

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

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Orlando, V., Edwards, S.G., Neilson, R., Prior, T., Roberts, D. and Back, M. 2020. Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.). *Nematology*.

2 October 2020

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

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35 **SUMMARY**

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

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65 **Keywords:** Diagnostics, DNA yield, glass beads, ITS rRNA, PCR, plant-parasitic nematodes,
66 *Pratylenchus crenatus*, *Pratylenchus neglectus*, *Pratylenchus penetrans*, *Pratylenchus thornei*,
67 proteinase K.

68 Nematodes are the most abundant phyla on earth with plant-parasitic nematodes in a global
69 context typically representing 25-30% of the total nematode community (Van den Hoogen, *et*
70 *al.*, 2019). Root-lesion nematodes (*Pratylenchus* spp. Filipjev, 1936) are migratory
71 endoparasitic nematodes of several crops with a worldwide distribution (Castillo & Vovlas,
72 2007; Jones *et al.*, 2013; Orlando *et al.*, 2020). Correct species diagnosis is central to supporting
73 agronomic management strategies to mitigate the impact of plant-parasitic nematodes on crop
74 yield and quality. Many species of *Pratylenchus* share similarities for some important
75 morphological characters that confound species identification (Castillo & Vovlas, 2007;
76 Geraert, 2013). Further, identification of *Pratylenchus* spp. by microscopy is time consuming
77 and requires well trained taxonomists that are diminishing in number (Coomans, 2000). Several
78 molecular techniques have been developed to assist with identification and to study the
79 intraspecific variability of root-lesion nematodes (Uehara *et al.*, 1998, 2001; Al-Banna *et al.*,
80 1997, 2004; Waeyenberge *et al.*, 2000, 2009; Subbotin *et al.*, 2008; Yan *et al.*, 2008, 2012,
81 2013; De Luca *et al.*, 2004, 2011; Oliveira *et al.*, 2017; Mokrini *et al.*, 2013, 2014; Fanelli *et*
82 *al.*, 2014, 2018; Peetz & Zasada, 2016; Janssen *et al.*, 2017a, b). Many of these diagnostic
83 methods have been summarised and discussed in a recent review by Orlando *et al.* (2020).

84 Effective molecular diagnostics depend upon efficient and robust extraction of DNA from
85 one or more target individuals. Nematodes can be crushed in a drop of water and the DNA
86 directly amplified by polymerase chain reaction (PCR) (Powers & Harris, 1993), or
87 alternatively, homogenised or cut into several pieces using a small blade or needle. However,
88 to enhance and ensure repeatability of DNA extraction, lysis via proteinase K (Tanha Maafi *et*
89 *al.*, 2003; Subbotin *et al.*, 2008), or worm lysis buffer (Holterman *et al.*, 2006; Waeyenberge
90 *et al.*, 2000, 2009; De Luca *et al.*, 2011; Peetz & Zasada, 2016) has been considered best
91 practice. Lysis buffers can easily be prepared and they usually release DNA in 2 to 3 h,
92 providing sufficient and clean DNA without any further DNA purification step. Alkaline lysis
93 with NaOH solution is another common protocol reported for nematode DNA extraction that
94 does not require previous disruption of the nematodes and require only 15 min at 95 °C for
95 lysis (Stanton *et al.*, 1998; Floyd *et al.*, 2002; Janssen *et al.*, 2016). There are also several
96 chemical treatments used for DNA purification and concentration such as phenol or phenol
97 with chloroform. A simple alternative is the use of commercially available DNA extraction
98 kits; however, they are typically more expensive if there are high numbers of samples to
99 process. The choice of the extraction method depends on the purpose of the study, equipment
100 available and the species targeted.

101 To our knowledge there are no studies testing the efficiency of DNA extraction methods for
102 *Pratylenchus* species and only a few have reported such data for other genera (Harris *et al.*,
103 1990; Stanton *et al.*, 1998; Adam *et al.*, 2007). Thus, the choice of DNA extraction method for
104 *Pratylenchus* spp. is not straightforward and may prove problematic for a new laboratory,
105 particularly in optimising DNA extraction from a single individual. The aim of the present
106 work is therefore to compare commonly used methods of DNA extraction to determine the
107 most efficient for extracting DNA from different *Pratylenchus* species and life stages.

