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Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.)

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Summary – Robust and accurate identification of root-lesion nematodes (*Pratylenchus* spp.) is an essential step for determining their potential threat to crop yields and, consequently, development of an efficient agronomic management strategy. It is recognised that DNA-based techniques provide rapid identification of a range of plant-parasitic nematodes including *Pratylenchus* spp. Efficient and repeatable DNA extraction is central to molecular methodologies. Here, six common DNA extraction protocols were compared to evaluate their efficiency to obtain quality DNA samples for *Pratylenchus penetrans*. Samples with five and ten individuals of *P. penetrans* were successfully extracted and amplified by all extraction methods tested, whereas samples with a single nematode presented challenges for DNA amplification. Among all methods tested, the DNA extraction protocol with glass beads proved to be efficient for *P. penetrans* and all other species tested (*P. crenatus*, *P. neglectus* and *P. thornei*), generating high quality DNA at comparatively low cost and with a rapid sample throughput.

Keywords – diagnostics, DNA yield, glass beads, ITS rRNA, PCR, plant-parasitic nematodes, *Pratylenchus crenatus*, *Pratylenchus neglectus*, *Pratylenchus penetrans*, *Pratylenchus thornei*, proteinase K.

Nematodes are the most abundant phyla on earth with plant-parasitic nematodes in a global context typically representing 25-30% of the total nematode community (van den Hoogen *et al.*, 2019). Root-lesion nematodes (*Pratylenchus* spp.) are migratory endoparasitic nematodes of several crops with a worldwide distribution (Castillo & Vovlas, 2007; Jones *et al.*, 2013; Orlando *et al.*, 2020). Correct species diagnosis is central to supporting agronomic management strategies to mitigate the impact of plant-parasitic nematodes on crop yield and quality. Many species of *Pratylenchus* share similarities for some important morphological characters that confound species identification (Castillo & Vovlas, 2007; Geraert, 2013). Further, identification of *Pratylenchus* spp. by microscopy is time consuming and requires well trained taxonomists that are diminishing in number (Coomans, 2000). Several molecular techniques have been developed to assist with identification and to study the intraspecific variability of root-lesion nematodes (Al-Banna *et al.*, 1997, 2004; Uehara *et al.*, 1998, 2001; Waeyenberge *et al.*, 2000, 2009; De Luca *et al.*, 2004, 2011; Subbotin *et al.*, 2008; Yan *et al.*, 2008, 2012, 2013; Mokrini *et al.*, 2013, 2014; Fanelli *et al.*, 2014, 2018; Peetz & Zasada, 2016; Janssen *et al.*, 2017a, b; Oliveira *et al.*, 2017). Many of these diagnostic methods have been summarised and discussed in a recent review by Orlando *et al.* (2020).

Effective molecular diagnostics depend upon efficient and robust extraction of DNA from one or more target individuals. Nematodes can be crushed in a drop of water and the DNA directly amplified by polymerase chain reaction (PCR) (Powers & Harris, 1993) or, alternatively, homogenised or cut into several pieces using a small blade or needle. However, to enhance and ensure repeatability of DNA extraction, lysis *via* proteinase K (Tanha Maafi *et al.*, 2003; Subbotin *et al.*, 2008) or worm lysis buffer (Waeyenberge *et al.*, 2000, 2009; Holterman *et al.*, 2006; De Luca *et al.*, 2011; Peetz & Zasada, 2016) has been considered best practice. Lysis buffers can easily be prepared and they usually release DNA in 2-3 h, providing sufficient and clean DNA without any further DNA

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purification step. Alkaline lysis with NaOH solution is another common protocol reported for nematode DNA extraction that does not require previous disruption of the nematodes and requires only 15 min at 95°C for lysis (Stanton *et al.*, 1998; Floyd *et al.*, 2002; Janssen *et al.*, 2016). There are also several chemical treatments used for DNA purification and concentration such as phenol or phenol with chloroform. A simple alternative is the use of commercially available DNA extraction kits; however, they are typically more expensive if there are high numbers of samples to process. The choice of the extraction method depends on the purpose of the study, equipment available and the species targeted.

To our knowledge there are no studies testing the efficiency of DNA extraction methods for *Pratylenchus* species and only a few have reported such data for other genera (Harris *et al.*, 1990; Stanton *et al.*, 1998; Adam *et al.*, 2007). Thus, the choice of DNA extraction method for *Pratylenchus* spp. is not straightforward and may prove problematic for a new laboratory, particularly in optimising DNA extraction from a single individual. The aim of the present work is therefore to compare commonly used methods of DNA extraction to determine the most efficient for extracting DNA from different *Pratylenchus* species and life stages.

