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Development and validation of four TaqMan real-time PCR diagnostics for the identification and quantification of *Pratylenchus crenatus*, *Pratylenchus neglectus*, *Pratylenchus penetrans* and *Pratylenchus thornei*

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Summary

Pratylenchus crenatus, *P. neglectus, P. penetrans* and *P. thornei* are globally the most commonly occurring species of root-lesion nematodes (RLN). Correct identification and quantification of these nematodes is important for strategic management interventions such as rotation choice and nematicide use. A real-time quantitative PCR can provide a fast and reliable alternative to morphological identification which requires significant taxonomic experience. A TaqMan hydrolysis probe method based on the 28S rDNA D2-D3 expansion region was developed and validated for the identification and quantification of these four root-lesion nematode species. Primers and TaqMan probes were designed for each species target. Standard curves for each target RLN species were generated by plotting known gene copy number, obtained by a 10-fold serial dilution of purified plasmids, with corresponding Ct values. Gene copy number ranged from 10^7 to 10^1, with a copy number of 10 being the lowest measurable standard. Each standard curve had a strong linear correlation ($R^2 \geq 0.9901$) between Ct value and gene copy number. There was consistent amplification of samples with target species from different geographic locations within the UK, whereas a lack of amplification was noted for selected non-target species *P. coffeae, P. pseudocoffeae, P. vulnu*s, *P. fallax*, *Globodera rostochiensis, Meloidogyne hapla, Trichodorus primitivus* and *Bitylenchus hispaniensis*. Specificity and sensitivity of the methods were confirmed by three experiments that explored different life stages, increasing the number of target species and had mixed *Pratylenchus* samples. Finally, estimates obtained by qPCR methods were compared with counting carried out by microscopy showing a good correlation ($R^2 = 0.789$). The TaqMan real-time PCR developed in this study provides a highly specific, fast and accurate quantification of *P. crenatus, P. neglectus, P. penetrans* and *P. thornei*.

Keywords: copy-number, D2-D3 expansion fragment, probe, root-lesion nematode.

Root-lesion nematodes (*Pratylenchus* spp.) are migratory endoparasitic nematodes, infecting a wide range of globally grown crops, including potato (Castillo and Volvas, 2007; Orlando *et al*., 2020a). Classical morphological identification using microscopy is time consuming and requires specialized training (Powers, 2004; Palomares-Rius *et al*., 2014). Moreover, morphological identification using light microscopy is not always possible due to minimal specimen recovered, intra- and inter-species taxonomic variability, and the presence of several *Pratylenchus* species isolated from the soil sample (Janssen *et al*., 2017 a,b). Thus, to overcome these issues and speed up the identification process a DNA-based diagnostic offers a potential solution, especially important for a high sample throughput phytosanitary laboratory.

While TaqMan real-time PCR offers a highly specific diagnostic, there are known limitations including false-positive reactions, DNA fragments from unknown species, or false-negative reactions due to intra-individual variation (Schrader *et al*., 2012). Several real-time PCR protocols for quantitative identification of several RLN species have been developed including for *P. crenatus* (Oliveira *et al*., 2017), *P. penetrans* (Sato *et al*., 2007, 2010; Yan *et al.*, 2008; Goto *et al*., 2011; Mokrini et al., 2013; Oliveira *et al*., 2017; Bandoo *et al*., 2017; Dauphinais *et al*., 2018), *P. thornei* (Yan *et al*., 2012; Mokrini *et al*., 2014; Lin *et al*., 2020), *P. neglectus* (Yan *et al.*, 2008, 2013; Oliveira *et al*., 2017; Lin *et al*., 2020), *P. vulnus* (Fanelli *et al*., 2014), *P. scribneri* (Arora *et al*., 2020) and *P. zeae* (Berry *et al*. 2008). The D2-D3 expansion segment of the large subunit 28S rDNA has previously been selected as a molecular marker for phylogenetic relationships to distinguish between closely related species of *Pratylenchus* giving a better resolution for species separation (Al-Banna *et al*., 1997, 2004; De Luca *et al*., 2004, 2010, 2011; Subbotin *et al*., 2008; Janssen *et al*., 2017 a, b). Consequently, this region seems to be promising molecular marker for the detection of *Pratylenchus* species by real-time PCR (Bandoo *et al*., 2017; Dauphinais *et al*., 2017; Lin *et al*. 2020). The aim of this work was to develop and validate a TaqMan real-time PCR method focusing on the D2-D3 expansion fragment for identification and quantification of *P. penetrans, P. crenatus, P. neglectus* and *P. thornei*.