108

109 **MATERIALS AND METHODS**

110

111 **Nematode population**

112 Initial populations of *P. penetrans*, *P. crenatus* and *P. thornei* were obtained from
113 established carrot-disk cultures supplied by East Malling Research (UK) and ILVO (Belgium)
114 and sub-cultured on other carrot discs following the method reported by Speijer and De Waele
115 (1997). A population of *P. neglectus* was recovered from infested potato roots (Shropshire,
116 UK) and also cultured on carrot discs. Nematodes were extracted from infested carrot discs
117 using a Baermann modified method (Hooper, 1986) with individual nematodes handpicked
118 using a sterile needle under a stereomicroscope (Mazurek Optical Service, Meiji EMT) and
119 transferred to sterile Eppendorf (500 µl) tubes for DNA extraction.

120

121 **Tests for comparison of six DNA extraction methods**

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

125 **Test 1.** DNA of one, five and ten females of *P. penetrans* was extracted, in three replicates for
126 each method. This comparison was used to determine the most suitable method for DNA
127 extraction.

128 **Test 2.** DNA of one juvenile, one female and one male of *P. penetrans* was extracted, in three
129 replicates for each method. This test aimed to identify any differences between DNA extraction
130 methods among life stages.

131 **Test 3.** The most consistent lysis method showing the greatest DNA amplification success rate
132 from Tests 1 and 2, was selected and used for DNA extraction and amplification of one, five

133 and ten specimens of *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei* with 3 replications.
134 This test aimed to detect differences of DNA extraction and amplification between species.

135 **Test 4.** The most consistent lysis method from Tests 1 and 2 was selected and used for DNA
136 extraction and amplification from one juvenile and one female of *P. crenatus*, *P. neglectus*, *P.*
137 *penetrans* and *P. thornei* with 3 replications. This test aimed to assess whether differences in
138 DNA extraction existed among life stages and species.

139 **DNA extraction methods**

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

142 (A) Manual cutting of nematodes under a binocular microscope based on a modification
143 of the method described by Tanha Maafi *et al.* (2003). One, five and ten specimens, depending
144 upon the test, were handpicked under a stereomicroscope (Mazurek Optical Service, Meiji
145 EMT) at 40X magnification using a sterile needle and then placed into 20 μl PCR water,
146 previously pipetted onto a glass slide. Each nematode was cut into 4-5 pieces using a scalpel
147 before the contents transferred with a pipette into an Eppendorf (500 μl) tube. Two μl of PCR
148 10X Buffer (GoTaq, Promega, UK), 3 μl proteinase K (600 $\mu\text{g ml}^{-1}$) and 5 μl PCR water were
149 added to the tube and incubated at 65 $^{\circ}\text{C}$ for 1h and 95 $^{\circ}\text{C}$ for 15 min. Samples were allowed
150 to cool before being centrifuged at 16000 g (Heraeus Pico 17 Ventilated Microcentrifuge,
151 Thermo Fisher Scientific).

152 (B) Heating and freezing before lysis extraction based on a method adapted from Williams
153 *et al.* (1992). Whole nematodes (one, five or ten) were placed into an Eppendorf tube with 20
154 μl PCR water. Tubes were incubated at 95 $^{\circ}\text{C}$ for 15 min and stored at -80 $^{\circ}\text{C}$ overnight.
155 Samples were thawed before 2 μl of PCR 10X Buffer (GoTaq, Promega, UK), 3 μl proteinase
156 K (600 $\mu\text{g ml}^{-1}$) and 5 μl PCR water were added to each tube. Samples were incubated at 65
157 $^{\circ}\text{C}$ for 1h and 95 $^{\circ}\text{C}$ for 15 min and cooled before being centrifuged at 16000 g .

158 (C) Utilisation of glass beads to cause mechanical disruption of nematodes, adapted from
159 Jesus *et al.*, (2016). Each specimen was handpicked using a needle and placed into a tube with
160 20 μl of 10X PCR buffer (GoTaq, Promega, UK). Three 1 mm glass beads (Thermo Fisher
161 Scientific) were added into each tube and homogenised using a Retsch M300 tissue disruptor
162 (Retsch, Germany) for 30 s at 30 Hz. Thereafter, 4 μl of proteinase K (100 $\mu\text{g ml}^{-1}$) and 1 μl
163 of 10X PCR buffer (GoTaq, Promega, UK) were added to each tube. Samples were incubated

164 at 60 °C for 1h, 95 °C for 15 min and 10 °C for 10 min. After DNA extraction, tubes were
165 centrifuged at 16000 g.

