Efficient quantification of Globodera pallida and G. rostochiensis (Tylenchida: Heteroderidae) in large amounts of soil using probe-based real-time PCR

Efficient quantification of *Globodera pallida* and *G. rostochiensis* (Tylenchida: Heteroderidae) in large amounts of soil using probe-based real-time PCR

Itaru Sakata*, Kenji Itou, Atsuhiko Kushida

Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-8555, Japan.

*Corresponding author: Itaru Sakata, sakatai387@affrc.go.jp

ORCID iDs: Itaru Sakata, 0000-0003-2563-5208; Atsuhiko Kushida, 0000-0002-4549-7861

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Abstract

Real-time quantitative polymerase chain reaction (qPCR) was used to estimate the population densities of the potato cyst nematodes *Globodera pallida* Stone and *Globodera rostochiensis* (Wollenweber) Skarbilovich (Tylenchida: Heteroderidae). Since it is difficult to extract nematode DNA from large amounts of soil (≥ 100 g, enough for quantification of cyst nematodes), cyst isolation is required before DNA extraction. However, when isolating cysts from the soil, various impurities are simultaneously isolated, and separating the cysts from these impurities is laborious. Although previous studies have reported methods for extracting DNA from mixtures of cysts and impurities, it is unclear whether such DNA can be used to estimate nematode densities using qPCR. To examine the effects of impurities on the accuracy of qPCR quantification, we extracted DNA from nematode eggs (*G. pallida* and *G. rostochiensis*) mixed with impurities and performed qPCR. The results suggested that the differences in the fields affected the quantification accuracy. Therefore, field-specific standard curves should be set, which are impractical for routine diagnosis. To propose a more practical method, we determined a fixed standard curve for each species and estimated the population densities in field soil samples by qPCR using the standard curves. The estimated population densities significantly correlated with those determined using conventional microscopic inspections. This study revealed that the population densities of *G. pallida* and *G. rostochiensis* can be estimated from large amounts of soil, probably only approximately, but efficiently, by qPCR using DNA extracted from mixtures of cysts and impurities.

Keywords: DNA extraction, *Globodera* spp., Impurity, Population density, Potato cyst nematodes, qPCR

Introduction

The potato cyst nematodes *Globodera pallida* Stone (Tylenchida: Heteroderidae) and *G. rostochiensis* (Wollenweber) Skarbilovich are the major potato (*Solanum tuberosum*) pests. In Japan, *G. rostochiensis* and *G. pallida* were first detected in 1972 and 2015, respectively (Narabu et al. 2016; Yamada et al. 1972) and now occur mainly in Hokkaido. These species cause a reduction in plant growth and potato yield. Yield loss is correlated with the initial nematode density (Trudgill et al. 1975a, 1975b). Although the *H1* gene, an excellent *G. rostochiensis*-resistance gene, has been introduced into many commercial potato cultivars, it is ineffective against *G. pallida*. As of 2023, only a few potato cultivars that are resistant to *G. pallida* are available in Japan. Therefore, to control *G. rostochiensis* and *G. pallida* appropriately, it is important to distinguish between the two species and estimate their population densities.

Traditionally, microscopic inspections have been performed in Japan to determine population densities of these nematodes. Because the distribution of cyst nematodes in the soil is not generally uniform, and some fields contain only a few cysts per 100 g of soil, the use of a small amount of soil (*e.g.*, 10–20 g) may lead to inaccurate quantification, especially at low densities. Therefore, in Japan, a sample mass of ≥ 100 g of dry soil per replicate is generally used for the estimation of cyst nematodes' population density. Microscopic inspection consisted of isolating the cysts from the soil, crushing them to release eggs and juveniles, and counting them under a microscope (Fig. 1). First, the field soil samples were spread on a tray and dried in the shade. Because dried cysts float on water, they can be isolated from the soil by stirring dry soil and water together and pouring floating cysts with the supernatant into a pair of sieves (*e.g.*, 850 μm over 212 μm aperture) (see Sakata et al. 2021a). The soil particles (sand, silt, and clay) were removed using this procedure. Although the cysts are collected on the lower sieve with 212 μm aperture, miscellaneous materials that float on water and are similar in size to the cysts are also collected. These materials (designated as "impurities") contain plant fragments, plant seeds, and insect carcasses, and other unidentifiable substances. Collected cysts and impurities were transferred onto filter paper, and the cysts were picked up using tweezers under a stereomicroscope and crushed in water using a homogenizer to release eggs and

juveniles. Population densities were determined by counting eggs and juveniles in the water using a microscope. Thus, this inspection procedure is laborious and too time-consuming to be widely used. In addition, because this method does not discriminate between species, additional procedures, such as polymerase chain reaction (PCR), are required.

Currently, several real-time PCR (qPCR) methods for simultaneous quantification of *G. pallida* and *G. rostochiensis* are available (Gamel et al. 2017; Madani et al. 2008; Nakhla et al. 2010; Papayiannis et al. 2013). However, these qPCR methods have not been verified using nematode-infected soil samples. Although the nematode DNA used for qPCR can be extracted directly from the soil (Goto et al. 2009, 2010; MacMillan et al. 2006; Min et al. 2011), the amount of soil used for DNA extraction is less than or equal to 20 g per sample, which is too small to accurately determine the population density of cyst nematodes. In addition, a method to extract nematode DNA directly and efficiently from large amounts (≥ 100 g) of soil has yet to be developed. Therefore, to inspect such large amounts of soil, cysts must be isolated from the soil to reduce the sample volume for DNA extraction, based on the method described above, and DNA extraction can then be performed. However, the selection of cysts from impurities is laborious.