Materials and methods

Nematode populations

Initial populations of *Pratylenchus penetrans*, *P. crenatus* and *P. thornei* were obtained from established carrot disc cultures supplied by East Malling Research (UK) and ILVO (Belgium), and sub-cultured on other carrot discs following the method reported by Speijer & De Waele (1997). A population of *P. neglectus* was recovered from infested potato roots (Shropshire, UK) and also cultured on carrot discs. Nematodes were extracted from infested carrot discs using a Baermann modified method (Hooper, 1986) with individual nematodes handpicked using a sterile needle under a stereomicroscope (Mazurek Optical Service, Meiji EMT) and transferred to sterile Eppendorf (500 μl) tubes for DNA extraction.

Tests for comparison of six DNA extraction methods

Four tests were performed to compare six different methods of DNA extraction. For each test, lysis was assessed by the success of ITS rRNA sequence amplification of *Pratylenchus* spp. DNA extracts.

Test 1

DNA of one, five and ten females of *P. penetrans* was extracted in three replicates for each method. This comparison was used to determine the most suitable method for DNA extraction.

Test 2

DNA of one juvenile, one female and one male of *P. penetrans* was extracted in three replicates for each method. This test aimed to identify any differences between DNA extraction methods among life stages.

Test 3

The most consistent lysis method showing the greatest DNA amplification success rate from Tests 1 and 2 was selected and used for DNA extraction and amplification of one, five and ten specimens of *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei* with three replications. This test aimed to detect differences of DNA extraction and amplification between species.

Test 4

The most consistent lysis method from Tests 1 and 2 was selected and used for DNA extraction and amplification from one juvenile and one female of *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei* with three replications. This test aimed to assess whether differences in DNA extraction existed among life stages and species.

DNA extraction methods

Six methods of DNA extraction were tested for their ability to lyse individuals of four target *Pratylenchus* species:

A: Manual cutting of nematodes under a binocular microscope based on a modification of the method described by Tanha Maafi *et al.* (2003). One, five and ten specimens, depending upon the test, were handpicked under a stereomicroscope (Mazurek Optical Service, Meiji EMT) at 40× magnification using a sterile needle and then placed into 20 μl PCR water previously pipetted onto a glass slide. Each nematode was cut into 4-5 pieces using a scalpel before the contents were transferred with a pipette into an Eppendorf (500 μl) tube. Two μl of PCR 10× Buffer (GoTaq, Promega), 3 μl proteinase K (600 μg ml^{-1}) and 5 μl PCR water were added to the tube and incubated at 65°C for 1 h and 95°C for 15 min.

Samples were allowed to cool before being centrifuged at 16 000 *g* (Heraeus Pico 17 Ventilated Microcentrifuge, Thermo Fisher Scientific).

B: Heating and freezing before lysis extraction based on a method adapted from Williams *et al.* (1992). Whole nematodes (one, five or ten) were placed into an Eppendorf tube with 20 μl PCR water. Tubes were incubated at 95°C for 15 min and stored at −80°C overnight. Samples were thawed before 2 μl of PCR 10× Buffer, 3 μl proteinase K (600 μg ml^{-1}) and 5 μl PCR water were added to each tube. Samples were incubated at 65°C for 1 h and 95°C for 15 min and cooled before being centrifuged at 16 000 *g*.

C: Utilisation of glass beads to cause mechanical disruption of nematodes, adapted from Jesus *et al.* (2016). Each specimen was handpicked using a needle and placed into a tube with 20 μl of 10X PCR buffer. Three 1 mm glass beads (Thermo Fisher Scientific) were added into each tube and homogenised using a Retsch M300 tissue disruptor (Retsch) for 30 s at 30 Hz. Thereafter, 4 μl of proteinase K (100 μg ml^{-1}) and 1 μl of 10× PCR buffer were added to each tube. Samples were incubated at 60°C for 1 h, 95°C for 15 min and 10°C for 10 min. After DNA extraction, tubes were centrifuged at 16 000 *g*.

D: Lysis of nematodes using worm lysis buffer (WLB) based on a method modified from Holterman *et al.* (2006). Whole nematodes (one, five or ten) were placed into a tube with 10 μl WLB (0.2 M NaCl, 0.2 M Tris-HCl pH 8.0, 0.1 M dithiotreitol) and 2 μl proteinase K (800 μg ml^{-1}). The mixture was incubated at 65°C for 1 h and 95°C for 15 min before being cooled and centrifuged at 16 000 *g*. Finally, 18 μl of PCR water was added to the tube.

E: DNA extraction using NaOH (0.05 M), adapted from the method reported by Janssen *et al.* (2016). Whole nematodes (one, five or ten) were handpicked with a needle and transferred to Eppendorf tubes (500 μl) with 10 μl NaOH (0.05 M) before 1 μl Tween 20 (4.5%) was added. Samples were incubated at 95°C for 15 min, and then allowed to cool down. Tubes were centrifuged at 16 000 *g* and 19 μl of PCR water was added.