166 (D) Lysis of nematodes using Worm Lysis Buffer (WLB) based on a method modified
167 from Holterman *et al.*, (2006). Whole nematodes (one, five or ten) were placed into a tube with
168 10 µl WLB (0.2 M NaCl, 0.2 M Tris-HCl pH 8.0, 0.1 M dithiotreitol) and 2 µl proteinase K
169 (800 µg ml⁻¹). The mixture was incubated at 65 °C for 1h and 95 °C for 15 min before being
170 cooled and centrifuged at 16000 g. Finally, 18 µl of PCR water was added to the tube.

171 (E) DNA extraction using NaOH (0.05 M), adapted from the method reported by Janssen
172 *et al.*, (2016). Whole nematodes (one, five or ten) were handpicked with a needle and
173 transferred to Eppendorf tubes (500 µL) with 10 µL NaOH (0.05 M) before 1 µL Tween 20
174 (4.5 %) was added. Samples were incubated at 95 °C for 15 min, and then allowed to cool
175 down. Tubes were centrifuged at 16000 g and 19 µL of PCR water was added.

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

181 **DNA Amplification and detection of PCR products**

182 The molecular target for DNA amplification was ITS rRNA, using the universal primers
183 VRAIN2F (CTT TGT ACA CAC CGC CCG TCG CT) and VRAIN2R (TTT CAC TCG CCG
184 TTA CTA AGG GAA TC) (Vrain *et al.*, 1992). Each PCR reaction contained: 5X PCR MyTaq
185 Red Reaction Buffer (Bioline, UK), 0.4 mM of each primer, 0.5 µL of MyTaq Red DNA
186 Polymerase (Bioline, UK), 2 µl of DNA sample and double sterile water for a total volume of
187 15 µl for each PCR reaction. PCR conditions were: denaturation at 95 °C for 5 min followed
188 by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72
189 °C for 1 min. A final extension was performed at 72 °C for 5 min. PCR products were separated
190 and visualised on a 1 % agarose gel using 6X GelRed loading buffer (Biotium).

191 **Statistical analysis**

192 DNA amplification data were expressed as the percentage of successful PCR
193 amplification. A two-way ANOVA with Bonferroni's test ($p < 0.05$) was used for Test 1,

194 whereas a Pearson Chi-squared test was carried out for Tests 2 - 4. All statistical analyses were
195 performed using Genstat (19th edition, VSN International Ltd, UK).

196

197 **RESULTS**

198 **DNA extraction and amplification of *P. penetrans***

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

205 Assessments of the DNA quality were made by PCR amplification of ITS rRNA
206 sequences. DNA extractions of five and ten nematodes were successful for all methods with
207 100 % DNA amplification, apart from DNA extracted with the commercial kit (Method F),
208 which had a lower efficiency compared to the other methods. Overall, DNA amplification for
209 one nematode was significantly lower ($p < 0.001$, $df = 34$, %CV = 34.9) than amplifications
210 for five and ten nematodes. Moreover, comparing each extraction method for increasing
211 nematode abundance, only Method B had a significantly lower ($p = 0.012$) success rate for
212 DNA extraction from a single nematode. Considering the data on individual nematodes,
213 Method A was the most successful with 100% DNA amplification. Method C, using glass
214 beads, was reasonably successful in amplifying the DNA from a single individual, and was
215 faster than Method A.

216 Except for Method B, all extraction methods resulted in successful amplification of DNA
217 for individual nematodes (Figure 2). There were no significant differences among life stages
218 ($p = 0.374$, $\chi^2 = 1.97$, d.f. = 2) (Figure 2). Whereas, significant differences ($p < 0.001$, $\chi^2 =$
219 24.92 , d.f. = 5) were observed between different DNA extraction methods (Figure 2). Method
220 B did not yield DNA amplification for any life stage, whereas Method D, with WLB, had lower
221 DNA amplification efficiency for males and females, and no amplification for juveniles.
222 Method E, with NaOH, yielded DNA amplification only for females but with low efficiency.
223 Method A, C and F were the most successful for DNA extraction from all life stages.

224

225 **DNA extraction and amplification of *P. crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei***

226 DNA was extracted with Method C from four species of root-lesion nematodes (*P.*
227 *crenatus*, *P. neglectus*, *P. penetrans* and *P. thornei*) with increasing number of individuals per
228 sample (Figure 3) and different life stages (Figure 4). When DNA was extracted from one, five
229 and ten individuals, there were no significant differences among different species ($p = 0.942$,
230 $\chi^2 = 0.39$, d.f. = 3) (Figure 3). Extraction using one female resulted in 100 % amplification of
231 *P. crenatus* replicates and 66.6 % amplification of *P. neglectus*, *P. thornei* and *P. penetrans*.
232 Similarly, considering single juveniles and females (Figure 4), Method C did not show
233 significant differences between species ($p = 0.528$, $\chi^2 = 2.22$, d.f. = 3), or among life stages (p
234 $= 0.178$, $\chi^2 = 1.82$, d.f. = 1).

