

**Species-specific detection of viable pale potato cyst nematode
(*Globodera pallida*) using real-time reverse transcription PCR**

Itaru SAKATA

Student ID No. 22701115

A doctoral thesis

Submitted to the Graduate School of Bio-Applications and Systems Engineering

Tokyo University of Agriculture and Technology (TUAT)

2023. 06

Content

Summary.....	1
Chapter 1. General introduction.....	3
1-1. Plant parasitic nematodes.....	3
1-2. Cyst nematodes.....	3
1-3. Potato cyst nematodes.....	5
1-4. Official control of <i>Globodera pallida</i> in Japan.....	10
1-5. Detection methods of viable <i>Globodera pallida</i> other than inoculation test.....	12
1-5-1. Visual inspection.....	12
1-5-2. Staining methods.....	12
1-5-3. Hatching tests.....	14
1-5-4. Trehalose method.....	15
1-5-5. DNA-based PCR.....	15
1-5-6. Reverse transcription PCR.....	16
1-6. Objective of this study.....	17
Chapter 2. Selection of housekeeping gene for detection of viable <i>Globodera pallida</i>.....	18
2-1. Introduction.....	18
2-2. Materials and methods.....	18
2-3. Results and discussion.....	21

Chapter 3. Design of *Globodera pallida*-specific primers on *Y45F10D.4* gene...22

3-1.	Introduction.....	22
3-2.	Materials and methods.....	23
3-2-1.	Nematode populations.....	23
3-2-2.	Design of primer sets.....	23
3-2-3.	Extraction of nucleic acids.....	23
3-2-4.	Confirmation of species specificity.....	29
3-2-5.	Detection sensitivity and standard curve.....	30
3-3.	Results.....	31
3-3-1.	Design of primer sets.....	31
3-3-2.	Confirmation of species specificity.....	31
3-3-3.	Detection sensitivity and standard curve.....	33
3-4.	Discussion.....	33

Chapter 4. Effect of disinfection with 1,3-dichloropropene on detectability of *Globodera pallida* DNA and RNA.....39

4-1.	Introduction.....	39
4-2.	Materials and methods.....	40
4-2-1.	RT-qPCR using RNA extracted from 1,3-dichloropropene-treated <i>Globodera pallida</i>	40
4-2-2.	Conventional DNA-based PCR.....	41
4-3.	Results.....	42
4-3-1.	RT-qPCR using RNA extracted from 1,3-dichloropropene -treated	

<i>Globodera pallida</i>	42
4-3-2. Conventional DNA-based PCR.....	42
4-4. Discussion.....	42
 Chapter 5. Comparison of detection sensitivity of <i>Globodera pallida</i> between the RT-qPCR and the cup test.....	47
5-1. Introduction.....	47
5-2. Materials and methods.....	47
5-3. Results.....	49
5-4. Discussion.....	49
 Chapter 6. General discussion and conclusion.....	54
 Acknowledgements.....	59
 References.....	60
 Appendix 1: DNA-based PCR using Y45_0803_F1 and Y45_0715_R4.....	78
 Appendix 2: Effect of RNA extraction methods on Ct values.....	80

Summary

Globodera pallida is a major pest of potatoes worldwide. In Japan, aiming at the eradication of *G. pallida*, control measures have been implemented in infested fields. To determine the necessity of control measures, the detection of viable *G. pallida* is required. However, the conventional inoculation test performed in Japan, named the ‘cup test,’ is time-consuming and some viable individuals may be overlooked. In this study, I developed an intercalator-based RT-qPCR method for the rapid detection of viable *G. pallida*.

I first assessed the expression levels of 10 housekeeping genes, *act-1*, *Ama-1*, *APC1*, *BUB3*, *cdc-42*, *eif4A*, *GR*, *Mce1*, *TUBG2*, and *Y45F10D.4* in *G. pallida* eggs by RT-qPCR. The gene with the lowest Ct value was *Y45F10D.4*, which encodes a putative iron-sulfur cluster assembly enzyme. This suggested that *Y45F10D.4* was highly expressed in *G. pallida* eggs and that this gene is suitable as a target gene for viable *G. pallida* detection.

Then, I designed a primer set for the partial cDNA sequence of the *Y45F10D.4* gene of *G. pallida*. This primer set successfully amplified *Y45F10D.4* mRNA of all tested *G. pallida* populations without any cross-reactions with other species. The *G. pallida*-specific primer pair was designed to sandwich a long (approximately 500 bp) intron to prevent amplification from DNA, resulting in prevention of false-positives derived from dead nematode’s DNA and reduction of labor and cost for DNA digestion. The RT-qPCR method detected RNA corresponding to a minimum of 3.9 *G. pallida* eggs, and a significant negative correlation was observed between the concentrations of RNA extracted from viable eggs and the Ct values.

In addition, no amplification by RT-qPCR was observed in *G. pallida* treated with 1,3-dichloropropene (1,3-D), whereas appropriate amplicons were obtained from untreated *G. pallida*. This result demonstrated that the RT-qPCR method detected viable *G. pallida* but not *G. pallida* died of 1,3-D. On the other hand, conventional DNA-based PCR methods detected DNA from *G. pallida* died of 1,3-D. This result confirmed that DNA-based PCR methods are not suitable for viable *G. pallida* detection after 1,3-D treatment.

I then compared the detection sensitivity between the cup test and RT-qPCR method using 24 soil samples. As a result, viable *G. pallida* was detected by both methods in 9 soil samples, but no viable *G. pallida* was detected by both methods in 10 soil samples. The cup test did not detect viable *G. pallida* in 5 soil samples, but the RT-qPCR obtained appropriate amplicons from the cysts contained in these soil samples. The additional pot test obtained new cysts by inoculating the cysts contained in these soil samples to potato. These results showed that the detection sensitivity of the RT-qPCR method was higher than that of the cup test.

This study showed that the RT-qPCR method enabled the rapid and reliable detection of viable *G. pallida*. This method is expected to contribute to appropriate *G. pallida* management.

Chapter 1. General introduction

1-1. Plant parasitic nematodes

Plant parasitic nematodes (PPNs) are important pests for various plants. Most PPNs species inhabit soil, have transparent and small (mostly < 2 mm) bodies, and penetrate host roots and deprive the host of water and nutrients. According to Sasser & Freckman (1987), annual crop losses caused by PPNs were estimated at 8.8 to 14.6% of total crop production. Nevertheless, the actual yield losses could potentially surpass the figures since there are no available data in countries lacking nematological expertise (Singh *et al.*, 2013). In addition, specific symptoms of PPNs are often lacking, therefore the damage by PPNs might be underestimated. In general, PPNs exhibit a relatively low mobility and are known to migrate between fields, regions, and countries, mostly by means of human activities, such as the transportation of seeds/seedlings, and agricultural machinery movement (Pickup *et al.*, 2018). In addition, some PPNs species are designated as quarantine pests in many countries (Pickup *et al.*, 2018). In Japan, 17 PPNs species are designated as quarantine pests (MAFF, 2022a).

