

## Species-specific detection of viable *Globodera pallida* using real-time reverse transcription PCR

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1 **Species specific detection of viable *Globodera pallida* using real-time reverse**  
2 **transcription PCR**

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10

11 **Summary –**

12 *Globodera pallida* is a major pest of potatoes worldwide. In Japan, aiming at eradication of *G.*  
13 *pallida*, control measures have been implemented in infested fields. To determine the necessity of  
14 control measures, the detection of viable *G. pallida* is required. However, the conventional  
15 inoculation test performed in Japan, named the ‘cup test,’ is time-consuming, and conventional  
16 PCR methods targeting DNA can detect dead individuals. In this study, we developed an  
17 intercalator-based RT-qPCR method for the rapid detection of viable *G. pallida*. We designed a  
18 primer set for the partial cDNA sequence of the *Y45F10D.4* gene of *G. pallida*. This primer set  
19 successfully amplified *Y45F10D.4* mRNA of all tested *G. pallida* populations without any  
20 cross-reactions with other species. The RT-qPCR method detected RNA corresponding to a  
21 minimum of 3.9 *G. pallida* eggs, and a significant negative correlation was observed between the  
22 concentrations of RNA extracted from viable eggs and the Ct values. In addition, no  
23 amplification by RT-qPCR was observed in *G. pallida* treated with 1, 3-dichloropropene,  
24 indicating that this method detected viable *G. pallida* specifically. We then compared the  
25 detection sensitivity between the cup test and RT-qPCR method using 24 soil samples, and the  
26 results showed that the detection sensitivity of the RT-qPCR method was higher than that of the  
27 cup test. The RT-qPCR method enabled the rapid and reliable detection of viable *G. pallida*.

28 **Keywords –** 1, 3-dichloropropene, eradication, intercalator, mRNA, potato cyst nematode,  
29 *Y45F10D.4* gene.

30

31 *Globodera pallida*, thought to be native to the Andes region of South America (Evans *et al.*,  
32 1975; Evans & Stone, 1977), is an important potato pest. Currently, *G. pallida* has been detected  
33 in more than 50 countries (EPPO, 2022). Eggs of the species can survive in the soil for more than  
34 10 years (Turner, 1996). However, in the presence of nearby host plants, hatching of the eggs is  
35 induced by hatching factors secreted from host roots (Devine *et al.*, 1996); subsequently, the  
36 hatched juveniles penetrate the host plant roots, resulting in a reduced yield (Trudgill *et al.*, 1975a,  
37 b). Many countries designate *G. pallida* as a quarantine species and prohibit seed potato  
38 production in infested fields (EPPO, 2022; Pickup *et al.*, 2018). In Japan, *G. pallida* was first  
39 detected in Hokkaido in 2015 (Narabu *et al.*, 2016) and official control of this species has been  
40 performed since 2016 (MAFF, 2022).

41 Fumigants such as isothiocyanates and 1, 3-dichloropropene (1, 3-D), non-fumigant  
42 nematicides such as fosthiazate and oxamyl, and nematode trap crops such as *Solanum*  
43 *sisymbriifolium* and *S. peruvianum* are effective against *G. pallida* (Dandurand & Knudsen, 2016;  
44 Dandurand *et al.*, 2019; Ito *et al.*, 2020; Minnis *et al.*, 2004; Whitehead *et al.*, 1979, 1994; Wood  
45 *et al.*, 2017). In Japan, 1, 3-D, and *S. peruvianum* have been used in the official control of *G.*  
46 *pallida* (Ito *et al.*, 2020); when viable individuals are no longer detected in an infested field,  
47 control measures are terminated. Therefore, the detection of viable individuals is important for  
48 appropriate management of *G. pallida*.

49 In Japan, a bioassay, named the ‘cup test,’ has been used to assess *G. pallida* viability (Narabu,  
50 2019). The procedure involves (1) placing approximately 200 ml of soil sample and a tuber of  
51 potato (*HI* conferred variety) in a transparent plastic cup, (2) incubating the cup at 18°C in dark  
52 for 60 to 70 days, and (3) counting the cysts and females visible on the roots through the wall and  
53 bottom of the cup. It is relatively easy to perform, but takes a long time. In addition, cysts formed  
54 inside the cup are not detected, which leads to overlooking viable *G. pallida*.

55 Other methods for determining egg viability include visual inspection under a microscope  
56 (EPPO, 2017), staining with Meldola's Blue (Ogiga & Estey, 1974; EPPO, 2017) or fluorescent  
57 dyes (Hajihassani & Dandurand, 2018; Pillai & Dandurand, 2019), hatching test (EPPO, 2017),  
58 and trehalose method (van den Elsen *et al.*, 2012; Ebrahimi *et al.*, 2015). However, all of these  
59 methods are also time- and labor-intensive. In addition, all of these methods, including the  
60 trehalose method, do not discriminate *Globodera* species, despite the fact that *G. rostochiensis*  
61 co-occurs in many *G. pallida*-infested fields in Japan (unpublished data). Therefore, additional  
62 procedures are required for species identification.

63 Conventional PCR and qPCR methods targeting DNA (Bulman & Marshall, 1997; Nakhla *et*  
64 *al.*, 2010; Reid *et al.*, 2015; Sakai *et al.*, 2019; Kushida & Sakai, 2022) are useful for  
65 discriminating *G. pallida* and *G. rostochiensis*. However, DNA can be amplified from dead  
66 nematodes (van den Elsen *et al.*, 2012; Kushida & Narabu, 2017), and thus, DNA-based PCR  
67 methods are not suitable for detecting viable *G. pallida*.

68 A study reported the use of RT-qPCR targeting the mRNA of housekeeping genes to  
69 specifically detect viable *G. pallida* (Benier *et al.*, 2014). In general, gene expression does not  
70 occur in dead cells. Moreover, mRNA has a shorter half-life than DNA (Eigner *et al.*, 1961) and  
71 is easily degradable by RNases. Therefore, mRNA expression is a good indicator of cell viability.  
72 However, while this method is time-saving (Benier *et al.*, 2014), it requires a locked nucleic acid  
73 probe, which is relatively costly.

74 In our preliminary study, we extracted RNA from 1000 *G. pallida* eggs and performed  
75 RT-qPCR using primers of 10 housekeeping genes (see Appendix). The gene with the lowest Ct  
76 value was *Y45F10D.4*, which encodes a putative iron-sulfur cluster assembly enzyme, suggesting  
77 that this gene was highly expressed in *G. pallida* eggs. Also, this gene was expressed in 2nd stage  
78 juveniles (J2s). In addition, we found some differences exist in the sequence of the gene between

79 *G. pallida* and *G. rostochiensis*.

80 The objective of this study was to develop an intercalator-based RT-qPCR method using  
81 *Y45F10D.4* gene for the detection of viable *G. pallida*. First, we designed a primer set specific to  
82 *G. pallida* and evaluated its performance. We then tested whether RNA could be detected in *G.*  
83 *pallida* treated with 1, 3-D. Finally, we compared the viable *G. pallida* detection sensitivity of  
84 this RT-qPCR method to that of the cup test.

85

## 86 **Materials and Methods**

### 87 NEMATODE POPULATIONS

88 Twenty populations of cyst nematode species were used in this study (Table 1). Nematode  
89 populations from outside Japan were imported in accordance with the official permits of the  
90 Ministry of Agriculture, Forestry, and Fisheries (49Y1213, 29Y1553, 2Y1139, 2Y1141, and  
91 2Y1142).

92

### 93 DESIGN OF PRIMER SETS

94 Because the primer set for *Y45F10D.4* gene developed by Sabeh *et al.* (2018) and used in our  
95 preliminary study (see Appendix) amplified *G. rostochiensis Y45F10D.4* gene also, we designed  
96 a new primer set specific to *G. pallida. Y45F10D.4* partial sequence of *Heterodera glycines*  
97 (GenBank accession no. BF014189) was searched in the *G. pallida*, *G. rostochiensis*, and *G.*  
98 *ellingtonae* reference genomes (GenBank accession no. GCA\_000724045, GCA\_018350315, and  
99 GCA\_001723225, respectively) using BLASTN in NCBI (<https://www.ncbi.nlm.nih.gov/>) to  
100 identify the orthologs. We aligned each sequence using MEGA-X v. 10.1.7, and designed primers  
101 (Y45\_0803\_F1 and Y45\_0715\_R4, Table 2, Fig. 1) for *G. pallida*. We also designed universal  
102 primers (Y45\_Com\_F1 and Y45\_Com\_R1, Table 2, Fig. 1) to confirm whether *Y45F10D.4*

103 mRNA had been extracted from all tested species.

