Final Report

Strategies for Quantifying and Controlling Free Living Nematode Populations and Consequent Damage by Tobacco Rattle Virus to Improve Potato Yield and Quality

Work package 1

(Nematode diagnostics)

Ref: R440

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The work described in this report was carried out as part of a five year research project "Strategies for Quantifying and Controlling Free Living Nematode Populations and Consequent Damage by Tobacco Rattle Virus to Improve Potato Yield and Quality". The project (292-249) is co-funded by Technology Strategy Board and Potato Council (now AHDB). The project partners were Cygnet Potato Breeders Ltd, McCain Foods (GB) Ltd, PepsiCo International, DuPont, Farmcare Ltd., Eden Research, Mylnefield Research Services Ltd, James Hutton Institute, SRUC, Plant Health Care UK Ltd, and Tozer Seeds Ltd., in conjunction with Harper Adams University College.

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1. SUMMARY

Accurate identification of free living nematodes can be problematic. The standard diagnostic technique to date has been microscopy/ physical appearance of the nematodes but this project has pursued the development of molecular diagnostic techniques. Specifically the objectives of the work package were:

i) Develop and validate a diagnostic primer for the genus *Longidorus* to be used with a previously published species specific primer for *Longidorus elongatus*;
ii) Develop and validate a quantitative PCR diagnostic primer suite for the common species of *Paratrichodorus* and *Trichodorus* relevant to the UK potato industry;
iii) Assess, for UK populations of *Pratylenchus penetrans*, the efficacy of a published diagnostic primer for the species.

1.1. Methods

Soil samples were taken from the project experimental sites before potato crops were planted. Nematodes were extracted from the soil samples using conventional techniques (modified Baermann funnel). The extracted nematodes were identified using microscopy and then freeze dried. Subsequently the freeze dried samples were used to develop/validate molecular diagnostic assays.

1.2. Results

i) Develop and validate a diagnostic primer for the genus *Longidorus* to be used with a previously published species specific primer for *Longidorus elongatus*.

Longidorus genus-specific primer pairs were designed, tested and shown to be able to detect low numbers of *Longidorus* consistent with levels of abundance typically found in UK agriculture. It is proposed that the genus-specific diagnostic would be used in conjunction with published primers that are specific for *L. attenuatus, L. elongatus, L. macrosoma, L. maximus, L. helveticus, L. profundorum* and *L. sturhani.* These primers are not quantitative and only provide information on presence/absence.

ii) Develop and validate a quantitative PCR diagnostic primer suite for the common species of *Paratrichodorus* and *Trichodorus* relevant to the UK potato industry.

Assays have been developed and there is a relationship between the counts derived from microscopy and the molecular diagnostics. On the whole the values generated by the molecular techniques are greater than manual counts due to the former being more sensitive. Unlike manual counts the molecular diagnostic includes eggs, sperm and gravid females. However, at low nematode abundance (≤ 10 target nematodes 200 g^{-1} soil) the results from the molecular diagnostic become less consistent. In terms of direct feeding this is not considered an issue (because low numbers are not considered a risk factor). In terms of virus transmission, the presence of a single target nematode is a risk so variability in the numbers generated by the molecular diagnostic is only a problem if the assay fails to detect the presence of the nematodes in a sample, which is not the case.

iii) Assess for UK populations of *Pratylenchus penetrans*, the efficacy of a published diagnostic primer for the species

The published primers are specific for *P. penetrans* but were developed for standard PCR protocols, i.e. they are not quantitative thus they only provide information on presence/absence.

Information has been generated on the variation in nematode numbers between repeat sampling occasions at the same site; and on the spatial distribution of nematodes within experimental plots.

2. INTRODUCTION

The phylum Nematoda represents a highly diverse group with over 26,000 described species (Hugot *et al*., 2001). It has been estimated that plant parasitic nematodes cause approximately 12 % of global crop losses (Sasser & Freckman, 1987) resulting in a conservative annual cost of $125 billion to global agricultural managing pathogenic nematode populations (Chitwood, 2003). In addition to direct damage, feeding by some plant parasitic nematodes can lead to secondary plant diseases including virus infection, and hence these are often referred to as virus-vector nematodes (Taylor & Brown, 1997). Free living plant parasitic nematodes (FLPPN) of the order Dorylaimida (specifically species of *Longidorus, Paralongidorus* and *Xiphinema)* transmit Nepoviruses whereas those of the order Triplonchida (specifically *Trichodorus* and *Paratrichodorus*) transmit Tobraviruses (Taylor & Brown, 1997). Tobacco Rattle Virus (TRV) can cause spraing (also known as corky ringspot) symptoms, appearing as brown, necrotic arcs or lines found within potato tuber flesh. This does not necessarily reduce the overall yield but greatly reduces the value of the crop. Relatively low levels (> 5 %) of TRV infections can render entire crops unsaleable, both for the fresh and the processing industries, causing an estimated loss to the UK potato industry of > £13m p.a. All sectors of the UK potato industry are affected by the impact of FLPPN.

Currently, species discrimination is based primarily on morphometrics but the high degree of overlap among species can increase the potential for mis-identification. This uncertainty over identification, due to population variation in their morphometrics, has led many taxonomists to regard *L. elongatus* as a species complex and it has been the subject of nine re-descriptions (Taylor & Brown, 1997). Root-lesion nematodes, *Pratylenchus spp*., are amongst the most economically damaging plant-parasitic nematodes (Al-Banna *et al*., 2004). They have a wide range of hosts and occur throughout temperate regions (MacGuidwin & Stanger, 1991); their geographic distribution being mainly dependant on the prevalence of host plants (Castillo & Vovlas, 2007). They exhibit little morphological diversity and yet present sufficient intraspecific variability in certain morphological characters so that the taxonomic separation of the various species is difficult (Roman & Hirschman, 1969).

FLPPN are able to travel vertically through the soil column (Boag, 1982; MacGuidwin & Stanger, 1991). Their movement is dependent on the presence of water films on the surface of soil particles but they are highly susceptible to desiccation or mechanical disturbance, leading to a rapid decrease in their numbers in the upper layers of soil during drought conditions. Stable populations can, however, persist below the level of

moisture or mechanical stress (below 20-30 cm). Whilst their lateral movement is typically less than 0.5 m per year (Cooper & Harrison, 1973) long-distance dispersal is possible by other means such as adherence to farm equipment, tillage and erosion events (Boag 1985, Taylor & Brown, 1997, Baxter *et al*., 2013). They have extensive host plant ranges and can survive for extended periods in plant-free soil and this ability to persist makes management difficult using crop rotation methods as populations do not diminish rapidly (Taylor & Brown, 1997).

As described above, accurate identification of FLPPN nematodes can be problematic and accurate observation of morphological characteristics and measurements of taxonomically indicative parameters is essential though this is compounded by an ever decreasing skill base (Coomans, 2002). However, with the advancement of molecular biology many of the taxonomic issues can be overcome by applying an integrated battery of molecular-based diagnostics. Typically, diagnostic primers are often based within the ribosomal DNA (rDNA) repeat unit as this is one of the most informative genomic regions for evolutionary and diagnostic purposes for a wide range of organisms (Boutsika *et al*., 2004) and extensive information for this region exists on public databases. A highly useful development in molecular biology was the ability to perform quantitative PCR (Q-PCR) which allows not only the qualitative detection of target organisms but also an estimated enumeration of the targets in a sample. For example, Holeva *et al.* (2006) developed a Q-PCR diagnostic for 2 species of trichodorid nematodes and for TRV producing accurate and sensitive molecular information on both virus and vector populations. Presence/absence diagnostics that did not provide quantification have also been developed for species of *Paratrichodorus* and *Trichodorus* prevalent in Portugal (Duarte *et al*., 2011).

The objectives of this work package (Work package 1) were:

iv) Develop and validate a diagnostic primer for the genus *Longidorus* to be used with a previously published species specific primer for *Longidorus elongatus*;
v) Develop and validate a quantitative PCR diagnostic primer suite for the common species of *Paratrichodorus* and *Trichodorus* relevant to the UK potato industry;
vi) Assess for UK populations of *Pratylenchus penetrans*, the efficacy of a published diagnostic primer for the species;

3. MATERIAL AND METHODS

3.1. Soil sample collection and storage

During the project lifespan, soil samples were taken from project experimental sites located in some of the key potato growing areas of the UK: Tayside, Yorkshire, Shropshire and East Anglia as well as soil samples sent to The James Hutton Institute as part of the nematode diagnostic service run by the institute. These samples were used to evaluate the diagnostics developed in-house as well as the published diagnostics.

For the project experimental sites, the actual area sampled for FLPPN was 1.83 m (1 bed) by 5 m long which represents the harvest bed (2 rows) from the centre of each plot. FLN sampling was done post ploughing, bedforming, destoning (if required) but before nematicide application/incorporation and planting. Similarly, soil samples were

taken from two experimental sites (Harper Adams and Scotland) used to evaluate the efficacy of alternative management strategies for free-living nematodes. From each plot, approximately 800 g soil was collected and thoroughly mixed, placed in re-sealable bags and transported to the James Hutton Institute (Dundee). On arrival, samples were stored in the dark at 4 °C until processing. The following protocol is recommended for soil sampling for free living plant parasitic nematodes.

3.1.1. Soil sampling protocol for trichodorid nematodes

1. If the 10 cm soil temperature has been at 0 ºC or below for three consecutive nights, do not sample.
2. Ideally use a grass plot sampler (Eijkelkamp, Giesbeek, Netherlands) for sampling. However, a trowel or sampling implement that can sample to a depth of 15 cm is acceptable.
3. The minimum recommended sampling rate is one composite sample ha^{-1}
4. Each composite sample should be comprised of at least 50, ideally 70 small cores (using grass plot sample). These figures are minimum values.
5. The area to be sampled should be walked in a W shape with sampling points randomly located along the W. Fixed sampling points along the W walk are not recommended.
6. At each sampling point, detritus on the soil surface such as dead plant material should be removed prior to sampling.
7. At each sampling point, for trichodorid nematodes, soil to a depth of 15 cm should be taken.
8. Soil should be placed in labelled plastic bags (ideally zip-lock bags) and moved to storage as soon as practicable.
9. Please also include a clearly written label (using an indelible pen) along with the soil as a backup label.
10. Prior to forwarding by mail, samples should be stored in a cold store at a temperature of 4 ºC.
11. Please note do not store samples in a fridge that has a freeze-thaw cycle. Many modern fridges have this cycle as default.
12. For mailing, please place the samples in a strong cardboard box with some loose packing material.

3.1.2. Nematode sampling – how representative at planting?

During a technical meeting in the spring of 2013, consortium members raised a question regarding whether the nematode sampling at planting of the experimental sites was representative of that at early plant growth a time when significant FLPPN was perceived to occur. It was therefore agreed that further limited sampling (24 plots) would occur at two dates after planting at the Harper Adams and Scotland main experimental sites. All methodologies to obtain the samples and nematode abundance data were identical to those described for the main sampling programme.