1-2. Cyst nematodes

Cyst nematode is a group of PPNs and contains 8 genera (*Betulodera*, *Cactodera*, *Dolichodera*, *Globodera*, *Heterodera*, *Paradolichodera*, *Punctodera*, and *Vittatidera*) and more than 120 species (Handoo & Subbotin, 2018). These species form a protective shell (i.e., cyst), containing hundreds of eggs. The lifecycle of cyst nematode is described in Fig. 1. In general, the hatching of cyst nematodes is promoted by hatching factors secreted from the host roots (Fenwick, 1949; Okada, 1971; Clarke & Perry, 1977;

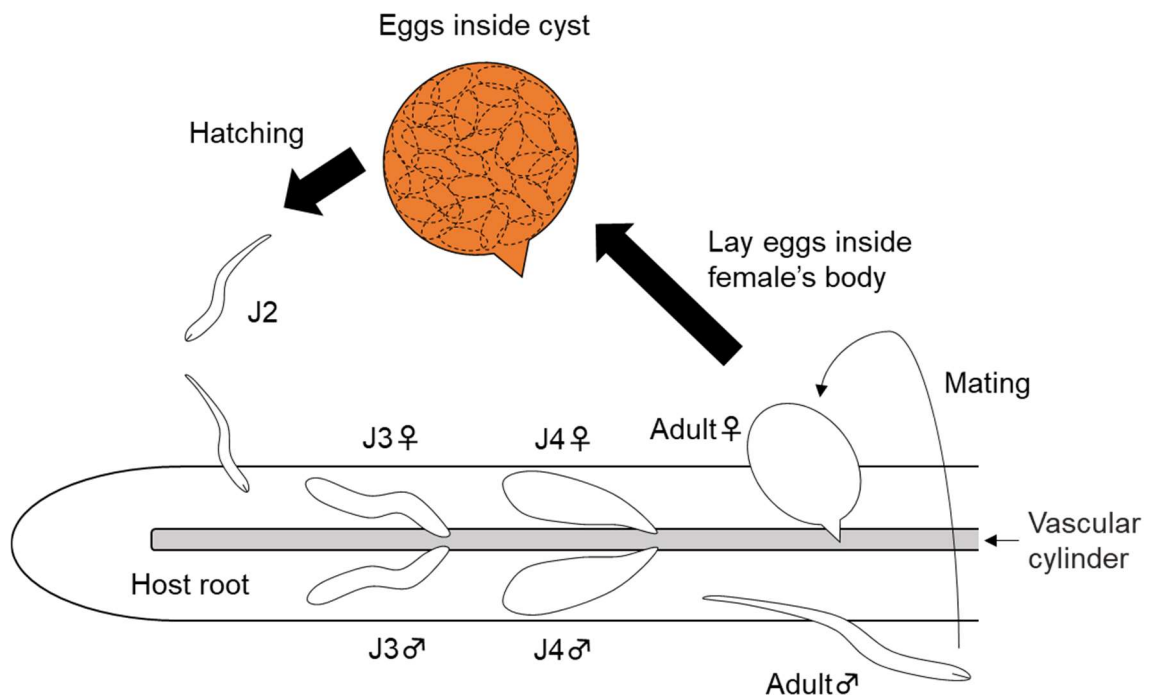


Fig. 1. Illustration of cyst nematodes' lifecycle. J2: second-stage juvenile; J3: third-stage juvenile; J4: forth-stage juvenile.

Greco, 1981; Tefft & Bone, 1985; LaMondia, 1995; Devine *et al.*, 1996). Hatched juveniles penetrate host roots and deprive the host of water and nutrients. Subsequently, the nematodes become sedentary adult females or motile vermiform adult males, except for some parthenogenetic species such as *Heterodera trifolii* (Mulvey, 1958). After mating, the adult females lay eggs inside their bodies and die holding hundreds of eggs, resulting in new cysts.

1-3. Potato cyst nematodes

Potato cyst nematodes (PCNs, Fig. 2) are major pests of potato (*Solanum tuberosum*) and are composed of three species, *Globodera rostochiensis*, *G. pallida*, and *G. ellingtonae*. In 1923, *Heterodera rostochiensis* was described (Wollenweber, 1923), and in 1959, this species was renamed *G. rostochiensis* (Skarbilovich, 1959). After that, *H. pallida* was described (Stone, 1973), but subsequently considered to belong to *Globodera*. In 2012, atypical PCNs were detected in Oregon and Idaho, the USA, and described as *G. ellingtonae* (Handoo *et al.*, 2012). PCNs are thought to be native to the Andes region (Evans *et al.*, 1975; Evans & Stone, 1977). As of 2022, both *G. pallida* and *G. rostochiensis* are detected in more than 50 countries (Fig. 3, EPPO, 2022) and thought to have invaded Europe, and then, spread worldwide (Evans *et al.*, 1975). Many countries designate *G. pallida* and *G. rostochiensis* as quarantine species and prohibit seed potato production in infested fields (EPPO, 2022; Pickup *et al.*, 2018). *G. ellingtonae* has been detected only in Argentina, Chile, and the USA so far (Handoo *et al.*, 2012; Lax *et al.*, 2014).

G. pallida and *G. rostochiensis* are known to cause stunted plant growth, and a decrease in both the number and size of tubers produced (Trudgill *et al.*, 1975a, 1975b).