Previous studies (Kushida and Sakai 2022; Reid et al. 2015) reported simple DNA extraction methods for mixtures of cysts and impurities. Researchers in these studies isolated cysts and impurities from the soil, dried them, collected them in plastic tubes, and extracted the DNA. By extracting DNA from a mixture of cysts and impurities, inspectors do not need to select cysts. In addition, Kushida and Sakai (2022) showed that a single egg added to 0.5 mL of impurities can be detected by endpoint multiplex PCR. However, no studies have tested whether these methods can be used to estimate the density of cystic nematodes by qPCR.

In this study, we developed a method to estimate the density of these nematode species by qPCR using DNA extracted in this manner. We first examined the effect of impurities on the accuracy of quantification of *G. pallida* and *G. rostochiensis* using qPCR. In addition, to test whether the population densities could be estimated without considering the effects of impurities, we established a fixed standard curve for each species and estimated their

92 population densities in the soils of various agricultural fields. We verified whether the densities estimated by qPCR
93 were significantly correlated with those determined by conventional microscopic inspection.
94
95 **Materials and methods**
96 **Nematodes**
97 We used *G. pallida* and *G. rostochiensis* populations originating from Hokkaido, Japan. The nematodes were
98 reared on potato plants in a greenhouse. After rearing, the soil containing the cysts was dried and stored at 4 °C
99 until further use. Cysts were isolated from the soil using the method described above and crushed to obtain eggs.
100
101 **Impurities**
102 To evaluate the effect of impurities on the Ct values, we prepared soil from agricultural fields in four
103 geographically isolated regions: Sapporo, Memuro (Hokkaido), Tsukuba (Ibaraki), and Koshi (Kumamoto) (Table
104 1). Neither *G. rostochiensis* nor *G. pallida* was present in these fields. Impurities were isolated from the soil in the
105 same way as the cysts and left to stand at 15–25 °C for 1–2 days to dry them. The impurity samples were designated
106 as S-im (Sapporo), M-im (Memuro), T-im (Tsukuba), and K-im (Koshi). S-im appeared to contain more plant
107 seeds than the other impurity samples, presumably because of weed overgrowth (Fig. 2). K-im seemed to contain
108 more plant fragments than the other impurity samples, presumably because the green manure was incorporated
109 just before soil sampling. No distinct differences in the impurities content were observed between M-im and T-im.
110
111 **DNA extraction and qPCR**
112 DNA was extracted from 0.1–0.6 mL of impurities with added *G. rostochiensis* or *G. pallida* eggs, or mixtures
113 of impurities and cysts obtained from *G. rostochiensis*- or *G. pallida*-infested fields (described later). The Wizard®
114 SV Genomic DNA Purification System (Promega Inc., WI, USA) was used to extract DNA as described in Kushida
115 and Sakai (2022) and Sakata et al. (2021b) with some modifications. Each sample was crushed in a 2 mL plastic

tube with ten stainless steel beads (2 mm diameter) using a multi-bead shocker (FastPrep-24™ 5G, MP Biomedicals, Solon, OH, USA). Then, 360 µL of Nuclei Lysis Solution, 45 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA), and 45 µL of 20% (w/v) skim milk were added to each tube, and the tubes were vortexed thoroughly. We added skim milk to the DNA extraction buffer to prevent DNA adsorption to impurities, as in previous studies that extracted DNA from soil (e.g., Ikeda et al. 2004; Takada-Hoshino and Matsumoto 2004; Volossiouk et al. 1995). Subsequently, 470 µL of SV Lysis Buffer was added to each tube, and the tubes were vortexed thoroughly again. After centrifugation of each tube for 2 min at 13,000 × *g*, each supernatant (approximately 650 µL) was transferred to a Wizard® SV Minicolumn Assembly. Subsequent column washing was performed according to the manufacturer's instructions. The DNA was eluted by adding 250 µL of nuclease-free water. The eluate was diluted twice with nuclease-free water and used as the template for qPCR.

The primer and probe sets are listed in Table 2. The qPCR reaction cocktail was prepared as follows: 5.0 µL of TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific, Inc., MA, USA), 0.2 µM of each primer (Table 2), 0.1 µM of each probe (Table 2), 1.0 µL of the DNA solution, and nuclease-free water to make up a total reaction volume of 10 µL. The qPCRs were performed on a Mic real-time PCR system (Bio Molecular Systems, Upper Coomera, QLD, Australia) under the following cycling conditions: one cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Nuclease-free water was also used as a negative template for each run. All qPCR reactions were performed in duplicate.

Effect of impurities on the Ct values

First, we investigated the influence of the volumes of impurities on the Ct values using S-im. In our preliminary study, we found 0.1–0.6 mL of impurities in 100 g of soil from most tested fields in Hokkaido (unpublished data). Therefore, 0.1, 0.2, 0.4, or 0.6 mL of S-im were added to a 2 mL plastic tube with 1, 10, 100, or 1000 eggs (of either *G. rostochiensis* or *G. pallida*). In addition, to investigate the influence of the difference in fields from which impurities originate, we added 0.4 mL of S-im, M-im, T-im, or K-im to a 2 mL plastic tube with

1, 10, 100, or 1000 eggs (of either *G. rostochiensis* or *G. pallida*). As a control, DNA was extracted from 0.4 mL of S-im, M-im, T-im, or K-im without *G. rostochiensis* and *G. pallida*. Two replicates were prepared for each sample. DNA extraction and qPCR were performed as described above. General linear models were constructed using Ct values as response variables. The explanatory variables were impurities (volume or field) and the common logarithm of the number of eggs. Model selection was based on the Akaike's Information Criterion (AIC) to examine the effects of each explanatory variable. R software v. 4.1.3 (R Development Core Team, 2023) was used for statistical analysis. The R package ggplot2 v. 3.3.5 was used to create the graphics.