MATERIALS AND METHODS

Nematode populations and cultures of Pratylenchus *spp.*

Initial populations of *P. penetrans* (PP), *P. thornei* (PT) and *P. crenatus* (PC) were obtained from established carrot-disk cultures supplied by ILVO (Belgium). *Pratylenchus neglectus* (PN) was extracted from potato soils (Norfolk, UK) and *P. thornei* (PTU) from soil samples taken from a field site at Harper Adams University (UK), where winter beans (*Vicia faba*) were grown. Nematodes were

93 extracted from infested carrot discs and soil samples using the Whitehead tray extraction method
94 (Hooper, 1986) and then sub-cultured following the method described by Speijer and De Waele
95 (1997) for monoxenic culture of *Pratylenchus* spp. in carrot discs. Nematodes were morphologically
96 identified under a light microscope (Zeiss Axiolab, ZEISS, Germany) at 100 X magnification using
97 taxonomic keys and monographs (Castillo and Vovlas, 2007; Geraert, 2013). Photographs of each
98 specimen were taken for identifying morphological key characteristics. Several other DNA samples
99 and specimens, obtained from different locations and sources, were included for validation assays
100 (Table 1). Single specimens from each population were then transferred into 1 mL Eppendorf tubes
101 for DNA extraction and PCR.

102 *DNA extraction*

103 Individuals of each *Pratylenchus* species were used for DNA extraction. Each specimen was
104 hand-picked with a sterilised needle under a stereomicroscope and transferred into a 200 µl
105 Eppendorf, containing 18 µl of 1X PCR buffer (GoTaq, Promega, UK). Four to five 1 mm glass beads
106 were added into each tube and homogenised in a Retsch M300 tissue disruptor for 30 seconds at 30
107 Hz. Then, 4 µl of proteinase K (100 µg ml^{-1}) and 1 µl of 1X PCR buffer were added to each tube.
108 Tubes were incubated at 60°C for 60 min, 95°C for 15 min and 10°C for 10 min. After DNA
109 extraction, tubes were centrifuged at 16,000 *g* (Heraeus Pico 17 Ventilated Microcentrifuge, Thermo
110 Fisher Scientific) and stored at −20°C.

111 *Conventional PCR and direct sequencing*

112 To confirm the morphological identification of the target nematodes, the D2-D3 expansion
113 fragment of 28S rDNA was amplified using the universal primers D2A (5'-ACA AGT ACC GTG
114 AGG GAA AGT TG-'3) and D3B (5'-TCG GAA GGA ACC AGC TAC TA'3) (De Ley *et al*., 1999)
115 and sequenced. For direct sequencing, each PCR reaction contained: 5X PCR GoTaq Buffer
116 (Promega, UK), 12.5 mM of each dNTP (Promega, UK), 0.4 mM of each primer, 1 unit of GoTaq
117 Polymerase (Promega, UK), 2 µl of DNA and double sterile water for a total volume of 15 µl for each
118 PCR reaction. PCR reactions were run on a G-Storm GS1 Thermal Cycler (Akribis Scientific
119 Limited) and conditions were: denaturation at 95°C for 5 min followed by 35 cycles of denaturation
120 at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension was
121 performed at 72°C for 5 min. PCR products were separated and visualised with Sybr Safe
122 (Thermofisher) on a 1% agarose gel. ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher
123 Scientific) was used for enzymatic clean-up of amplified PCR products according to the
124 manufacturer's instructions and incubated at 37°C for 30 min followed by 10 min at 80°C and 5 min

125 at 10°C. PCR products were sequenced by the James Hutton Institute in-house sequencing service, in
126 one direction using the D2A forward primer.