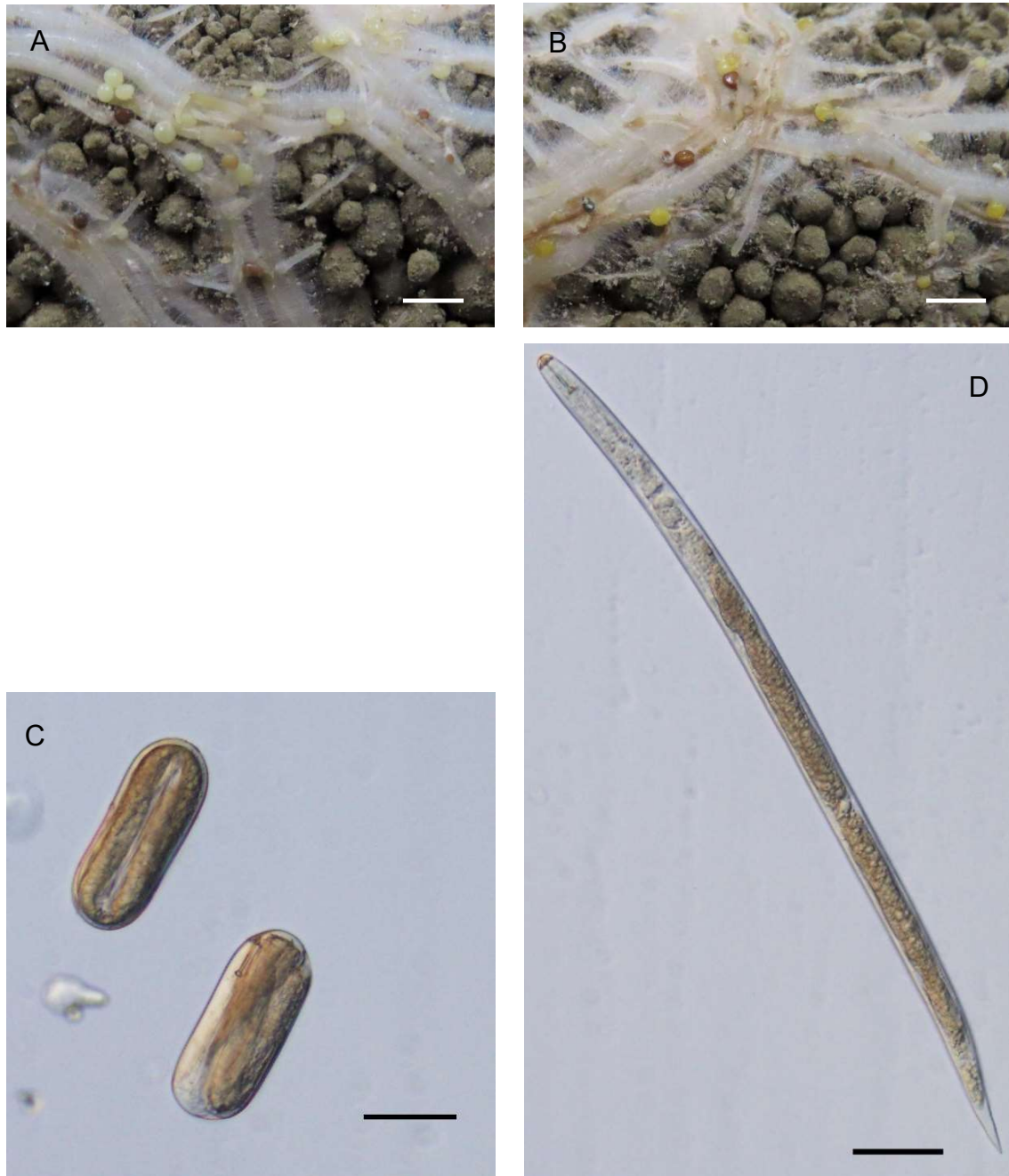


Fig. 2. Images of potato cyst nematodes. A: *Globodera pallida* cysts on potato roots. B: *G. rostochiensis* cysts on potato roots. C: *G. pallida* eggs. D: *G. pallida* 2nd stage juvenile. Bars represent 2 mm (A and B) and 50 μ m (C and D), respectively.

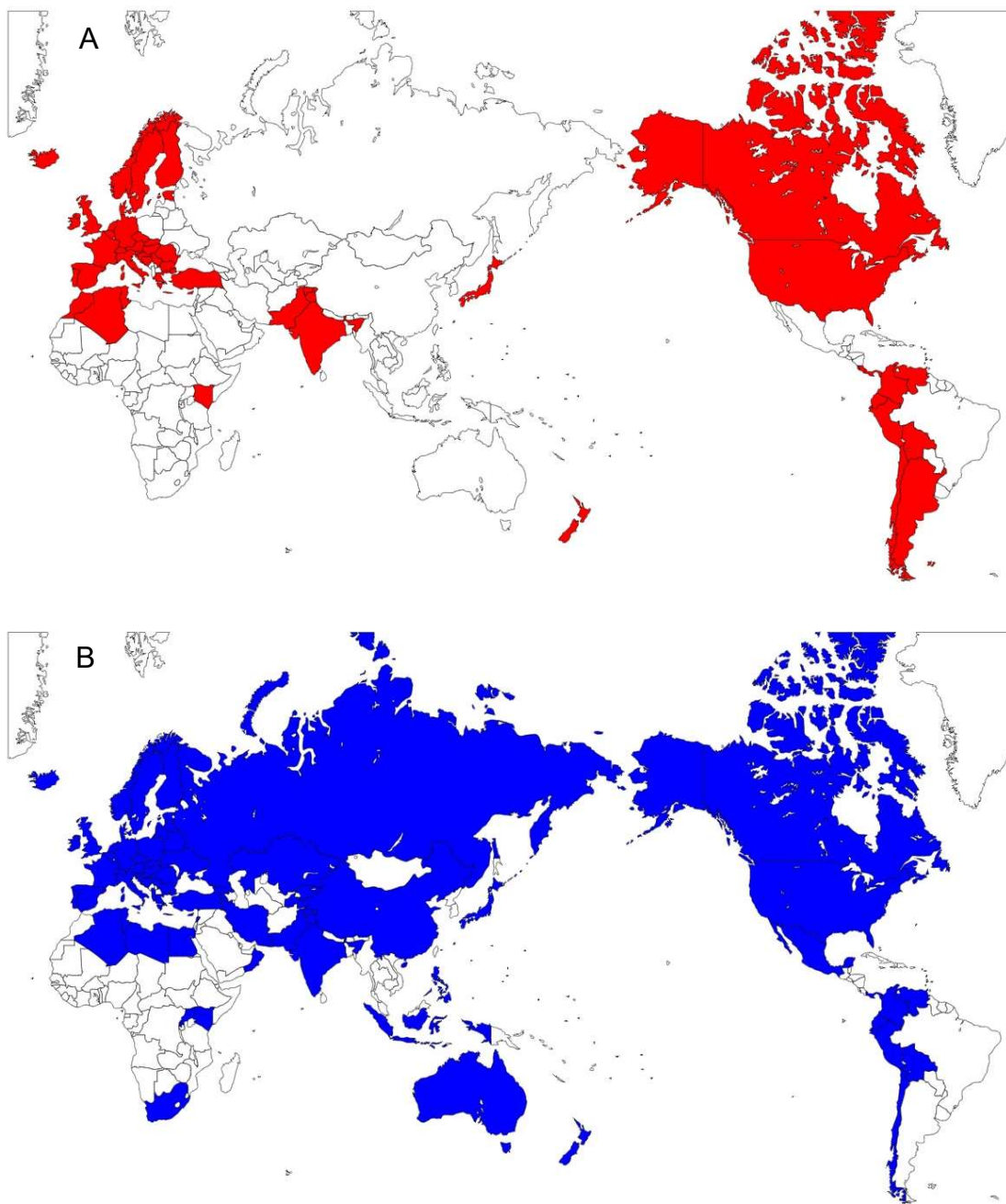


Fig. 3. Distribution map of PCNs as of 2022 (EPPO, 2022). A: *Globodera pallida*, B: *G. rostochiensis*