3.1.3. Nematode extraction and identification

Nematodes were extracted from a representative sub-sample of 200 g soil using a modified Baermann funnel for 48 h (Brown & Boag, 1988). Briefly this involves suspending 200 g soil in at least 4 l water before sieving through a 2 mm sieve to remove large debris. The soil suspension was then passed through a 250 µm sieve and any captured material (including nematodes) washed off the sieve into a container. The soil suspension was then passed through a 150 µm sieve and the captured material recovered and added to the first container. The soil suspension was then passed sequentially through 75 µm and 53 µm sieves and the captured material collected and combined as for the first sieve pair. The recovered material from the first sieve pair was poured onto a 95 µm nylon support suspended in a funnel full of water. The recovered material from the second pair was poured into a second 1 mm mesh nylon support lined with 2 ply Kleenex, suspended in a funnel full of water. The funnels were left for 48 h to allow any viable nematodes to pass through the supports and migrate to the bottom of the funnel. After 48 h the bottom 10 ml of water was collected from each funnel. Collected nematodes were identified (to genus level) and enumerated using low-powered light microscopy. Once enumerated, samples were preserved by freeze-drying and stored at -20 °C for later molecular analyses. Nematode abundance data was passed to Andy Evans (SRUC), WorkPackage 2 leader, for subsequent analyses in conjunction with field crop data.

3.1.4. Nematode DNA extraction

Total DNA was extracted from freeze dried nematode extracts using a variation of the method of Donn *et al.*, (2008). Briefly this involved the extraction of DNA using a Purelink Genomic DNA extraction kit (Invitrogen) following the instructions listed by the manufacturer. DNA was eluted in 100 µl water and stored at -20 °C until ready for amplification. Total DNA was extracted from individual nematodes using a method based on proteinase K digestion. Individual nematodes were placed in 20 µl TE and frozen overnight in a PCR tube. 2 x 1 mm glass beads were added and the tube beaten for 2 minutes at max speed. 2 µl Proteinase K was added before incubating at 55 °C for 2 h followed by 10 minutes at 85 °C. The tube was then centrifuged for 5 minutes at max speed. The extracted DNA was diluted 1:10 in water and 2 µl used as template for PCR. Extracted DNA was stored at -20 °C short term or -80 °C long-term.

3.1.5. Sequence database creation/searching

Public sequence databases were mined for all sequences of the relevant gene targets from all members of the target genera plus any available sequences from closely related organisms. These sequences were aligned using Clustal X2 (www.ebi.ac.uk). Aligned sequences are imported into TOPALi (www.topali.org) for phylogenetic analyses including creation of phylogenetic trees which allowed identification of unusual sequences and allocation of unknown sequences to groups of species or genera with known confidence levels.

3.1.6. Primer design

All PCR targets are multi-copy DNA regions that provided a greater sensitivity to any diagnostic. Full-length DNA sequences (obtained in both directions to minimise sequencing errors) were aligned with published sequence data and analysed to identify

regions unique to the target species. This was done for all available sequences of longidorids as well as trichodorids. Once these regions were identified, primers were designed to amplify target species only. These species-specific (or genus-specific, in the case of *Longidorus*) assays were tested on samples of known nematode populations.

3.1.7. Primer testing

A number of potentially *Longidorus* genus-specific primer pairs were designed. These primer pairs were first tested against purified *Longidorus* DNA to confirm compatibility (and to test for self-priming) before testing against both environmental samples and soil samples known to contain *Longidorus* and others believed to be *Longidorus* negative by microscopic analysis. Soil samples were processed as above and analysed using light microscopy to detect and enumerate any *Longidorus* present before extracting total DNA and amplifying with the putative *Longidorus* genus-specific primer pairs. Primers that passed this initial testing process were tested further against more soil samples as they become available. Soils believed to be *Longidorus* negative by microscopic analysis produced no PCR product with the tested primers with one exception. Sequence analysis of this PCR product confirmed it was from *Longidorus*. This was likely to be from a first stage juvenile and demonstrated the ability of these primers to detect low numbers of *Longidorus* likely to be undetectable by simple microscopic analysis.

In accordance with the project remit, PCR primers specific to *Pratylenchus penetrans*, *P. neglectus, P. scribneri, P. thornei* and *P. vulnus* (Al-Banna *et al*., 2004) were tested to determine their utility against UK populations of *P. penetrans*. These primers were based within the large subunit of the ribosomal gene region; specifically a region called the D2 – D3 expansion region, and utilized a common reverse primer (D3). Furthermore, these primers provided a presence/absence analysis rather than a quantitative measure from Q-PCR primer/probe combinations. Individual *Pratylenchus* nematodes were extracted from soil samples and their DNA extracted as described above. DNA from these individual *Pratylenchus* was amplified using the primer-combination specific for *P. penetrans*. Any PCR products obtained were sequenced to confirm their species identity. Once it was established that this published species-specific primer was specific it was tested further against total DNA extracted from soil samples.

3.1.8. Sequencing

Sequencing was performed by the James Hutton Institute in-house sequencing service using Big Dye Version 3.1 (Applied Biosystems) according to instructions listed by the manufacturer in a 10 µl final volume.

3.1.9. Q-PCR or Real-time PCR

Q-PCR is a modification of standard PCR protocols that use specifically targeted primers plus a probe associated with a fluorescent reporter and a quencher molecule. The probe binds specifically to target sequences and initially the fluorescent signal is quenched. During the extension stage of the Q-PCR the probe is cleaved, physically separating the reporter molecule from the quencher which allows the signal from the reporter to be detected by a spectrophotometer. Fluorescence is recorded after the

extension step in each cycle (Figure 3.1). Each Q-PCR run also includes standards of known copy number of the target gene to allow calibration of the signal from samples (Figure 3.2). The signal generated is proportional to the number of copies present and can be correlated with the initial number of target gene copies in the Q-PCR. With care and sufficient knowledge regarding the number of copies of the target gene present in each target species, this can be correlated with the number of nematodes present in the initial sample. The earlier a signal is detected above background level, the more DNA template that was present in the initial sample.

3.1.10. Determining gene copy number for trichodorid targets.

In order to fully utilise a molecular diagnostic it is necessary to understand the number of copies of the gene target that is present in each of the four target nematode species to calibrate the Q-PCR results in the context of nematode abundance. Initial estimations were based on information for *Caenorhabditis elegans* that has *c.* 1000 cells, each containing 10 copies per cell, giving in the order of 10,000 copies of the target gene per individual nematode (Griffiths, personal communication) but it would be unwise to make the assumption that all species of nematodes have the same cell number. The number of copies of the target gene can vary in Eukaryotes from 10's of copies per cell to 10000's of copies per cell (Zhu *et al.,* 2005). For the few nematode species for which the copy number has been studied, the number varies from 10's to 100's (Bik *et al.,* 2013, Darby *et al.,* 2013). In order to determine the copy-number per target species we performed a Q-PCR using the relevant diagnostic on representative individuals of the target species.

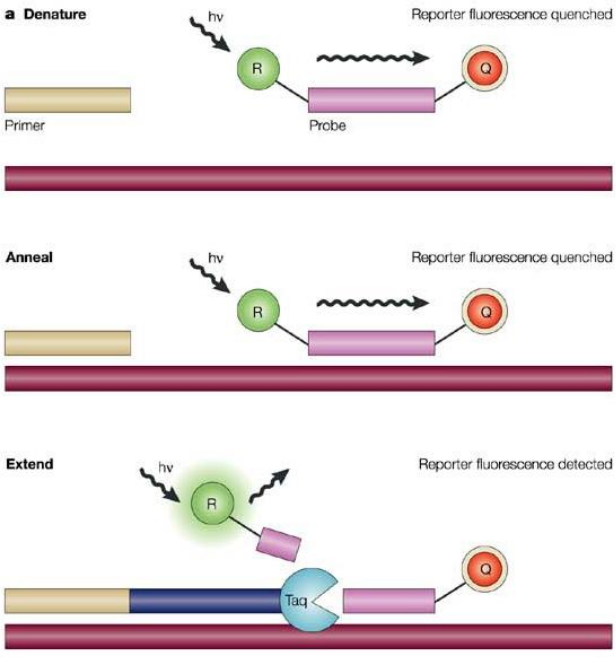

Figure 3.1. Overview of the Q-PCR process. After denaturing, the primer and probe bind to the template strand. During the extension stage the reporter (R) molecule is cleaved from the quencher (Q) molecule and a fluorescent signal is emitted and detected. After each round of amplification the signal generated from the amplification products will increase exponentially.

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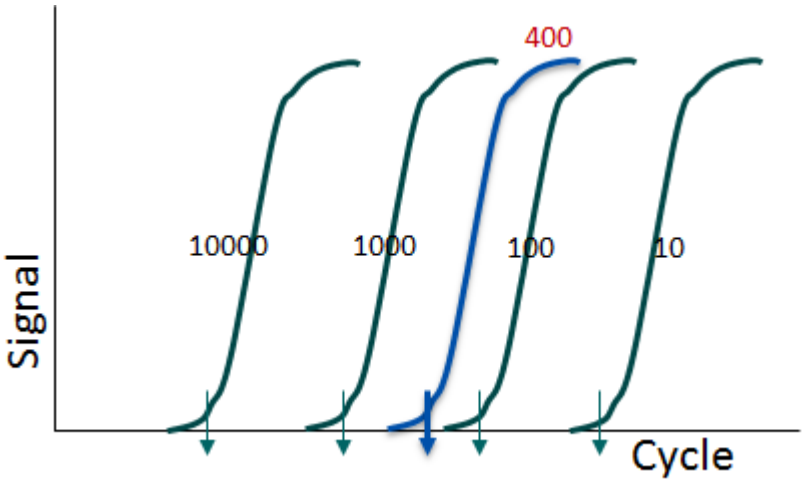

Figure 3.2. Comparing Q-PCR from samples to standards. In this example four reaction wells contain known numbers (copies) of the Q-PCR template, known as standards. In this case standards of 10 copies, 100 copies, 1000 copies and 10,000 copies are included (green curves). The stage at which signal is detected from these standards (known as the CT or threshold cycle) is inversely dependent on the number of copies present in each standard. This allows a standard curve to be generated against which the CT of any unknown sample (shown as a blue curve in the figure) can be compared to calculate the number of copies of the target-gene that was present in the original sample, 400 copies in this example (shown in red).

3.1.11. Validation of trichodorid diagnostics

Typically, there are 4 primary stages in the validation of a new, molecular diagnostic.

