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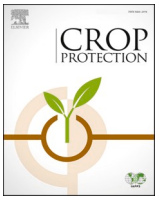

Real time PCR assays to detect and quantify the nematodes *Pratylenchus vulnus* and *Mesocriconema xenoplax*

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ABSTRACT

Economically damaging populations of lesion nematode and ring nematode are managed in tree crops largely through pre-plant chemical fumigation, the use of which is increasingly restricted due to human health and environmental concerns. Reducing the use of fumigants requires precise knowledge of pest nematodes' density and distribution, however; extensive sampling is costly due to the time intensive process of nematode counting and identification. In this study, species specific primers were designed and real time PCR (qPCR) assays developed separately for both species of nematodes. The assays successfully detected each species and did not show significant amplification of non-target nematode groups. Both assays related well with microscopic counts of prepared solutions of nematodes, as well as solutions extracted from field samples. Such high-throughput molecular quantification could reduce diagnostic costs, allowing a more accurate picture of nematode populations in the field.

1. Introduction

Close to 80% of the world's almonds and 75% of walnuts are produced in the Central Valley of California (Beede 1998; California Walnut Board, 2019; Almond Board of California, 2017). Over 400,000 ha of almonds are produced in California with an estimated annual economic impact of 21.5 billion dollars (California Department of Food and Agriculture, 2018; Sumner et al., 2014). Although smaller by comparison, the walnut industry recently generated 1.4 billion dollars in annual revenue from 161,874 ha (California Department of Food and Agriculture, 2019). Two of the main nematodes of concern in these production systems are ring nematode, *Mesocriconema xenoplax*, and root lesion nematode, *Pratylenchus vulnus* (Micke, 1996; Beede 1998). While other species of *Pratylenchus* and *Mesocriconema* are also occasionally present in California almond and walnut orchards (Siddiqui et al., 1973), they are considered less of an economic concern. *P. vulnus* are migratory plant endoparasites, and produce black necrotic lesions throughout the cortex of infected roots (Jones et al., 2013). In contrast, *M. xenoplax* are migratory ectoparasites, living in the soil and feeding on root tips, reducing root mass by up to 85% (Micke, 1996). To controls these nematodes, growers use resistant rootstocks and chemical fumigation (UCIPM 2017), although annual fumigant applications have recently been restricted due to public health concerns (Marks, 2016).

To minimize fumigant use, growers need to know precisely the density and distribution of nematodes present. However, currently, it is difficult to estimate the extent of nematode problems because accurately representing their often-patchy distributions requires extensive soil sampling (Goodell and Ferris 1980). This leads directly into problems with nematode diagnostics. Decisions must be made about how many soil samples to take for a given area, with some recommendations citing extensive soil sampling in as small as 0.1 ha blocks to be sure that pest nematodes are not present and that fumigation treatments are unnecessary (Schneider and Hanson 2009). Since increasing the number of soil samples submitted to a laboratory for testing increases diagnostic fees, soil samples are often composited from as large an area as is practical. The benefits of compositing samples from a large area must be weighed against the variation potentially introduced by incorporating additional samples, which could affect reliability (Ferris et al., 1981).

The most labor and time intensive part of nematode quantification involves their extraction from soil, as well as identification and counting under the microscope (Ferris et al., 1981). Compounding the problem, the extraction efficiency of nematodes from soil is often low and counts can vary greatly between laboratories (Duncan and Phillips 2009). Once isolated, specialized training is additionally needed to correctly identify

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nematodes, making quantification a lengthy and expensive process, with costs ranging from $30-$125 per sample. Molecular methods of identification using real time PCR (qPCR) can overcome some of these drawbacks, and predict damage more accurately and consistently than traditional methods of quantification (Yan et al., 2013; Berry et al., 2007; Atkins et al., 2005), potentially at a lower cost. For example, while it can take up to 30 min to identify pest nematodes from a single sample under the microscope, 26–43 samples can be analyzed in 2 h using a qPCR approach, depending on the number of technical replicates. The reduced time required for identification (6–10 times less) could potentially make up for increased reagent costs (approximately $6 per sample).

Building on polymerase chain reaction (PCR), qPCR simultaneously quantifies and identifies nematode populations by comparing the intensity of the amplified signal to a standard curve calculated from known densities (Berry et al., 2007). Although few qPCR assays exist to quantify and detect *M. xenoplax*, several have been developed for *Pratylenchus* spp., but none for *P. vulnus* specifically. Yan et al. (2013) designed a qPCR assay that detected and quantified *Pratylenchus neglectus* from DNA extracts of 0.5 g soil, while Berry et al. (2008) developed qPCR methods for *Pratylenchus zeae* extracted DNA from nematode solutions which were enriched from larger 200 cm^3 samples. For other nematodes groups, such as *M. incognita*, there has also been work on direct quantification of nematodes from dried, pulverized soil samples (Min et al., 2012), although it remains to be examined how quantification from small soil samples (<1g) interacts with field heterogeneity and the sample size limitations discussed above. qPCR methods can be quite sensitive. For example Sato et al. (2007) designed PCR primers for *P. penetrans* which were able to detect a single target nematode in over 800 non-target nematodes. Such methods have also occasionally been tested in the field with good relationships between microscopic counts and qPCR predictions for *P. thornei* (Yan et al., 2012). Despite these advances, nematodes are largely still quantified commercially using traditional microscopic counting by analytical labs, although notable exceptions have existed (Ophel-Keller et al., 2008).