F: DNA extraction using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific). As previously, individual nematodes were handpicked and transferred into an Eppendorf tube (2 ml) with 40 μl of PCR water. All steps were performed according to the instructions listed by the manufacturer, with DNA eluted in 40 μl genomic elution buffer (10 mM Tris-HCl pH 9.0, 0.1 mM EDTA).

DNA amplification and detection of PCR products

The molecular target for DNA amplification was ITS rRNA, using the universal primers VRAIN2F (CTT TGT ACA CAC CGC CCG TCG CT) and VRAIN2R (TTT CAC TCG CCG TTA CTA AGG GAA TC) (Vrain *et al.*, 1992). Each PCR reaction contained: 5X PCR MyTaq Red Reaction Buffer (Bioline), 0.4 mM of each primer, 0.5 μl of MyTaq Red DNA Polymerase (Bioline), 2 μl of DNA sample and double sterile water for a total volume of 15 μl for each PCR reaction. PCR conditions were: denaturation at 95°C for 5 min followed by 35 cycles of denaturation 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 performed at 72°C for 5 min. PCR products were separated and visualised on a 1 % agarose gel using 6X GelRed loading buffer (Biotium).

Statistical analysis

DNA amplification data were expressed as the percentage of successful PCR amplification. A two-way ANOVA with Bonferroni's test ($P < 0.05$) was used for Test 1, whereas a Pearson Chi-squared test was carried out for Tests 2-4. All statistical analyses were performed using Genstat (19th edition, VSN International).

Results

DNA extraction and amplification of *P. penetrans*

Six methods of DNA extraction were tested with increasing numbers (one, five and ten females) (Fig. 1) and different life stages (Fig. 2) of *P. penetrans*. It took *ca* 15 min of microscope work to prepare a set of five samples for DNA extraction methods B-F. Method A, however, required *ca* 30 min for picking and cutting of nematode specimens. Overall, the estimated total time for each method was: 2 h for method A, 24 h for method B, 1 h 40 min for method C, 1 h 30 min for method D, 30 min for method E and 3 h for method F.

Assessments of the DNA quality were made by PCR amplification of ITS rRNA sequences. DNA extractions of five and ten nematodes were successful for all methods with 100% DNA amplification, apart from DNA extracted with the commercial kit (method F), which had a lower efficiency compared to the other methods. Overall, DNA amplification for one nematode was significantly lower

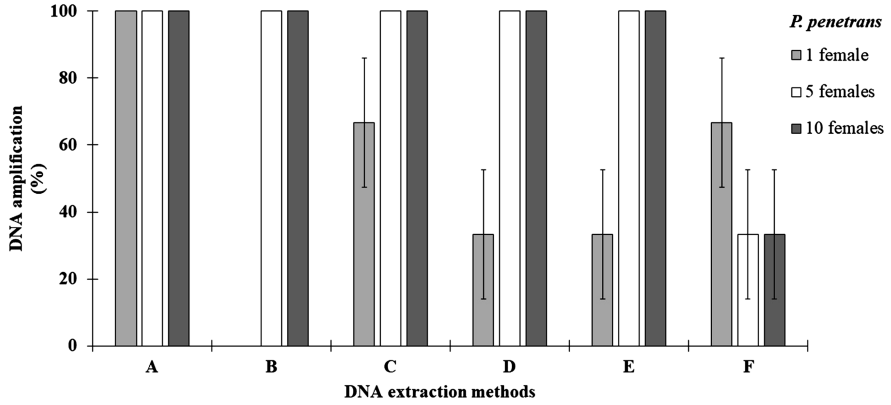

Fig. 1. DNA amplification (%) of one (n = 3), five (n = 3) and ten (n = 3) *Pratylenchus penetrans* females using six DNA extraction methods. A: Manual cut of nematode; B: Heating and freezing; C: Glass beads; D: Worm lysis buffer; E: NaOH; F: PureLink DNA extraction kit. Error bars show the standard error of the mean.