Estimation of population densities using fixed standard curves

We pooled Ct values from the above experiments and constructed standard curves. We collected 28 and 16 field soil samples infested with *G. pallida* and *G. rostochiensis,* respectively, in Hokkaido, Japan. We determined the nematode densities in the soil samples using the conventional method (microscopic inspection) and compared the densities with those estimated by qPCR using standard curves (flowcharts of the two methods are shown in Fig. 1). In the conventional method, cysts were isolated from 50 g or 100 g of each dried soil sample. Cysts were picked using tweezers, collected in water, and crushed using a homogenizer (G-1010; Dremel, USA). The numbers of eggs and juveniles were counted under a microscope. Microscopic inspection was performed in two (100 g) or three replicates (50 g). For qPCR, cysts were isolated from 100 g of each dry soil sample in duplicate. The cysts and impurities were left to stand at 15–25 °C for 1–2 days to dry them and then collected in a 2 mL plastic tube. DNA extraction and qPCR were performed as described above. As positive templates, we used DNA solutions prepared from 10 cysts of each species, using the method described by Sakata et al. (2021b). Nematode density was estimated using standard curves. One *G. pallida*-infected soil sample (100 g) contained > 0.6 mL of cysts and impurities. The cysts and impurities were split in half and each half was subjected to DNA extraction and qPCR.

Results

Effect of impurities on the Ct values

The relationships between the Ct values, volume of impurities, and common logarithm of the number of eggs are shown in Fig. 3a and b. The model that used only the common logarithm of the number of eggs as an explanatory variable had the lowest AIC values (Table 3 and Online Resource 1). The relationships between the Ct values, fields from which the impurities originated, and the common logarithm of the number of eggs are shown in Fig. 3c and d. The model that employed fields and the common logarithm of the number of eggs as explanatory variables had the lowest AIC values (Table 3, Online Resource 1). No amplicons were obtained from 0 eggs added with S-im, M-im, T-im, or K-im (data not shown).

Estimation of population densities using fixed standard curves

We pooled the Ct values obtained from the above experiments and constructed standard curves (Fig. 3e and f). The Ct values (y) and common logarithm of the number of eggs (x) were significantly negatively correlated ($y = -3.2457x + 32.823$ [$R^2 = 0.9781$, $P < 0.001$; *G. pallida*] and $y = -3.7801x + 33.336$ [$R^2 = 0.9798$, $P < 0.001$; *G. rostochiensis*]). Using these equations, we estimated the nematode population densities in soil samples from the infested fields. Some soil samples were infected with both species. However, the Ct value of one species were lower than that of the other species at least by 7. Therefore, we regarded the soil samples as infested by the nematode species with the lower Ct value. The densities estimated by qPCR (x) and those determined by microscopic inspection (y) were significantly and positively correlated (*G. pallida*: $y = 0.7654x$ [$R^2 = 0.7275$, $P < 0.001$]; *G. rostochiensis*: $y = 0.8935x$ [$R^2 = 0.9101$, $P < 0.001$]; Fig. 4).

Discussion

To efficiently control *G. pallida* and *G. rostochiensis*, it is important to estimate their population densities in agricultural fields. Although qPCR is a useful method for quantifying nematodes, simplifying the sample preparation and DNA extraction from large amounts of soil (≥ 100 g) is essential for practical use. Although

188 previous studies (Kushida and Sakai 2022; Reid et al. 2015) have reported DNA extraction methods from mixtures
189 of cysts and impurities isolated from such large amounts of soil, it is unclear whether such DNA samples can be
190 used to estimate cyst nematode densities.

191 As we detected DNA from a single *G. rostochiensis* or *G. pallida* egg mixed with impurities (Fig. 3), the
192 sensitivity of this method was sufficiently high. In the experiment assessing the effect of impurities volume on the
193 Ct values, the lowest AIC values were obtained when the models employed only the common logarithm of the
194 number of eggs as the explanatory variable. This result suggests that the volume of impurities has little or no effect
195 on the quantification accuracy. In contrast, in the experiment assessing the effect of different fields from which
196 impurities originated on the Ct values, the lowest AIC values were obtained when the model employed the fields
197 and common logarithm of the number of eggs as explanatory variables. This result suggests that the difference in
198 fields has a significant effect on quantification accuracy. Because the soil particles were not contained in the
199 impurities, they must not have been relevant to the results. Therefore, this may have been caused by PCR inhibitors
200 extracted from impurities or DNA adsorption, and the degree of these effects may differ among fields. However,
201 in our supplemental experiment, the Ct values obtained by qPCR using twice diluted DNA samples (100 *G. pallida*
202 eggs mixed with 0.4 mL of S-im, M-im, or K-im) were approximately 1 value (0.9–1.1) higher than those of their
203 original samples (data not shown). These results suggested that PCR inhibition did not occur. Therefore,
204 differences in the degree of DNA adsorption to impurities (or their chemical components) among fields may have
205 led to the differences in Ct values, despite the use of skim milk, an inhibitor of DNA adsorption (see Materials and
206 Methods). Consequently, standard curves should be set for each field, but this procedure is impractical for routine
207 diagnosis. Since the differences in Ct values obtained for each number of eggs between fields were not very large
208 (< 1.5, Fig. 3c and d), it may be possible to roughly estimate the density using fixed standard curves derived from
209 all the Ct value data.