1 *In silico*. This first stage involves testing possible primer (or primer + probe combinations) against published sequence information for target nematodes as well as any non-target, but closely related nematodes or other, non-related nematodes that are likely to be present in tested soil samples. This process is greatly facilitated by access to publically available, searchable databases. Using the BLAST (Basic Local Alignment Search Tool, Altschul *et al*., 1990) sequences of putatively diagnostic primers can be used as search terms to search all known DNA sequences for homology. This way it is possible to eliminate most cross-reactions (which would give false positive results) before synthesising the primers. This stage is not fully robust but does allow us to exclude most non-suitable primer-probe combinations before the expensive synthesis stage.

2 Test against plasmids. The second stage is to test the designed primers against DNA extracted from identified nematodes. Specific target genes are cloned from the target nematodes and the diagnostic tested against known numbers of plasmids. Thus specificity of the diagnostics against known levels of closely-related, but non-target nematode DNA is determined as well as the sensitivity of the diagnostic against known numbers of target DNA. At this stage the level of sensitivity against nematodes is not known, only against purified DNA.

3 Test against known and type nematodes. DNA extracted from single nematodes (target and non-target) was used to test the developed diagnostic. This allowed an

estimation of the copy number of the gene target for each nematode. Thus the theoretical limit of detection of the diagnostic in terms of actual nematodes can be estimated. This stage and the latter are often omitted by diagnostic development teams.

4 Determine the fidelity of scaling. In order to determine if the diagnostic continued to show a linear relationship between target-nematode abundance and the signal generated by the Q_PCR, known numbers of trichodorids were hand-picked from nematode populations extracted from random soil samples into tubes containing water. Each tube contained either: 10, 20, 40 or 80 trichodorid nematodes and replicated three times. These were treated in the same manner as previously, frozen, freeze-dried, total-DNA extracted and thereafter subjected to Q-PCR analysis to determine the abundance of trichodorids as assessed by the diagnostic.

5 Screen large numbers of field samples. The final validation stage was an extensive screening of *c.* 2400 field samples to ascertain whether the diagnostic has utility in a commercial arena. This provided an understanding of the limits of detection in field samples and an understanding of potential confounding factors that could influence the developed diagnostic.

We utilised a two-fold approach for the final validation. Firstly, microscope derived counts from samples sourced from the project and manually derived nematode counts from field samples sent to the nematode diagnostic service of the James Hutton Institute to Q-PCR data obtained from the same samples. The latter samples were a more realistic validation as it encompassed a range of soil-types from a large geographic area rather than a handful of managed experimental plots. Furthermore, during the project, we had access to soil samples derived from the East of Scotland Farm Network (Hawes *et al*., 2010) that provided a further unique opportunity to test the diagnostic.

4. RESULTS

4.1.1. Testing of the *Longidorus* genus-specific primer set.

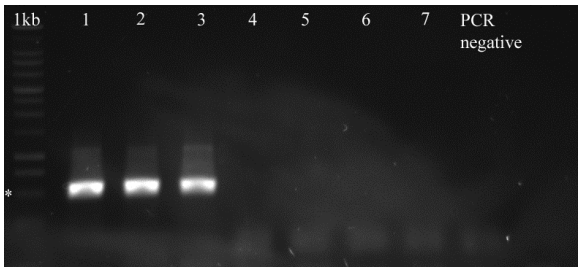

Figure 4.1. Validation of *Longidorus* genus-specific primer from field samples. Lanes 1-3 contain *Longidorus* whereas lanes 4-7 are *Longidorus* deficient. * Indicates position of the 500bp size standard.

A number of potentially *Longidorus* genus-specific primer pairs were designed and tested. These primer pairs were first tested against purified *Longidorus* DNA to confirm compatibility and to test for self-priming. Thereafter, primers were tested against environmental samples, with a mix of soil samples known to contain *Longidorus* and others believed to be *Longidorus* negative by microscopic analysis. Soil samples were processed as above and analysed using light microscopy to detect and enumerate any

Longidorus present before extracting total DNA and amplifying with the putative *Longidorus* genus-specific primers.

The selected genus-specific *Longidorus* PCR primers have utility as a genus diagnostic. Soils containing *Longidorus* gave PCR products; soils believed to be *Longidorus* negative produced no PCR product with one exception (Figure 4.1). This exception was likely to be from a juvenile nematode or an egg-mass and demonstrated the ability of the selected primer to detect low numbers of *Longidorus* consistent with levels of abundance typically found in UK agriculture. This is common to all molecular diagnostics that act as a proxy for microscope counts and is discussed later. It is proposed that the genus-specific diagnostic would be used in conjunction with the published primers of Hubschen *et al*. (2004) that are specific for *L. attenuatus, L. elongatus, L. macrosoma, L. maximus, L. helveticus, L. profundorum* and *L. sturhani.* These primers are not quantitative but ascertain the presence/absence of the target species.

4.1.2. Testing of the published *Pratylenchus penetrans* primers.

Species-specific PCR primers for five separate *Pratylenchus* species (Al-Banna *et al*., 2004) were used in conjunction with an universal reverse primer D3B (Nunn *et al*., 1996) and used to detect the presence of the following *Pratylenchus* species:

P. neglectus, P. penetrans, P. scribneri, P. thornei and *P. vulnus*.

The utility of the *P. penetrans* primer was tested during this project on individual *Pratylenchus* nematodes hand-picked from a range of UK soil samples. These single nematodes were amplified using the specific *P. penetrans* primer and any nematodes that produced a PCR product were then sequenced to confirm their species identity. Overall, the primer was found to be useful in that it specifically amplified only *P. penetrans* (Figure 4.2). However, the use of this primer as a diagnostic does suffer from the limitation that these primers are not quantitative but only ascertain the presence/absence of the target species.

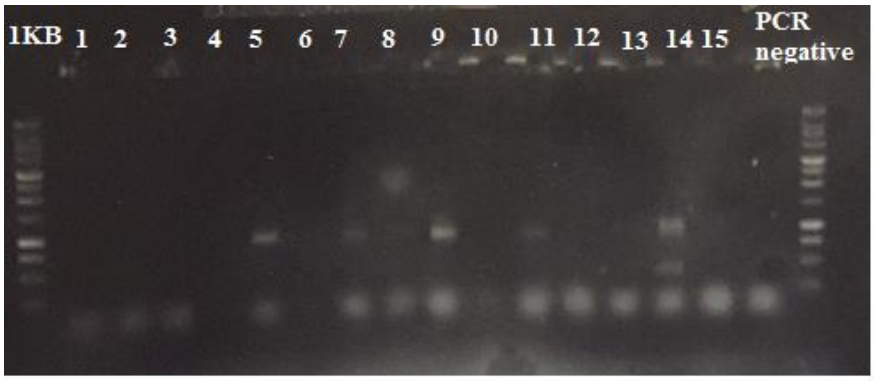

Figure 4.2. Amplification of *Pratylenchus* with *P. penetrans* species-specific primers (Al-Banna *et al*., 2004). The identity of the amplified nematodes (lanes 5, 7, 8, 9, 11 & 14) was confirmed separately by sequence analysis.

4.1.3. Testing of the *Trichodorus* species-specific, quantitative diagnostic

The quantitative, Q-PCR diagnostic developed in WP1 was extensively tested during the project to assess both its sensitivity and its specificity. A different gene target was initially proposed to complement existing, published Q-PCR diagnostics for two of the target trichodorid species (Holeva *et al*., 2006). Detailed *in silico* assessment of these primers and other proposed diagnostic regions utilising the same gene target showed that the previously published gene target was unsuitable for the target species of this project. A second gene target was identified and the process of designing diagnostic primers for all four target species initiated.

4.1.3.1. Testing the specificity of the developed diagnostics

The specificity of each trichodorid diagnostic was determined by creating serial dilutions of known copies of the target DNA for each species. These serial dilutions were later used as calibrators (standards) to quantify target DNA(s) copy number in soil samples. Each species-specific diagnostic was initially tested against serial dilutions of DNA from the intended target species and then tested against serial dilutions of DNA from the other three target species of trichodorids to determine the level of any cross-reaction (Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.6, Figure 4.7, Figure 4.8, Figure 4.9).

Analysis of the specificity of the *T. primitivus* diagnostic against target (*T. primitivus*) and non-target trichodorid species is shown below. A linear relationship was found between target *T. primitivus* DNA copy number and the cycle at which a Q-PCR signal was generated (Figure 4.2). Furthermore, no cross-reaction was noted for the *T. primitivus* diagnostic against the other target trichodorid species (Figure 4.3).

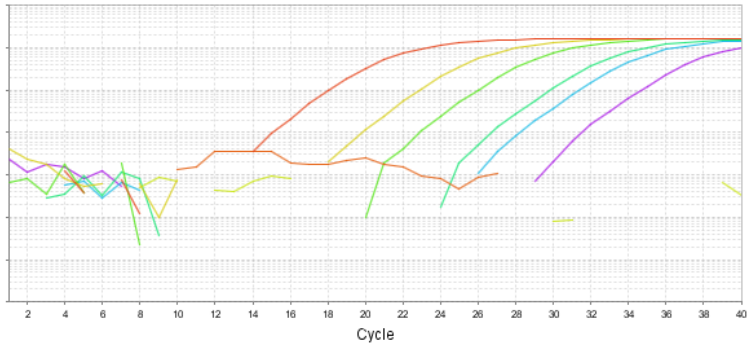

Figure 4.3 *T. primitivus* diagnostic against target DNA.

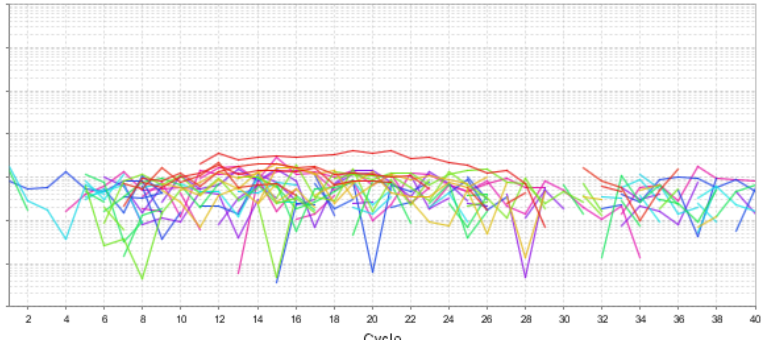

Figure 4.4 *T. primitivus* diagnostic against non-target DNA.