104

#### 105 EXTRACTION OF NUCLEIC ACIDS

106 RNA was extracted from five cysts of each population. Because *G. rostochiensis* was the most  
107 likely non-target species in *G. pallida*-infested fields, we also prepared a deep RNA solution  
108 from 50 cysts of *G. rostochiensis* from Kucchan. Each sample was crushed in a 2 ml plastic tube  
109 with 1 g zirconium dioxide beads (1.5 mm diameter), 350  $\mu$ l of RA1 Buffer (Takara Bio Inc.,  
110 Shiga, Japan), and 3.5  $\mu$ l of 1 M dithiothreitol (FUJIFILM Wako Pure Chemical Co., Osaka,  
111 Japan) using a multi-beads shocker (FastPrep-24™ 5G, MP Biomedicals Inc., Solon, OH, USA).  
112 Each lysate was centrifuged at 11,000  $\times$  *g* for 2 min and the supernatants were collected. Then,  
113 330  $\mu$ l of 70% ethanol (FUJIFILM Wako Pure Chemical Co.) was added to each supernatant, and  
114 the mixture was applied to a NucleoSpin® RNA Column (Takara Bio Inc.) with a collection tube.  
115 After centrifugation (11,000  $\times$  *g*, 2 min), each column was washed using 250  $\mu$ l of Buffer RA2  
116 (Takara Bio Inc.) and then 600  $\mu$ l and 250  $\mu$ l of Buffer RA3 (Takara Bio Inc.). RNA was eluted  
117 from each column using 60  $\mu$ l of nuclease-free water. Finally, the eluate was diluted 4 times with  
118 nuclease-free water (a total of the volume of the RNA solution became 240  $\mu$ l). Since DNase  
119 treatment was not performed, the RNA solution contained DNA. Therefore, the species identity  
120 of each RNA sample was confirmed using PCR-RFLP of ribosomal RNA internal spacer (ITS)  
121 regions (Orui, 1997; Amiri *et al.*, 2002; Subbotin *et al.*, 1999, 2000, 2011). Subspecies identity of  
122 each *G. tabacum* population was confirmed by PCR-RFLP of CLE peptide coding genes (Alenda  
123 *et al.*, 2013).

124 Sixty microliters of the 240  $\mu$ l RNA sample were utilized to measure the total RNA yield of  
125 each sample. Prior to RNA quantification, DNA was degraded as follows. Seven microliters of  
126 DNase solution (10% (w/w) rDNase in Reaction Buffer for rDNase, both reagents were contained

127 in the Nucleospin<sup>®</sup> RNA) were added to the 60  $\mu$ l of total RNA solution. The mixture was heated  
128 at 37°C for one hour. Next, 170  $\mu$ l of 99.5% ethanol, 1  $\mu$ l of Dr. GenTLE Precipitation Carrier  
129 (Takara Bio Inc.), and 7  $\mu$ l of 3 M sodium acetate (pH 5.2, Takara Bio Inc.) were added to the  
130 mixture and mixed well. Then, the mixture was centrifuged for 10 min at 12,000  $\times$  g at 4°C. The  
131 supernatant was discarded, 500  $\mu$ l of 70% ethanol was added to the pellet, which was then  
132 centrifuged again for 5 min at 7,500  $\times$  g at 4°C. The supernatant was discarded, and the pellet  
133 was dried and dissolved in 15  $\mu$ l of nuclease-free water. The RNA concentration of each sample  
134 was measured using NanoPhotometer<sup>®</sup> N60 (Implen GmbH, Germany). The yield of total RNA  
135 was then calculated as follows: Yield (ng) = Concentration (ng/ $\mu$ l)  $\times$  15 ( $\mu$ l)  $\times$  4 (240  $\mu$ l divided  
136 by 60  $\mu$ l). The RNA detection range of the NanoPhotometer<sup>®</sup> N60 is not declared, but the dsDNA  
137 detection range is 1–16,500 ng/ $\mu$ l. Therefore, if the RNA concentration was lower than 1 ng/ $\mu$ l,  
138 the RNA yield was considered to be < 60 ng (i.e., 1 (ng/ $\mu$ l)  $\times$  15 ( $\mu$ l)  $\times$  4).

139 To test whether RT-qPCR detected *G. pallida* DNA, a DNA solution extracted from 10 cysts of  
140 *G. pallida* from Abashiri (Gp Abashiri) was prepared as described by Sakata *et al.* (2021a). The  
141 DNA extraction procedure included removal of the RNA, which was performed by adding RNase  
142 A to the cyst lysate and then heating the lysate at 55°C for 30 min. The species identity of this  
143 DNA sample was confirmed using multiplex PCR, as developed by Sakai *et al.* (2019).

144

#### 145 CONFIRMATION OF SPECIES SPECIFICITY

146 RT-qPCR was performed on a Mic real-time PCR system (Bio Molecular Systems, Upper  
147 Coomera, QLD, Australia) using a One Step TB Green<sup>®</sup> PrimeScript<sup>™</sup> PLUS RT-PCR Kit  
148 (Takara Bio Inc.). The reaction cocktail contained 5.0  $\mu$ l of 2  $\times$  One Step TB Green RT-PCR  
149 Buffer 4, 0.6  $\mu$ l of Takara Ex Taq HS Mix, 0.2  $\mu$ l PrimeScript PLUS RTase Mix, 0.4  $\mu$ l each of  
150 Y45\_0803\_F1 (10  $\mu$ M) and Y45\_0715\_R4 (10  $\mu$ M), 2.4  $\mu$ l of 7.4% (w/w) polyvinylpyrrolidone

151 (PVP, Merck KGaA, Germany, average Mw ~29,000) solution, and 1.0  $\mu\text{l}$  of the RNA solution  
152 prepared previously (not treated with DNase). PVP was added to mitigate the effects of the PCR  
153 inhibitors (Koonjul *et al.*, 1999; Monpoeho *et al.*, 2000). The cycling conditions were one cycle  
154 at 42°C for 5 min and 95°C for 10 s, and 40 cycles at 95°C for 5 s and 63°C for 35 s. The  
155 specificity of the RT-qPCR reaction for each amplified product was confirmed by melting curve  
156 analysis, which was carried out as follows: 95°C for 15 s, 60°C for 1 min, and then ramped to  
157 95°C (at 0.3°C s<sup>-1</sup> transition rate). Threshold cycles (Ct) were automatically determined using the  
158 micPCR software v. 2.8.10. A negative control was prepared using nuclease-free water as the  
159 template for each run. The RT-qPCRs were carried out in duplicate, and two independent assays  
160 were performed.

161 To confirm whether *Y45F10D.4* mRNA had been successfully extracted from all tested species,  
162 an additional endpoint RT-PCR was performed using PrimeScript™ OneStep RT-PCR Kit Ver. 2  
163 (Takara Bio, Inc., Shiga, Japan). The reaction cocktail contained 0.4  $\mu\text{l}$  of PrimeScript 1 step  
164 Enzyme Mix, 5.0  $\mu\text{l}$  of 2 × 1 step Buffer, 0.4  $\mu\text{l}$  each of Y45\_Com\_F1 (10  $\mu\text{M}$ ) and Y45\_Com\_R1  
165 (10  $\mu\text{M}$ ), 2.8  $\mu\text{l}$  of nuclease-free water, and 1.0  $\mu\text{l}$  of the RNA solution of each population (not  
166 treated with DNase). Thermal cycling was performed under the following conditions: one cycle  
167 at 50°C for 30 min and 94°C for 2 min, and 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C  
168 for 10 s, using an Applied Biosystems® SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific,  
169 Inc., MA, USA). The PCR products were electrophoresed on a 2% agarose gel in 1 × TAE at 100  
170 V for 30 min. The gel was stained with GelGreen™ (Biotium Inc., CA, USA) and visualized  
171 under cyan LED illumination.

172

#### 173 DETECTION SENSITIVITY AND STANDARD CURVE

174 We extracted RNA from 1000 eggs of Gp Abashiri using the method described above. The

175 RNA samples were diluted to 1/4, 1/16, 1/64, 1/128, 1/256, and 1/512 (corresponding to 250,  
176 62.5, 15.6, 7.8, 3.9, and 2.0 eggs, respectively) with nuclease-free water; these diluted RNA  
177 samples were used in RT-qPCR. Three replicates were prepared for each sample.