210 We constructed fixed standard curves for *G. pallida* and *G. rostochiensis* using the Ct values obtained in the
211 above experiment. There were significant and strong ($R^2 > 0.97$) correlations between the common logarithm of

the number of eggs and the Ct values. Therefore, we validated whether we could estimate the population densities in infested field soils using these curves. The results showed that the population densities estimated by qPCR using fixed standard curves significantly correlated with the observed densities, which were determined by microscopic inspection, demonstrating that *G. pallida* and *G. rostochiensis* population densities can be estimated to a certain extent using fixed standard curves.

For conventional microscopic inspection, the total time required to collect and crush cysts, and count eggs and juveniles was > 20 h per 24 samples (calculated from Fig. 1). In contrast, in our qPCR method, the total time required to collect cysts and impurities in plastic tubes, extract DNA, and perform qPCR was < 6 h per 24 samples (ditto). Although it is necessary to dry the cysts and impurities to place them into the tube efficiently, inspectors can perform other tasks during this time. Therefore, the qPCR method is less laborious than the conventional microscopic inspection.

It should be noted that the qPCR method tended to overestimate the nematode population densities, given that the slopes in Fig. 4 were less than the theoretical value (i.e.,1). Several studies have shown that DNA can be detected in dead *G. pallida* and *G. rostochiensis* (Kushida and Narabu 2017; Sakata et al. 2023; van den Elsen et al. 2012). Although all soil samples were collected from fields that had not, to the best of our knowledge, been treated with fumigants, samples with overestimated nematode population densities may have contained a considerable number of dead eggs. In some overestimated soil samples, many eggs with dark and disintegrated contents were observed. Such eggs should be dead but may have residual DNA, and the DNA may have caused an overestimation of the densities by qPCR.

In conclusion, the population densities of *G. pallida* and *G. rostochiensis* in the soil of agricultural fields could be estimated to a certain extent by qPCR using DNA extracted from mixtures of cysts and soil-derived impurities. This method enabled the efficient estimation of *G. pallida* and *G. rostochiensis* population densities in large amounts of soil. Further studies are required to improve quantification accuracy.

Declarations and statements

Ethics declaration

No approval from the research ethics committees was required to accomplish the goals of this study because the experimental work was conducted with an unregulated invertebrate species.

Author contributions

Conceptualization: Itaru Sakata, Kenji Itou, and Atsuhiko Kushida; sample collection and investigation: Itaru Sakata and Kenji Itou; writing of the original draft: Itaru Sakata; and review and editing: Itaru Sakata, Kenji Itou, and Atsuhiko Kushida.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare no competing interests relevant to the contents of this article.

References

Gamel S, Letort A, Fouville D, Folcher L, Grenier E (2017) Development and validation of real-time PCR assays based on novel molecular markers for the simultaneous detection and identification of *Globodera pallida*, *G. rostochiensis* and *Heterodera schachtii*. Nematology 19:789–804. https://doi.org/10.1163/15685411-00003086

Goto K, Sato E, Toyota K (2009) A novel detection method for the soybean cyst nematode *Heterodera glycines* using soil compaction and real-time PCR. Nematol Res 39:1–7. https://doi.org/10.3725/jjn.39.1

Goto K, Sato E, Gang LF, Toyota K, Sugito T (2010) Comparison of calibration curves prepared by soil compaction and ball milling methods for direct quantification of the potato cyst nematode *Globodera rostochiensis* in soil. Nematol Res 40:41–45. https://doi.org/10.3725/jjn.40.41

Ikeda S, Watanabe KN, Minamisawa K, Ytow N (2004) Evaluation of soil DNA from arable land in Japan using a modified direct-extraction method. Microbes Environ 19:301–309. https://doi.org/10.1264/jsme2.19.301

Kushida A, Narabu T (2017) The sterilizing effects of heat or ethanol treatment on cysts of potato cyst nematodes. Ann Rept Plant Prot North Japan 68:150–154. https://doi.org/10.11455/kitanihon.2017.68_150 **(in Japanese with English abstract)**

Kushida A, Sakai H (2022) Development of a simple method for simultaneous detection and differentiation of *Globodera pallida* and *G. rostochiensis*. J Gen Plant Pathol 88:251–258. https://doi.org/10.1007/s10327-022-01065-6

MacMillan K, Blok V, Young I, Crawford J, Wilson MJ (2006) Quantification of the slug parasitic nematode *Phasmarhabditis hermaphrodita* from soil samples using real time qPCR. Int J Parasitol 36:1453–1461. https://doi.org/10.1016/j.ijpara.2006.08.005

Madani M, Ward LJ, De Boer SH (2008) Identifying potato cyst nematodes, *Globodera pallida* and *Globadera rostochiensis*, and the tobacco cyst nematode, *Globadera tabacum*. Can J Plant Pathol 30:554–564. https://doi.org/10.1080/07060660809507555