Analysis of the specificity of the *T. similis* diagnostic against target (*T. similis*) and non-target trichodorid species is shown below. A linear relationship was found between target *T. similis* DNA copy number and the cycle at which a Q-PCR signal was generated (Figure 4.5). For the *T. similis* diagnostic, minor cross-reaction was seen for *P. pachydermus* but at 1000-fold reduction (Figure 4.6) thus 1000 *P. pachydermus* would appear as a single *T. similis.*

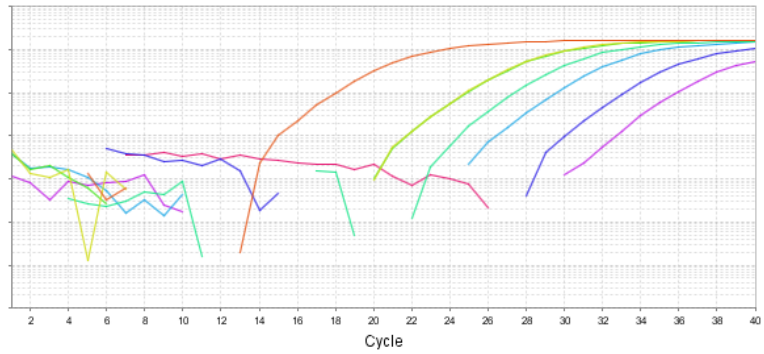

Figure 4.5 *T. similis* diagnostic against target DNA.

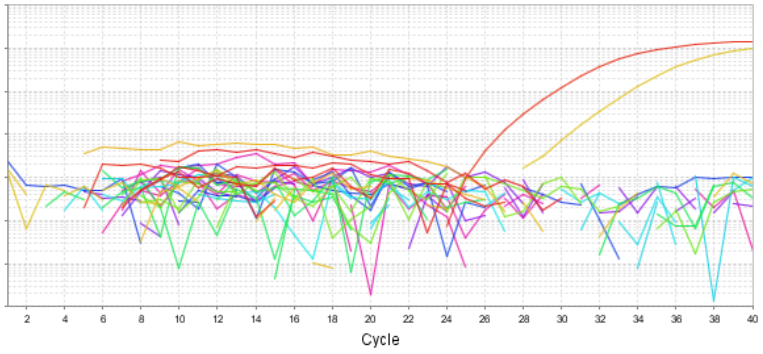

Figure 4.6 *T. similis* diagnostic against no-target DNA.

Analysis of the specificity of the *P. anemones* diagnostic against target (*P. anemones*) and non-target trichodorid species is shown below. A linear relationship was found between target *P. anemones* DNA copy number and the cycle at which a Q-PCR signal was generated (Figure 4.8). For the *P. anemones* diagnostic, minor signals are detected for other targets at a 10000-fold reduction but it is likely that this was simply the diagnostic giving slight self-complimentary reaction after 32 cycles (Figure 4.8).

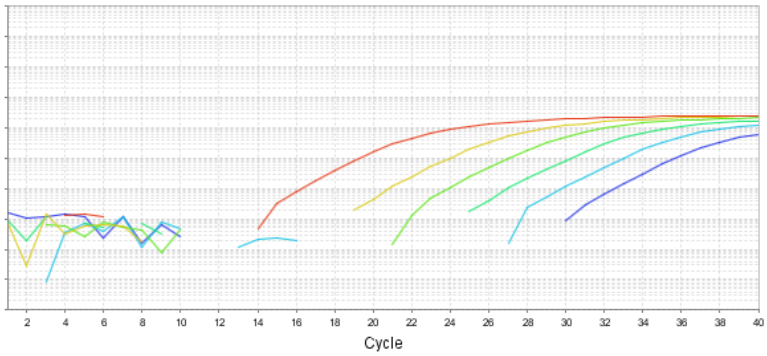

Figure 4.7 *P. anemones* diagnostic against target DNA.

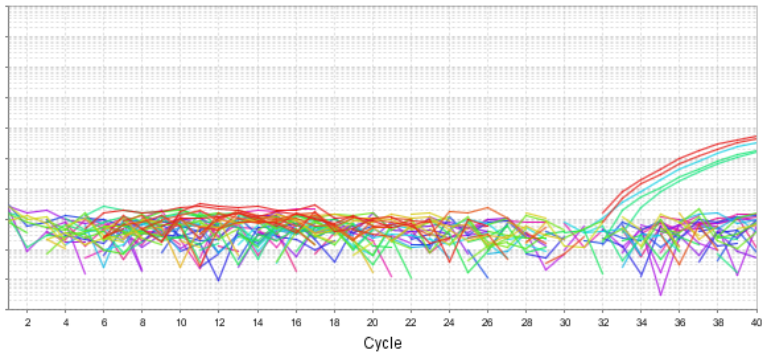

Figure 4.8 *P. anemones* diagnostic against non-target DNA.

Analysis of the specificity of the *P. pachydermus* diagnostic against target (*P. pachydermus*) and non-target trichodorid species is shown below. A linear relationship was found between target *P. pachydermus* DNA copy number and the cycle at which a Q-PCR signal was generated (Figure 4.10). For the *P. pachydermus* diagnostic, minor signals are detected for other targets at a 10000-fold reduction but it is also likely that this was simply the diagnostic giving slight self-complimentary reaction after 32 cycles (Figure 4.10).

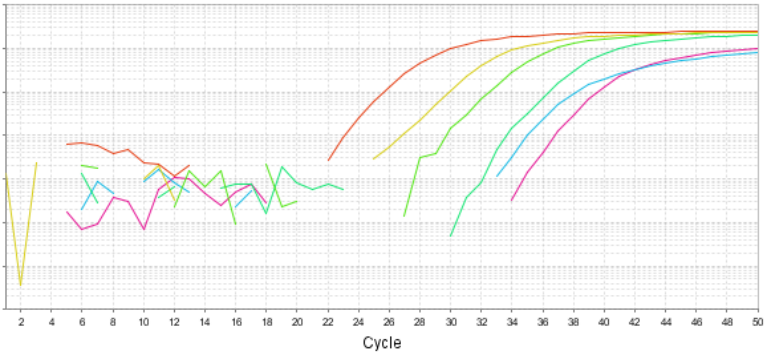

Figure 4.9 *P. pachydermus* against target DNA.

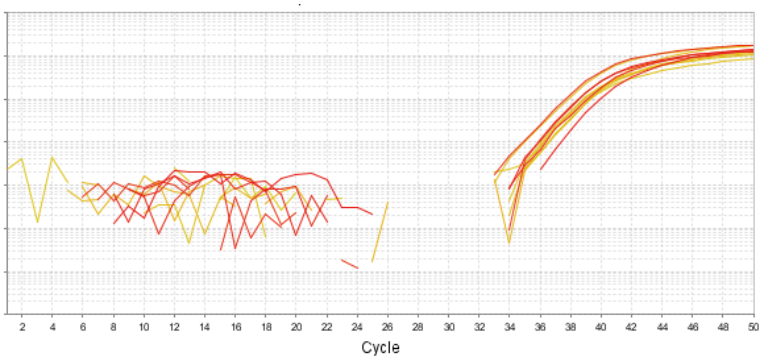

Figure 4.10 *P. pachydermus* against non-target DNA.

4.1.3.2. Calibrating the quantitative diagnostic to number of nematodes.

An important step in the design of any molecular diagnostic is to assess the number of copies of the gene target present in the target species. Without this, assumptions have to be made which can introduce errors leading to over or under estimation of the true abundance. Accurate estimations of target gene copy number were established for

three of the four target nematodes in this study (*T. primitivus, T. similis* and *P. pachydermus*).

Unfortunately, despite exhaustive efforts, it has not been possible to obtain specimens of *P. anemones* from any of the experimental sites within the project or numerous soil samples external to the project. This means that gene copy number has not been established for *P. anemones*, despite repeated attempts. This is consistent with *P. anemones* having a restricted geographic distribution within the UK, primarily the Vale of York (Boag & Alphey, 1977). Sites in Yorkshire were identified as likely sources of *P. anemones* and soil samples were obtained and tested. Signal for *P. anemones* was detected in one of these soil samples and additional 200 g sub-samples were processed and 150 trichodorids individually picked from each sub-sample. All individuals were identified as *T. primitivus*. This has been repeated with 6 soil samples but no individual *P. anemones* was discovered.

For the three other target species, gene copy numbers have been established and were within an order of magnitude of each other. In the absence of data for *P. anemones*, gene copy number for *P. anemones* was estimated as that for *P. pachydermus*. Whilst this does leave the Q-PCR diagnostic with a slight vulnerability, the restricted geographic distribution of *P. anemones* means that this assumption would only be invoked on few occasions.

Copy number for each target nematode was applied as a calibrator to convert data on the abundance of the target gene (determined by the Q-PCR diagnostic) into an estimation of target species.

4.1.3.3. Fidelity of scaling

Tubes containing 10, 20, 40 or 80 trichodorid nematodes were freeze-dried, DNA extracted and subjected to Q-PCR analysis to assess the abundance of trichodorids using the developed diagnostic. The relationship between the number of hand-picked nematodes and the Q-PCR diagnostic (replicated 3 times) was close to linear (Figure 4.11).

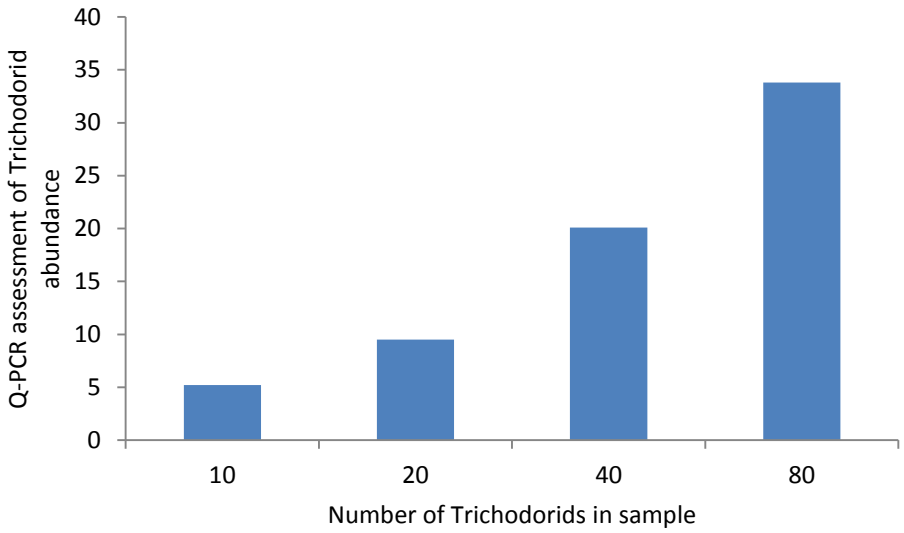

Figure 4.11. Fidelity of scaling of the trichodorid Q-PCR diagnostic determined by quantifying replicates of known numbers of trichodorid nematodes.