127 *Conventional PCR and Cloning*

128 To generate products for cloning, PCR reactions were prepared with 1X PCR High Fidelity
129 buffer (Invitrogen, UK), 2 mM $MgSO_4$ (Invitrogen, UK), 0.2 mM of each dNTP (Promega, UK), 0.4
130 mM of each primer, 1 unit of High-Fidelity Platinum Taq polymerase (Invitrogen, UK), 2 µl of DNA
131 and double sterile water for a total volume of 15 µl for each PCR reaction. PCR conditions were:
132 denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at
133 55°C for 30 s and extension at 68°C for 1 min. A final extension was performed at 68°C for 5 min.
134 PCR products were purified with gel purification using the QIAquick Gel extraction Kit (Qiagen)
135 according to the protocol listed by the manufacturer. Before ligation, additional dATP was added to
136 each purified PCR product to enhance the ligation into the vector: 2 µl GoTaq Buffer (5X), 2 µl dATP
137 (0.2 mM), 1 µl GoTaq Flexi Polymerase, 4 µl purified PCR products and nuclease-free water to a
138 final volume of 10 µl; samples were incubated at 72°C for 15 min. Two µl of purified PCR products
139 were ligated into pGEM-T Easy® vector (Promega, UK) following the manufacturer's protocol.
140 Resulting plasmids were transformed into *Escherichia coli* DH5α competent cells (Invitrogen, UK).
141 White colonies with recombinant plasmids were selected and DNA purified using QIAprep Spin
142 Miniprep kit (Qiagen, UK) according to the manufacturer's instructions. Purified plasmids were
143 sequenced using universal M13F and M13 primers (Messing, 1983), in forward and reverse direction
144 by the James Hutton Institute in-house sequencing service. Contigs were assembled using DNA baser
145 sequencing assembler (http://www.dnabaser.com). Each D2-D3 sequence obtained was then
146 compared with published reference sequences, mainly from Subbotin *et al*., (2008) and Janssen *et al*.,
147 (2017a,b). Sequences had 98-100% similarity with reference sequences for each species.

148 *Primers and probe design*

149 All sequences generated in this study, along with sequences of D2-D3 expansion fragment of
150 28S rDNA for *Pratylenchus* spp. are available in the GenBank database and were used to generate an
151 alignment of 667 sequences (Supplementary results) using ClustalW (http://www.genome.jp/tools-
152 bin/clustalw) for the detection of potential conserved regions useful for primers and probe design.
153 New reference sequences (with associated GenBank accession numbers) are provided in
154 Supplementary results (Table S1). Jalview Version 2 was used to visualize variable regions among
155 species (Waterhouse *et al*., 2009). Four primer/probe sets for each target species were designed using
156 the genscript on-line tool for real-time PCR TaqMan primer design
157 (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool) and synthesised by

158 PrimerDesign, Southampton, UK. Probes were labelled at the 5'-end with FAM reporter dye and at
159 the 3'-terminal with TAMRA quencher.

160 *Real time PCR optimisation*

161 A gradient of annealing temperatures from 58°C to 70°C was tested using a Biorad CFX96
162 Touch Real-Time PCR Detection System (Biorad). Combinations of different concentrations of
163 primers (0.3 µM, 0.6 µM and 0.9 µM) and probe (0.25 µM and 0.5 µM) were also tested to determine
164 the optimal conditions for specificity and efficiency of each assay.

165 *Standard curves*

166 The DNA concentration of each plasmid was measured by spectrophotometer (Beckman
167 Coulter DU 640 spectrophotometer) and used to calculate the corresponding number of copies of each
168 target using an online tool gene copy number and dilution calculator (http://scienceprimer.com/copy-
169 number-calculator-for-realtime-pcr) based on the sum of length of the p-GEMT vector (3015 bp) and
170 our DNA inserts (~ 780 bp). Gene copy number for each plasmid was calculated with the following
171 formula reported by Daniell *et al.* (2012):

172 $$\text{Number of copies} = (\text{amount (ng)} * 6.022\text{x}10^{23}) / (\text{length (bp)} * 1\text{x}10^{9} * 650)$$