Creating a qPCR diagnostic tool for *P. vulnus* and *M. xenoplax* could lead to new knowledge of their precise densities and distributions in orchards, allowing growers to make more informed management decisions. As a step towards this goal, the objectives of this study were: 1) to develop qPCR assays for identifying and quantifying *P. vulnus* and *X. xenoplax* and 2) to compare the method's performance to standard microscopic methods on field samples with native nematode populations.

2. Methods

2.1. DNA extraction from pure cultures and soil

For experiments and standard curves, separate cultures of *M. xenoplax* and *P. vulnus* were maintained on grape root tissue cultures (Cultivar 'French Colombard') at room temperature. The nematodes were extracted from the media into solution, and known concentrations removed in a small volume of water (<200 μl) using a glass pipette. These nematodes were then immediately placed in bead beating tubes and extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For primer specificity experiments, DNA was extracted from individual nematodes which were cut in half and added to 1X Amplitaq buffer (Fisher Scientific, Waltham, MA) and digested using 2 mg ml^{-1} Proteinase K (Fisher Scientific, Waltham, MA) by incubating at 56 °C for 1 h, followed by 100 °C for 8 min.

To test the accuracy of the qPCR assays, nematodes were extracted from 200 ml field soil using a sieving and decanting technique followed by sugar centrifugation (Barker, K.R., Carter, C.C., 1985) and concentrated into a 4 ml solution. The sample was vortexed for 5 s to mix nematodes and water thoroughly, then a glass pipette was used to load 2 ml of liquid/nematodes onto a plastic Hawksley slide with a 1 ml field of view. Nematodes on the slide were identified using morphological characteristics according to Bongers (1988). The other 2 ml of solution remaining was further concentrated to 0.5 ml and transferred to a DNeasy PowerSoil tube for DNA analysis. For field samples, 10 μl of a reducing agent, β-mercaptoethanol (BME, Qiagen, Hilden, Germany), was added to the bead beating tube prior to DNA extraction to reduce the degree of DNA inhibition in the soil extracts, as recommended by others (Mondino et al., 2015).

2.2. Primer design and specificity

Primers were developed targeting Internal Transcribed Spacer Region 2 (ITS2), located between the nuclear 5.8S and 28S ribosomal DNA genes, based on sequences for *P. vulnus* (MG372806.2) and *M. xenoplax* (FN433849.1) retrieved from GenBank (NCBI 2016). This region is known to show phylogenetic variability between nematode species and is well represented in Genbank sequences (Powers et al., 1997). Target amplicons in each region were initially identified by aligning all deposited sequences for the respective nematode species. Genomic regions that yielded low homology scores with closely related nematodes using NCBI Primer-BLAST algorithms were selected for primer design (Table 1). The amplicon size for the primer set PVF/PVR was 111 base pairs and 94 base pairs for MXF/MXR.

For primer specificity testing, isolates were obtained from the DNA collection of the California Department of Food and Agriculture (CDFA), tissue cultures described above, and field samples collected from corn, grapes and pecans (Table 2). The qPCR cycle threshold (Ct) values, which measure the signal intensity of the fluorescent DNA marker, were compared between DNA extracted from these nematodes and the intended target species. To expand tests of primer specificity to include nematodes that could not be obtained from culture or by requesting DNA, primer specificity was also assessed *in-silico* by performing sequence alignments with all nematode sequences available in GenBank.

The identity of field isolates was determined by obtaining and sequencing PCR products of gene fragments amplified with the end point 28S rRNA primers 391/501 (Holovachov et al., 2015). Sequencing was performed at the UC DNA Sequencing Facility at the University of California Davis. Amplifying samples using these primers also served as positive controls, ensuring that nematode DNA was present and that the lack of qPCR signal in these cases was due to primer specificity and not failure of the DNA extraction. End point PCR reactions (25 μl) consisted of 0.5 μM of each primer, 12.5 μl 10X buffer, 5.8 μl water and 0.2 μl KOD DNA Polymerase (MilliporeSigma Burlington, MA). Amplified products were purified using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). Contigs were assembled using Aligner (Version 3.6.1) and an online BLAST search performed on the final consensus sequence (Altschul et al., 1990).

The qPCR assay was performed using a Bio-Rad CFX thermocycler (model C1000) (Bio-Rad Laboratories, Inc. Hercules, CA. U.S.A.) and data analyzed using Bio-Rad CFX Manager Software (v3.1). Sso Advances Universal SYBR Green Supermix (Bio-Rad) was used with 1.0 μl DNA template and 10 μM primer concentration. The cycling conditions were as follows: incubation at 95 °C for 5 min, 39 cycles of 95 °C for

Table 1
Real time PCR (qPCR) primers used to detect and quantify *Pratylenchus vulnus* and *Mesocriconema xenoplax* targeting the Internal Transcribed Spacer Region 2 (ITS2).