4.1.3.4. Validation of the Q-PCR diagnostic against soil samples.

In total, *c.* 2400 soil samples were used for the validation of the developed diagnostic. These included samples generated through the project as well as a large number of samples sent to The James Hutton Institute nematode diagnostic unit. This extensive validation process facilitated the identification of improvements to all aspects of the methodology and potential limitations of the diagnostic. All soil samples were first assessed by microscope and the abundance of trichodorids determined. Samples were processed as above and the abundance of each of the four trichodorid species determined using the Q-PCR diagnostic(s). An estimate of the total abundance of all four trichodorids was calculated and compared to the microscope-derived assessments as shown in Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15, Figure 4.16, Figure 4.17.

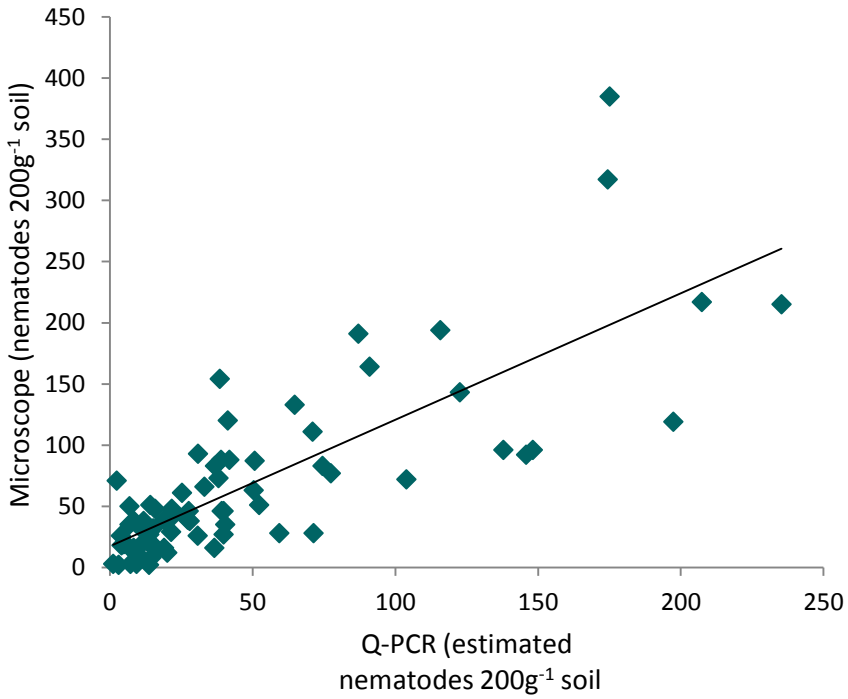

Figure 4.12. Comparison of estimations of total trichodorid abundance (200 g soil) supplied to JHI for commercial soil testing in 2012 by microscope and Q-PCR analysis.

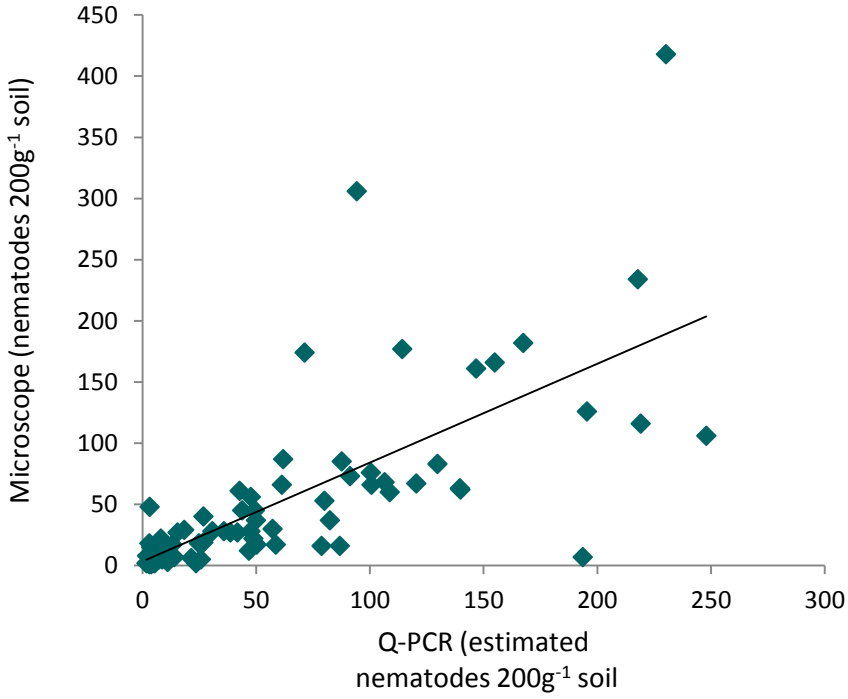

Figure 4.13. Comparison of estimations of total trichodorid abundance (200 g soil) supplied to JHI for commercial soil testing in 2013 by microscope and Q-PCR analysis.

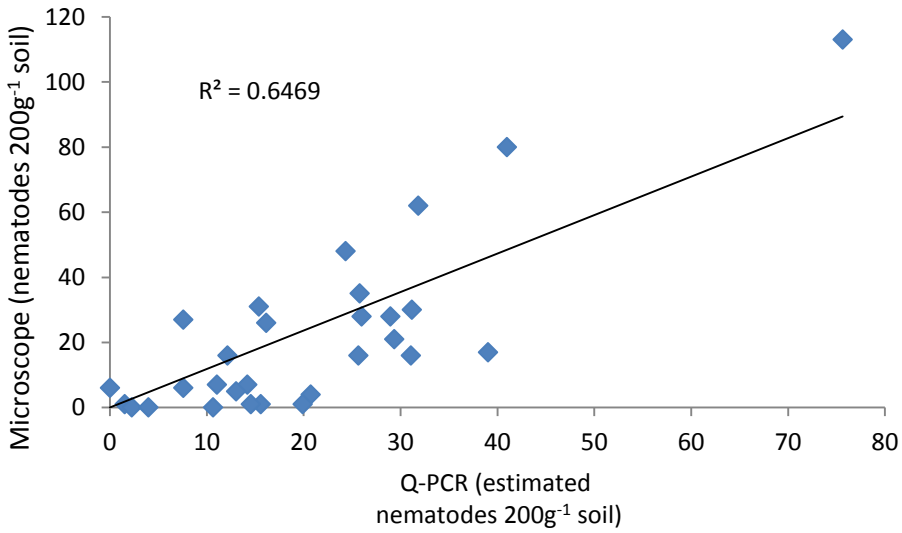

Figure 4.14. Comparison of nematode abundance of (*Para*)*Trichodorus* species derived from classical microscopy and estimated by Q-PCR molecular diagnostics from the 2014 Harper Adams alternatives experiment.

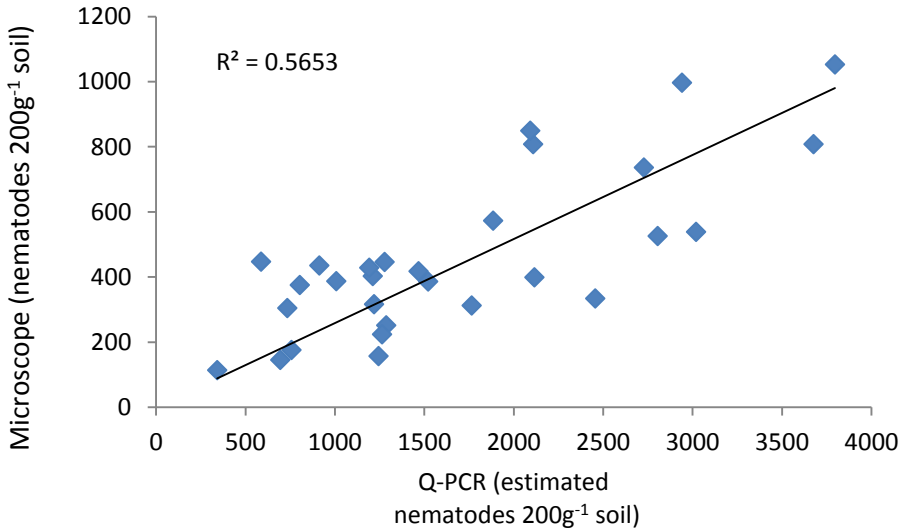

Figure 4.15. Comparison of nematode abundance of (*Para*)*Trichodorus* species derived from classical microscopy and estimated by Q-PCR molecular diagnostics from the 2014 Scotland alternatives experiment.

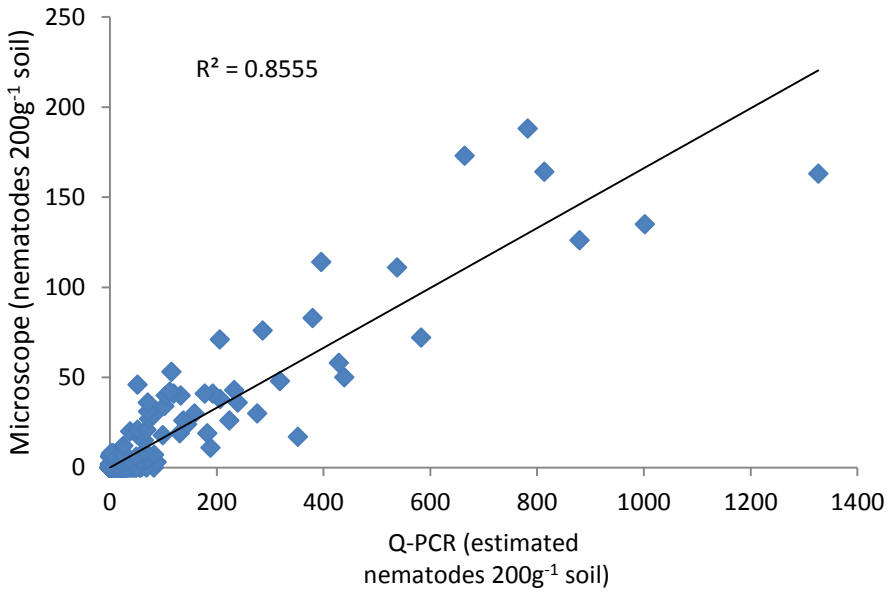

Figure 4.16 Comparison of nematode abundance of (*Para*)*Trichodorus* species derived from classical microscopy and estimated by Q-PCR molecular diagnostics from the soil samples sourced from the 2014 re-sampling of the East of Scotland farm network (n=350, Hawes *et al*., 2010). Samples from the East of Scotland farm network were not limited to potato growing fields and represent soil samples from a full arable rotation and the occasional grassland.