178

179 RT-qPCR USING RNA EXTRACTED FROM 1, 3-D-TREATED *G. PALLIDA*

180 We sampled *G. pallida*-infested soil from a field in Shari, Hokkaido, Japan. Approximately 300  
181 cysts and 10,000 eggs in the cysts were found in 100 ml of the soil. We placed 50 ml of the soil in  
182 50 ml glass vials and injected it with 0, 15, or 50  $\mu$ l of 1, 3-D (mixture of cis- and trans-, Tokyo  
183 Chemical Industry Co., Ltd., Tokyo, Japan). The actual application amount in the field (400 l/ha)  
184 approximately corresponds to 15  $\mu$ l/50 ml. The vial was tightly capped and incubated at 20–25°C  
185 for two weeks. Then, the soil in the vial was degassed by spreading it on a plastic tray in a fume  
186 hood for several hours and then incubated at 18°C for 2 weeks in a polyethylene bag.  
187 Subsequently, cysts in the soil were separated according to the method described by Sakata *et al.*  
188 (2021b). RNA was extracted from half of the cysts, quantified using NanoPhotometer<sup>®</sup> N60, used  
189 as a template in RT-qPCR as described previously. The other cysts were used in an inoculation  
190 test to determine their viability. The cysts were packed in a nylon mesh bag (3 × 3 cm, 100  $\mu$ m  
191 aperture). The bag was put in a quadrangular 250 ml plastic cup (MH-3, MKTEC Co. Ltd.,  
192 Toyama, Japan) with a tuber of the potato cultivar ‘Pearl Starch’ (conferring *H1* gene) and 125 ml  
193 of culture soil (0.374 g N, 1.485 g P<sub>2</sub>O<sub>5</sub>, 0.242 g K<sub>2</sub>O, and 0.165 g MgO per kg; Hokusan Co.,  
194 Ltd., Hokkaido, Japan). A cup without cyst bag was prepared as a control. Each cup was  
195 incubated at 18°C in the dark and watered as required. After 15 weeks, the soil in the cup was  
196 dried and the newly formed cysts were isolated from the soil and counted using a binocular  
197 microscope. Each treatment was replicated three times.

198

199 COMPARISON OF RT-QPCR AND THE CUP TEST

200 A total of 24 soil samples from 10 fields in Abashiri city (A01-A10), 12 fields in Shari town  
201 (S01-S12), and 2 fields in Kiyosato town (K01 and K02) were prepared. Fields A07–A09,  
202 S01–S12, K01, and K02 were infested with *G. pallida* and the other fields were applied with 1,  
203 3-D and cultivated with *S. peruvianum*. For the cup test, a tuber of potato cultivar ‘Kita-akari’  
204 (conferring *H1* gene) was planted in a 430 ml plastic cup (Matsuyoshi & Co., Ltd., Tokyo, Japan)  
205 with 200 ml of each soil sample (Fig. 2A). Four holes (approximately 4 mm in diameter) were  
206 created in the cover for watering. The cover was attached to a cup with gummed tape. Each cup  
207 was incubated at 18°C in the dark and watered, as required. After 60–70 days, the cysts visible on  
208 the roots through the transparent wall and the bottom of the cup were counted (Fig. 2B). Five  
209 replicates of each soil sample were prepared (1000 ml each).

210 For RT-qPCR, cysts were extracted from 200 ml of each soil sample. RNA was extracted from  
211 the cysts as described above; however, we used EconoSpin® RNA Mini Spin Columns (Epoch  
212 Life Science Inc., TX, USA) instead of NucleoSpin® RNA Columns to reduce the cost of RNA  
213 extraction. This change in the RNA extraction method did not affect the Ct values (data not  
214 shown) in our preliminary experiments. Five replicates of each soil sample were prepared (1000  
215 ml each). RT-qPCRs were performed once in duplicate for each sample. Nuclease-free water and  
216 RNA from five cysts of *Gp* Abashiri (the same sample used in the species specificity test) were  
217 used as controls for each run.

218 To examine the presence/absence of viable *G. pallida* in the soil samples A08, A09, S04, S11,  
219 and K02 more thoroughly, an additional pot test was performed. Cysts were isolated from another  
220 1000 ml of the soil samples and packed in a nylon mesh bag (3 × 3 cm, 100 µm aperture). Each  
221 bag was inoculated to potato cultivar ‘Sanju-maru’ (conferring *H1* gene) in a plastic pot (11.3 cm  
222 diameter and 14.0 cm height) filled with approximately 1000 ml of culture soil. After 15 weeks,

223 the soil in the pot was dried and the newly formed cysts were isolated. Cysts were counted using  
224 a binocular microscope. The test was performed once for each soil sample.

225

## 226 **Results**

### 227 DESIGN OF PRIMER SETS

228 The BLAST search revealed that orthologs of each species were hit (*G. pallida*; base no.  
229 173619–173996 and 174512–174663 in scaffold 77, *G. rostochiensis*; base no. 536749–537126  
230 and 537636–537766 in scaffold 35, *G. ellingtonae*; base no. 328503–328880 and 329340–329468  
231 in scaffold 39). By comparing these sequences with the cDNA sequence of the *Y45F10D.4* gene  
232 for *H. glycines*, two introns (approximately 40 and 500 bp, respectively) were identified in each  
233 of the *Globodera* sequences (Fig. 1). Then, we designed *G. pallida*-specific primers  
234 (Y45\_0803\_F1 and Y45\_0715\_R4) and universal primers for *Globodera* spp. and *H. glycines*  
235 (Y45\_Com\_F1 and Y45\_Com\_R1) (Table 2) to sandwich the approximately 500 bp intron  
236 between the forward and reverse primers to prevent amplification of *Y45F10D.4* genomic DNA  
237 (Fig. 1). Additionally, the intron divides Y45\_Com\_R1 into two parts. To prevent primer dimer  
238 formation and improve species specificity, the 4th base from the 3' end of Y45\_0715\_R4 was  
239 designated thymine instead of adenine (Table 2).

240

### 241 CONFIRMATION OF SPECIES SPECIFICITY

242 The RNA samples contained 95–580 ng (per 5 cysts) or 1400 ng (per 50 cysts) of total RNA  
243 (Table 3). The universal primers, Y45\_Com\_F1 and Y45\_Com\_R1, generated approximately 150  
244 bp amplicons from the RNA of all tested nematode populations (Fig. 3). RT-qPCR using the *G.*  
245 *pallida* specific primer set, Y45\_0803\_F1 and Y45\_0715\_R4, yielded an amplicon (367 bp)  
246 whose melting temperature ( $T_m$ ) was 86.3–86.4°C for the RNA of all tested *G. pallida*

247 populations (Table 3, Fig. 4). However, no amplicons or inappropriate amplicons ( $< 83^{\circ}\text{C}$   $T_m$   
248 value) were obtained from the RNA of the other *Globodera* species or DNA of *G. pallida*.

249

#### 250 DETECTION SENSITIVITY AND STANDARD CURVE

251 Amplification was successful for all replicates of the undiluted and 1/2–1/256 diluted RNA  
252 samples. However, one out of the three replicates failed when 1/512 diluted RNA samples were  
253 used; average Ct value of the two replicates was 32.5. This result indicated that RT-qPCR  
254 detected RNA corresponding to a minimum of 3.9 eggs.  $T_m$  values of appropriate amplicons  
255 ranged from 86.4 to 86.8°C.

256 The dilution series of RNA extracted from 1000 *G. pallida* eggs generated a standard curve  
257 (Fig. 5); the relationship between the Ct values ( $y$ ) and the common logarithm of the number of  
258 eggs ( $x$ ) was  $y = -3.635x + 34.157$  ( $R^2 = 0.978$ ,  $P < 0.001$ ,  $E = 88.4\%$ ).

259

#### 260 RT-QPCR USING RNA EXTRACTED FROM 1, 3-D-TREATED *G. PALLIDA*

261 The total RNA yield per half of the cysts isolated from 50 ml of the soil treated with 0  $\mu\text{l}$  of 1,  
262 3-D was  $524 \pm 61$  ng (mean  $\pm$  SE), whereas that treated with 50  $\mu\text{l}$  of 1, 3-D was  $< 60$  ng for all  
263 replicates. For *G. pallida* treated with 15  $\mu\text{l}$  of 1, 3-D, the total RNA yield was  $< 60$  ng for two  
264 out of the three replicates and 64 ng for the other replicate. When RNA was extracted from *G.*  
265 *pallida* treated with 0 or 15  $\mu\text{l}$  of 1, 3-D, amplification was successful for all replicates (Table 4).  
266 The Ct values of untreated *G. pallida* were lower than those of *G. pallida* treated with 15  $\mu\text{l}$  of 1,  
267 3-D.  $T_m$  values of appropriate amplicons ranged from 86.3 to 86.5°C. No appropriate amplicon  
268 was obtained from RNA extracted from *G. pallida* treated with 50  $\mu\text{l}$  of 1, 3-D. In the inoculation  
269 test, *G. pallida* treated with 0 or 15  $\mu\text{l}$  of 1, 3-D produced new cysts (Table 4). In contrast, *G.*  
270 *pallida* treated with 50  $\mu\text{l}$  of 1, 3-D did not reproduce.