173 A pre-dilution in 10mM Tris (pH 8.0) of each plasmid generated a concentration stock of 1 x 10^{10}
174 copies μl^{-1}. Two microlitres of this pre-dilution stock was linearized by digestion with 2 µl of *NotI*
175 enzyme (Promega, UK) in 2 µl of buffer and 16 µl sterile UV-treated water and incubated at 37°C
176 for 90 min and at 65°C for 15 min (Daniell *et al*., 2012), to obtain a final concentration of 1 x 10^{9}
177 copies μl^{-1}, for each plasmid. A serial dilution ranging from 10^{8} to 10^{1} copies μl^{-1} in 10mM Tris (pH
178 8.0) was prepared in triplicate and used to generate standard curves for real time PCR. These standards
179 were included in all real-time PCR reactions to allow quantification of the target nematodes.
180

181 *Real-time PCR*

182 PCR amplifications were performed initially using a StepOnePlus Real-Time PCR System (Applied
183 Biosystems) for development of methods, and thereafter a Biorad CFX96 Touch Real-Time PCR
184 Detection System (Biorad) for the optimization and validation of methods. Each reaction contained:
185 10 μl SensiFast Probe Hi-Rox Mix (Bioline Reagents), 0.25 μM of probe, 0.6 μM of primers, UV-
186 treated sterile distilled water to give a total volume of 18 µL. Additional primers were required to
187 enhance the specificity of *P. penetrans* assay, using the same concentration 0.6 μM for each primer.
188 Thereafter, 2 μL of template DNA were added to each reaction. The amplification conditions were:

95˚C for 3 min followed by 35 cycles at 95°C for 10 s with 68-69°C for 60 s. Positive controls with plasmids and negative controls with sterilized water were included for each assay performed.

Specificity of primers and probe to detect each species target

After optimization of annealing temperature, primer and probe sets were tested for their specificity. Eight different *Pratylenchus* spp. were used (*P. crenatus*, *P. penetrans, P. thornei, P. neglectus, P. coffae, P. pseudocoffae, P. vulnus* and *P. fallax*), as well as other non-target nematode species (*Globodera rostochiensis, Meloidogyne hapla, Trichodorus primitivus* and *Bitylenchus hispaniensis)*. Each sample was tested in triplicate. Reactions and amplification conditions were as described above.

Specificity and sensitivity of each diagnostic PCR

Three different tests were performed to test the specificity of primers and probe for detection of the four target RLM species, *P. crenatus, P. neglectus*, *P. penetrans* and *P. thornei*.

Test 1. The specificity of each target species in samples with mixed species was tested. One female of *P. penetrans, P. neglectus, P. crenatus* and *P. thornei* was hand-picked with a sterilised needle and transferred to a 500 µL Eppendorf tube. The same procedure was applied for preparing samples with 10 females of each species in separate tubes per species and negative controls without species target. Positive controls were also prepared with one and ten females of pure species target per tube. Five replicates for each species were prepared for DNA extraction and real-time PCR. Negative controls (n=3) were included using 2 µL distilled water as a replacement for DNA template.

Test 2. The sensitivity of each diagnostic PCR was tested with different life stages of each target species. Each specimen was hand-picked with a sterilised needle and transferred individually to a 500 µL Eppendorf tube containing 20 µL of 10X PCR buffer for DNA extraction. Ten replicates for each life stage (juveniles, females and males) were hand-picked with a sterilised needle as before and transferred into a 200 µl Eppendorf, containing 18 µl of 1X PCR buffer (GoTaq, Promega, UK). Real-time PCR was performed following the real-time PCR conditions as detailed above. Gene copy number per life stage was estimated by multiplying gene copy number obtained for each reaction with the total volume of each sample. Mean and standard error of the mean (S.E.M.) were calculated for adults and juveniles. Gene copy number per individual was estimated based on the average gene copy number for adults and juveniles. Negative controls were as described for Test 1.

Test 3. The sensitivity of each species diagnostic was tested with increasing numbers of target *P. penetrans, P. thornei* and *P. crenatus* in separate Eppendorf tubes, with three replicates of each. For

P. penetrans one, ten, 100 and 1000 females were prepared in separate tubes, whereas for *P. thornei* one, ten and 1000 and one, ten and 50 nematodes for *P. crenatus*. Negative controls were as described for Test 1.