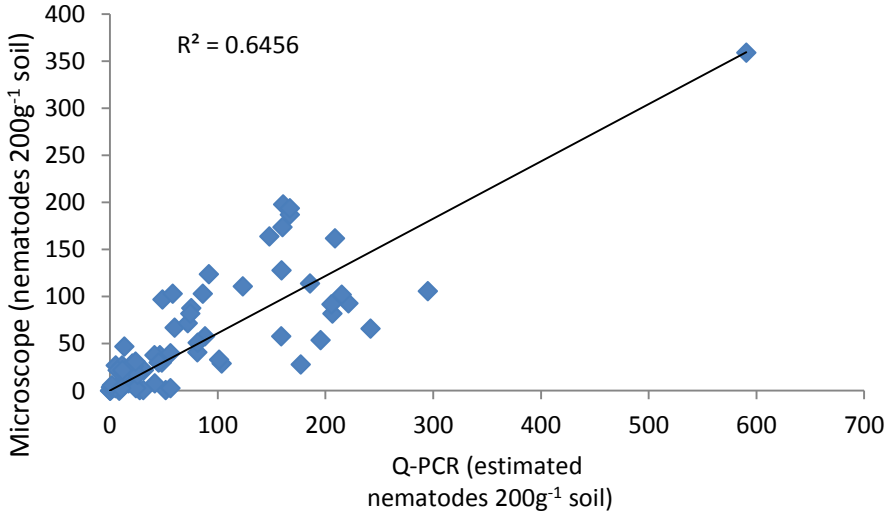

Figure 4.17. Comparison of estimations of total trichodorid abundance (200 g soil) supplied to JHI for commercial soil testing in 2014 by microscope and Q-PCR analysis.

4.1.4. Nematode sampling – how representative at planting?

The consortium technical group wished to explore whether sampling for FLPPN at planting was representative of the nematode burden during early growth stage of the potato crop. Consequently, 24 plots were sampled on two additional occasions at two sites (Harper Adams and Scotland). At Harper Adams, nematode abundance as a percentage of the abundance at planting was 86 % and 75 % for sampling date 1 and

2, respectively (Figure 4.18). In Scotland (Figure 4.19), the equivalent values were 103 % and 91 %, respectively. The lower abundance at Harper on sampling date 2 was likely to be a consequence of the onset of hot, dry weather. These values masked considerable variation at the plot level of both sites.

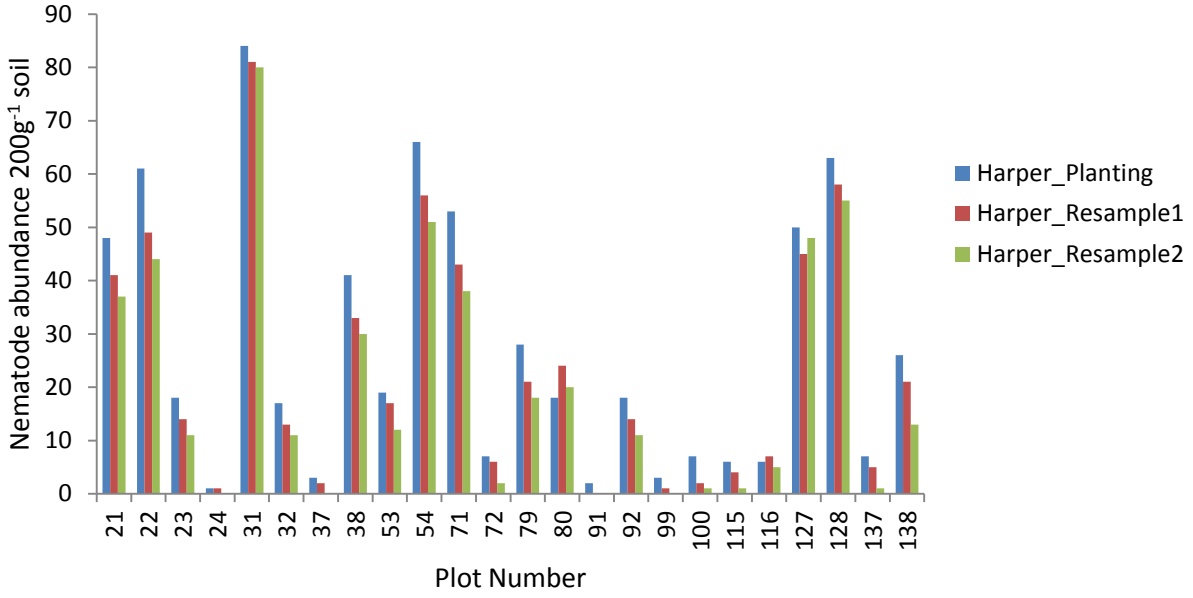

Figure 4.18. Nematode abundance of (*Para*)*Trichodorus* from selected plots at the Harper Adams main experimental site during planting and two subsequent sampling events.

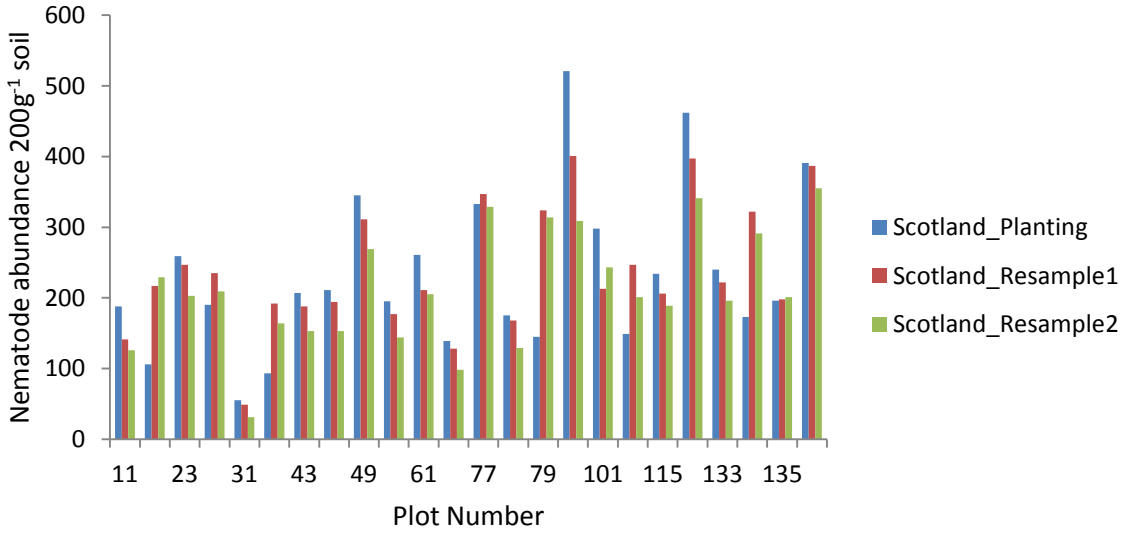

Figure 4.19. Nematode abundance of (*Para*)*Trichodorus* from selected plots at the Scotland main experimental site during planting and two subsequent sampling events.

4.1.5. Spatial distribution of (*Para*)*Trichodorus* at the main experimental sites

Trichodorids exhibited an aggregated distribution for all four main experimental sites during the period 2011-2013 (Figure 4.20, Figure 4.21, Figure 4.22, Figure 4.23, Figure 4.24, Figure 4.25). Across all sites and years, discrete foci were apparent emphasising the importance of an appropriate sampling strategy to capture the in-field variability of nematode abundance. The majority of the experimental sites had significant areas of the experimental area with relatively low and uniform abundance of trichodorids (Figure 4.20, Figure 4.21, Figure 4.22, Figure 4.23, Figure 4.24, Figure 4.25).

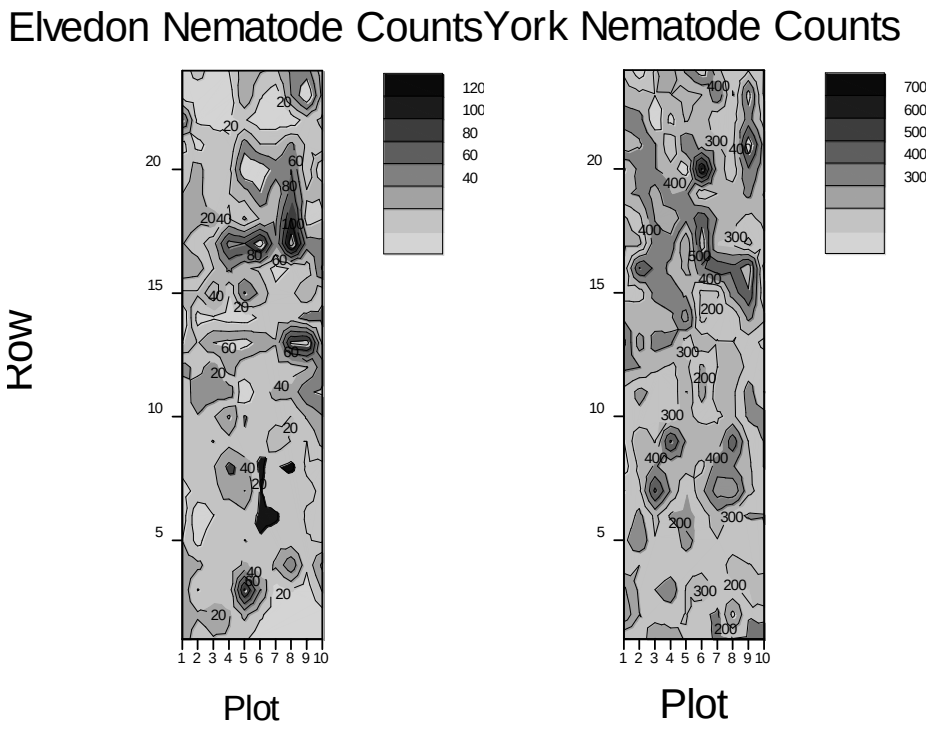

Figure 4.20. Spatial distribution of (*Para*)*Trichodorus* across the main experimental plots at a) Elvedon and b) York in 2011. Legend: nematode abundance expressed as 200 g^{-1} soil.