271

## 272 COMPARISON OF RT-QPCR AND THE CUP TEST

273 In all trials, appropriate amplicons were obtained from five cysts of Gp Abashiri (average Ct  
274 value was 20.7), whereas none were obtained from nuclease-free water. Newly formed cysts were  
275 detected in soil samples A07, S02, S03, S05, S06, S08, S09, S10, and K01 using the cup test  
276 (Table 5). In addition, appropriate amplicons were obtained from the RNA extracted from the  
277 cysts contained in these soil samples. The cup test did not detect newly formed cysts from soil  
278 samples A08, A09, S04, S11, and K02, whereas RT-qPCR yielded appropriate amplicons from  
279 the RNA of cysts contained in these soil samples. No newly formed cysts were detected in the  
280 other soil samples, and no appropriate amplicons were obtained.  $T_m$  values of appropriate  
281 amplicons ranged from 86.1 to 86.9°C.

282 The additional pot test found new cysts in pots inoculated with cysts isolated from soil samples  
283 A08, A09, S04, S11, and K02 (Table 5).

284

## 285 Discussion

286 Rapid and specific detection of viable eggs is important for the control of *G. pallida*. The  
287 conventional cup test takes a relatively long time to assess *G. pallida* viability, while the visual  
288 inspection, staining, hatching test, and trehalose methods do not distinguish *G. pallida* and *G.*  
289 *rostochiensis*. Conventional PCR methods can detect DNA even in dead nematodes. This led us  
290 to develop an RT-qPCR-based detection method for viable *G. pallida*.

291 We found that the *Y45F10D.4* gene was highly expressed in *G. pallida* eggs, and there were  
292 some sequence differences in the *Y45F10D.4* gene between *G. pallida* and *G. rostochiensis* in our  
293 preliminary study. Consequently, we designed a primer set for this gene generating a 367 bp  
294 amplicon. The optimal amplicon length for qPCR is thought to be between 75–150 bp (Thornton

295 & Basu, 2015). However, a previous study evaluating the validity of *Legionella pneumophila*  
296 using PCR analyses found that longer amplicons were more indicative of viability than shorter  
297 ones (McCarty & Atlas, 1993). This led us to design a *G. pallida*-specific primer set that  
298 generates a relatively long amplicon (367 bp).

299 This primer set amplified all tested *G. pallida* populations without cross-reactivity to other  
300 tested species. Additionally, we prepared RNA from 50 cysts of *G. rostockiensis*, based on the  
301 assumption that a lot of cysts of this species can be also present in the samples. However, no  
302 amplicons were obtained for this RNA sample. These results confirmed the species specificity of  
303 this primer set. For *G. pallida*, we used only Japanese and European populations. However, *G.*  
304 *pallida* populations in Algeria, North America, and New Zealand are phylogenetically close to  
305 European and Japanese *G. pallida* populations (Skanter *et al.*, 2007; Madani *et al.*, 2010; Tirchi *et*  
306 *al.*, 2016; Ohki *et al.*, 2018; Subbotin *et al.*, 2020). Therefore, this primer set may be applicable  
307 to *G. pallida* populations outside of Japan and Europe.

308 DNA is often co-extracted with RNA, and the DNA remaining in the RNA sample may cause  
309 false-positive signals derived from dead organisms. However, DNase treatment is time- and  
310 labor-intensive. To solve this problem, Leal *et al.* (2013) designed a reverse primer for an  
311 exon-exon junction. However, we designed a *G. pallida*-specific primer set sandwiching a long  
312 (approximately 500 bp) intron. If the primers hybridize with the genomic DNA, the elongation  
313 step requires a relatively long time. Under our experimental conditions, where the  
314 annealing/extension step was set to 35 s, amplification of *G. pallida* DNA did not occur. However,  
315 amplification from DNA may occur with a longer annealing/extension step. In other words, if the  
316 annealing/extension step is extended, the primer set may be useful for *G. pallida*-specific  
317 detection by conventional DNA-based PCR.

318 The RT-qPCR method was sensitive enough to detect RNA corresponding to 3.9 *G. pallida*

319 eggs. Many DNA-based detection methods using the ITS region or mitochondrial cytochrome *b*  
320 gene can detect a single egg or juvenile (Nakhla *et al.*, 2010; Sakai *et al.*, 2019; Kushida and  
321 Sakai, 2022), indicating that the detection sensitivity of our method is lower than that of these  
322 DNA-based methods. The expression level of *Y45F10D.4* and the differences in copy number  
323 among genes might account for the differences in sensitivity. However, the detection threshold of  
324 the probe-based RT-qPCR method is 30 eggs (Benier *et al.* 2014). Therefore, our method is more  
325 sensitive than the previous RT-qPCR methods. In addition, we drew a standard curve and showed  
326 a significant negative correlation between the concentration of RNA and Ct values, illustrating  
327 that the density of *G. pallida* could be estimated.

328 In many studies that developed methods to assess nematode viability, heat treatment has been  
329 used to prepare dead individuals (Leal *et al.*, 2013; Ebrahimi *et al.*, 2015; Pillai & Dandurand,  
330 2019). In our preliminary study, conducted in July and August of 2020 and 2021, we measured  
331 soil temperature (10 cm deep) at 30- or 60 min intervals in a *G. pallida*-infested field. The  
332 average daily soil temperature ranged from 15.0 to 27.9°C, and the maximum soil temperature  
333 was 31.8°C (unpublished data). Considering that many *G. pallida* eggs were still alive after  
334 heating at 40°C for 64 min (Stone & Webley, 1975), it is unlikely that *G. pallida* in Hokkaido's  
335 fields died of heat. Therefore, we used 1, 3-D, which is used in the official control, to prepare  
336 dead *G. pallida*. We showed that *G. pallida*, treated with a sufficient amount of 1, 3-D, did not  
337 reproduce, and its RNA was not detected by RT-qPCR. The total RNA yield of *G. pallida* treated  
338 with 50  $\mu$ l of 1, 3-D was much lower than that treated with 0  $\mu$ l of 1, 3-D, suggesting that the  
339 RNA was degraded after death and this resulted in no amplification in the RT-qPCR. Our results  
340 demonstrated that the RT-qPCR method did not detect dead *G. pallida*. To the best of our  
341 knowledge, this is the first study to show the validity of reverse transcription PCR for the  
342 detection of viable nematodes after 1, 3-D use. In our experiment, RNA extraction was performed

343 2 weeks after 1, 3-D treatment lasting for 2 weeks. Thus, the mRNA of the *G. pallida* Y45F10D.4  
344 gene appears to be undetectable 2 to 4 weeks after death. A previous study showed that mRNA of  
345 the *Bursaphelenchus xylophilus* Hsp70 gene was detectable after 11 days of heat treatment, but  
346 was undetectable after 14 days (Leal *et al.*, 2013). Our study yielded similar data to those of a  
347 previous study on the durability of nematode mRNA after death. *G. pallida* treated with 15  $\mu$ l of  
348 1, 3-D produced several cysts and generated appropriate amplicons with Ct values of  
349 approximately 30. This result suggested that a small number of individuals survived after the 1,  
350 3-D treatment, and such viable individuals were detected by RT-qPCR. In addition, conventional  
351 DNA-based PCR methods (Bulman & Marshall, 1994; Sakai *et al.*, 2019) detected DNA from *G.*  
352 *pallida* treated with 50  $\mu$ l of 1, 3-D (data not shown). Therefore, DNA-based PCR methods are  
353 not useful for viable *G. pallida* detection, as demonstrated by van den Elsen *et al.* (2012).

354 The RT-qPCR method yielded appropriate amplicons from all samples in which newly formed  
355 cysts were detected using the cup test. The cup test did not detect newly formed cysts in the soil  
356 samples A08, A09, S04, S11, and K02. However, RT-qPCR obtained appropriate amplicons from  
357 the cysts contained in these soil samples. The additional pot test showed that cysts from these soil  
358 samples produced new cysts, which is clear evidence that these soil samples contained viable *G.*  
359 *pallida*. These results showed that the sensitivity of the RT-qPCR method was higher than that of  
360 the cup test. In the cup test, we only checked the cysts visible through the wall and bottom of the  
361 cup. Therefore, in soil samples A08, A09, S04, S11, and K02, it is possible that some individuals  
362 formed new cysts inside the cup, which we did not detect. Furthermore, the additional pot test  
363 was performed by gathering cysts in one place (i.e., mesh bag) and inoculating them. Therefore,  
364 mating might have occurred more easily during these tests. Although fields S01, S07, and S12  
365 were infested with *G. pallida*, this study did not detect viable individuals. This could be caused  
366 by a low population density and the patchy distribution of *G. pallida* in these fields, and the

367 samples we collected may not have contained a detectable number of viable individuals.