Total potato yield losses exceeding 50% have been reported (Trudgill, 1986; Trudgill *et al.*, 2014). Also, a recent study showed that *G. ellingtonae* can cause up to a 40% loss in potato yield (Zasada *et al.*, 2019). Furthermore, these species are capable of parasitizing tomato (*S. lycopersicum*) (Graham, 1966; Ellis, 1968; Ellis & Smith, 1971; Hesling & Ellis, 1972; Peetz *et al.*, 2019).

Based on the multiplication ability on different potato clones, Kort *et al.* (1977) identified three pathotypes for *G. pallida* (Pa1, Pa2, and Pa3) and five for *G. rostochiensis* (Ro1, Ro2, Ro3, Ro4, and Ro5) (see Table 1). Ro1 is believed to be the most widespread pathotype of *G. rostochiensis* outside South America (Turner & Evans, 1998). Breeding of potato cultivars resistant to *G. rostochiensis* pathotypes Ro1 and Ro4 is feasible by introducing *H1*, an excellent resistant gene derived from *S. tuberosum* spp. *andigena* CPC 1673. *H1* conferred potato varieties prevent invaded juveniles of *G. rostochiensis* pathotype Ro1 and Ro4 from maturing into cysts (Rice *et al.*, 1985). In contrast, the other *G. rostochiensis* pathotypes and *G. pallida* can multiply on *H1*-conferred potato varieties.

In Japan, *G. rostochiensis* was identified in 1972 (Yamada *et al.*, 1972). Currently, *G. rostochiensis* has spread to five prefectures, Hokkaido, Aomori, Mie, Nagasaki, and Kumamoto (Iseki & Sekimoto, 2012; MAFF, 2022b). However, so far, Ro1 is the only *G. rostochiensis* pathotype identified in Japan (Kushida & Momota, 2005). Therefore, the use of *H1*-conferred potato varieties is an excellent control method for *G. rostochiensis* in Japan. However, *G. pallida* was first detected in Hokkaido in 2015 (Narabu *et al.*, 2016) and has been identified in four cities/towns (Abashiri, Oozora, Kiyosato, and Syari) (MAFF, 2022c). Official control of this species has been performed since 2016 (MAFF, 2022c).

Table 1. Pathotyping scheme of *Globodera rostochiensis* and *G. pallida* (Kort *et al.*, 1977).

Potato clones	<i>G. rostochiensis</i>					<i>G. pallida</i>		
	Ro1	Ro2	Ro3	Ro4	Ro5	Pa1	Pa2	Pa3
<i>S. tuberosum</i> spp. <i>andigena</i> CPC 1673	–	+	+	–	+	+	+	+
<i>S. kurtzianum</i> KTT 60.21.19	–	–	+	+	+	+	+	+
<i>S. vernei</i> GLKS 58.1642.4	–	–	–	+	+	–	+	+
<i>S. multidissectum</i> P55/7	+	+	+	+	+	–	+	+
<i>S. vernei</i> Vt 62.33.3	–	–	–	–	+	–	–	+

Pathotype discrimination is performed by inoculating the PCNs population to the potato clones above. +: multiplication rate (population density after inoculation test divided by inoculated population density) ≥ 1 , –: multiplication rate < 1

1-4. Official control of *Globodera pallida* in Japan

In Japan, the production of potato and tomato is prohibited in *G. pallida*-infested fields to prevent an increase in *G. pallida* population density in the fields. In addition, the movement of underground parts of other plants, such as sugar beets and carrots, is restricted. Different measures are taken to reduce the *G. pallida* population density. Fumigants such as isothiocyanates (Wood *et al.*, 2017) and 1,3-dichloropropene (1,3-D) (Whitehead *et al.*, 1979; Ito *et al.*, 2020), non-fumigant agrochemicals such as fosthiazate and oxamyl (Whitehead *et al.*, 1994; Minnis *et al.*, 2004), and nematode trap crop such as sticky nightshade (*Solanum sisymbriifolium*) (Dandurand *et al.*, 2019) and *S. peruvianum* (Ito *et al.*, 2020) are effective against *G. pallida*. In Japan, 1,3-D and *S. peruvianum* are commonly used for the control of *G. pallida* (Ito *et al.*, 2020) until viable individuals are not detected in a field, and then the control measures are terminated. Therefore, the detection of viable *G. pallida* is important for appropriate management of *G. pallida*.

In Japan, a bioassay, named the ‘cup test’, has been used to assess *G. pallida* viability (Narabu, 2019). The procedure involves (1) placing approximately 200 ml of soil sample and a tuber of potato (*HI* conferred cultivar) in a transparent plastic cup, (2) incubating the cup at 18°C in dark for 60 to 70 days, and (3) counting the cysts and females which are visible on the roots through the wall and bottom of the cup (Fig. 4). Because *HI*-conferred cultivars are used in this test, and Ro1 is the only *G. rostochiensis* pathotype identified in Japan, when new cysts are formed, they are determined as *G. pallida*. It is relatively easy to perform the assay but takes a long time. In addition, cysts formed inside the cup are not detected, meaning that some viable *G. pallida* individuals may be overlooked.