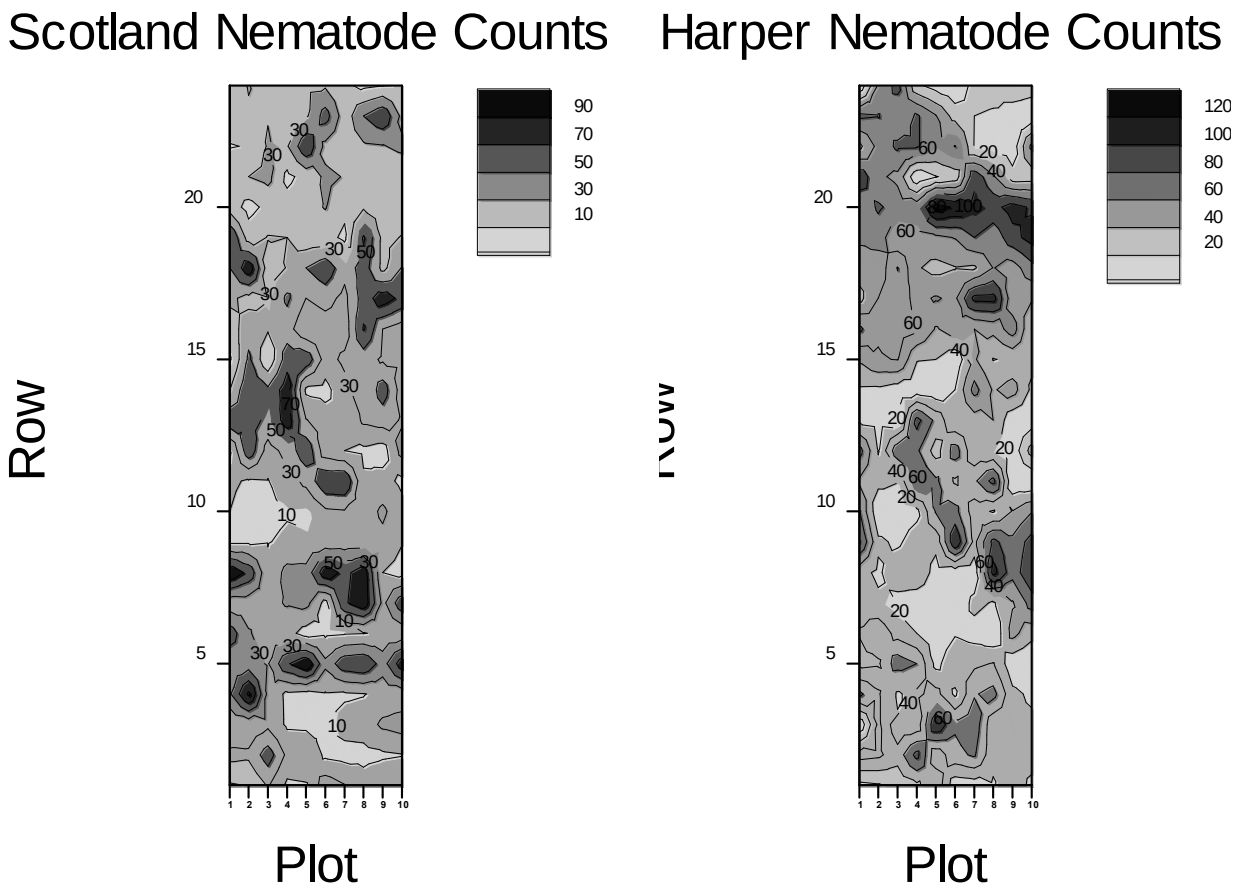

Figure 4.21. Spatial distribution of (*Para*)*Trichodorus* across the main experimental plots at a) Scotland and b) Harper Adams in 2011. Legend: nematode abundance expressed as 200 g^{-1} soil.

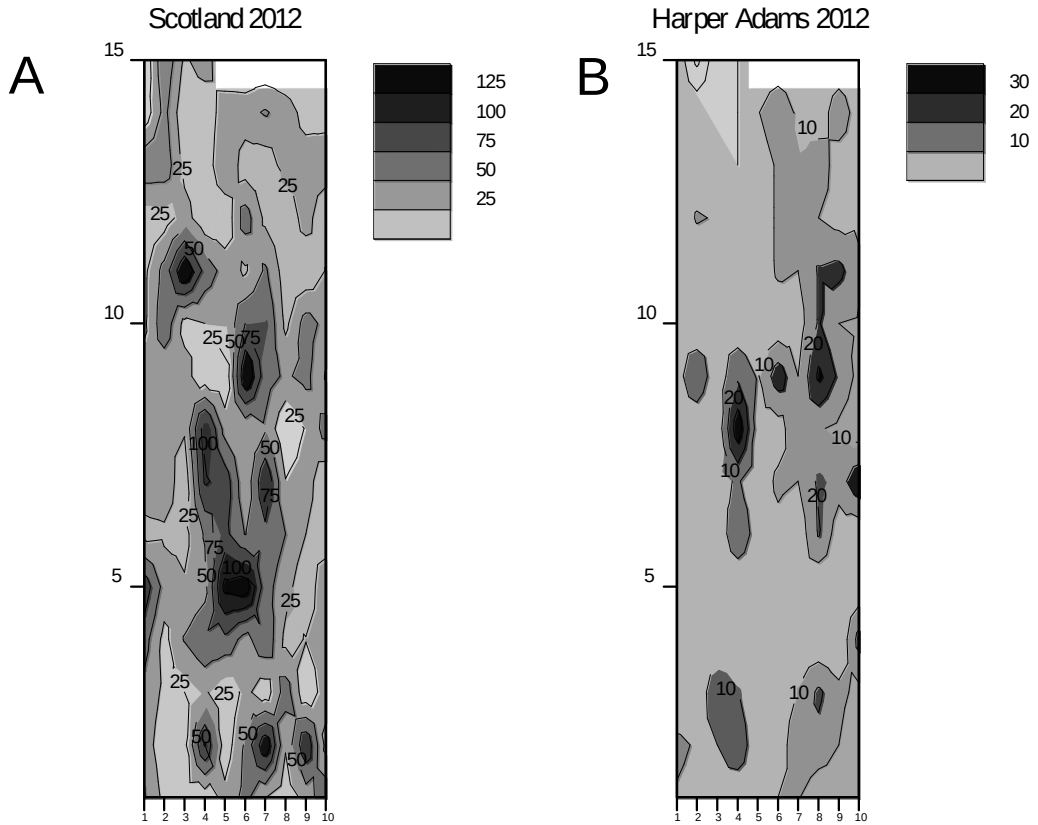

Figure 4.22. Spatial distribution of (*Para*)*Trichodorus* across the main experimental plots at a) Scotland and b) Harper Adams in 2012. Legend: nematode abundance expressed as 200 g^{-1} soil.

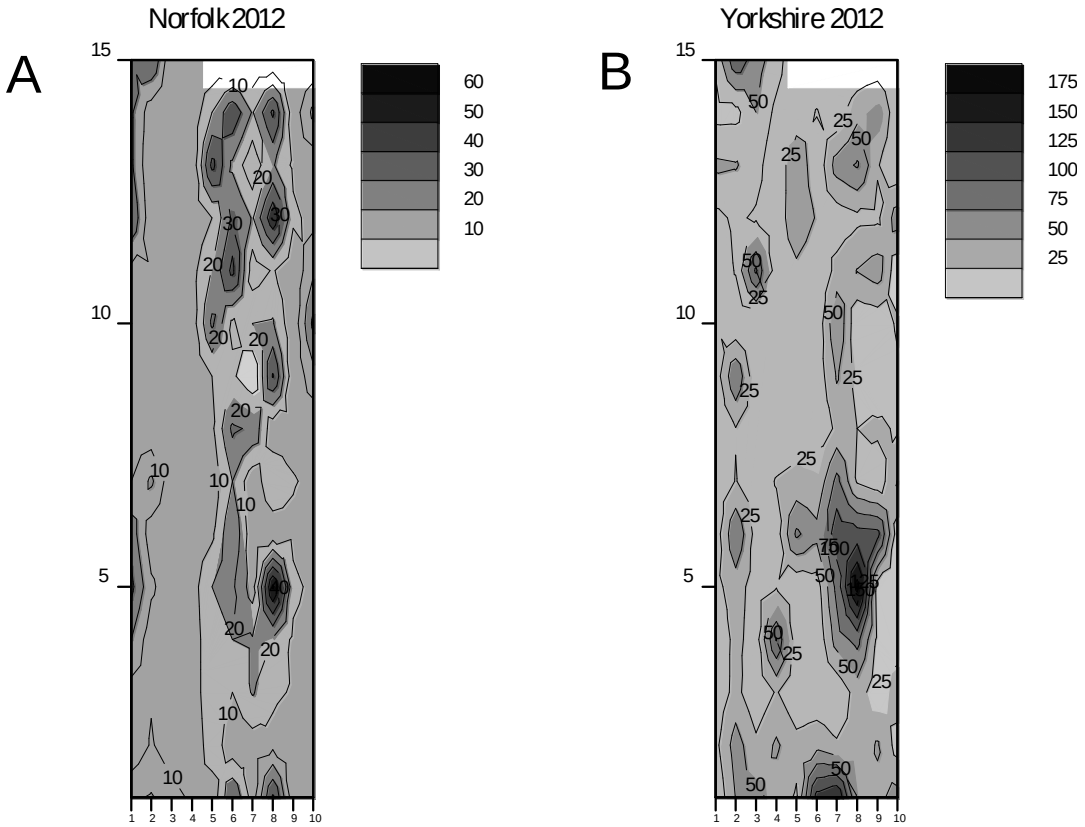

Figure 4.23. Spatial distribution of (*Para*)*Trichodorus* across the main experimental plots at a) Norfolk and b) Yorkshire in 2012. Legend: nematode abundance expressed as 200 g^{-1} soil.

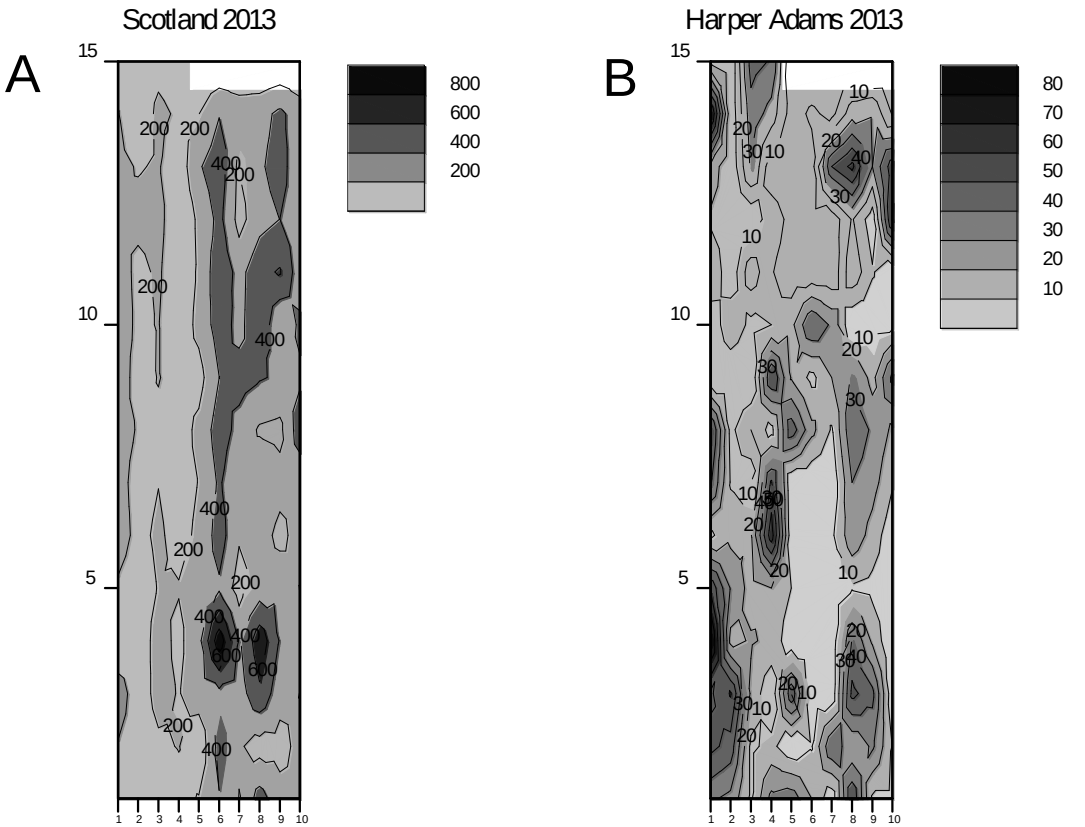

Figure 4.24. Spatial distribution of (*Para*)*Trichodorus* across the main experimental plots at a) Scotland and b) Harper Adams in 2013. Legend: nematode abundance expressed as 200 g^{-1} soil.