Primer name	Target	Gene	Sequence (5' → 3')
PVF	*P. vulnus*	ITS2	TGCGGATGTGAGAAGTATGAG
PVR	*P. vulnus*	ITS2	ATGAATTTGGCCATGATTGG
MXF	*M. xenoplax*	ITS2	TGACTGCGCTTTTCAAACAC
MXR	*M. xenoplax*	ITS2	AAGCAATTGTGCTCAACACG

Table 2
Nematode taxa used to test the specificity of primer sets for two species, *P. vulnus* (PV) and *M. xenoplax* (MX) and cycle threshold values, where (−) indicates no detected signal. The presence of nematode DNA was confirmed (+) by end point PCR with the general nematode primers 391F/501R. Isolates were obtained from laboratory tissue culture, the DNA collection of the California Department of Food and Agriculture (CDFA) and field samples. For field and culture isolates, DNA was extracted from single nematodes, while for CDFA isolates DNA was extracted from several nematodes together.

Species	Ct value qPCR (PV)	Ct value qPCR (MX)	End point PCR (391/501)	Origin
Pratylenchus vulnus	27.86	–	+	Culture - *Vitis vinifera*
Pratylenchus scribneri	–	–	+	Field - *Zea mays*
Pratylenchus zeae	–	–	+	CDFA - *Miscanthus* sp.
Pratylenhcus bolivianus	–	–	+	CDFA - unknown plant
Pratylenchus coffeae	–	–	+	CDFA - *Alocasia* sp.
Mesocriconema xenolax	–	24.24	+	Culture - *Vitis vinifera*
Mesocriconema xenoplax	–	23.51	+	Field - *Vitis vinifera*
Mesocriconema xenoplax	–	21.3	+	CDFA - *Prunus* sp.
Mesocriconema sphaerocephalum	–	39.42	+	CDFA - *Prunus* sp.
Mesocriconema ornatum	–	–	+	CDFA - *Prunus* sp.
Criconemoides spp.	–	–	+	Field - *Carya illinoinensis*

0.05 s, and 60 °C for 30 s. Non-template controls, using distilled H_2O instead of DNA in the PCR reaction, were run for all tests. The Ct values were determined using the programs default settings. Reactions were run in technical triplicates for each sample and averaged. Separate standard curves were run on each 96 well plate for all experiments, which consisted of serially diluted DNA, which was extracted from either 200 or 1000 nematodes of the target species (10,000 fold change), depending on expected densities in the sample. Nematodes used for standard curves were isolated from pure cultures and concentrated similarly to soil samples as described above.

2.3. In vitro experiments

In order to determine the assay's limit of detection, two experiments were conducted for each species with solutions prepared from known numbers of nematodes. In the first experiment, 200 adult nematodes from sterile tissue culture were transferred to DNA extraction tubes in small amounts of water (<200 μl). For each species, three biological replicates of DNA extraction tubes were prepared and then pooled together to minimize variability. These were then serially diluted four times to give concentrations of 200, 20, 2, 0.2 and 0.02 nematodes $tube^{-1}$. Three technical replicates of qPCR reactions were then performed for each concentration and averaged. The DNA from each sample was extracted and qPCR performed as described above. After plotting the Ct values and concentrations on log scale and generating a linear regression curve, primer efficiency was calculated as $E = -1+10^{(-1/slope)}$. The second experiment focused on how much variability existed between solutions of individually prepared samples. Solutions of nematodes were individually prepared at concentrations of 200, 100, 50, 25, 5, and 1 nematodes $tube^{-1}$, with three separate tubes prepared as biological replicates of each concentration. Each biological replicate was repeated in three separate technical qPCR reactions and the resulting Ct values averaged. Standard deviation was calculated between the averaged values of the three biological replicates to measure variability.

2.4. Field experiments

To validate the assay in field samples, nematode counts predicted by qPCR were compared to microscopic counts of nematodes. Nematodes were extracted from soil at three sites and two cropping systems, almond and walnut. Samples were collected from two almond orchard sites in the fall of 2018 near Ballico CA, USA. Almond site 1 was planted in 2015 on sandy soil with 0.6% organic matter at a spacing of 4.9 × 6.7 m, and was irrigated using a combination of single in-line drip tubing and solid set sprinklers. Almond site 2 was planted in 2015 on loamy sand soil with 0.9% organic matter on similar spacing and was irrigated with double in-line drip tubing. Both sites were part of ongoing cooperative extension trials examining the effects of fumigation with Telone C35, which was applied in December 2014. Samples were additionally taken from a walnut orchard in Winters, CA in the spring of 2018. The walnuts had been planted in 2010 on sandy clay loam type soil with 1.8% organic matter on a 7.8 × 7.9 m spacing and irrigated by microsprinkler. The site was used to experimentally evaluate non-fumigant nematicides such as Fluensulfone (NIMITZ, Adama). Nematode samples were prepared, DNA extracted and qPCRs performed as described above in sections 2.1 and 2.2.

2.5. Statistical analysis

For all experiments, predictions of nematode counts were generated from qPCR Ct values using the log scale standard curve, where nematode numbers in the standard curve dilution (1000, 100, 10, 1, 0.1) were transformed into log form (3, 2, 1, 0, −1) and then plotted against the Ct values obtained. Predicted nematode counts for test samples were then calculated based on their Ct values according to the equation of the standard curve. Linear models were constructed to determine to what degree qPCR related to nematode counts determined by microscopic evaluation. Assumptions of normality and homoscedasticity were assessed by visual inspection of residual plots and data were square root transformed in models to meet these assumptions (graphs report non-transformed values). ANOVAs were used to compare square root transformed nematode abundances between sites. All analyses were performed in the statistical program R v. 3.5.1 (R Core Team, 2012).