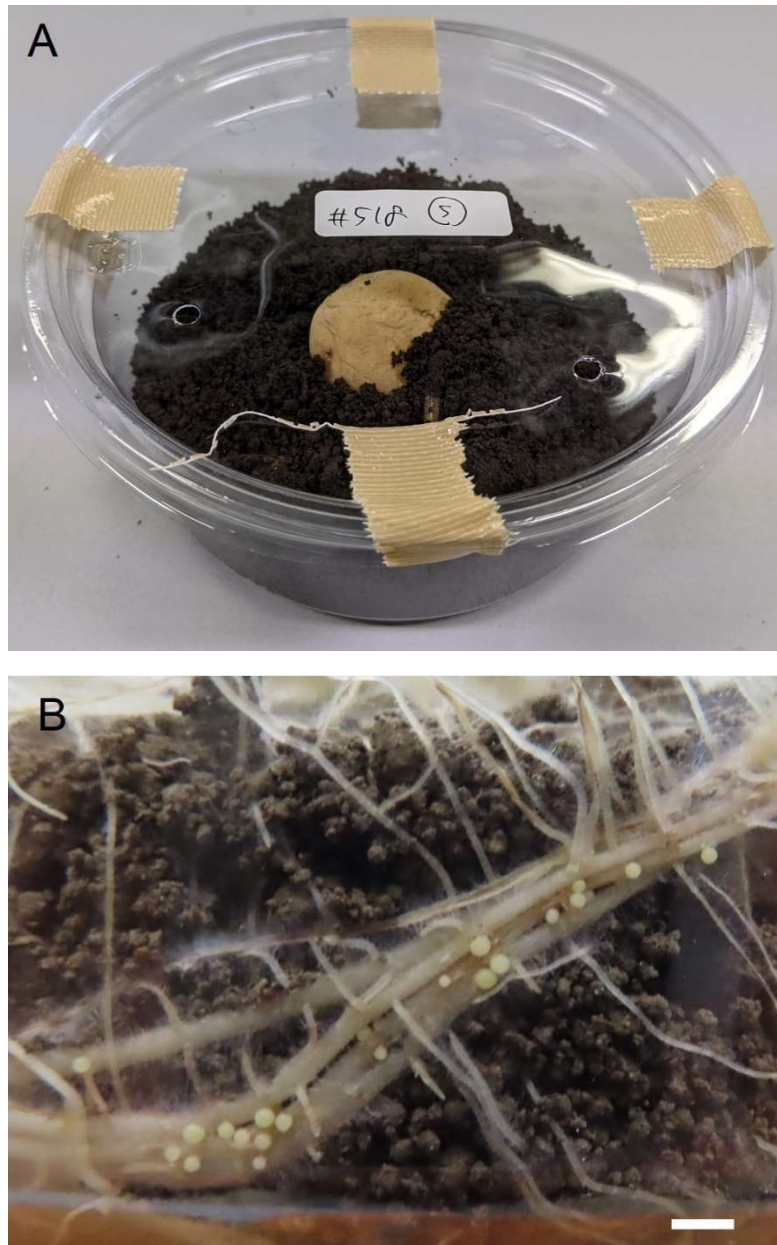


Fig. 4. Illustrations of cup test. A: The cup just after planting. B: Developing cysts visible on the roots (approximately 2 months after planting). The bar in image B represents 2 mm.

1-5. Detection methods of viable *Globodera pallida* other than inoculation tests

In Germany, Austria, and Norway, inoculation tests akin to the cup test are performed to detect viable PCNs (EPPO, 2017). However, as well as the cup test, these inoculation tests are also lengthy (6 weeks to 3 month). Several detection methods for viable *G. pallida* have been developed to replace the conventional inoculation tests.

1-5-1. Visual inspection

Visual inspection (reviewed in EPPO, 2017) is a classical method to assess egg viability. This involves crushing the cysts and observing the contained eggs under a microscope. A viable egg contains a curled juvenile (Fig. 5). Also, a clear stylet of the juvenile can be seen in the egg (EPPO, 2017). In contrast, a dead egg contains dark or disintegrated content or a shrunk juvenile (EPPO, 2017, Fig. 5). However, differentiating between viable and dead eggs requires specialized expertise and extensive labor. Furthermore, this method does not discriminate between different species.

1-5-2. Staining methods

Staining methods are also used to distinguish viable and dead eggs. These methods take advantage of the permeability change of eggshells or cell membranes after death. Traditionally, Meldora's blue was often utilized as a dye to evaluate egg viability. Nematodes are exposed to a 0.01 to 0.025% Meldora's blue solution for several hours, and then washed and examined under a microscope (Ogiga & Estey, 1974; Christoforou *et al.*, 2014). Viable eggs are not stained or partially stained, whereas dead eggs are uniformly stained dark blue or purple (Ogiga & Estey, 1974; Christoforou *et al.*, 2014). Additionally, recent studies reported the usage of fluorescent dyes, such as SYTOX



Fig. 5. Comparison of viable or dead *Globodera pallida* eggs. A and B: viable *G. pallida*. C and D: dead *G. pallida*. A viable egg contains a curled juvenile. In contrast, a dead egg contains dark or disintegrated content (C) or a shrunk juvenile (D). Bars represent 50 μm .

Green and acridine orange (Hajihassani *et al.*, 2018; Pillai & Dandurand, 2019, 2021). Nematodes are exposed to appropriate concentrations of solutions for several hours to one day. Then, the nematodes are washed and examined under a fluorescence microscope using exciting light with an appropriate wavelength. Dead eggs stained with SYTOX Green or acridine orange emit green fluorescence (Hajihassani *et al.*, 2018; Pillai & Dandurand, 2019). However, currently, it is not easy to obtain Meldola's blue (EPPO, 2017), and all staining methods also require microscopic observation, which is time- and labor-consuming. Furthermore, these techniques also do not discriminate between different species.

1-5-3. Hatching tests

Hatching tests are used in Norway, Sweden, and France to detect viable PCNs (EPPO, 2017). PCNs respond to host root-secreted hatching factors, and potato root diffusate (PRD) is commonly used as a hatching agent. There are a lot of methods to prepare PRD. In most cases, the roots of potato are gently washed and immersed in water for several hours to one day, then the water is filtered to remove debris and microorganisms. Cysts are then exposed to PRD for several weeks, and upon the detection of hatched juveniles, inspectors determine that viable individuals are present within the cysts.

However, PRD's hatching-stimulation activity is influenced by multiple factors, including the root growth and age of plants used for PRD preparation, as well as PCNs' storage conditions (Rawsthorne & Brodie, 1986; Salazar & Ritter, 1993; Byrne *et al.*, 2001). Additionally, PCNs can enter a state of diapause, an arrested development phase that enables them to survive unfavorable external conditions such as low temperatures

(Janssen *et al.*, 1987; Perry, 1989). PCNs in diapause do not hatch even in the presence of hatching factors (Devine & Jones, 2001), leading to viable individuals being overlooked. Moreover, the hatching tests also do not discriminate between different species.