277 Min YY, Toyota K, Goto K, Sato E, Mizuguchi S, Abe N, Nakano A, Sawada E (2011) Development of a direct
278 quantitative detection method for *Meloidogyne incognita* in sandy soils and its application to sweet potato
279 cultivated fields in Tokushima prefecture, Japan. Nematology 13:95–102.
280 https://doi.org/10.1163/138855410X504916
281 Nakhla MK, Owens KJ, Li W, Wei G, Skantar AM, Levy L (2010) Multiplex real-time PCR assays for the
282 identification of the potato cyst and tobacco cyst nematodes. Plant Dis 94:959–965.
283 https://doi.org/10.1094/PDIS-94-8-0959
284 Narabu T, Ohki T, Onodera K, Fujimoto T, Ito K, Maoka T (2016) First report of the pale potato cyst nematode,
285 *Globodera pallida*, on potato in Japan. Plant Dis 100:1794. https://doi.org/10.1094/PDIS-12-15-1515-PDN
286 Papayiannis LC, Christoforou M, Markou YM, Tsaltas D (2013) Molecular typing of cyst‐forming nematodes
287 *Globodera pallida* and *G. rostochiensis*, using real‐time PCR and evaluation of five methods for template
288 preparation. J Phytopathol 161:459–469. https://doi.org/10.1111/jph.12091
289 R Development Core Team (2023) R: A Language and environment for statistical computing. R Foundation for
290 Statistical Computing, Vienna, Austria. https://www.R-project.org/. (Accessed 3 September 2023)
291 Reid A, Evans F, Mulholland V, Cole Y, Pickup J (2015) High-throughput diagnosis of potato cyst nematodes in
292 soil samples. In: Lacomme C (ed) Plant pathology: techniques and protocols, methods in molecular biology.
293 Humana Press, New York, pp 137–148
294 Sakata I, Kushida A, Tanino K (2021a) The hatching-stimulation activity of solanoeclepin A toward the eggs of
295 *Globodera* (Tylenchida: Heteroderidae) species. Appl Entomol Zool 56:51–57.
296 https://doi.org/10.1007/s13355-020-00707-5
297 Sakata I, Sakai H, Kushida A (2021b) Subspecies identification of the Japanese population of *Globodera tabacum*.
298 Nematol Res 51:37–40. https://doi.org/10.3725/jjn.51.37
299 Sakata I, Kushida A, Toyota K (2023) Species-specific detection of viable *Globodera pallida* using real-time
300 reverse transcription PCR. Nematology 25:411–426. https://doi.org/10.1163/15685411-bja10228

301 Takada-Hoshino Y, Matsumoto N (2004) An improved DNA extraction method using skim milk from soils that
302 strongly adsorb DNA. Microbes Environ 19:13–19. https://doi.org/10.1264/jsme2.19.13
303 Trudgill DL, Evans K, Parrott DM (1975a) Effects of potato cyst nematodes on potato plants. I. Effects in a trial
304 with irrigation and fumigation on the growth and nitrogen and potassium contents of a resistant and a
305 susceptible variety. Nematologica 21:169–182
306 Trudgill DL, Evans K, Parrott DM (1975b) Effects of potato cyst nematodes on potato plants. II. Effects on haulm
307 size, concentration of nutrients in haulm tissue and tuber yield of a nematode resistant and a nematode
308 susceptible potato variety. Nematologica 21:183–191
309 van den Elsen S, Ave M, Schoenmakers N, Landeweert R, Bakker J, Helder J (2012) A rapid, sensitive, and cost-
310 efficient assay to estimate viability of potato cyst nematodes. Phytopathology 102:140–146.
311 https://doi.org/10.1094/PHYTO-02-11-0051
312 Volossiouk T, Robb EJ, Nazar RN (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. Appl
313 Environ Microbiol 61:3972–3976. https://doi.org/10.1128/aem.61.11.3972-3976.1995
314 Yamada E, Takakura S, Tezuka H (1972) On the occurrence of the potato cyst nematode, *Heterodera rostochiensis*
315 Wollenweber in Hokkaido, Japan. Jpn J Nematol 2:12–15 https://doi.org/10.14855/jjn1972.2.12
316

Figure legends

Fig. 1

Flowchart of the conventional method (microscopic inspection) and qPCR method developed in this study. The procedure required for each method is described below. The required time is indicated in blue.

Fig. 2

Impurities used in this study (S-im, M-im, T-im, and K-im). They were isolated from approximately 100 g dry soil of each location. The black granules on the edge of the impurities were thought to be plant seeds. Bars represent 5 mm.