3. Results

3.1. Assay specificity

When compared to closely related species, both primers did not cross amplify (Table 2). The PVF/PVR primers did not detect signal for *Pratylenchus scribneri*, *Pratylenhcus bolivianus* or *Pratylenchus coffeae*, despite nematode DNA being detected in these samples using the more general 391F/501R primers. The MXF/MXR primers effectively amplified DNA from *M. xenoplax* isolated from tissue culture, grape field samples, and isolates of *M. xenoplax* from Florida, USA. In contrast, signal of *Mesocriconema sphaerocephalum*, a non-target species, was near detection limits (Ct = 39.42), suggesting poor amplification, although no signal was observed for other non-target species or negative controls. Other than the intended target species, searches of BLAST found no plant-parasitic nematodes that matched both forward and reverse primer sets. The primers did match several species not commonly encountered in soil samples, such as *Haemonchus contortus*, an animal parasite (CP035804), as well as *Caenorhabditis inopinata* (AP018154) and *Caenorhabditis elegans* strains (CP038191). However, compared to target taxa, the E values indicated a higher statistical probability that these database matches were due to chance (Altschul and Gish, 1996).

3.2. Assay limit of detection

The assays for both species of nematodes were able to detect a qPCR signal at low dilutions of DNA, such as 0.02 nematode, or −1.7 of the log

starting concentration (Fig. 1). For *P. vulnus*, a serial dilution of 200 nematodes showed a range of Ct values from 22.27 to 38.57, indicating that the assay could detect some signal from the lowest level tested (0.02 nematode). According to the standard curve, the primers showed 105% efficiency ($y = -3.93x + 30.46$; $R^2 = 0.97$, $P < 0.01$). For *M. xenoplax*, the primers had a similar efficiency (105%) and the relationship between the log concentration of nematodes and Ct value was $y = -2.99x + 27.22$ ($R^2 = 0.99$, $P < 0.01$). The limit of detection for *M. xenoplax* was also 0.02 nematodes and Ct values ranged from 20.09 to 31.88. For both species, no signal was detected in negative control samples were no nematodes were present.

The assay was able to differentiate quantities from 1 to 200 nematodes in solution and no signal was observed for samples where nematodes were not added (Fig. 2). For *P. vulnus*, the relationship between the number of nematodes added to solution and those predicted by the qPCR assay was strong (Fig. 2A; $R^2 = 1.0$, $P < 0.01$) and the predicted values were very close to those observed by microscopic counting. For *M. xenoplax*, the assay tended to overestimate the number of nematodes added to solution (Fig. 2B) and this effect became more pronounced at higher densities, but the overall correlation between the actual number of nematodes added and those predicted by the assay was still high ($R^2 = 0.99$, $P < 0.01$).

3.3. Field experiments

The qPCR assays were able to accurately detect nematodes in two different field sites and cropping systems. For the soils collected from the walnut orchard, the qPCR assay showed strong agreement with microscopic counts of the nematodes (Fig. 3). The walnut orchard had high populations of both *P. vulnus* ($x = 265.6 \pm 50$ standard error of measurement (SEM)) and *M. xenoplax* ($x = 222.6 \pm 70$ SEM) but also showed heterogeneity, with microscopic counts ranging from 14 to 900 nematodes 200 ml^{-1} soil, and 0 and 1253 nematodes 200 ml^{-1} soil, respectively. The model relating microscopic counts to those predicted by the assay was statistically significant for *P. vulnus* ($R^2 = 0.87$, $F = 122.3$ (1,19), $P < 0.01$) as was the model for *M. xenoplax* ($R^2 = 0.82$, $F = 85.4$ (1,19), $P < 0.01$). For *M. xenoplax*, nematode predictions using the qPCR assay became more variable above 200 nematodes sample $^{-1}$, which was the limit of the standard curve. There were no statistically significant differences, though, between the densities of nematodes quantified by microscopy compared to qPCR for both nematode species.

In the almond orchards sampled, the qPCR assay's predictions related well to the number of nematodes counted under the microscope (Fig. 4). This relationship was strong for both *P. vulnus* ($R^2 = 0.87$, $F = 79$ (1,12).3, $P < 0.01$) and *M. xenoplax* ($R^2 = 0.90$, $F = 105.9$ (1,12), $P < 0.01$). The two almond sites sampled had very different concentrations of nematodes according to microscopic counts. The six samples from almond site 1 had populations of *P. vulnus* ranging from 72 to 725 nematodes 200 ml $soil^{-1}$ with an average of 283.03 ± 115.41. *M. xenoplax* populations ranged from 24 to 632 nematodes 200 ml $soil^{-1}$ with an average of 196.56 ± 49.64. The abundance of nematodes in the eight samples from almond site 2 for both species was much lower for the two species ($P < 0.01$, $F = 14.4$; $P < 0.01$, $F = 21.6$, respectively) with populations ranging from 0 to 132 for *P. vulnus* (26.27 ± 17.78) and

