profile has evolved rapidly over the past decades and will continue to do so, given that the climate models for the coming decades predict even warmer conditions. Under controlled conditions, *P. alleni* was shown to reproduce more than *P. penetrans* at temperatures above 25°C (Acosta and Malek 1979; Dickerson 1979). Based on current climatological models, it is most likely that *P. alleni* densities will increase and *P. penetrans* will decline as the soil temperature exceeds the optimum level for the latter species. The method described in this article will surely be a valuable tool for monitoring such a shift in the coming decades and for adapting management techniques to the new nematological reality of Quebec.

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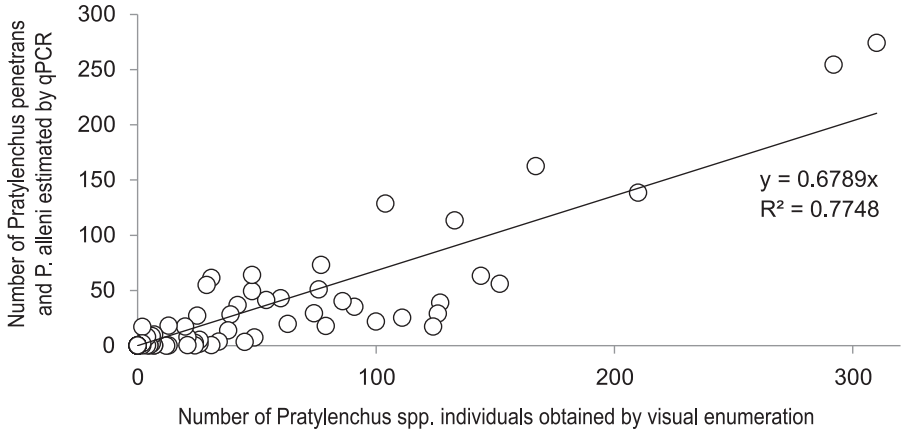

Fig. 4. Comparison of the number of *Pratylenchus* spp. individuals (any species) observed in field samples with the total number of *Pratylenchus penetrans* and *P. alleni* estimated by quantitative polymerase chain reaction (qPCR).

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