Comparison of real-time PCR quantification with microscope counts

Fifteen soil samples from potato fields were used. Briefly, nematode suspensions were counted using a binocular microscope at 40 X magnification (Mazurek Optical Service, Meiji EMT) before the contents were transferred to a 2 mL Eppendorf tubes and freeze dried. DNA was extracted using a Purelink DNA (Invitrogen, UK) extraction kit. All four TaqMan PCR assays were performed on each DNA sample. The estimated total number of each species per sample was calculated by dividing the total DNA copy number per sample with the average DNA copy number per individual for each species. The total estimated number of *Pratylenchus* per sample was the sum of qPCR numbers for each of the 4 target-species present in the sample. Microscope counts were then compared with real-time PCR data.

Statistical analysis

Ct values and gene copy number for each test were subjected to a two-way ANOVA with Tukey's test ($P < 0.05$). Data obtained by microscopy and by real-time PCR were analysed with a linear regression model. All statistical analyses were performed using Genstat (19th edition, VSN International).

RESULTS

Primer/probe design for P. penetrans*,* P. thornei*.* P. neglectus *and* P. crenatus *PCR diagnostic*

A total of 121 new sequences were generated in this study and coupled with sequences obtained from Genbank were used to form an alignment of 667 sequences. Based on this alignment, primer and probe sets for *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei* were designed (Table 2). Based on the Ct values, the optimal annealing temperature for *P. crenatus* and *P. penetrans* was 68°C, whereas it was 69°C for *P. neglectus* and *P. thornei* (data not shown). Standard curves for all four target species, *P. penetrans*, *P. neglectus*, *P. thornei* and *P. crenatus* had a strong linear correlation ($R^2 > 0.9901$) between cycle threshold and gene copy number, with a limit of detection of 10 copies μL^{-1} (Figure 1 A-D). No amplification was detected for either negative controls, samples with non-species targets or water (NTC).

Specificity of primers and probe to detect each species target

Based on Ct values no inter-population variability was detected for three of the target species, *P. penetrans*, *P. neglectus* and *P. thornei* ($P > 0.05$). However, for *P. crenatus* a sample from Scotland (code 452) had a Ct value significantly lower ($P = 0.037$; 28.8 ± 0.12) than a population from England (30.1 ± 0.3; code PC). No significant differences were observed between sample 452 and another Scottish sample (Je2), nor between Je2 and PC. No amplification was detected for either non-target *Pratylenchus* spp. (*P. penetrans, P. thornei, P. neglectus, P. coffae, P. pseudocoffae, P. vulnus* and *P. fallax*) or other nematode species tested (*B. hispaniensis, G. rostochiensis, M. hapla, T. primitivus*).

Specificity and sensitivity of diagnostic assays

Test 1 – Specificity of primers and probe in mixtures of Pratylenchus spp.

A single female *P. penetrans* was detected ($Ct = 29.8 \pm 0.09$) in a mixture comprising of the other three target species (*P. crenatus*, *P. neglectus* and *P. thornei*), with no significant differences ($P > 0.05$) compared to samples of one individual *P. penetrans* ($Ct = 30.8 \pm 0.6$) (Table 4). The same pattern was also reflected for ten specimens of *P. penetrans* when combined with a mixture of 30 individuals of the other three species (Table 4). Similar results were obtained for *P. thornei* and *P. neglectus* (Table 4). However, one female of *P. crenatus* in a mixture of *P. neglectus*, *P. thornei* and *P. penetrans* had Ct values that were significantly greater ($P < 0.05$) than Ct values from a single *P. penetrans*. All samples with mixed non-target species and negative controls did not amplify.

Test 2 – Amplification of different life stages

Mean gene copy number per individual of each *Pratylenchus* species were: 7775 ± 199 for *P. crenatus*, 9555 ± 297 for *P. penetrans*, 5292 ± 266 for *P. neglectus* and 3624 ± 109 for *P. thornei* (Table 5). Ct values for each life stage were similar among the different species showing gene copy number in female specimens was significantly greater than juveniles ($P < 0.05$). *Pratylenchus penetrans* was the only species with males and consequently their presence with the inclusion of sperm may have this increased the overall copy number for individuals compared to the other tested *Pratylenchus* species.