368 RT-qPCR requires isolation of cysts from the soil. During this process, floating debris, such as  
369 plant fragments, seeds, and dead organisms similar in size to cysts are also isolated (Kushida &  
370 Sakai, 2022). Because the debris might act as a PCR inhibitor, the cysts must be sorted from the  
371 debris. Reid *et al.* (2015) and Kushida & Sakai (2022) reported DNA-based PCR methods using  
372 DNA extracted from cysts with floating debris. To reduce the labor and time for sample  
373 preparation, it is necessary to similarly develop an RNA extraction method for cysts with floating  
374 debris.

375 In summary, we have successfully developed an intercalator-based RT-qPCR method for the  
376 detection of viable *G. pallida*. This method consists of cyst extraction, RNA extraction, and  
377 RT-qPCR and requires only one week approximately, even considering the time required to dry  
378 soil samples to isolate cysts. Therefore, this method saves significantly more time than the cup  
379 test. Verification using other *Globodera* and *Heterodera* species and populations will provide  
380 more confirmatory data regarding the effectiveness of this method.

381

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564

565 **Table 1.** Nematodes used in this study

Code	Species	Pathotype	Origin	Source
Gp Abashiri	<i>Globodera pallida</i>	Pa3	Abashiri, Hokkaido, JP	This study
Gp Shari	<i>G. pallida</i>	Not determined	Shari, Hokkaido, JP	This study
Gp Chavornay	<i>G. pallida</i>	Pa3	Chavornay, CH	E. Grenier <sup>3</sup>
Gp UK	<i>G. pallida</i>	Pa1	UK	F. G. W. Jones <sup>1</sup>
Gr Kucchan	<i>G. rostochiensis</i>	Ro1	Kucchan, Hokkaido, JP	This study
Gr Shari	<i>G. rostochiensis</i>	Ro1	Shari, Hokkaido, JP	This study
Gr Nagasaki	<i>G. rostochiensis</i>	Ro1	Nagasaki, JP	This study
Gr Aomori	<i>G. rostochiensis</i>	Ro1	Aomori, JP	This study
Gr Ecosse	<i>G. rostochiensis</i>	Ro1	Ecosse, UK	E. Grenier <sup>3</sup>
Gr UK	<i>G. rostochiensis</i>	Ro1	UK	F. G. W. Jones <sup>1</sup>
Ge 29Y1553	<i>G. ellingtonae</i>	-	US	T. Prior <sup>2</sup>
Ge 2Y1141	<i>G. ellingtonae</i>	-	US	I. Zasada <sup>3</sup>
Gtt Kochi	<i>G. tabacum tabacum</i>	-	Kochi, JP	This study
Gtt Landes	<i>G. tabacum tabacum</i>	-	Landes, FR	E. Grenier <sup>3</sup>
Gts 75140	<i>G. tabacum solanacearum</i>	-	MX	E. Grenier <sup>3</sup>
Ga	<i>G. artemisiae</i>	-	Nagasaki, JP	This study

Hg	<i>Heterodera glycines</i>	-	Kyougoku, Hokkaido, JP	This study
Ht	<i>H. trifolii</i>	-	Nanae, Hokkaido, JP	This study
Hs	<i>H. schachtii</i>	-	Nagano, JP	H. Okada <sup>3</sup>
He	<i>H. elachista</i>	-	Chiba, JP	H. Sakai <sup>3</sup>

---

566 <sup>1</sup> Gp UK and Gr UK were provided in 1974 and have been propagated in our laboratory.

567 <sup>2</sup> Ge 29Y1553 was provided in 2017 and has been propagated in our laboratory.

568 <sup>3</sup> Cysts of these populations were provided in 2020 to 2022 and directly used in this study.

569

570 **Table 2.** Primers designed in this study

Target species	Primer name	Sequence (5'=>3')
<i>Globodera pallida</i>	Y45_0803_F1	CAAAAATGACCCATCGGTTG
	Y45_0715_R4	GCAATGAATGCAACG <u>IT</u> CG
Cyst nematodes	Y45_Com_F1	GGTCAGCAATTGCGAGTTC
	Y45_Com_R1	TTGCGTCTTGAGCCAACAT

571 Underlined portion represents an intentionally replaced base.

572 **Table 3.** Species specificity of the primer set, Y45\_0803\_F1 and Y45\_0715\_R4

Code	Number of cysts	Nucleic acid type	Ct values	Total RNA yield (ng) <sup>1</sup>
Gp Abashiri	5	RNA	19.5	181
	10	DNA	nd <sup>2</sup>	- <sup>3</sup>
Gp Shari	5	RNA	18.6	580
Gp Chavornay	5	RNA	20.6	235
Gp UK	5	RNA	19.9	254
Gr Kucchan	5	RNA	nd	238
	50	RNA	nd	1400
Gr Shari	5	RNA	nd	182
Gr Nagasaki	5	RNA	nd	313
Gr Aomori	5	RNA	nd	258
Gr Ecosse	5	RNA	nd	200
Gr UK	5	RNA	nd	208
Ge 29Y1553	5	RNA	nd	389
Ge 2Y1141	5	RNA	nd	216
Gtt Kochi	5	RNA	nd	104
Gtt Landes	5	RNA	nd	174
Gts 75140	5	RNA	nd	101
Ga	5	RNA	nd	157
Hg	5	RNA	nd	228
Ht	5	RNA	nd	424

Hs	5	RNA	nd	344
He	5	RNA	nd	95

573 <sup>1</sup> Per 50 cysts (Gr Kucchan 50 cysts) or 5 cysts (the others), quantified using Nanophotometer<sup>®</sup>

574 N60

575 <sup>2</sup> nd: not detected

576 <sup>3</sup> -: not quantified

577

578 **Table 4.** Effect of 1, 3-dichloropropene (1, 3-D) treatment of infested soil on the detectability by  
 579 the RT-qPCR method and evaluation of viability of *Globodera pallida* cysts by the inoculation  
 580 test

Quantity of 1, 3-D ( $\mu$ l) added to 50 ml soil	RT-qPCR (Ct values) <sup>1</sup>	Corresponding egg number <sup>1,2</sup>	Inoculation test (The number of newly formed cysts) <sup>1,3</sup>
0	21.5 $\pm$ 0.4	3196 $\pm$ 772	167.3 $\pm$ 27.7
15	29.1 $\pm$ 0.5	27 $\pm$ 8	1.7 $\pm$ 0.9
50	nd <sup>4</sup>	0	0
Without cysts	- <sup>5</sup>	-	0

581 Ct values were determined using half of the *G. pallida* cysts in 50 ml soil treated with 1, 3-D for  
 582 two weeks. Inoculation tests were performed by inoculating the other cysts in the soil to the  
 583 potato cultivar ‘Pearl Starch’ in plastic cups. An inoculation test without cysts was performed as  
 584 the negative control.

585 <sup>1</sup> Data represent mean  $\pm$  SE.

586 <sup>2</sup> Calculated using the regression equation in Fig. 5,  $y = -3.635x + 34.157$  (where  $y$  represents Ct  
 587 values and  $x$  represents the common logarithm of the number of eggs)

588 <sup>3</sup> Cysts were isolated from the entire soil in the cups and counted using a binocular microscope.