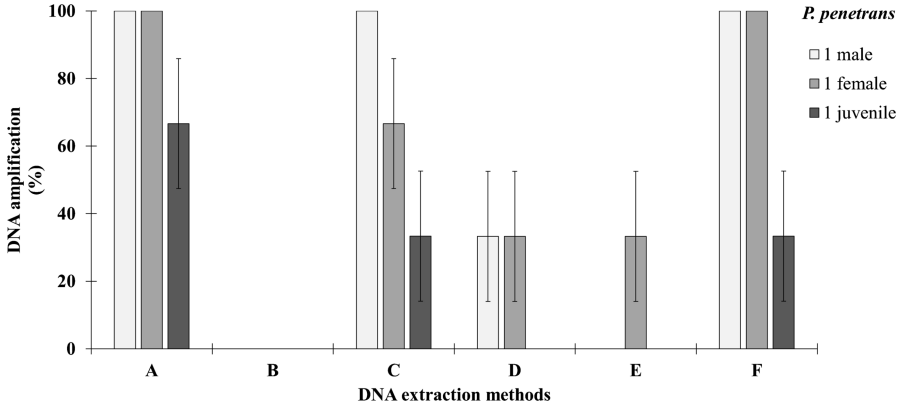

Fig. 2. DNA amplification (%) of one male (n = 3), one female (n = 3) and one juvenile (n = 3) of *Pratylenchus penetrans* using six DNA extraction methods. A: Manual cut of nematode; B: Heating and freezing; C: Glass beads; D: Worm lysis buffer; E: NaOH; F: PureLink DNA extraction kit. Error bars show the standard error of the mean.

($P < 0.001$, df = 34, %CV = 34.9) than amplifications for five and ten nematodes. Moreover, comparing each extraction method for increasing nematode abundance, only method B had a significantly lower ($P = 0.012$) success rate for DNA extraction from a single nematode. Considering the data on individual nematodes, method A was the most successful with 100% DNA amplification. Method C, using glass beads, was reasonably successful in amplifying the DNA from a single individual, and was faster than method A.

Except for method B, all extraction methods resulted in successful amplification of DNA for individual nematodes (Fig. 2). There were no significant differences among life stages ($P = 0.374$, $\chi^2 = 1.97$, df = 2) (Fig. 2), whereas significant differences ($P < 0.001$, $\chi^2 = 24.92$, df = 5) were observed between different DNA extraction methods (Fig. 2). Method B did not yield DNA amplification for any life stage, whereas method D, with WLB, had lower DNA amplification efficiency for males and females and no amplification for juveniles. Method E, with NaOH, yielded DNA amplification only for females but with low efficiency. Methods A, C and F were the most successful for DNA extraction from all life stages.

DNA EXTRACTION AND AMPLIFICATION OF *P. CRENATUS*, *P. NEGLECTUS*, *P. PENETRANS* AND *P. THORNEI*

DNA was extracted with method C from four species of root-lesion nematodes (*P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei*) with increasing number of individuals per sample (Fig. 3) and different life stages (Fig. 4).

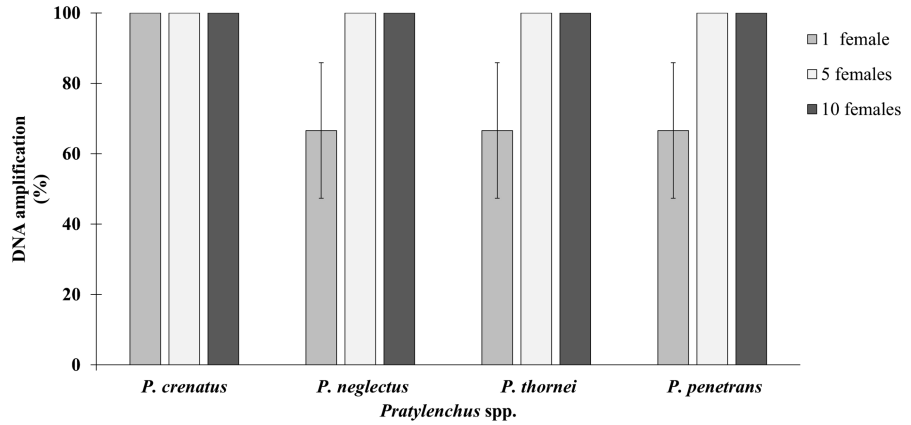

Fig. 3. DNA amplification (%) of one female (n = 3), five females (n = 3) and ten females (n = 3) of *Pratylenchus crenatus*, *P. neglectus*, *P. thornei* and *P. penetrans* using a glass bead DNA extraction method (method C). Error bars show the standard error of the mean.

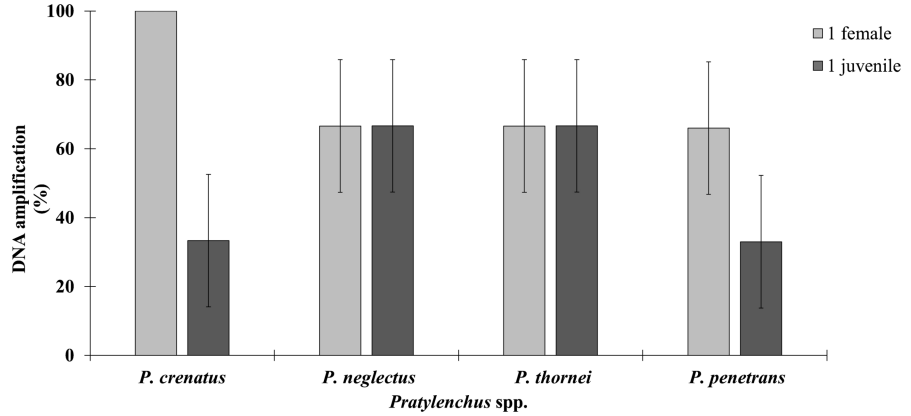

Fig. 4. DNA amplification (%) of one female (n = 3) and one juvenile (n = 3) of *Pratylenchus crenatus*, *P. neglectus*, *P. thornei* and *P. penetrans* using a glass bead extraction method (method C). Error bars show the standard error of the mean.