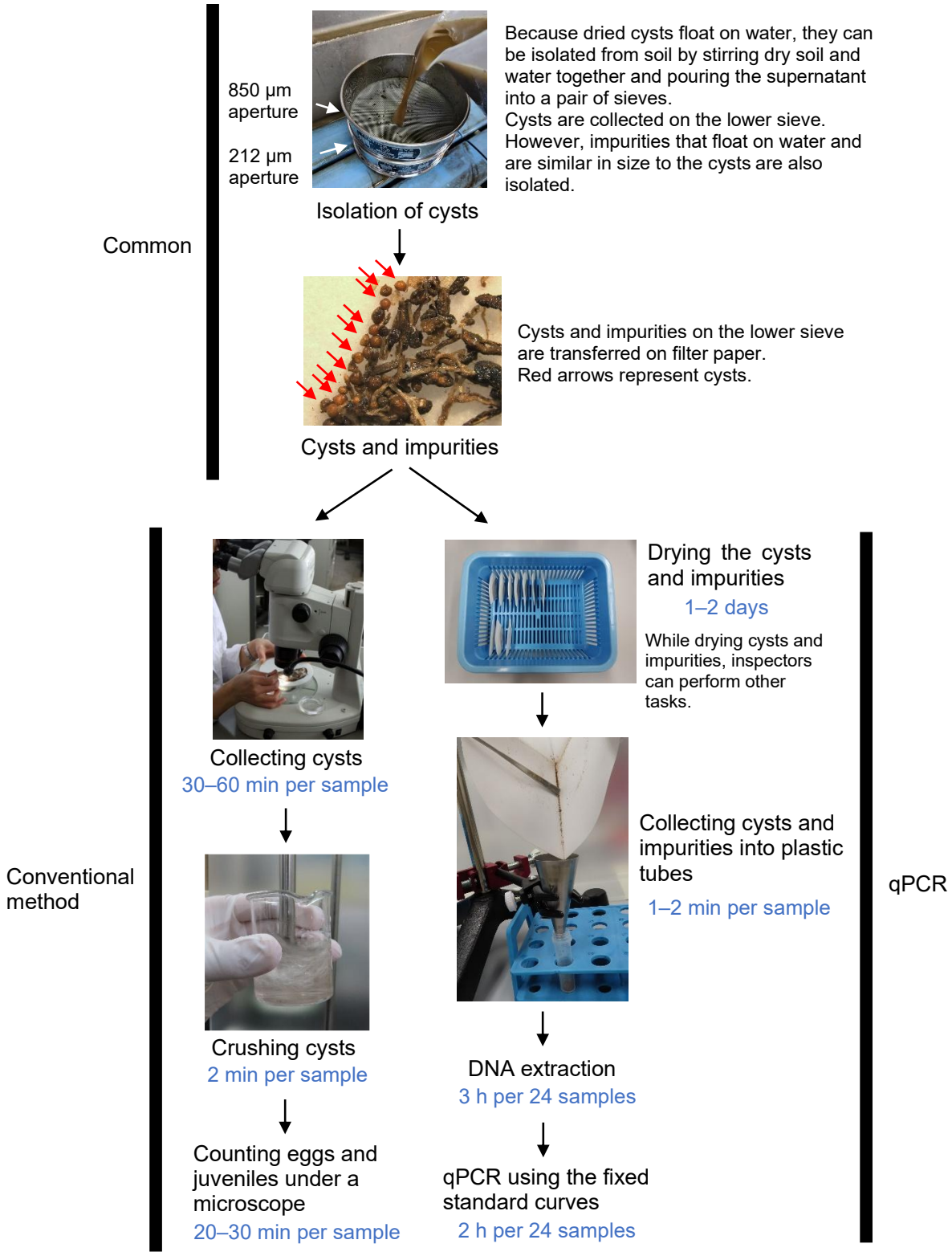
Fig. 3

Relationships between Ct values and the common logarithm of the number of eggs. **a**, **c**, and **e**: *Globodera pallida*; **b**, **d**, and **f**: *G. rostochiensis*. **a** and **b**: Ct values for volumes of impurities. DNA was extracted from 1, 10, 100, and 1000 eggs and added to 0.1, 0.2, 0.4, or 0.6 mL of S-im. **c** and **d**: Ct values for impurities which originated from different fields. The DNA was extracted from 1, 10, 100, or 1000 eggs added to 0.4 mL of S-im, M-im, T-im, or K-im. **e**: Ct values shown in **a** and **c** were pooled. **f**: The Ct values shown in **b** and **d** were pooled.

Fig. 4

Relationships between the estimated and observed nematode population densities. **a**: *Globodera pallida*; **b**: *G. rostochiensis.* The estimated population densities were calculated from the Ct values using equations shown in Fig. 3e and f. The observed population densities were determined by the conventional method (counting nematode eggs and juveniles under a microscope).