Test 3 – Assessing the response of the quantitative assay over increasing target abundance

The sensitivity and linearity of the assay was tested with increasing numbers of nematodes for *P. penetrans* (1, 10, 100, 1000 individuals) (Figure 2), and a strong linear relationship ($R^2 = 0.998$) was found between number of nematodes and cycle thresholds. Due to limited numbers of individuals

283 available from cultures, a maximum of 50 and 100 individuals were used in the same test for *P.*
284 *crenatus* and *P. thornei*, respectively. As with *P. penetrans*, strong linear relationships were also
285 found between nematode number and Ct values for *P. thornei* (Figure S2; R^2=0.97) and *P. crenatus*
286 (Figure S3; R^2=0.972).*Pratylenchus neglectus* were not included in this test. All negative controls
287 with water (NTC) did not amplify.
288
289 *Comparison of nematode counts obtained from microscopy with real-time PCR*
290
291 RLN abundance from fifteen soil samples collected as part of a ware potato survey across England
292 (Orlando, 2021) were determined using traditional counts. Following quantification, the nematode
293 suspensions in their entirety, from the same samples, were used for DNA extraction. Total
294 *Pratylenchus* abundance was determined by real-time PCR and calculated as the sum of the four
295 diagnostics. Soil samples contained between 4 and 343 *Pratylenchus* nematodes. All samples tested
296 positive for the presence of *Pratylenchus* in both real-time PCR and microscopy. There was a
297 significant ($P < 0.001$) and strong relationship ($R^2 = 0.789$) between real-time PCR estimates and
298 microscopy counts (Figure 3) indicating that real-time PCR estimates were similar to results obtained
299 from microscopy, although the analysis was limited to 15 observations. At low densities, counts by
300 microscopy were slightly higher compared to real-time PCR estimates, whereas for six samples at
301 densities greater than 100 nematodes 200 g^{-1} soil, real time PCR overestimated compared to results
302 obtained by microscopy.

DISCUSSION

Molecular diagnostic methods can help the rapid identification and quantification of nematodes including different species of *Pratylenchus* and enhance their management where infested fields occur. In the current study, four TaqMan real-time PCR protocols for the identification and quantification of *P. crenatus, P. penetrans, P. neglectus* and *P. thornei* based on the D2-D3 region of the large subunit rDNA were developed. After optimization of primer/probe concentration and annealing temperature, standard curves were constructed for identification and quantification of each species target, ranging from 10^7 to 10^1 copies μl^{-1}, showing a strong linearity ($R^2 \geq 0.9901$) for all four diagnostics. The real-time PCR methods successfully discriminated each isolate of *P. penetrans, P. crenatus, P. neglectus* and *P. thornei* from seven non-target *Pratylenchus* species and four other non-target genera tested. Overall, the specificity of primers and probe for each diagnostic was confirmed by the absence of amplification for non-target species/genera. Adults of each target *Pratylenchus* species from several locations, mainly from UK and Europe, were tested with each of developed diagnostic primer/probe sets. While *P. penetrans, P. neglectus* and *P. thornei* showed no statistical differences ($P > 0.05$) between Ct values from different populations, one *P. crenatus* isolate from Scotland (code 452) had lower Ct values ($P < 0.05$) than the other tested isolates. This may be due to the presence of more than one individual/juvenile accidentally picked up during preparation of samples or the presence of a gravid female(s) with eggs or a full spermatheca that may have decreased Ct values compared to the other samples (Roberts *et al*., 2016; Oliveira *et al*., 2017; Bandoo *et al*., 2017; Arora *et al*., 2020). Although there is no evidence of this from the current sequences available, it is also possible that *P. crenatus* exhibits intra-specific variation for either the D2-D3 fragment or copy-number among different populations. Kim *et al*. (2019) showed that several *P. crenatus* populations from Dokdo island (Korea) had a 3.7% variation in D2-D3 sequences. Each *Pratylenchus* species yielded a similar gene copy number, with only *P. thornei* having a lower copy number than the other species. Moreover, the presence of sperm in males or gravid females may increase the estimate of gene copy number of *P. penetrans* per individual. Additionally, the degree of inter-specific variation within the D2-D3 segment may differ the gene copy number per species. Lin *et al*. (2020) reported that the intraspecific variation of the D2-D3 expansion fragment for *P. neglectus* and *P. thornei* ranged from 0.1–2% and 0.0–1.7% respectively, while interspecific variations between these two species ranged from 14.7% to 20.3%. Furthermore, Sato et al. (2007) reported significantly lower Ct values of female (23 ± 0.3) and male (22 ± 0.7) *P. penetrans* than juveniles (25.1 ± 0.4), which agrees with our study. Juveniles of *P. thornei, P. neglectus* and *P. crenatus* were statistically different in terms of gene copy number compared to females. A possible