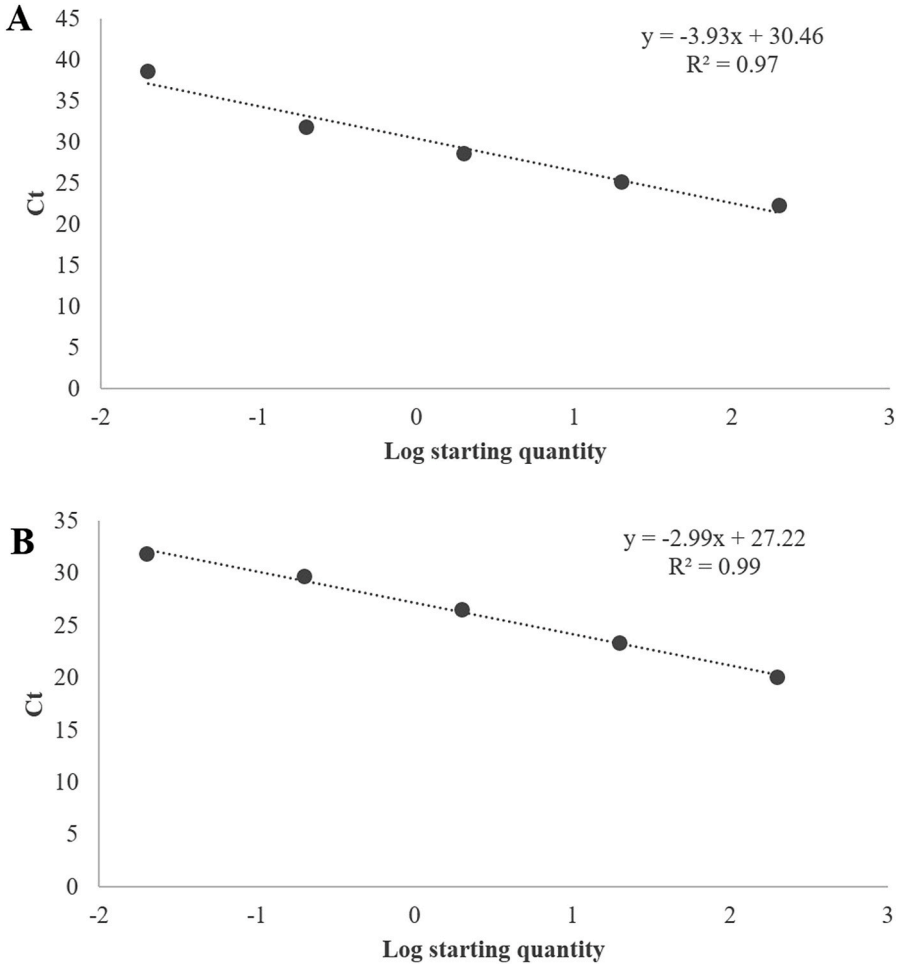

Fig. 1. Standard curve of Ct values plotted against the log starting quantity of (A) *Pratylenchus vulnus* and B) *Mesocriconema xenoplax*. The primer efficiency for both curves was 105%.

