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

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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. Filipjev, 1936) 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 (Uehara *et al*., 1998, 2001; Al-Banna *et al*., 1997, 2004; Waeyenberge *et al*., 2000, 2009; Subbotin *et al*., 2008; Yan *et al*., 2008, 2012, 2013; De Luca *et al*., 2004, 2011; Oliveira *et al*., 2017; Mokrini *et al*., 2013, 2014; Fanelli *et al*., 2014, 2018; Peetz & Zasada, 2016; Janssen *et al*., 2017a, b). 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 (Holterman *et al*., 2006; Waeyenberge *et al*., 2000, 2009; 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 to 3 h, providing sufficient and clean DNA without any further DNA 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 require 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 population

Initial populations of *P. penetrans*, *P. crenatus* and *P. thornei* were obtained from established carrot-disk cultures supplied by East Malling Research (UK) and ILVO (Belgium) and sub-cultured on other carrot discs following the method reported by Speijer and 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 3 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 3 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 40X 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 transferred with a pipette into an Eppendorf (500 µl) tube. Two µl of PCR 10X Buffer (GoTaq, Promega, UK), 3 µl proteinase K (600 µg ml^{-1}) and 5 µl PCR water were added to the tube and incubated at 65 °C for 1h and 95 °C for 15 min. Samples were allowed to cool before being centrifuged at 16000 *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 10X Buffer (GoTaq, Promega, UK), 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 1h and 95 °C for 15 min and cooled before being centrifuged at 16000 *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 (GoTaq, Promega, UK). Three 1 mm glass beads (Thermo Fisher Scientific) were added into each tube and homogenised using a Retsch M300 tissue disruptor (Retsch, Germany) for 30 s at 30 Hz. Thereafter, 4 µl of proteinase K (100 µg ml^{-1}) and 1 µl of 10X PCR buffer (GoTaq, Promega, UK) were added to each tube. Samples were incubated

at 60 °C for 1h, 95 °C for 15 min and 10 °C for 10 min. After DNA extraction, tubes were centrifuged at 16000 *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 1h and 95 °C for 15 min before being cooled and centrifuged at 16000 *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 16000 *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, UK), 0.4 mM of each primer, 0.5 µL of MyTaq Red DNA Polymerase (Bioline, UK), 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 Ltd, UK).

RESULTS

DNA extraction and amplification of *P. penetrans*

Six methods of DNA extraction were tested with increasing numbers (one, five and ten females) (Figure 1) and different life stages (Figure 2) of *P. penetrans.* It took ca.15 minutes 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: 2h for Method A, 24 h for Method B, 1h and 40 min for Method C, 1h and 30 min for method D, 30 min for Method E and 3h 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 ($p < 0.001$, 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 (Figure 2). There were no significant differences among life stages ($p = 0.374$, $\chi^2 = 1.97$, d.f. = 2) (Figure 2). Whereas, significant differences ($p < 0.001$, $\chi^2 = 24.92$, d.f. = 5) were observed between different DNA extraction methods (Figure 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. Method 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 (Figure 3) and different life stages (Figure 4). When DNA was extracted from one, five and ten individuals, there were no significant differences among different species ($p = 0.942$, $\chi^2 = 0.39$, d.f. = 3) (Figure 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 (Figure 4), Method C did not show significant differences between species ($p = 0.528$, $\chi^2 = 2.22$, d.f. = 3), or among life stages ($p = 0.178$, $\chi^2 = 1.82$, d.f. = 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 (1h 40 min) than Method A (2h) 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 (24h) to complete the procedure respect to the other protocols. Method with WLB (Method D) required a total time of 1h and 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.3h) 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 contaminations 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 fifteen extraction methods, including commercial kits from different companies. In their study, pre-treatment in liquid nitrogen followed by 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) presented 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 mostly focus 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 juvenile 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 *Meloidogyne 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. In contrast, successful DNA extraction from one individual was dependent upon the method used. *P. penetrans* DNA was successfully amplified by PCR for all methods tested, with 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. In 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 provide 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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FIGURES

Figure 1: DNA amplification (%) of one (n=3), five (n=3) and ten (n=3) *P. 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.

Figure 2: DNA amplification (%) of one male (n=3), one female (n=3) and one juvenile (n=3) of *P. 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.

Figure 3: DNA amplification (%) of one female (n=3), five females (n=3) and ten females (n=3) of *P. 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.

Figure 4: DNA amplification (%) of one female (n=3) and one juvenile (n=3) of *P. 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.