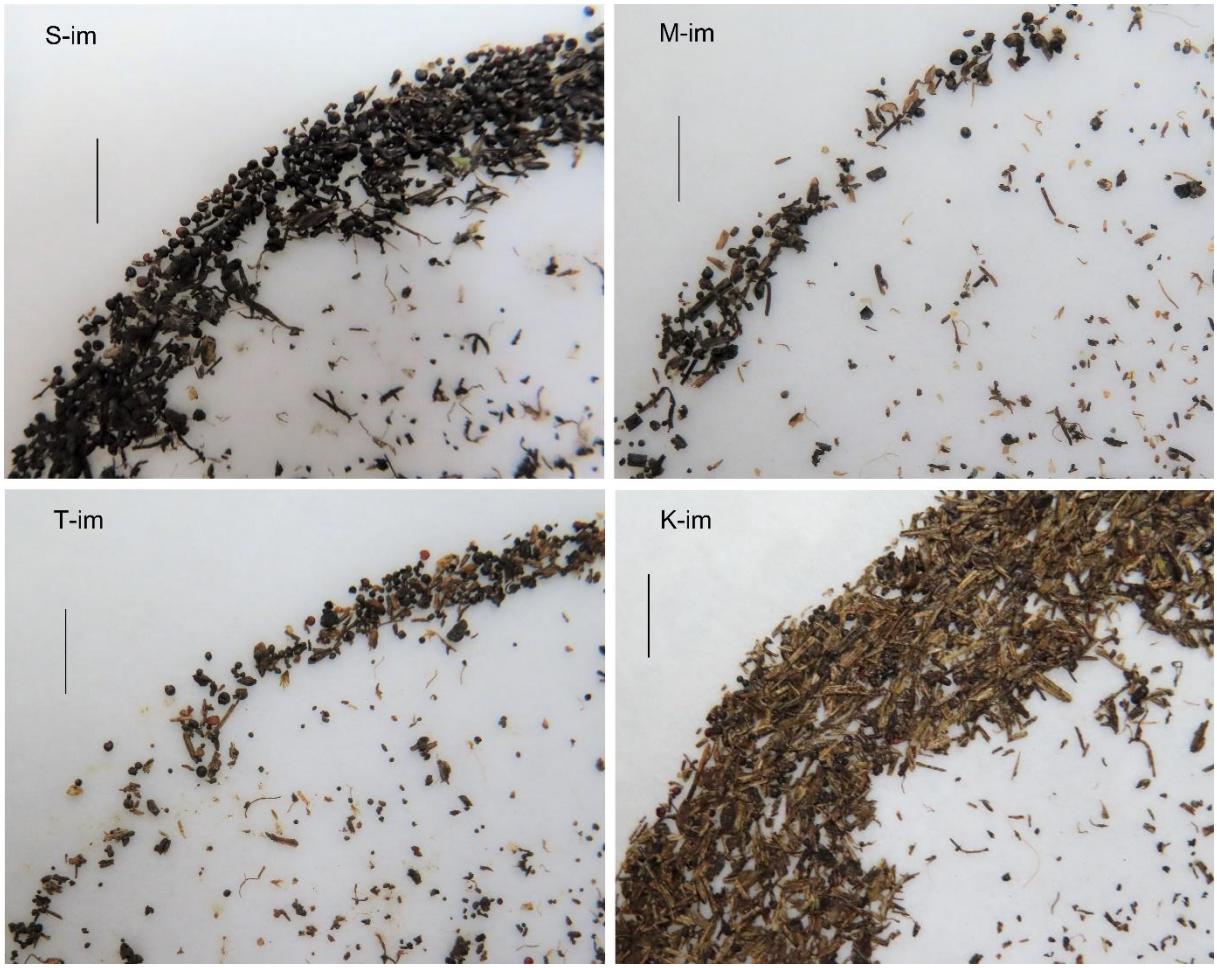
341 **Fig. 1**



342

343

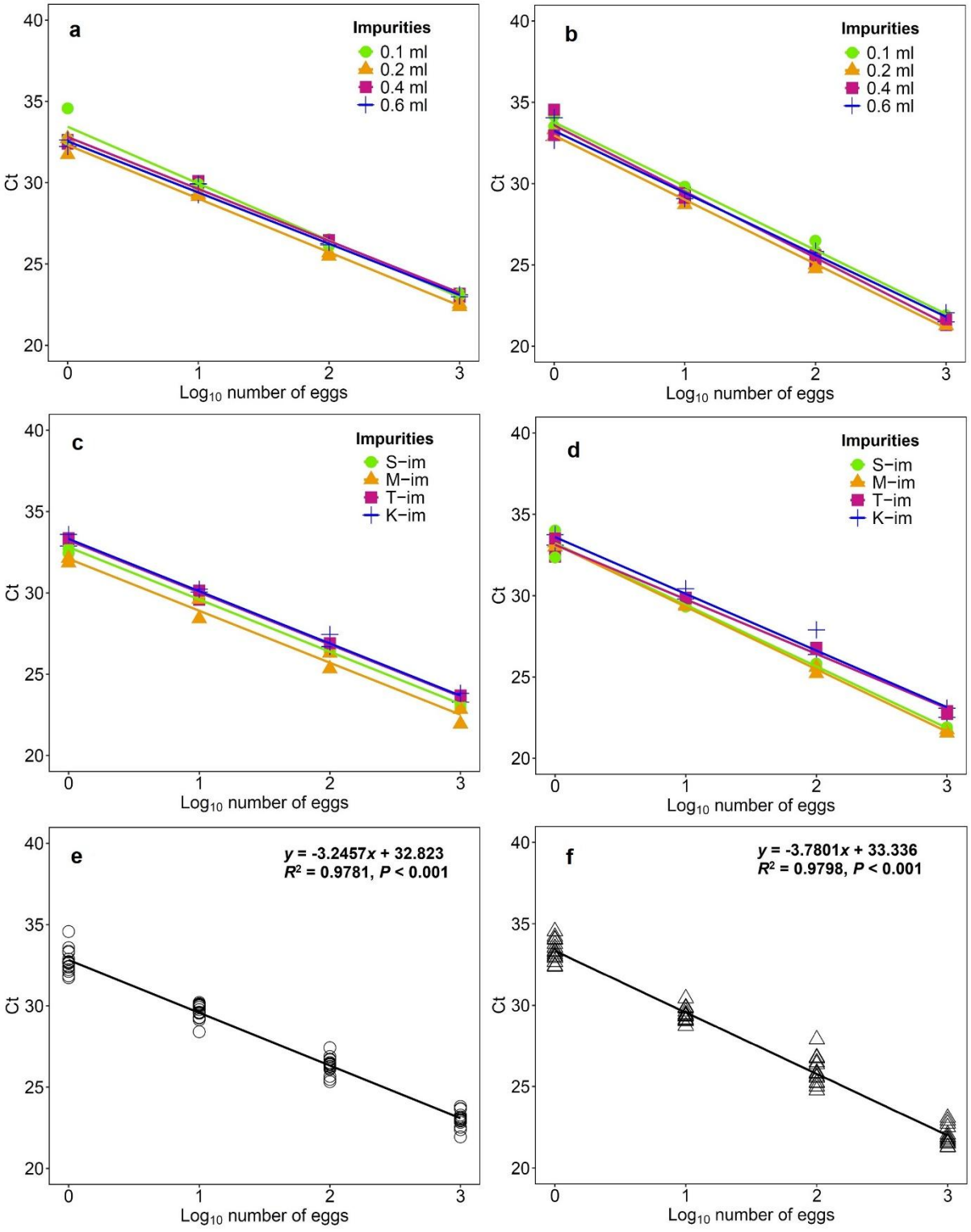
344 **Fig. 2**



345

346

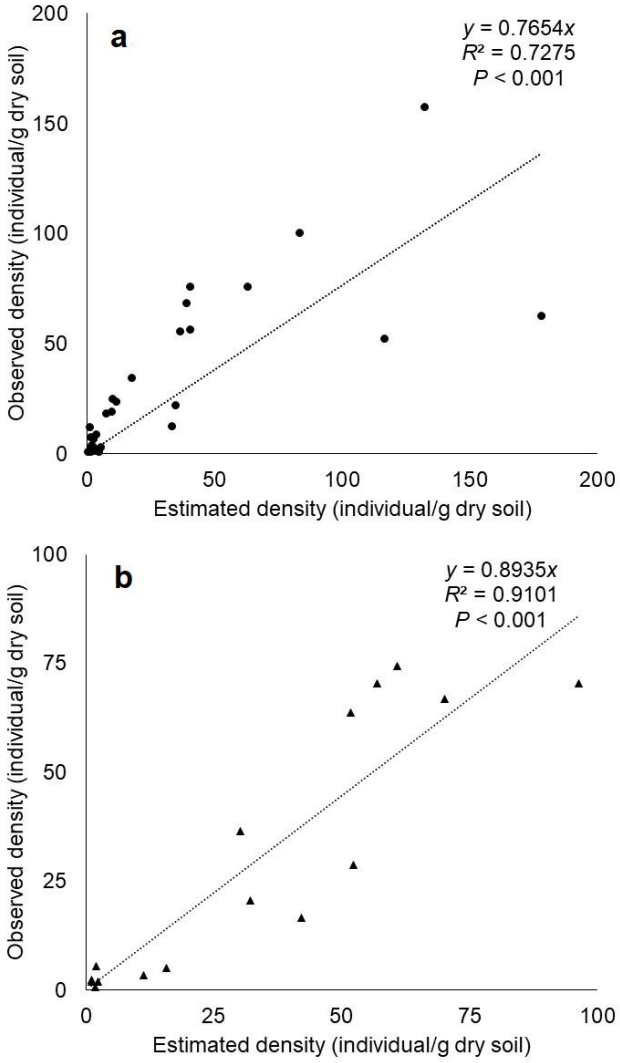
347 **Fig. 3**



348

349

Fig. 4


a
y = 0.7654x
R² = 0.7275
P < 0.001
Observed density (individual/g dry soil)
Estimated density (individual/g dry soil)
0
50
100
150
200
b
y = 0.8935x
R² = 0.9101
P < 0.001
Observed density (individual/g dry soil)
Estimated density (individual/g dry soil)
0
25
50
75
100

354 **Table 1** Impurities used in this study.

Name	Location of soil sampling	Year and month sampled
S-im	Sapporo, Hokkaido 43°00'31"N, 141°24'34"E	Oct. 2022
M-im	Memuro, Hokkaido 42°53'25"N, 143°04'22"E	Unknown
T-im	Tsukuba, Ibaraki 36°01'24"N, 140°06'16"E	Oct. 2022
K-im	Koshi, Kumamoto [a] 32°52'31"N, 130°44'26"E; 32°52'30"N, 130°44'26"E; and 32°52'58"N, 130°44'27"E	Jan. 2022

355 [a] Soil samples derived from the three locations were combined.

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Table 2 Primers and probes used in this study

Target species	Name	Description	Sequence (5′→3′)
Globodera pallida	PITSpf [a]	Forward primer	ACGGACACATGCCCGCTA
	PITSp4 [a]	Reverse primer	ACAACAGCAATCGTCGAG
	GFAMp (MGB) [b]	Probe	FAM/ACATGAGTGTTGGGGTGTAAC/MGB-NFQ
G. rostochiensis	PGrtf [a]	Forward primer	TCTGTGCGTCGTTGAGC
	Prostor [a]	Reverse primer	CGCAGACATGCCGCAA
	GNEDp (MGB) [c]	Probe	NED/CGCAGATATGCTAACATGGAGTGTAG/MGB-NFQ

[a] Used by Nakhla et al. (2010).

[b] GFAMp (Nakhla et al. 2010) with the quencher changed from BHQ-1 to MGB-NFQ.

[c] GTETp (Nakhla et al. 2010) with the quencher changed from BHQ-2 to MGB-NFQ, and the reporter changed from TET to NED.

362 **Table 3** Akaike Information Criterion (AIC) values for the following models of Ct values