1-5-4. Trehalose-detection method

Trehalose, a disaccharide, is contained in the perivitelline fluid, which surrounds the juvenile inside the egg. The trehalose concentration is reported to be 0.34 M in *G. rosotochiensis* eggs (Clarke *et al.*, 1978). It protects the juveniles in the eggs from desiccation (Perry, 1989). After death, the permeability of the eggshells is altered, resulting in the release of trehalose from the eggs. Consequently, viable eggs can be detected by boiling the cysts or eggs and measuring the amount of trehalose leached from eggs (van den Elsen *et al.*, 2012; Beniers *et al.*, 2014; Ebrahimi *et al.*, 2015). The lowest limit for egg detection in the method is five eggs (Ebrahimi *et al.*, 2015). However, to achieve precision, it is essential to crush the cysts without damaging the eggs to eliminate residual trehalose (presumably derived from dead eggs) in cysts before measurement (Beniers *et al.*, 2014; Ebrahimi *et al.*, 2015). In addition, this method also does not discriminate between different species.

1-5-5. DNA-based PCR

Routine diagnosis of PCNs inevitably relies on DNA-based detection methods (Bulman & Marshall, 1997; Nakhla *et al.*, 2010; Reid *et al.*, 2015; Sakai *et al.*, 2019; Kushida & Sakai, 2022). In general, DNA is gradually degraded after death. Min *et al.* (2011) reported that the DNA of root-knot nematode *Meloidogyne incognita* was

detectable up to 10 days after heat-treatment but became undetectable after 14 days. Similarly, MacMillan *et al.* (2006) reported that the DNA of slug parasitic nematode *Phasmarhabditis hermaphrodita* was detectable up to 6 days after heat-treatment but became undetectable after 8 days. However, the DNA of *G. pallida* was still detectable 51 days after heat-treatment (van den Elsen *et al.*, 2012). The eggshells and cyst shells may act as barriers against DNA degradation.

A recent study reported a detection method of viable PCNs eggs by PCR in combination with propidium monoazide (PMA, Christoforou *et al.*, 2014). PMA is a photoreactive membrane-impermeable dye that penetrates cells through compromised membranes after death (Nocker *et al.*, 2006). When exposed to bright white light, PMA forms covalent bonds with DNA molecules, preventing PCR amplification (Nocker *et al.*, 2006; Brescia *et al.*, 2009). Therefore, this method can detect viable organisms and identify species. Christoforou *et al.* (2014) detected and quantified viable PCNs by this method. However, concerning cyst nematodes, cyst shells may act as barriers against exposure to the light, which is inevitable for viable nematode detection. Therefore, it is required to release eggs from cysts and remove cyst shells thoroughly, which is extremely labor-intensive.

1-5-6. Reverse transcription PCR

In general, gene expression does not occur in dead cells. Moreover, mRNA has a shorter half-life than DNA (Eigner *et al.*, 1961) and is easily degradable by RNases. Therefore, mRNA expression is a good indicator of cell viability, and reverse transcription PCR (RT-PCR) is used for the detection of viable protozoa (Alum *et al.*, 2012), bacteria (Yaron & Matthews, 2002), and nematodes (Leal *et al.*, 2013; Beniers *et*

al., 2014; Mimee *et al.*, 2017). Moreover, RT-PCR is more time-saving than inoculation tests, microscopic inspections, and hatching tests. A study reported the use of a quantitative RT-PCR (RT-qPCR) method to detect viable PCNs (Mimee *et al.*, 2017). However, this method does not discriminate *G. pallida*, *G. rostochiensis*, and *G. ellingtonae*. Another study developed an RT-qPCR method to specifically detect viable *G. pallida* and *G. rostochiensis* (Beniers *et al.*, 2014). However, it requires locked nucleic acid probes, which are relatively costly. Moreover, a previous study showed that the Ct values of probe-based qPCR were higher than those of intercalator-based qPCR even using the same primers and templates (Lin *et al.*, 2020). Therefore, it is favorable to develop an intercalator-based RT-qPCR method.

In addition, to detect viable nematodes sensitively by RT-qPCR, it is important to select a highly expressed gene as a target. However, information about gene expression levels in *G. pallida* eggs was lacking.

1-6. Objective of this study

The study aimed to develop an intercalator-based RT-qPCR method for detecting viable *G. pallida*. In Chapter 2, the objective was to select an optimal gene for detecting viable *G. pallida*. In Chapter 3, I aimed to design a primer set specific to *G. pallida* and evaluate its performance. In Chapter 4, to clarify the detectability of nucleic acids of dead *G. pallida*, I performed the RT-qPCR and DNA-based PCR methods using nucleic acids of *G. pallida* treated with 1,3-D. Lastly, in Chapter 5, to demonstrate the validity of the RT-qPCR, the sensitivity of this RT-qPCR method in detecting viable *G. pallida* was compared to that of the cup test.

Chapter 2. Selection of housekeeping gene for detection of viable *Globodera pallida*

2-1. Introduction

mRNA expression is a reliable indicator of viability due to its shorter half-life compared to DNA (Eigner *et al.*, 1961) and its susceptibility to degradation by RNases. Recent studies reported the use of RT-qPCR for testing the viability of nematodes (Leal *et al.*, 2013; Beniers *et al.*, 2014; Mimee *et al.*, 2017). To detect viable nematodes sensitively using RT-qPCR, the selection of a highly expressed gene is essential. Housekeeping genes, which are generally thought to be highly expressed, are potential candidates for target of the RT-qPCR. However, previous studies reported that the expression levels of some housekeeping genes can be considerably low (Dheda *et al.*, 2004; Expósito-Rodríguez *et al.*, 2008; Hu *et al.*, 2009). In addition, information on the expression levels of housekeeping genes in *G. pallida* eggs was lacking.

The objective of this chapter was to select a gene suitable for the detection of viable *G. pallida*. For this end, I assessed the expression levels of 10 housekeeping genes (*act-1*, *Ama-1*, *APC1*, *BUB3*, *cdc-42*, *eif4A*, *GR*, *Mce1*, *TUBG2*, and *Y45F10D.4*) in *G. pallida* eggs by RT-qPCR.