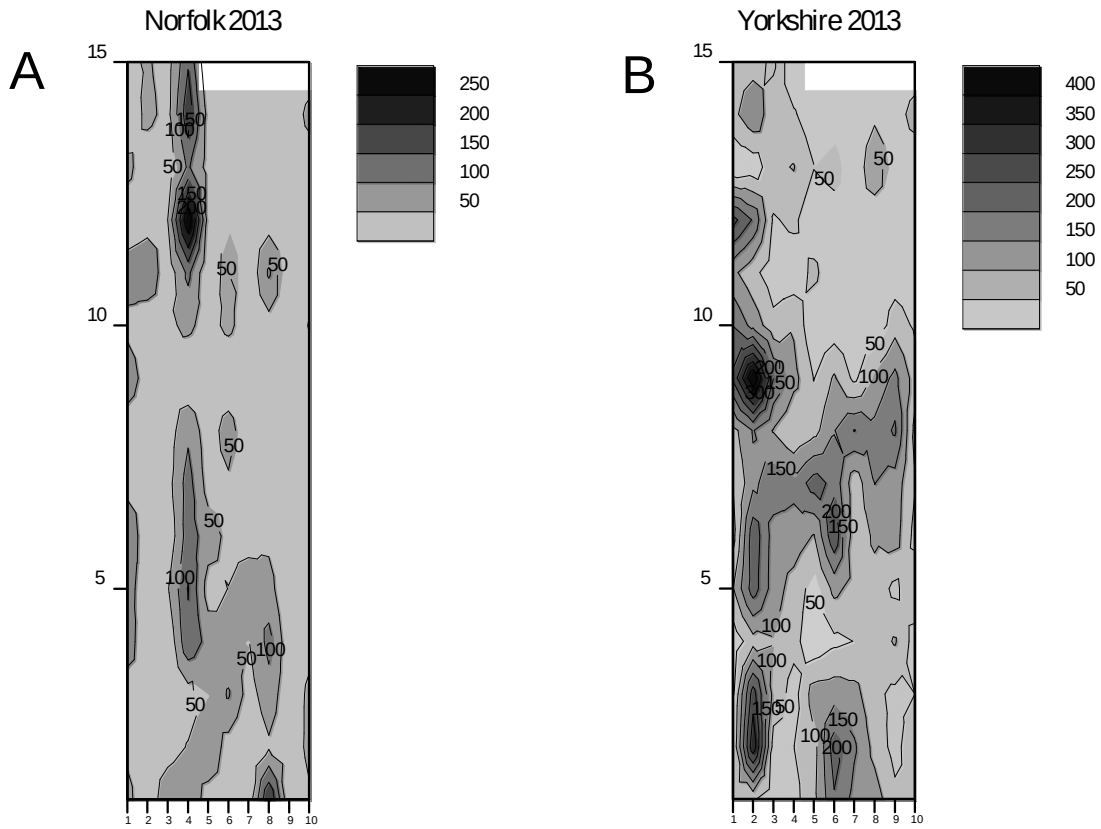

Figure 4.25. Spatial distribution of (*Para*)*Trichodorus* across the main experimental plots at a) Norfolk and b) Yorkshire in 2013. Legend: nematode abundance expressed as 200 g^{-1} soil.

5. DISCUSSION

Overall, the quantitative diagnostics developed during this project have proven to give results that align with results obtained by microscope identification. However, we do believe that a proportion of all samples processed by the molecular diagnostic should also be checked by microscope analysis (roughly 10 % is suggested) to monitor the process.

Whilst both the *Longidorus* genus-level diagnostic and the published species-level, *Pratylenchus penetrans* diagnostic both appear to be useful we recognise that they are not quantitative and can therefore give no information regarding abundance of these nematodes. If either species in the future becomes a major issue for potato production in the UK, for example, the onset of potato early dying disease mediated by *Pratylenchus penetrans*, a high throughput Q-PCR diagnostic may be required to be developed.

5.1. Considerations on use of molecular diagnostic as a proxy for microscopic identification and enumeration of nematodes

Identification of nematodes to species based on their morphology is a difficult task, and is especially difficult for juvenile stages. In addition, eggs/egg masses are not identified (and counted) by this method. A molecular diagnostic is based on detection of specific DNA sequences of target taxa. Successful detection is irrespective of developmental stage. This has a number of likely effects on the comparisons between microscopic and molecular data:

- Early juvenile stages that may not be recognised (and therefore not counted) by microscopy would generate a signal with the Q-PCR diagnostic.
- Eggs and egg-masses would also be detected by the Q-PCR diagnostic but not by classical microscopy.
- Gravid females would generate a stronger signal than a non-gravid female.
- Males containing sperm will generate a higher signal than non-gravid females or males without sperm.

The above would likely result in a signal generated by the Q-PCR diagnostic that would be greater than nematode abundance estimated by microscopy. However, the first three points explain how the Q-PCR diagnostic can ‘count’ the ‘potential level of nematodes’ in the soil sample, assuming all eggs and juveniles reach feeding-maturity and so is a good indicator of the potential risk from nematode (or nematode-mediated) damage.

We have identified a potential constraint associated with the Q-PCR diagnostic. During the validation process we have found consistency and agreement that at low nematode abundance (≤ 10 target nematodes 200 g^{-1} soil) results become stochastic. In terms of direct feeding this is not considered an issue. In terms of virus transmission the fact that the presence of a single target nematode is a problem further reduces the impact of this stochastic result.

To further complicate comparisons between microscope and molecular data, soil contains a number of compounds that can inhibit the activity of DNA polymerases and thus reduce the efficiency of any PCR. This could result in an artificially *lowered* signal

being generated with the Q-PCR diagnostic from a 'dirty' sample. Consequently, we modified a component of the extraction procedure to minimise where possible excessive dirt could enter the process and at each stage of the process post nematode extraction from soil we reviewed the experimental protocols. It was evident from the lack of extreme outliers during the validation process that the refinement of the protocols was successful.

The focus of this project was the four most prevalent virus-transmitting species (*T. primitivus, T. similis, P. anemones* and *P. pachydermus*) in the UK. However, other trichodorids (*P. teres, T. cylindricus T. velatus* and *T. virulferus*) have a limited UK distribution (Boag & Alphey, 1977). These non-target *Trichodorus* species would be counted by microscope analysis but would not be detected by the Q-PCR diagnostic.

Notwithstanding this, direct comparisons (Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15, Figure 4.16, Figure 4.17) showed a relationship between the counts derived from microscopy and the Q-PCR diagnostics, indicating that the Q-PCR diagnostic is a suitable proxy.

To date, Q-PCR diagnostics have been developed for sedentary endo- or semi endo-parasitic nematode species such as *Globodera, Heterodera* and *Meloidogyne* (Madani *et al*., 2008; Goto *et al*., 2011; de Weerdt *et al*., 2011; Lopez-Nicora *et al*., 2012). However to our knowledge, we are unaware of a published nematode Q-PCR diagnostic that has been calibrated against gene-copy number to provide robust data with regard to estimated nematode abundance. Thus, previous studies have at worst demonstrated an enhanced capability to provide a presence/absence indicator or at best coupled with presence/absence data been able to suggest that nematode abundance in one sample was greater or less than that in another sample. Here we report one of the first Q-PCR diagnostics for an economically important group of free-living parasitic nematodes, namely trichodorids that is calibrated against gene-copy number and thus aims to provide a true quantitative measure of abundance.

As outlined above, WP1 undertook to test the efficacy of currently published primers for *Pratylenchus penetrans*. The published primers are specific for *P. penetrans* and were developed for standard PCR protocols, i.e. they are not quantitative thus they only provide information on presence/absence. *Pratylenchus* species are semi-endoparasitic thus they have a component of their life cycle within the plant host. The wound that is caused when the nematode enters the plant is a site for potential subsequent *Verticillium dahliae* infection which leads to potato early dying disease. This contrasts with trichodorids whose life cycle is fully external of the plant host. This leads to significant implications for the sampling of *Pratylenchus*. If sampling is poorly timed at the point when the nematodes are within the plant host, only sampling the soil may generate a false negative. Thus correct protocols for sampling for *Pratylenchus* include taking plant samples for subsequent nematode extraction. However due to health and safety reasons concerning *Legionella* contamination the relevant equipment for extracting nematodes from plant parts has been removed from UK nematology laboratories. Thus soil sampling is only available for large scale sampling of *Pratylenchus*. However, it is possible at low scale (petri-dish) to establish *Pratylenchus* extraction from plant parts. Hence, irrespective of the efficacy of any diagnostic the key is accurate timing of sampling to ensure that the nematode is not within the plant host.

Notwithstanding these important biological issues, we have tested the published primers and they appear to work well for UK populations of *P. penetrans*. We conducted confirmatory sequencing from individual nematodes that were putatively described as *P. penetrans* and yielded a diagnostic band when using the published primers.

6. CONCLUSIONS

iv) Modification and refinement of the protocol post nematode extraction from soil has removed the presence of extreme outliers. The inclusion of control systems to allow identification of samples where external factors have influenced DNA-extraction efficiency or PCR efficiency has further reduced the impact of 'dirty' samples.

v) A consistent relationship between microscope derived trichodorid counts and Q-PCR data has been maintained during all validation rounds however, a key post-project task is to establish the gene copy number for *P. anemones*.

vi) On the whole Q-PCR data is greater than manual counts due to being more sensitive and thus unlike manual counts includes eggs, sperm and gravid females.

vii) Published primers for a standard, non-quantifiable, *P. penetrans* molecular diagnostic have been successfully tested against UK populations of *P. penetrans*. However, a caveat is required with respect to the correct timing of sampling for *P. penetrans* given that it is a semi-endoparasitic species.

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