explanation might be that gravid females containing eggs within their body, or males with sperm, will generate lower Ct values than non-gravid females or males without sperm (Oliveira *et al*., 2017).

No fluorescent signals were detected for non-target species, suggesting a high degree of specificity of the diagnostics developed in this study. For example, although *P. fallax* is a species closely related to *P. penetrans* (Ibrahim *et al*., 1995; Waeyenberge *et al*., 2000; Carta *et al*., 2001; Handoo *et al*., 2001; Janssen *et al*., 2017b), and can be morphologically misidentified, this study showed that the real-time PCR diagnostic developed for *P. penetrans* was specific with no amplification for *P. fallax*. All four diagnostics allowed the detection and correct quantification of a single *Pratylenchus* in samples with a mixture of other non-target *Pratylenchus*, even at ten times as many *Pratylenchus* non-targets as target nematodes. Although it is indicative of its specificity in mixed samples of *Pratylenchus* species, a further test including samples with 2000-3000 nematodes might be performed to reproduce the field representative populations. Ct values were comparable with pure samples with one or ten females for each species, demonstrating the consistency of the method in mixed samples. The only exception was represented by the diagnostic for *P. crenatus* that showed Ct values for mixed species with one female of *P. crenatus* in a mixture of species (33.7 ± 0.15) significantly ($P < 0.05$) higher than Ct values for one pure nematode (29.3 ± 0.14). A lack of amplification of four replicates of juveniles in test 2 also occurred, which may be due to a decrease of efficiency for the selected DNA extraction method. Similar to this study, Bandoo *et al*. (2017) reported a real-time PCR method for detection of a single *P. penetrans* juvenile in 1 g of soil, but the real-time PCR assay overestimated nematode numbers when compared to microscopy. Bandoo *et al*. (2017) considered this also may be due to a reduction of DNA extraction efficiency from soil and/or variation in DNA among different life stages. In contrast, a TaqMan real-time PCR method reported by Dauphinais *et al*. (2017) for identification and quantification of *Pratylenchus alleni* and *P. penetrans* showed high specificity and sensitivity, although the real-time PCR underestimated when compared to microscope counts.

Recently, Lin *et al*. (2020) developed a duplex real-time PCR assay based on the 28 rDNA D2-D3 expansion region for the simultaneous identification and quantification of *P. neglectus* and *P. thornei*. Two different species of *Pratylenchus* were detected and quantified simultaneously with a multiplex real-time PCR, and in samples with up to 100 non-target nematodes. In this regard, our diagnostic assays could be developed into a multiplex real-time PCR for the simultaneous detection of all four species in one reaction, subject to further optimisation and validation. . It is important to acknowledge that DNA extraction efficiency, especially from nematode suspensions or directly from soil, can vary according to the method selected (Orlando *et al*., 2020b; Donn *et al*., 2008), which may reduce the sensitivity of the molecular assay for nematode identification and quantification (Roberts