Experiment	Species	Explanatory variables incorporated into models			
		Intercept only	Log_{10} number of eggs	Impurities' volume/field	Log_{10} number of eggs + Impurities' volume/field
Effect of the impurities' volume on Ct values	*Globodera pallida*	178.61	<u>51.24</u>	180.61	53.19
	G. rostochiensis	190.12	<u>49.80</u>	192.12	51.70
Effect of the difference in fields from which impurities originate	*G. pallida*	177.35	60.93	182.79	<u>29.03</u>
	G. rostochiensis	184.97	65.23	190.67	<u>53.58</u>

363 Underlined portions indicate the lowest AIC values.

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Online Resource 1 Parameter estimates of selected models

Experiments	Species	Parameters	Estimates	Standard error	*t*	*P*	R^2 adjusted by degrees of freedom
Effect of the impurities' volume on Ct values	*Globodera pallida*	Intercept	32.77907	0.14989	218.68	< 0.001	0.9819
		Log_{10} number of eggs	-3.28371	0.08012	-40.98	< 0.001	
	G. rostochiensis	Intercept	33.39318	0.14655	227.87	< 0.001	0.9879
		Log_{10} number of eggs	-3.94213	0.07833	-50.33	< 0.001	
Effect of the difference in fields from which impurities originate	*G. pallida*	Intercept (S-im)	32.80103	0.14633	224.152	< 0.001	0.9913
		Impurities K-im	0.50911	0.17186	2.962	0.006	
		Impurities M-im	-0.68642	0.17186	-3.994	< 0.001	
		Impurities T-im	0.43726	0.17186	2.544	0.017	

		Log_{10} number of eggs	-3.20759	0.05435	-59.020	< 0.001	
	G. rostochiensis	Intercept (S-im)	32.96965	0.21476	153.521	< 0.001	
		Impurities K-im	0.82713	0.25222	3.279	0.003	
		Impurities M-im	-0.14611	0.25222	-0.579	0.567	0.9853
		Impurities T-im	0.55583	0.25222	2.204	0.036	
		Log_{10} number of eggs	-3.61814	0.07976	-45.364	< 0.001	

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