2-2. Materials and methods

I evaluated the expression levels of the housekeeping genes listed in Table 2 in *G. pallida* eggs. I prepared a *G. pallida* population derived from Abashiri city. One thousand eggs of the *G. pallida* population were homogenized in a 2 ml plastic tube with one hundred 1 mm diameter zirconium oxide beads, twenty 2 mm diameter zirconium

Table 2. Primers used in expression analysis of housekeeping genes and Ct values of each gene

Gene	Sequence (5'=>3')	Gene description	Reference	Ct values ¹	Melting temperature (°C) of each amplicon
<i>act-1</i>	Forward: CTTCTTGGGCATGGAGTCGG Reverse: AATGCCCCGGGTACATCGTC	actin 1	Jones <i>et al.</i> , (2018)	18.6 ± 0.1	86.4–86.5
<i>Ama-1</i>	Forward: CTCCAAGCTCTCCACGTTATT Reverse: GGCGAAGTTGGACTGTATGT	amanitin resistant family member	Sabeh <i>et al.</i> , (2018)	23.9 ± 0.2	83.8–84.0
<i>APC1</i>	Forward: CCGCTGGACTTGTTGGTAAT Reverse: TAAGCTGCAGCACATCCAAC	anaphase-promoting complex subunit 1	Palomares-Rius <i>et al.</i> , (2016)	23.7 ± 0.3	83.8–83.9
<i>BUB3</i>	Forward: CACACAGATGGGGTGAGATG Reverse: GTACACTCTGTCCCCGCATT	mitotic checkpoint protein bub3	Palomares-Rius <i>et al.</i> , (2016)	22.5 ± 0.1	86.7–86.7
<i>cdc-42</i>	Forward: GCATCATTGTACGAAGACTCCA Reverse: TGGGCTTCATTTTGTCTTTGC	cell division control protein 42 homolog	Jones <i>et al.</i> , (2018)	21.1 ± 0.1	83.6
<i>ef4A</i>	Forward: CGAAACAGGACCAACAAATG Reverse: GTTCAGATCAGCTCCCCAAT	translation initiation factor	Valdes <i>et al.</i> , (2012)	18.7 ± 0.1	80.2–80.3
<i>GR</i>	Forward: TTGAGAGACCATGCCGATTAC Reverse: GAGTTGAGACGCCGAATGT	glutathione reductase	Sabeh <i>et al.</i> , (2018)	20.8 ± 0.1	81.5–81.7
<i>Mce1</i>	Forward: CCCGCATAAACTCCCATCTT Reverse: CTTACACCGATTTGCCTTTC	mRNA capping enzyme	Sabeh <i>et al.</i> , (2018)	20.8 ± 0.1	80.1–80.2
<i>TUBG2</i>	Forward: ATTGGCATTTCGACCTG Reverse: GTCATTGGTCCGACTTTGGT	tubulin γ -2 chain	Palomares-Rius <i>et al.</i> , (2012)	22.8 ± 0.1	80.8
<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 ± 0.1	82.0–82.1

¹ Data represent mean ± SE (n = 3).

oxide beads, 350 μ l of RA1 buffer (in Nucleospin[®] RNA (Takara Bio Inc., Shiga, Japan)), and 3.5 μ l of 1 M of dithiothreitol (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) using a multi-beads shocker (FastPrep-24[™] 5G, MP Biomedicals Inc., Solon, OH, USA). Total RNA extraction was carried out using a Nucleospin[®] RNA according to the manufacturer's instructions. The total RNA was dissolved in 60 μ l of nuclease-free water. To degrade DNA, the DNase treatment was carried out as follows. Seven microliters of DNase solution (10% (w/w) rDNase in Reaction Buffer for rDNase, both reagents were contained in the Nucleospin[®] RNA) were added to the total RNA solution (60 μ l). The mixture was heated at 37°C for one hour. Next, 170 μ l of 99.5% ethanol (FUJIFILM Wako Pure Chemical Co.), 1 μ l of Dr. GenTLE Precipitation Carrier (Takara Bio Inc.), and 7 μ l of 3 M sodium acetate (pH 5.2, Takara Bio Inc.) were added to the mixture and mixed well. Then, the mixture was centrifuged for 10 min at 12,000 \times g at 4°C. The supernatant was discarded, 500 μ l of 70% ethanol (FUJIFILM Wako Pure Chemical Co.) was added to the pellet, which was then centrifuged again for 5 min at 7,500 \times g at 4°C. The supernatant was discarded, and the pellet was dried and dissolved in 60 μ l of nuclease-free water. Three replicates were prepared for each sample.

RT-qPCRs were performed on a Mic real-time PCR system (Bio Molecular Systems, Upper Coomera, QLD, Australia) using a One Step TB Green[®] PrimeScript[™] PLUS RT-PCR Kit (Takara Bio Inc.). The reaction cocktail contained 5.0 μ l of 2 \times One Step TB Green RT-PCR Buffer 4, 0.6 μ l of Takara Ex Taq HS Mix, 0.2 μ l PrimeScript PLUS RTase Mix, 0.4 μ l each of forward and reverse primers (10 μ M, Table 2), 2.4 μ l of nuclease-free water, and 1.0 μ l of the total RNA solution prepared above. The cycling conditions 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 for 30 s. The specificity of the RT-qPCR reaction for each

amplified product was verified by melting curve analysis, which was carried out as follows: 95°C for 15s, 60°C for 1 min, and then ramping to 95°C (at 0.15°C s⁻¹ transition rate). Threshold cycles (Ct) were automatically determined using the micPCR software v. 2.8.10. The RT-qPCRs were carried out in duplicate.

2-3. Results and discussion

The Ct values for each gene were shown in Table 2. The gene with the lowest Ct value was *Y45F10D.4*, which encodes a putative iron-sulfur cluster assembly enzyme, suggesting that this gene was highly expressed in *G. pallida* eggs. Sabeh *et al.* (2018) assessed the expression levels of housekeeping genes in *G. rostochiensis* eggs and juveniles and showed that *Y45F10D.4* was the gene with the lowest Ct value. Therefore, *G. pallida* and *G. rostochiensis* might have similar expression profiles of housekeeping genes.

Because the primer set for *Y45F10D.4* gene developed by Sabeh *et al.* (2018) and used in this chapter amplified *G. rostochiensis Y45F10D.4* gene also. Therefore, the design of a new primer set was required.

Chapter 3. Design of *Globodera pallida*-specific primers on *Y45F10D.4* gene

3-1. Introduction

In Japan, 14 cyst nematode species, *G. artemisiae*, *G. pallida*, *G. rostochiensis*, *G. tabacum*, *H. avenae*, *H. elachista*, *H. glycines*, *H. humuli*, *H. koreana*, *H. latipons*, *H. schachtii*, *H. sojae*, *H. trifolii*, and *Cactodera cacti* have been recorded (Yamada *et al.*, 1972, Shimomoto *et al.*, 2000; Momota, 2004; Narabu *et al.*, 2016; Sekimoto *et al.*, 2016, 2017, 2019, Sakai & Kushida, 2019). To achieve precise detection of *G. pallida*, it is important to avoid detecting other cyst nematode species. Notably, *G. rostochiensis* is frequently detected in *G. pallida*-infested fields (unpublished data).