When DNA was extracted from one, five and ten individuals, there were no significant differences among different species ($P = 0.942$, $\chi^2 = 0.39$, df = 3) (Fig. 3). Extraction using one female resulted in 100% amplification of *P. crenatus* replicates and 66.6% amplification of *P. neglectus*, *P. thornei* and *P. penetrans*. Similarly, considering single juveniles and females (Fig. 4), method C did not show significant differences between species ($P = 0.528$, $\chi^2 = 2.22$, df = 3), or among life stages ($P = 0.178$, $\chi^2 = 1.82$, df = 1).

Discussion

DNA extraction is an important step for molecular identification of nematodes. Several protocols for nematode DNA extraction have been published. Depending on the purpose of the study, DNA extraction can be performed on a single specimen or from the whole suspension extracted from soil or roots. There are also protocols for direct DNA extraction from soil or infested roots. Each method can have an impact on yield and purity of DNA, influencing DNA amplification and further molecular analysis.

Six common protocols were tested for their efficiency of DNA extraction and amplification of *P. penetrans*. Methods that used manual cutting of the nematodes (method A) and the use of glass beads (method C) were the most efficient for extracting DNA from a single nematode. Thus, mechanical disruption of the cuticle and body of the nematode appears to be an important step to achieve successful and consistent DNA amplification.

Method C was slightly faster (1 h 40 min) than method A (2 h) and less laborious because it did not require the step of manual cutting, which can be time-consuming and impractical with either large numbers of nematodes per sample or many samples to process. The method involving heating and freezing before lysis (method B) did not generate successful amplification for samples with one individual, possibly due to less disruption of the cuticle and cell membranes and subsequently less DNA released. Moreover, it required more time (24 h) to complete the procedure compared with the other protocols. The method with WLB (method D) required a total time of 1 h 30 min to complete the protocol but was less efficient for individual nematodes compared to methods A, C and F. Despite being the quickest to perform, the protocol with NaOH (method E) had low efficiency and only resulted in DNA amplification from females. Lastly, the commercial kit (method F) was relatively quick to perform (*ca* 3 h) but had lower efficiency with DNA extraction from five and ten individuals of *P. penetrans*.

In our study, DNA extraction methods did not include a DNA purification step and crude DNA extracts were directly used for PCR amplification. Before performing any molecular assays, it is important to remember that many compounds used for DNA extraction can inhibit DNA amplification, in addition to the inhibitors present in soil (Schrader *et al.*, 2012). As a consequence of PCR inhibition, the sensitivity of any molecular assay will be decreased (Roberts *et al.*, 2016). The failure of PCR amplification for some methods tested, like extractions with NaOH or WLB buffers, could have been caused by PCR inhibitors within the buffers such as Tween 20, dithiothreitol or proteinase K. Some PCR inhibitors may degrade DNA samples or disrupt the annealing of the primers to DNA templates, whereas others can directly degrade the DNA polymerase or inhibit its activity. Chemicals such as Nonidet P-40, Tween 20, EDTA, dithiothreitol, dimethyl sulphoxide or mercaptoethanol may be necessary for efficient cell lysis but, at high concentrations, they can cause PCR inhibition (Schrader *et al.*, 2012).

There are several DNA extraction and purification methods and commercial kits available that have been tested for individual nematodes and nematode communities. However, the efficiency of DNA extraction may vary between commercial kits depending on the buffers and the matrix used (Schrader *et al.*, 2012). Donn *et al.* (2008) compared five different extraction methods including three commercial kits for nematode communities. DNA extraction with phenol chloroform purification and a Purelink PCR purification kit were the most efficient methods yielding consistently high-quality DNA templates (Donn *et al.*, 2008). While NaOH extractions gave the highest yields as measured by absorbance, they were not amplified by PCR. The authors suggested the possibility of protein contamination leading to the high recorded values for absorbance. Also, Waeyenberge *et al.* (2019) showed the variation of DNA extraction efficiency on nematode species richness comparing 15 extraction methods, including commercial kits from different companies. In their study, pre-treatment in liquid nitrogen followed by the Qiagen method was the most successful with greatest DNA yield. Similarly, four DNA extraction protocols (chelex, worm lysis buffer method, Holterman lysis buffer method and FastDNA kit) were tested to compare the efficiency of DNA extraction and amplification of *Meloidogyne javanica* (Carvalho *et al.*, 2019). Extraction with the FastDNA provided low DNA concentration and failure on PCR amplification, whereas the WLB method was the most efficient for extracting DNA, confirming that efficiency varied among different methods (Carvalho *et al.*, 2019). In our results, DNA extracted with Purelink commercial kit (method F) gave a low efficiency for five and ten individuals of *P. penetrans* and a relatively greater efficiency than the other methods for one individual.