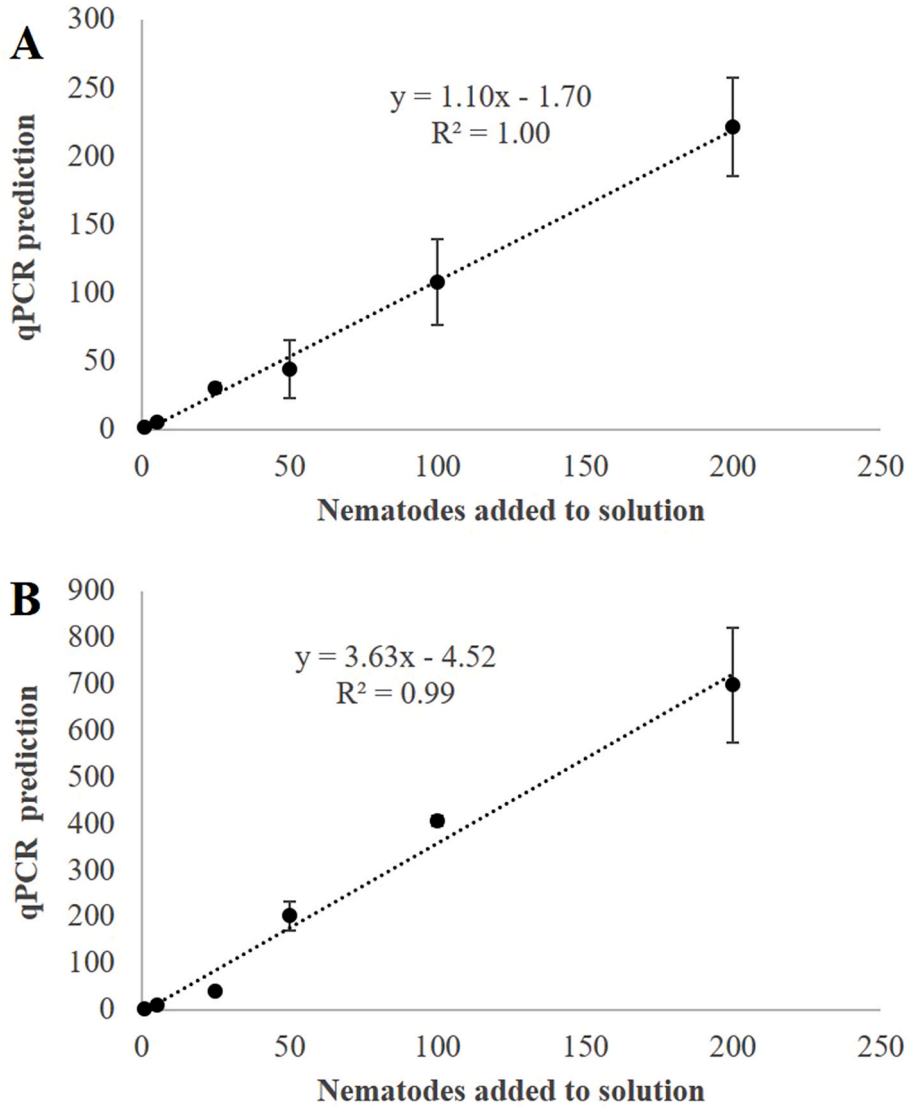

Fig. 2. Relationship between the number of nematodes inoculated into solution and nematodes predicted from the resulting Ct values for A) *Pratylenchus vulnus* and B) *Mesocriconema xenoplax*. Bars are standard deviation of three biological replicates.

0 to 77 for *M. xenoplax* (22.57 ± 9.33). This variability gave a wide range of nematode densities to test with the assay. Linear models did not detect statistically significant differences between the densities of nematodes quantified by microscopy and qPCR.

4. Discussion

The designed primers successfully differentiated the two target pests from other nematode species. To demonstrate specificity for the qPCR assays, template DNA was tested from seven other species of plant parasitic nematodes. While low Ct values were seen with the primers for target species, indicating a strong signal, Ct values for the non-target nematode species were either undetectable, or had Ct values so high that they could be considered a negative result (as in the case of *M. sphaerocephalum*). Other free living nematodes were observed to be abundant in all field samples, which suggests that the assay did not cross-react with any common non plant parasitic nematode species.

Non-target matching primer sequences found through BLAST searches corresponded to species not expected to be found in large numbers in diagnostic samples extracted from soil. These included the animal parasite, *H. contortus*, and *Caenorhabditis* species that typically colonize decaying plant material or are arthropod/gastropod associates (Zajac 2006; Felix and Braendle 2010; Kiontke and Sudhaus 2006). However, the E values given by the BLAST algorithm were higher in these cases, indicating a higher statistical probability that these database matches were due to chance (Altschul and Gish, 1996), potentially because of the long sequence lengths of these entries and the short lengths of the primers. If the primers do, indeed, amplify these species, potential cross reactions could occur in soil samples containing raw manure or unfinished compost, although neither of these would be likely in commercial agricultural settings due to food health concerns.

The qPCR assays for *P. vulnus* and *M. xenoplax* were sensitive, able to detect as little as 1/50th of a nematode in prepared serial dilutions. This is in line with previous studies that have found limits of detection as low as 1/128th nematode for *Pratylenchus scribneri* (Huan and Yan 2016) and 0.5 nematodes per 0.5 g of soil in studies with *P. neglectus* and *P. thornei* (Yan et al. 2012, 2013). Sensitivities of one nematode in mixed solutions were found for *P. penetrans* (Mokrini et al., 2013) as well as the cyst nematode, *Globodera rostochiensis,* and root knot nematode, *M. incognita* (Toyota et al., 2008), although since only one nematode was added to the solution in these cases the actual sensitivity may be lower. Molecular assay sensitivity may vary with the presence of soil inhibitors (Yan et al., 2012) as well as nematode life history stage (Peng et al., 2013; Sato et al., 2007). However, Yan et al. (2013) found no differences in Ct