235 **DISCUSSION**

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

242 Six common protocols were tested for their efficiency of DNA extraction and
243 amplification of *P. penetrans*. Methods that used manual cutting of the nematodes (Method A)
244 and the use of glass beads (Method C) were the most efficient for extracting DNA from a single
245 nematode. Thus, mechanical disruption of the cuticle and body of the nematode appears to be
246 an important step to achieve successful and consistent DNA amplification. Method C was
247 slightly faster (1h 40 min) than Method A (2h) and less laborious because it did not require the
248 step of manual cutting, which can be time-consuming and impractical with either large numbers
249 of nematodes per sample or many samples to process. The method involving heating and
250 freezing before lysis (Method B) did not generate successful amplification for samples with
251 one individual, possibly due to less disruption of the cuticle and cell membranes and
252 subsequently less DNA released. Moreover, it required more time (24h) to complete the
253 procedure respect to the other protocols. Method with WLB (Method D) required a total time
254 of 1h and 30 min to complete the protocol but was less efficient for individual nematodes
255 compared to Methods A, C and F. Despite being the quickest to perform, the protocol with
256 NaOH (Method E) had low efficiency and only resulted in DNA amplification from females.

257 Lastly, the commercial kit (Method F) was relatively quick to perform (ca.3h) but had lower
258 efficiency with DNA extraction from five and ten individuals of *P. penetrans*.

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

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

291 (Method F) presented a low efficiency for five and ten individuals of *P. penetrans* and a
292 relatively greater efficiency than the other methods for one individual.

293 Few studies have assessed DNA extraction methods for plant-parasitic nematodes, and those
294 that have mostly focus on *Meloidogyne* spp. (Harris *et al.*, 1990; Stanton *et al.*, 1998; Adam *et*
295 *al.*, 2007; Carvalho *et al.*, 2019). Adam *et al.* (2007) used a combination of worm lysis buffer
296 and manual cutting of single second-stage juvenile of *Meloidogyne* spp. and PCR amplification
297 products were obtained from 95 % of the extracts. Harris *et al.* (1990) reported a comparison
298 of different lysis protocols on juveniles and eggs of *Meloidogyne incognita*, *M. hapla*, *M.*
299 *javanica*, and *M. arenaria*. These authors included methods such as squashing the specimen
300 with a micropipette tip, a proteinase K method and freezing and thawing protocol. However,
301 only methods which included manual disruption of individuals provided consistent DNA
302 amplification (50 %), whereas the other methods were less efficient. Furthermore, a lysis
303 method using NaOH (24 h) showed consistent results with 81 % amplification for *Meloidogyne*
304 juveniles, whilst squashing of the nematodes resulted in 50 % amplification and a proteinase
305 K protocol, without nematode squashing gave 20 % amplification efficiency (Stanton *et al.*,
306 1998). In our study, the NaOH protocol had low efficiency and only resulted in DNA
307 amplification from *P. penetrans* females.

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

320

321 **ACKNOWLEDGEMENTS**

322

323 Valeria Orlando is receipt of a PhD studentship from AHDB Potatoes (Ref. 11120009). The
324 James Hutton Institute receives financial support from support from the Scottish Government,

325 Rural and Environment Science and Analytical Services Division. The authors declare no
326 conflict of interest with the content of this review. The authors thank Nancy de Sutter (ILVO,
327 Belgium) for providing carrot discs and useful advice for *Pratylenchus* spp. cultures.

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480 **FIGURES**

481

482 Figure 1: DNA amplification (%) of one (n=3), five (n=3) and ten (n=3) *P. penetrans* females
483 using six DNA extraction methods: (A) manual cut of nematode; (B) heating and freezing; (C)
484 glass beads; (D) Worm lysis buffer; (E) NaOH; (F) PureLink DNA extraction kit. Error bars
485 show the standard error of the mean.

486

487 Figure 2: DNA amplification (%) of one male (n=3), one female (n=3) and one juvenile (n=3)
488 of *P. penetrans* using six DNA extraction methods: (A) manual cut of nematode; (B) heating
489 and freezing; (C) glass beads; (D) Worm lysis buffer; (E) NaOH; (F) PureLink DNA extraction
490 kit. Error bars show the standard error of the mean.

491

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

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

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