Few studies have assessed DNA extraction methods for plant-parasitic nematodes, and those that have focused mostly on *Meloidogyne* spp. (Harris *et al.*, 1990; Stanton *et al.*, 1998; Adam *et al.*, 2007; Carvalho *et al.*, 2019). Adam *et al.* (2007) used a combination of worm lysis buffer and manual cutting of single second-stage juveniles of *Meloidogyne* spp. and PCR amplification products were obtained from 95% of the extracts. Harris *et al.* (1990) reported a comparison of different lysis protocols on juveniles and eggs of *M. incognita*, *M. hapla*, *M. javanica* and *M. arenaria*. These authors included methods such as squashing the specimen with a micropipette tip, a proteinase K method and freezing and thawing protocol. However, only methods which included manual disruption of individuals provided consistent DNA amplification (50%), whereas the other methods were less efficient. Furthermore, a lysis method using NaOH (24 h) showed consistent results with 81% amplification for *Meloidogyne* juveniles, whilst squashing of the nematodes resulted in 50% amplification and a proteinase K protocol without nematode squashing gave 20% amplification efficiency (Stanton *et al.*, 1998). In our study, the NaOH

protocol had low efficiency and only resulted in DNA amplification from *P. penetrans* females.

Our results showed that the six DNA extraction methods did not differ regarding the amplification of DNA extracted from five or ten *P. penetrans* adults. By contrast, successful DNA extraction from one individual was dependent upon the method used. *Pratylenchus penetrans* DNA was successfully amplified by PCR for all methods tested, with the exception of method B where amplification for one single nematode was unsuccessful. Manually cutting nematodes (method A) was the most successful method but it is laborious and time-consuming. By contrast, method C, using glass beads, was easy to use and effective for successful PCR amplifications. The glass beads mechanically disrupt cells, facilitating DNA extraction and provides a simple, rapid and relatively affordable extraction method that favours DNA extraction from single nematodes. This was the most consistent method among different life stages, increasing numbers of specimens, and species of *Pratylenchus* tested (*P. penetrans*, *P. crenatus*, *P. neglectus* and *P. thornei*).

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References

Adam, M.A.M., Phillips, M.S. & Blok, V.C. (2007). Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). *Plant Pathology* 56, 190-197. DOI: 10.1111/j.1365-3059.2006.01455.x

Al-Banna, L., Williamson, V. & Gardner, S.L. (1997). Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Molecular Phylogenetics and Evolution* 7, 94-102. DOI: 10.1006/mpev.1996.0381

Al-Banna, L., Ploeg, A.T., Williamson, V.M. & Kaloshian, I. (2004). Discrimination of six *Pratylenchus* species using PCR and species-specific primers. *Journal of Nematology* 36, 142-146.

Carvalho, V.R., Wilcken, S.R.S., Wilcken, C.F., Castro, B.M.C.E., Soares, M.A. & Zanuncio, J.C. (2019). Technical and economic efficiency of methods for extracting genomic DNA from *Meloidogyne javanica*. *Journal of Microbiological Methods* 157, 108-112. DOI: 10.1016/j.mimet.2018.12.022

Castillo, P. & Vovlas, N. (2007). Pratylenchus *(Nematoda: Pratylenchidae): diagnosis, biology, pathogenicity and management*. *Nematology Monographs and Perspectives 6* (Series Editors: Hunt, D.J. & Perry, R.N.). Leiden, The Netherlands, Brill.

Coomans, A. (2000). Nematode systematics: past, present and future. *Nematology* 2, 3-7. DOI: 10.1163/156854100508845

De Luca, F., Fanelli, E., Di Vito, M., Reyes, A. & De Giorgi, C. (2004). Comparison of the sequences of the D3 expansion of the 26S ribosomal genes reveals different degrees of heterogeneity in different populations and species of *Pratylenchus* from the Mediterranean region. *European Journal of Plant Pathology* 110, 949-957. DOI: 10.1007/s10658-004-0813-4

De Luca, F., Reyes, A., Troccoli, A. & Castillo, P. (2011). Molecular variability and phylogenetic relationships among different species and populations of *Pratylenchus* (Nematoda: Pratylenchidae) as inferred from the analysis of the ITS rDNA. *European Journal of Plant Pathology* 130, 415-426. DOI: 10.1007/s10658-011-9763-9