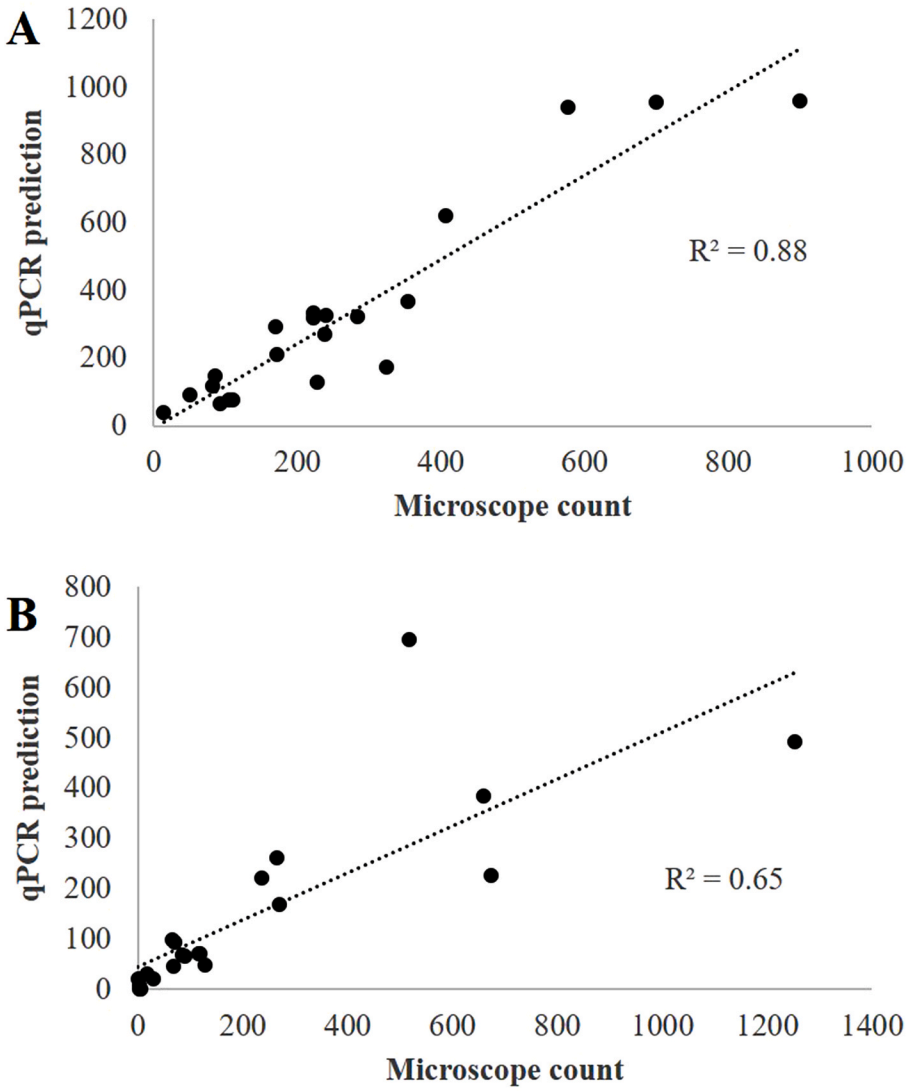

Fig. 3. Relationship at the walnut orchard site between numbers of A) *Pratylenchus vulnus* and B) *Mesocriconema xenoplax* estimated from morphological quantification under the microscope (microscope count - x axis) and nematode numbers predicted by qPCR (qPCR prediction - y axis). R^2 values are based on square root transformed data.

values between life history stages of *P. neglectus*.

In our study, microscopic counts of nematodes in prepared laboratory solutions related well to those predicted by the assay. In these experiments, nematodes were estimated from a standard curve calculated from serially diluted nematode DNA. Other studies have used standard curves calculated from individually prepared solutions of nematodes (Berry et al., 2008) or known numbers of nematodes added to soil (Yan et al., 2012; Min et al., 2012). Sato et al. (2007) found more variation in DNA samples extracted from different numbers of *P. penetrans* than serially diluted DNA samples. Despite serial dilution providing a more reliable standard curve, it has been argued that samples prepared individually more accurately represent the variability present in actual soil samples (Berry et al., 2008). Commercial diagnostic labs, however; could likely not maintain cultures of nematodes to prepare for standards, and obtaining nematode DNA could also prove problematic. One practical solution could be to use qPCR standards prepared from the target sequence cloned into a plasmid or double-stranded DNA that can be ordered for any desired sequence (for example gBlocks, IDT), although the validity of these approaches would need to be tested.

When nematodes were extracted from field soil, the qPCR assays related well to microscopic counts of the two species. This was true for a sandy clay loam type soil in the walnut orchard and from both sandy and loamy sand type soils taken from two almond orchards. It should be noted that these sites had relatively low organic matter and low clay contents, which can reduce the efficacy of molecular assays through binding with extracted DNA (Frostegard et al., 1999; Miller et al., 1999). Although there were no statistical differences between the microscopic counts and those predicted by either assay, interesting differences were observed between them. Primers for *P. vulnus* tended underestimate nematode numbers by 28.9%, on average, at the walnut site and 43.6% at the almond site, while primers for *M. xenoplax* performed on the same samples overestimated nematode counts by 93.5% and 168%, respectively. While cases of underestimation of nematode numbers using molecular assays have been attributed to inhibitors (Yan et al., 2012), other studies have also found that qPCR tends to overestimate nematode populations (Toyota et al., 2008; Min et al., 2012; Goto et al., 2009), which has been hypothesized to be due to the qPCR method amplifying DNA from eggs and non-mobile life history strategies (Yan et al., 2012; Sapkota et al., 2015). However, in the current study, a sugar centrifugation method was used, which suggests that discrepancies may be due to other factors such as primer efficiency or the increased ability of the assay to correctly identify nematodes in mixed solutions. Further validation across a range of soil types is needed to determine if the assays behave consistently.