589 <sup>4</sup> nd: not detected

590 <sup>5</sup> -: not tested

591

592 **Table 5.** Comparison of detection sensitivity of *Globodera pallida*

Soil sample	Status of <i>G. pallida</i> infection <sup>1</sup>	RT-qPCR			Cup test		Additional pot test
		Ct values <sup>2</sup>	No. positives/No. replicates	Corresponding egg number <sup>2,3</sup>	The number of cysts <sup>2</sup>	No. positives/No. replicates	The number of cysts <sup>4</sup>
A01	–	nd <sup>5</sup>	0/5	0	0	0/5	– <sup>6</sup>
A02	–	nd	0/5	0	0	0/5	–
A03	–	nd	0/5	0	0	0/5	–
A04	–	nd	0/5	0	0	0/5	–
A05	–	nd	0/5	0	0	0/5	–
A06	–	nd	0/5	0	0	0/5	–
A07	+	27.2 ± 1.0	4/5	129 ± 58	9.6 ± 2.5	5/5	–
A08	+	30.3 ± 0.9	5/5	27 ± 20	0	0/5	50
A09	+	32.3	1/5	3	0	0/5	48
A10	–	nd	0/5	0	0	0/5	–
S01	+	nd	0/5	0	0	0/5	–
S02	+	33.5 ± 0.4	5/5	2 ± 0	6.8 ± 1.2	5/5	–
S03	+	32.2 ± 0.2	4/5	4 ± 1	6.0 ± 1.5	5/5	–

S04	+	31.1 ± 1.0	5/5	15 ± 8	0	0/5	46
S05	+	31.3 ± 1.0	5/5	13 ± 6	0.8 ± 0.5	2/5	-
S06	+	31.6 ± 0.7	5/5	7 ± 3	4.4 ± 1.6	4/5	-
S07	+	nd	0/5	0	0	0/5	-
S08	+	25.2 ± 0.8	5/5	442 ± 181	18.4 ± 4.7	5/5	-
S09	+	23.2 ± 0.7	5/5	1377 ± 399	63.6 ± 14.5	5/5	-
S10	+	25.5 ± 0.7	5/5	332 ± 117	50.2 ± 7.5	5/5	-
S11	+	28.4	1/5	39	0	0/5	3
S12	+	nd	0/5	0	0	0/5	-
K01	+	28.1 ± 0.8	4/5	66 ± 27	1.6 ± 0.7	3/5	-
K02	+	31.2	1/5	6	0	0/5	12

593 <sup>1</sup> -: Fields applied with 1, 3-dichloropropene and cultivated with *Solanum peruvianum*. +: *G. pallida* infected fields

594 <sup>2</sup> Data represent mean ± SE.

595 <sup>3</sup> Calculated using the regression equation in Fig. 5,  $y = -3.635x + 34.157$  (where  $y$  represents Ct values and  $x$  represents the common  
596 logarithm of the number of eggs)

597 <sup>4</sup> Cysts were isolated from the entire soil in the pots and counted using a binocular microscope.

598 <sup>5</sup> nd: not detected

599 <sup>6</sup> -: not tested

600

601

602 **Figure legends**

603 **Fig. 1.**

604 Partial sequences of *Y45F10D.4* gene for *Globodera pallida*, *G. rostochiensis*, *G. ellingtonae*,  
605 and *Heterodera glycines*. The sequence of *H. glycines* represents cDNA, whereas the other  
606 sequences represent genomic DNA. The position of the primers is indicated by arrows. Hyphens  
607 indicate deletion of the corresponding base and the middle dots indicate the same base as *G.*  
608 *pallida*.

609

610 **Fig. 2.**

611 Illustrations of cup test. A: The cup just after planting. B: Developing cysts visible on the roots  
612 (approximately 2 months after planting).

613

614 **Fig. 3.**

615 Endpoint RT-PCR results using the universal primer set (Y45\_Com\_F1 and Y45\_Com\_R1). The  
616 RNA was extracted from 5 and/or 50 cysts of each nematode population. M: 100 bp DNA ladder  
617 (DM2100, SMOBIO Technology, Taiwan); 1: Gp Abashiri; 2: Gp Shari; 3: Gp Chavornay; 4: Gp  
618 UK; 5: Gr Kucchan (5 cysts); 6: Gr Shari; 7: Gr Nagasaki; 8: Gr Aomori; 9: Gr Ecosse; 10: Gr  
619 UK; 11: Ge 29Y1553; 12: Ge 2Y1141; 13: Gtt Kochi; 14: Gtt Landes; 15: Gts 75140; 16: Ga; 17:  
620 Hg; 18: Ht; 19: Hs; 20: Gr Kucchan (50 cysts); 21: He; NC: negative control (nuclease-free  
621 water).

622

623 **Fig. 4.**

624 Results examples of the RT-qPCR using the *G. pallida*-specific primer set (Y45\_0803\_F1 and  
625 Y45\_0715\_R4). Nucleic acid samples of nematode populations listed in Table 3 were used. A:

626 Amplification plots obtained from nematodes used in this study. B: Melting plots obtained from

627 Gp Abashiri, Gp Shari, Gp Chavornay, and Gp UK.

628

629 **Fig. 5.**

630 Standard curve of the RT-qPCR method calculated with the Ct values and number of eggs.

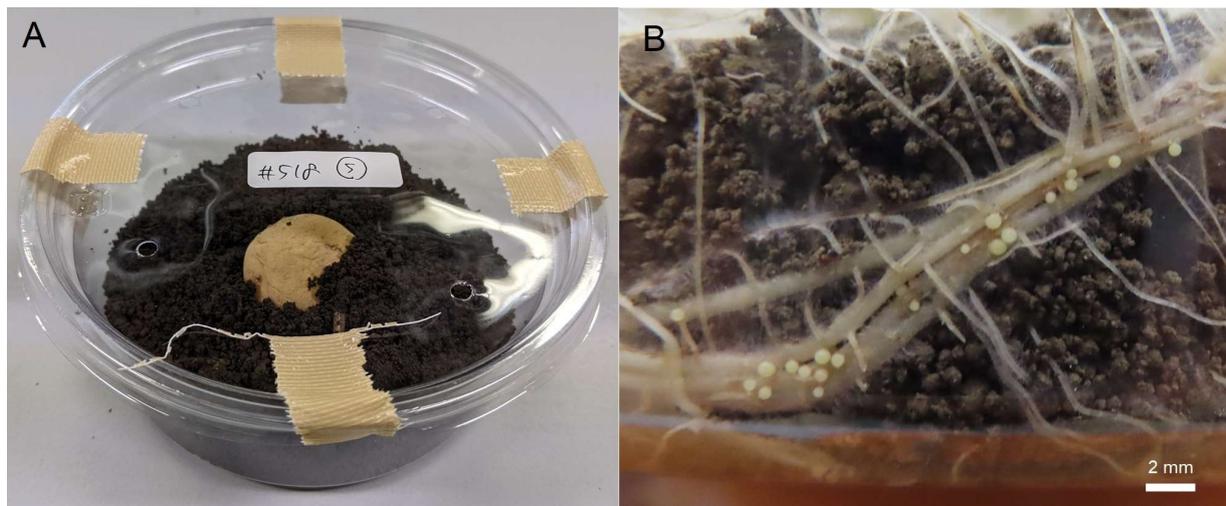
631

<i>G. pallida</i> gDNA	-----	0
<i>G. rostochiensis</i> gDNA	-----	0
<i>G. ellingtonae</i> gDNA	-----	0
<i>H. glycines</i> cDNA	TTCTTTTTTCGCTGACGCGACTTTTCCCCACTTTGGTCTTCCACCGTCATGGTAACATTCA	60
<i>G. pallida</i> gDNA	ACTGCAGCAGATTGCGGGGTACCACGAGAAGGTCATTGACCATTATGAAAATCCAAGAAA	60
<i>G. rostochiensis</i> gDNA	.....A.....G.....	60
<i>G. ellingtonae</i> gDNA	.....G.....	60
<i>H. glycines</i> cDNA	.....T...A.....CT...T...A.....C.....C.....	120
	Y45_0803_F1 ----->	
<i>G. pallida</i> gDNA	TGTTGGCTCTTTGGACAAAAATGACCCATCGGTTGGCACTGGGGTTGTTGGAGCGCCGGC	120
<i>G. rostochiensis</i> gDNA	...C.....	120
<i>G. ellingtonae</i> gDNA	...C.....C.....	120
<i>H. glycines</i> cDNA	...G.....T...G...T...T...A...A...C...G...A.....	180
	----- ----- Intron -----	
<i>G. pallida</i> gDNA	TTGTGGCGATGTTATGAAATTGCAAATCAAGGTTTGAGGAAAACAGGTTTGCTATATGGG	180
<i>G. rostochiensis</i> gDNA	.....CC...C.....T.....T.....	180
<i>G. ellingtonae</i> gDNA	.....CC...C.....T.....	180
<i>H. glycines</i> cDNA	...C...A...A...C...T...T.....	214
	----- -----	
<i>G. pallida</i> gDNA	TTTGTGTGTTTAAGGTGGATGCAAATGGCAAAATAATCGACGCGAAGTTTAAGACGTTTCG	240
<i>G. rostochiensis</i> gDNA	.....C.....	240
<i>G. ellingtonae</i> gDNA	.....	240
<i>H. glycines</i> cDNA	-----C...G...C...AC.....T.....A...A...C.....T.....	257
	Y45_Com_F1 ----->	
<i>G. pallida</i> gDNA	GATGCGGGTCAGCAATTGCGAGTTCGTCACTCGCCACCGAATGGATAAAAAGGGCAGAATT	300
<i>G. rostochiensis</i> gDNA	.....G.....A.....	300
<i>G. ellingtonae</i> gDNA	.....A.....	300
<i>H. glycines</i> cDNA	.G...T.....T...GT...G.....T.....G.....	317
<i>G. pallida</i> gDNA	TGGACTATGCCAGCAAGGTCAAGAACCAGCAGATTGCCAAGGAACTATCGCTTCTCTCCCG	360
<i>G. rostochiensis</i> gDNA	.....T.....A.....	360
<i>G. ellingtonae</i> gDNA	.....A.....	360
<i>H. glycines</i> cDNA	...A...GC...AA...A...A...A...C...A...G...T...A...G...A...A...	377
	Y45_Com_R1 -----<	
<i>G. pallida</i> gDNA	TCAAGCTGCACTGTTTCGA	405
<i>G. rostochiensis</i> gDNA	.....	405
<i>G. ellingtonae</i> gDNA	.....	405
<i>H. glycines</i> cDNA	.T.....C.....	422
	Approx. 500 bp intron	
<i>G. pallida</i> gDNA	CACTGAGTGATTATCAGAAGAAGCAAGAAGCGCGCATTGAAAAGACTTGAACGCCAATTG	465
<i>G. rostochiensis</i> gDNA	.....G.....	464
<i>G. ellingtonae</i> gDNA	.....G.....A.....	464
<i>H. glycines</i> cDNA	.G...A...C.....T...A...G...A.....TTGTGAC	482
	Y45_0715_R4 -----<	
<i>G. pallida</i> gDNA	T-CGATCGTTGCATTTCATTGCAAAACATTTA-----CAATAAACCCGGCGGATAAAATTTA	518
<i>G. rostochiensis</i> gDNA	..T.....A.....G.....	509
<i>G. ellingtonae</i> gDNA	..T.....A.....G.....	507
<i>H. glycines</i> cDNA	.AT...TA...C...CAA...A...A...AGCCCGGCGGATTT...TTTT...ATTAA...TT...GCG	542
<i>G. pallida</i> gDNA	TTTTTACTCATT-----	530
<i>G. rostochiensis</i> gDNA	-----	509
<i>G. ellingtonae</i> gDNA	-----	507
<i>H. glycines</i> cDNA	CAA...T...TC...GTTTCGATATTTCTCCTGA	571

632

633 **Fig. 1**

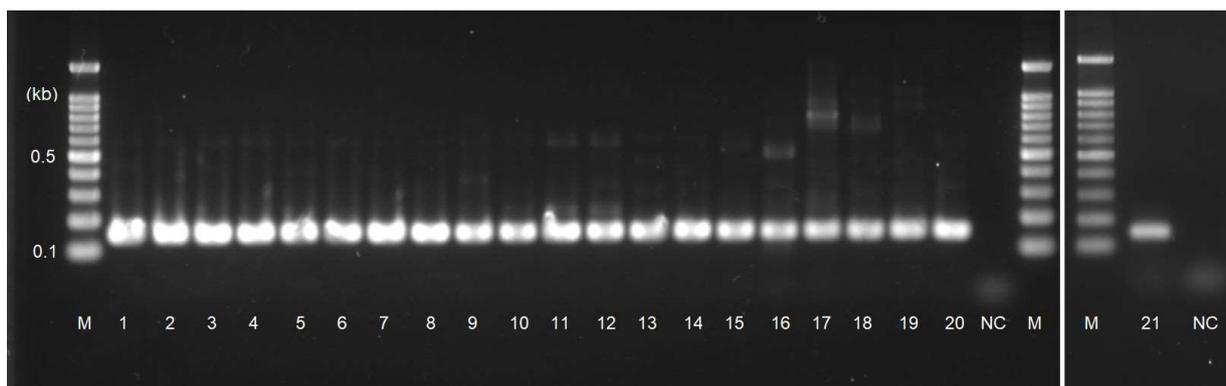
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635

636 **Fig. 2**

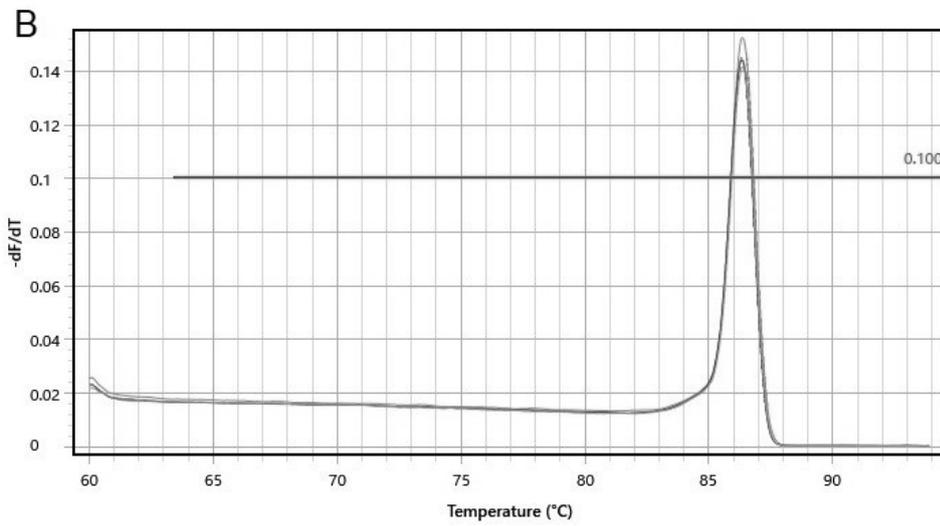
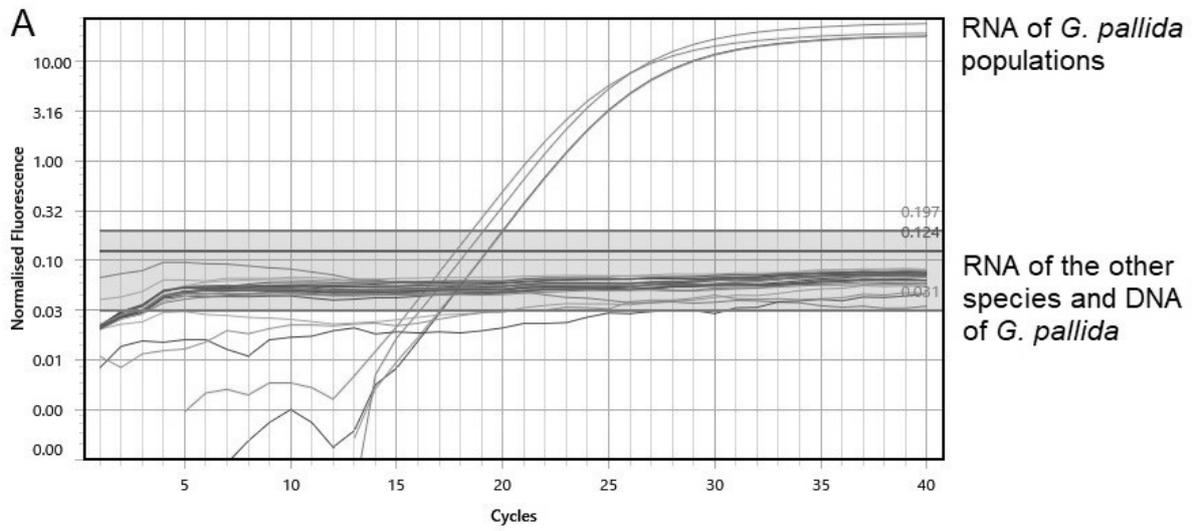
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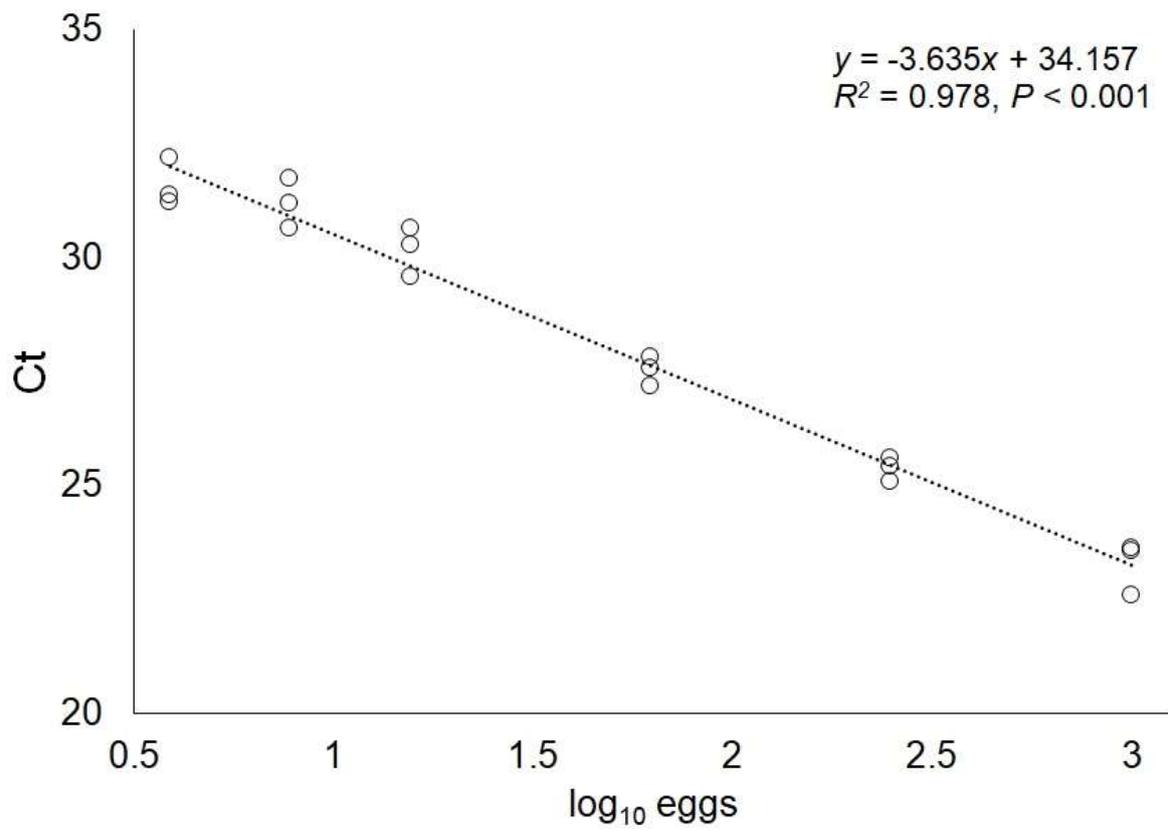
639 **Fig. 3**