Donn, S., Griffiths, B.S., Neilson, R. & Daniell, T.J. (2008). DNA extraction from soil nematodes for multi-sample community studies. *Applied Soil Ecology* 38, 20-26. DOI: 10.1016/j.apsoil.2007.08.006

Fanelli, E., Troccoli, A., Picardi, E., Pousis, C. & De Luca, F. (2014). Molecular characterization and functional analysis of four β-1,4-endoglucanases from the root-lesion nematode *Pratylenchus vulnus*. *Plant Pathology* 63, 1436-1445. DOI: 10.1111/ppa.12222

Fanelli, E., Troccoli, A., Capriglia, F., Lucarelli, G., Vovlas, N., Greco, N. & De Luca, F. (2018). Sequence variation in ribosomal DNA and in the nuclear *hsp*90 gene of *Pratylenchus penetrans* (Nematoda: Pratylenchidae) populations and phylogenetic analysis. *European Journal of Plant Pathology* 152, 355-365. DOI: 10.1007/s10658-018-1480-1

Floyd, R., Abebe, E., Papert, A. & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology* 11, 839-850. DOI: 10.1046/j.1365-294X.2002.01485.x

Geraert, E. (2013). *The Pratylenchidae of the world – identification of the family Pratylenchidae (Nematoda: Tylenchida)*. Ghent, Belgium, Academia Press.

Harris, T.S., Sandall, L.J. & Powers, T.O. (1990). Identification of single *Meloidogyne* juveniles by polymerase chain reaction amplification of mitochondrial DNA. *Journal of Nematology* 22, 518-524.

Holterman, M., Van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J. & Helder, J. (2006). Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. *Molecular Biology and Evolution* 23, 1792-1800. DOI: 10.1093/molbev/msl044

Hooper, D.J. (1986). Extraction of free-living stages from soil. In: Southey, J.F. (Ed.). *Laboratory methods for work with plant and soil nematodes*. London, UK, Her Majesty's Stationery Office, pp. 5-30.

Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. & Bert, W. (2016). Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Scientific Reports* 6, 22591. DOI: 10.1038/srep22591

Janssen, T., Karssen, G., Couvreur, M., Waeyenberge, L. & Bert, W. (2017a). The pitfalls of molecular species identification: a case study within the genus *Pratylenchus* (Nematoda: Pratylenchidae). *Nematology* 19, 1179-1199. DOI: 10.1163/15685411-00003117

Janssen, T., Karssen, G., Orlando, V., Subbotin, S. & Bert, W. (2017b). Molecular characterization and species delimiting of plant parasitic nematode of the genus *Pratylenchus* from the *penetrans* group (Nematoda: Pratylenchidae). *Molecular Phylogenetics and Evolution* 117, 30-48. DOI: 10.1016/j.ympev.2017.07.027

Jesus, D.J., Oliveira, C.M.G., Roberts, D., Blok, V., Neilson, R., Prior, T., Balbino, H.M., MacKenzie, K.M. & Oliveira, R.A.L. (2016). Morphological and molecular characterisation of *Aphelenchoides besseyi* and *A. fujianensis* (Nematoda: Aphelenchoididae) from rice and forage grass seeds in Brazil. *Nematology*, 1-20. DOI: 10.1163/15685411-00002962

Jones, J.T., Haegeman, A., Danchin, E.G.J., Gaur, H.S., Helder, J., Jones, M.G.K., Kikuchi, T., Manzanilla-López, R.H., Palomares-Rius, J.E., Wesemael, W.M.L. *et al.* (2013). Top 10 plant-parasitic nematodes in molecular plant pathology. *Molecular Plant Pathology* 14, 946-961. DOI: 10.1111/mpp.12057

Mokrini, F., Waeyenberge, L., Viaene, N., Andaloussi, F.A. & Moens, M. (2013). Quantitative detection of the root-lesion nematode, *Pratylenchus penetrans*, using qPCR. *European Journal of Plant Pathology* 137, 403-413. DOI: 10.1007/s10658-013-0252-1

Mokrini, F., Waeyenberge, L., Viaene, N., Andaloussi, F.A. & Moens, M. (2014). The β-1,4-endoglucanase gene is suitable for the molecular quantification of the root-lesion nematode, *Pratylenchus thornei*. *Nematology* 16, 789-796. DOI: 10.1163/15685411-00002808

Oliveira, C.M.G., Blok, V., Neilson, R., Mróz, T. & Roberts, D. (2017). Hydrolysis probe-based PCR for detection of *Pratylenchus crenatus*, *P. neglectus* and *P. penetrans*. *Nematology* 19, 81-91. DOI: 10.1163/15685411-00003033

Orlando, V., Grove, I.G., Edwards, S.G., Prior, T., Roberts, D., Neilson, R. & Back, M. (2020). Root-lesion nematodes of potato: current status of diagnostics, pathogenicity and management. *Plant Pathology* 69, 1-13. DOI: 10.1111/ppa.13144

Peetz, A.B. & Zasada, I.A. (2016). Species-specific diagnostics using a β-1,4-endoglucanase gene for *Pratylenchus* spp. occurring in the Pacific Northwest of North America. *Nematology* 18, 1-11. DOI: 10.1163/15685411-00003026

Powers, T.O. & Harris, T.S. (1993). A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* 25, 1-6.