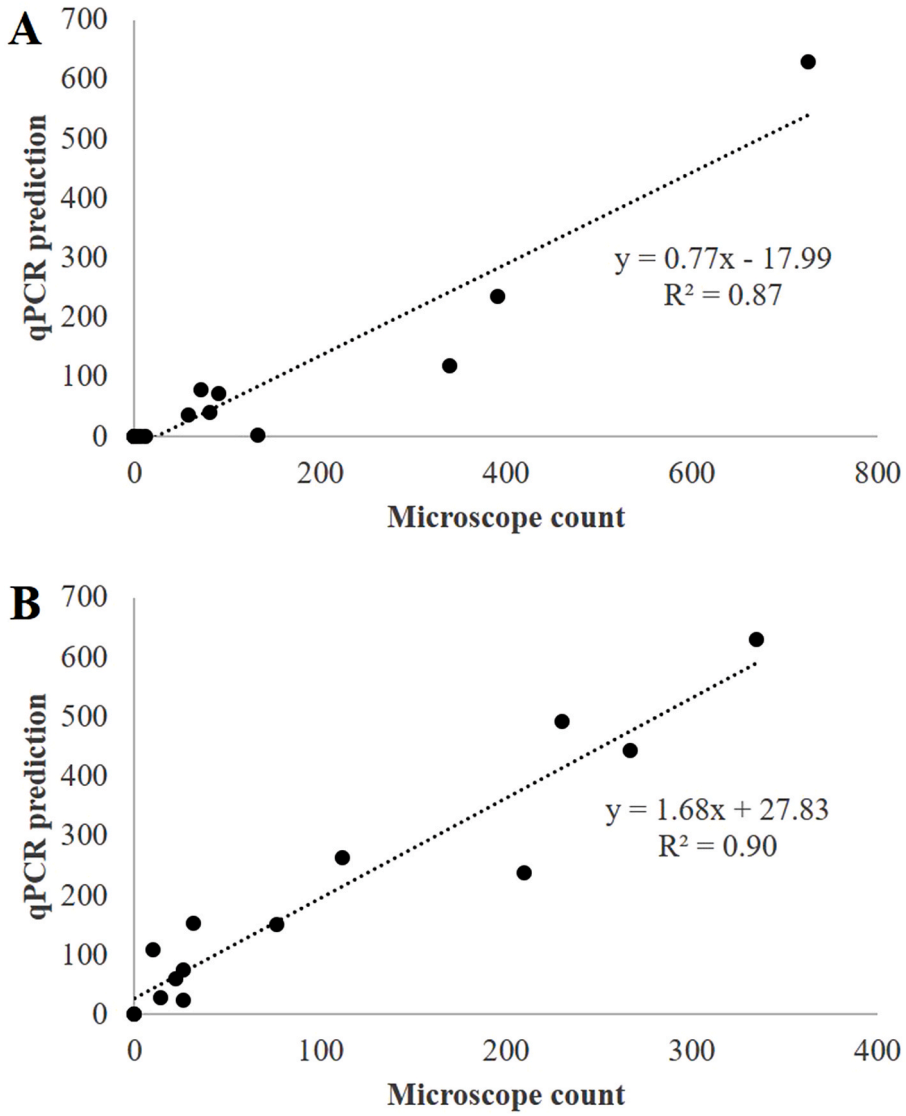

Fig. 4. Relationship at the almond orchard sites between numbers of A) *Pratylenchus vulnus* and B) *Mesocriconema xenoplax* estimated from morphological quantification under the microscope (microscope count - x axis) and nematode numbers predicted by qPCR (qPCR prediction - y axis). R^2 values are based on square root transformed data.

The issue remains for molecular diagnostics of what amount of soil accurately represents nematode populations. While some studies have employed methods of enriching samples for nematodes such as Baermann funnel (Toyota et al., 2008), elutriation (Berry et al., 2008) or sugar centrifugation (Huan and Yan 2016), others have either extracted nematode DNA directly from fresh soil samples (Yan et al., 2012) or from a dried, homogenized sub sample (Sapkota et al., 2015; Min et al., 2011; Watanabe et al., 2013). The low sample sizes inherent in extracting nematode DNA directly from soil has been hypothesized to be responsible for some of the discrepancies between qPCR methods and microscopic counts (Yan et al., 2012). Strong agreement between microscopic and qPCR methods has been seen, though, in cases where nematode DNA was extracted directly from dried subsamples of 0.5 g (Watanabe et al., 2013) and also using enrichment methods where nematode DNA was extracted from solutions prepared by Baermann funnel (Toyota et al., 2008). Others have developed a method to extract nematode DNA from a blended soil buffer solution (Tan 2012). One advantage of extracting nematode DNA directly from soil or buffer solutions is that samples can be processed more quickly than the Baermann funnel method, which typically takes 24–48 h (Barker 1985). However, sugar centrifugation can be completed relatively quickly in the laboratory using standard equipment and has the added advantage that nematodes are rinsed after extraction in sugar solution, which removes inhibitors which are likely retained in the Baermann funnel and direct soil methods (Qui et al., 2006).

Standard extraction methods also enrich nematodes from soil, allowing a larger volume of soil to be processed. Determining the appropriate amount of subsample for diagnostic determination is important because results are being used to estimate nematode populations on large areas of land. The method described here uses a subsample typically considered representative (200 ml) taken from a 3 L homogenized field sample. This subsample could be processed in its entirety, with nematodes extracted into solution, pelleted, and this pellet transferred in a small amount of water to the DNA extraction tube.

This study adds to a growing body of literature suggesting that molecular diagnostics can efficiently quantify and identify pest nematodes. As management options for nematodes become more restrictive, the commercial need for rapid, cheap nematode testing can only be expected to increase. The described qPCR assays for *P. vulnus* and *M. xenoplax* could dramatically reduce the cost per diagnostic sample by eliminating the time intensive step of nematode counting and identification under the microscope. This would allow growers to take more samples,

enabling a more accurate picture of nematode populations in the field.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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