640



641

642 **Fig. 4**



643

644 **Fig. 5**

645

646 **Appendix**

647 EXPRESSION LEVEL OF HOUSEKEEPING GENES IN *G. PALLIDA*

648 We evaluated the expression levels of the housekeeping genes listed in Table A1 in *G. pallida*  
649 (Gp Abashiri). To prepare J2s, cysts were immersed in hydroponic solution of potatoes (collected  
650 from Hokkaido-Chuo Station, Center for Seeds and Seedlings, NARO, Kitahiroshima, Hokkaido)  
651 for a week at 16°C. One thousand eggs or J2s were homogenized in a 2 ml plastic tube with one  
652 hundred 1 mm diameter zirconium oxide beads, twenty 2 mm diameter zirconium oxide beads,  
653 350  $\mu$ l of RA1 buffer (in Nucleospin<sup>®</sup> RNA), and 3.5  $\mu$ l of 1 M dithiothreitol (FUJIFILM Wako  
654 Pure Chemical Co.) using a multi-beads shocker (FastPrep-24<sup>™</sup> 5G). Total RNA extraction was  
655 carried out using a Nucleospin<sup>®</sup> RNA according to the manufacturer's instructions. The total  
656 RNA was dissolved in 60  $\mu$ l of nuclease-free water. Three replicates were prepared for each  
657 sample. To degrade DNA, the DNase treatment was carried out as described in Materials and  
658 Methods, but the resulting RNA pellets were dissolved in 60  $\mu$ l of nuclease-free water.

659 RT-qPCRs were performed on a Mic real-time PCR system using a One Step TB Green<sup>®</sup>  
660 PrimeScript<sup>™</sup> PLUS RT-PCR Kit. The reaction cocktail contained 5.0  $\mu$ l of 2  $\times$  One Step TB  
661 Green RT-PCR Buffer 4, 0.6  $\mu$ l of Takara Ex Taq HS Mix, 0.2  $\mu$ l PrimeScript PLUS RTase Mix,  
662 0.4  $\mu$ l each of forward and reverse primers (10  $\mu$ M, Table A1), 2.4  $\mu$ l of nuclease-free water, and  
663 1.0  $\mu$ l of the total RNA solution prepared above. Primer sequences are summarized in Table A1.  
664 For J2s, the RT-qPCR was performed using only *Y45F10D.4* primer set. The cycling conditions  
665 were one cycle at 42°C for 5 min and 95°C for 10 s, and 40 cycles each at 95°C for 5 s and 60°C  
666 for 30 s. The specificity of the RT-qPCR reaction for each amplified product was verified by  
667 melting curve analysis, which was carried out as follows: 15 s at 95°C, 1 min at 60°C, and then  
668 ramping to 95°C (at 0.15°C s<sup>-1</sup> transition rate). Threshold cycles (Ct) were automatically  
669 determined using the micPCR software v. 2.8.10. The RT-qPCRs were carried out in duplicate.

670 The Ct values for each gene was shown in Table A1. For eggs, the gene with the lowest Ct  
671 value was *Y45F10D.4*, which encodes a putative iron-sulfur cluster assembly enzyme, suggesting  
672 that this gene was highly expressed in *G. pallida* eggs. The Ct values of *Y45F10D.4* using RNA  
673 of *G. pallida* J2s were  $19.0 \pm 0.4$  (Mean  $\pm$  SE), indicating that this gene was expressed to a  
674 certain degree in J2s also.

675

676 **Table A1.** Primers used in expression analysis of housekeeping genes and Ct values of each gene

Gene	Sequence (5'=>3')	Gene description	Reference	Ct values <sup>1</sup>
<i>act-1</i>	Forward: CTTCTTGGGCATGGAGTCGG Reverse: AATGCCCGGGTACATCGTC	actin 1	Jones <i>et al.</i> , 2018	18.6 ± 0.1
<i>Ama-1</i>	Forward: CTCCAAGCTCTCCACGTTATT Reverse: GGCGAAGTTGGACTGTATGT	amanitin resistant family member	Sabeh <i>et al.</i> , 2018	23.9 ± 0.2
<i>APC1</i>	Forward: CCGCTGGACTTGTTGGTAAT Reverse: TAAGCTGCAGCACATCCAAC	anaphase-promoting complex subunit 1	Palomares-Rius <i>et al.</i> , 2016	23.7 ± 0.3
<i>BUB3</i>	Forward: CACACAGATGGGGTGAGATG Reverse: GTACTCTGTCCCCGCATT	mitotic checkpoint protein bub3	Palomares-Rius <i>et al.</i> , 2016	22.5 ± 0.1
<i>cdc-42</i>	Forward: GCATCATTGTACGAAGACTCCA Reverse: TGGGCTTCATTTTGTCTTTGC	cell division control protein 42 homolog	Jones <i>et al.</i> , 2018	21.1 ± 0.1
<i>eif4A</i>	Forward: CGAAACAGGACCAACAAATG Reverse: GTTCAGATCAGCTCCCCAAT	translation initiation factor	Valdes <i>et al.</i> , 2012	18.7 ± 0.1
<i>GR</i>	Forward: TTGAGAGACCATGCCGATTAC Reverse: GAGTTGAGACGCCGAATGT	glutathione reductase	Sabeh <i>et al.</i> , 2018	20.8 ± 0.1
<i>Mce1</i>	Forward: CCCGCATAAACTCCCATCTT Reverse: CTTACACCGATTTGCCTTTC	mRNA capping enzyme	Sabeh <i>et al.</i> , 2018	20.8 ± 0.1

<i>TUBG2</i>	Forward: ATTGGCATTTCGACCTG Reverse: GTCATTGGTCCGACTTTGGT	tubulin $\gamma$ -2 chain	Palomares-Rius <i>et al.</i> , 2012	22.8 $\pm$ 0.1
<i>Y45F10D.4</i>	Forward: CCAAGCAGCACTGAGTGATTA Reverse: CATGATCCGCCGGGTTTATT	iron-binding protein involved in Fe-S cluster formation	Sabeh <i>et al.</i> , 2018	17.9 $\pm$ 0.1

677 <sup>1</sup> Data represent mean  $\pm$  SE. This column represents the Ct values using RNA extracted from eggs.

678