371 *et al*., 2016). For example, Donn *et al*. (2008) found variation between samples when five different
372 DNA extraction methods, including three commercial kits for nematode communities, were
373 compared. In addition, several studies have reported an overestimation of real-time PCR Ct values
374 compared to numbers estimated by microscopy after using a Whitehead tray extraction method (Sato
375 *et al*., 2010; Yan *et al*., 2012, 2013; Bandoo *et al*., 2017). Different approaches to nematode extraction
376 may result in variations in extraction efficiency and therefore discrepancies in the final counts (EPPO,
377 2013). For instance, results between laboratories can vary greatly due to differences in equipment
378 used, specific adaptations in methodology made by each laboratory or different levels of experience
379 in operators (EPPO, 2013). Ideally, a standardised protocol for nematode extraction should be used
380 in all nematology laboratories around the world (Cesarz *et al*., 2019). Implementing real-time PCR
381 with DNA extraction directly from soil would reduce time and avoid potential variability between
382 different nematode extraction methods. However, currently available methods for direct DNA
383 extraction from soil are limited to samples of 1-10 g^{-1} of soil, and this may not be comparable with
384 the standard volume of 200-300 g^{-1} soil used for nematode extraction (Wiesel *et al.*, 2015). Also,
385 residual soil particles may inhibit the amplification of DNA template reducing real-time PCR
386 efficiency (Schrader *et al*., 2012). Indeed, Sato *et al*. (2007) tested two types of soils, andosol and
387 clay soil, containing the same number of root-lesion nematodes and they found significant differences
388 in Ct values, revealing that soil type may also influence the efficiency of the DNA extraction and
389 quality of PCR products. An alternative would be to count specimens from the suspension under
390 microscope and then use current diagnostics for a quick species identification. The four species-
391 specific diagnostics developed in this study will facilitate discrimination and quantification of *P.*
392 *penetrans, P. crenatus, P. neglectus* and *P. thornei* within mixed nematode extracts from soil,
393 providing a tool for rapid diagnostics when required.
394
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548

549 **TABLES AND FIGURES**
550

551 Table 1: Origin, host/sources and population codes of *Pratylenchus* spp. and other nematode
552 genera used in this study

553 Table 2: Primer/probe sets for diagnostic PCR of *Pratylenchus crenatus*, *P. neglectus, P.*
554 *penetrans* and *P. thornei*.

555 Table 3: Population codes, host/sources and origin of *Pratylenchus* spp. and other nematode
556 species used in this study, together with the Ct values (mean ± SEM). Each sample was tested
557 in three replicates.

558 Table 4: Specificity of each diagnostic for detection and quantification of one (n=6) and ten
559 (n=6) target species in a mixture of *Pratylenchus* spp. Ct values obtained by real-time PCR are
560 reported by mean ± SEM. Data with different letters are significantly different according to
561 Tukey's test ($P \leq 0.05$).

562 Table 5: Sensitivity of each assay for detection and quantification of different life stages of *P.*
563 *penetrans* (n=10), *P. neglectus* (n=10), *P. thornei* (n=10) and *P. crenatus* (n=6 for juvenile,
564 and n=10 for adults). Data with different letters in the same column are significantly different
565 according to Tukey's test ($P \leq 0.05$*).* Data for copy number per individual is the average of
566 DNA copy number of the life stages analysed.

567 Figure 1: Standard linear curves of cycle threshold (Ct) values plotted against log-transformed
568 DNA copy number of A: *P. crenatus*; B: *P. neglectus*; C: *P. penetrans* and D: *P. thornei*. The
569 standard curves were run in triplicate for each *Pratylenchus* species.

570 Figure 2: Linear regression of the real-time PCR cycle threshold (Ct) for *Pratylenchus*
571 *penetrans* against the log number of *P. penetrans* individuals (1, 10, 100, 1000) (n = 3).

572 Figure 3: The relationship between estimated total number of *Pratylenchus* spp. determined by
573 microscopy (morphological characteristics) and real-time PCR for 15 soil samples collected
574 from arable fields in England.

575

576 **SUPPLEMENTARY DATA**
577

578 Table S1: Clones code, host/sources, origin, GenBank accession numbers of *Pratylenchus*
579 spp. used for the alignment.

580 Figure S2: Linear regression of the real-time PCR cycle threshold (Ct) for *Pratylenchus*
581 *thornei* against the log number of *P. thornei* individuals (1, 10, 1000) (n = 3).

582 Figure S3: Linear regression of the real-time PCR cycle threshold (Ct) for *Pratylenchus*
583 *crenatus* against the log number of *P. crenatus* individuals (1, 10, 50) (n = 3).