Roberts, D., Oliveira, C.M.G., Neilson, R. & Blok, V. (2016). Diagnose molecular de nematoides parasitos de plantas. In: Oliveira, C.M.G., Santos, M.A. & Castro, L.H.S. (Eds). *Diagnose de fitonematoides*. *Campinas, Brazil, Millennium Editora 14*, pp. 281-324.

Schrader, C., Schielke, A., Ellerbroek, A.L. & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *Journal of Applied Microbiology* 113, 1014-1026. DOI: 10.1111/j.1365-2672.2012.05384.x

Speijer, P.R. & De Waele, D. (1997). *Screening of* Musa *germplasm for resistance and tolerance to nematodes*. INIBAP Technical Guidelines N°1, Montpellier, France, INIBAP.

Stanton, J.M., McNicol, C.D. & Steele, V. (1998). Non-manual lysis of second-stage *Meloidogyne* juveniles for identification of pure and mixed samples based on the polymerase chain reaction. *Australasian Plant Pathology* 27, 112-115. DOI: 10.1071/AP98014

Subbotin, S.A., Ragsdale, E.J., Mullens, T., Roberts, P.A., Mundo-Ocampo, M. & Baldwin, J.G. (2008). A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): evidence from 18S and D2-D3 expansion segments of 28S ribosomal RNA genes and morphological characters. *Molecular Phylogenetics and Evolution* 48, 491-505. DOI: 10.1016/j.ympev.2008.04.028

Tanha Maafi, Z., Subbotin, S.A. & Moens, M. (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences. *Nematology* 5, 99-111. DOI: 10.1163/156854102765216731

Uehara, T., Mizukubo, T., Kushida, A. & Momota, Y. (1998). Identification of *Pratylenchus penetrans* (Cobb) by PCR using ITS-based species-specific primers. *Japanese Journal of Nematology* 28, 1-7.

Uehara, T., Kushida, A. & Momota, Y. (2001). PCR-based cloning of two β-1,4-endoglucanases from the root-lesion nematode *Pratylenchus penetrans*. *Nematology* 3, 335-341. DOI: 10.1163/156854101317020259

van den Hoogen, J., Geisen, S., Routh, D., Ferris, H., Traunspurger, W., Wardle, D.A., de Goede, R.G.M., Adams, B.J., Ahmed, W., Andriuzzi, W.S. *et al.* (2019). Soil nematode abundance and functional group composition at a global scale. *Nature* 572, 194-198. DOI: 10.1038/s41586-019-1418-6

Vrain, T.C., Wakarchuk, D.A., Lévesque, A.C. & Hamilton, R.I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.

Waeyenberge, L., Ryss, A., Moens, M., Pinochet, J. & Vrain, T.C. (2000). Molecular characterisation of 18 *Praty-*

lenchus species using rDNA restriction fragment length polymorphism. *Nematology* 2, 135-142. DOI: 10.1163/156854100509024

Waeyenberge, L., Viaene, N. & Moens, M. (2009). Species-specific duplex PCR for the detection of *Pratylenchus penetrans*. *Nematology* 11, 847-857. DOI: 10.1163/156854109X428016

Waeyenberge, L., de Sutter, N., Viaene, N. & Haegeman, A. (2019). New insights into nematode DNA-metabarcoding as revealed by the characterization of artificial and spiked nematode communities. *Diversity* 52, 1-22. DOI: 10.3390/d11040052

Williams, B.D., Schrank, B., Huynh, C., Shownkeen, R. & Waterston, R.H. (1992). A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* 131, 609-624.

Yan, G.P., Smiley, R.W., Okubara, P.A., Skantar, A., Easley, S.A., Sheedy, J.G. & Thompson, A.L. (2008). Detection and discrimination of *Pratylenchus neglectus* and *P. thornei* in DNA extracts from soil. *Plant Disease* 92, 1480-1487. DOI: 10.1094/PDIS-92-11-1480

Yan, G.P., Smiley, R.W. & Okubara, P.A. (2012). Detection and quantification of *Pratylenchus thornei* in DNA extracted from soil using real-time PCR. *Phytopathology* 102, 14-22. DOI: 10.1094/PHYTO-03-11-0093

Yan, G.P., Smiley, R.W., Okubara, P.A., Skantar, A.M. & Reardon, C.L. (2013). Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. *Plant Disease* 97, 757-764. DOI: 10.1094/PDIS-08-12-0729-RE