In addition, DNA can be co-extracted with RNA. As DNA has a longer half-life than RNA (Eigner *et al.*, 1961), the co-extracted DNA derived from dead nematodes might cause false positives. Although DNA can be removed using DNase, digestion of DNA is time- and labor-consuming. Therefore, primer sets sandwiching a long intron or primers on exon-exon junctions are desirable to eliminate amplification from DNA (Markou *et al.*, 2021).

In Chapter 2, I suggested that the *Y45F10D.4* gene, coding for a putative iron-sulfur cluster assembly enzyme, was highly expressed in *G. pallida* eggs. Therefore, in this chapter, I aimed to design a *G. pallida*-specific primer set on *Y45F10D.4* gene and evaluate the species-specificity of the primers. Additionally, a common primer set on the same gene was designed to ensure the successful extraction of RNA from all nematode populations. These primer sets were designed not to amplify DNA. Subsequently, sensitivity and amplification efficacy were assessed using the *G. pallida*-specific primer

set.

3-2. Materials and methods

3-2-1. Nematode populations

Twenty populations of cyst nematode species were used in this study (Table 3). Nematode populations from outside Japan were imported in accordance with the official permits of the Ministry of Agriculture, Forestry, and Fisheries (49Y1213, 29Y1553, 2Y1139, 2Y1141, and 2Y1142).

3-2-2. Design of primer sets

Y45F10D.4 partial sequence of *Heterodera glycines* (GenBank accession no. BF014189) was searched in the *G. pallida*, *G. rostochiensis*, and *G. ellingtonae* reference genomes (GenBank accession no. GCA_000724045, GCA_018350315, and GCA_001723225, respectively) using BLASTN in NCBI (<https://www.ncbi.nlm.nih.gov/>) to identify the orthologs. I aligned each sequence using MEGA-X v. 10.1.7, and designed primers (Y45_0803_F1 and Y45_0715_R4, Table 4, Fig. 6) for *G. pallida*. I also designed universal primers (Y45_Com_F1 and Y45_Com_R1, Table 4, Fig. 6) to confirm whether the RNA extraction of all tested species had succeeded.

3-2-3. Extraction of nucleic acids

RNA was extracted from five cysts of each population. Because *G. rostochiensis* was the most likely non-target species in *G. pallida*-infested fields, I also prepared a deep RNA solution from 50 cysts of *G. rostochiensis* from Kutchan (Gr Kutchan). Each

Table 3. Nematodes used in this study

Code	Species	Pathotype	Origin	Source
Gp Abashiri	<i>Globodera pallida</i>	Pa3	Abashiri, Hokkaido, Japan	This study
Gp Shari	<i>G. pallida</i>	Not determined	Shari, Hokkaido, Japan	This study
Gp Chavornay	<i>G. pallida</i>	Pa3	Chavornay, Switzerland	E. Grenier ³
Gp UK	<i>G. pallida</i>	Pa1	UK	F. G. W. Jones ¹
Gr Kutchan	<i>G. rostochiensis</i>	Ro1	Kutchan, Hokkaido, Japan	This study
Gr Shari	<i>G. rostochiensis</i>	Ro1	Shari, Hokkaido, Japan	This study
Gr Nagasaki	<i>G. rostochiensis</i>	Ro1	Nagasaki, Japan	This study
Gr Aomori	<i>G. rostochiensis</i>	Ro1	Aomori, Japan	This study
Gr Ecosse	<i>G. rostochiensis</i>	Ro1	Ecosse, UK	E. Grenier ³
Gr UK	<i>G. rostochiensis</i>	Ro1	UK	F. G. W. Jones ¹
Ge 29Y1553	<i>G. ellingtonae</i>	-	USA	T. Prior ²
Ge 2Y1141	<i>G. ellingtonae</i>	-	USA	I. Zasada ³
Gtt Kochi	<i>G. tabacum</i>	-	Kochi, Japan	This study
Gtt Landes	<i>G. tabacum</i>	-	Landes, France	E. Grenier ³
Gts 75140	<i>G. tabacum</i>	-	Mexico	E. Grenier ³
Ga	<i>G. solanacearum</i>	-	Nagasaki, Japan	This study
Hg	<i>G. artemisiae</i>	-	Nagasaki, Japan	This study
Ht	<i>Heterodera glycines</i>	-	Kyougoku, Hokkaido, Japan	This study
Hs	<i>H. trifolii</i>	-	Nanae, Hokkaido, Japan	This study
Hs	<i>H. schachtii</i>	-	Nagano, Japan	H. Okada ³
He	<i>H. elachista</i>	-	Chiba, Japan	H. Sakai ³

¹ Gp UK and Gr UK were provided in 1974 and have been propagated in our laboratory.

² Ge 29Y1553 was provided in 2017 and has been propagated in our laboratory.

³ Cysts of these populations were provided in 2020 to 2022 and directly used in this study.

Table 4. 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>T</u> TCG
Cyst nematodes	Y45_Com_F1	GGTCAGCAATTGCGAGTTC
	Y45_Com_R1	TTGCGTCTTGAGCCAACAT

Underlined portion represents an intentionally replaced base.

<i>G. pallida</i> gDNA	-----	0
<i>G. rostochiensis</i> gDNA	-----	0
<i>G. ellingtonae</i> gDNA	-----	0
<i>H. glycines</i> cDNA	TTCTTTTTCGCTGACGCGACTTTTCCCCACTTTGGTCTTCCACCGTCATGGTAACATTCA	60
<i>G. pallida</i> gDNA	ACTGCAGCAGATTGCGGGGTACACGAGAAGGTCATTGACCATTATGAAAATCCAAGAAA	60
<i>G. rostochiensis</i> gDNAA.....G.....	60
<i>G. ellingtonae</i> gDNAG.....	60
<i>H. glycines</i> cDNAT...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
<i>G. pallida</i> gDNA	TTGTGGCGATGTTATGAAATTGCAAATCAAGGTTTGAGGAAAAACAGGTTTGCTATATGGG	180
<i>G. rostochiensis</i> gDNACC...C.....T.....T.....	180
<i>G. ellingtonae</i> gDNACC...C.....T.....	180
<i>H. glycines</i> cDNA	...C...A...A...A...C..T.....T.....	214
Intron		
<i>G. pallida</i> gDNA	TTTGTGTGTTTAAGGTGGATGCAAATGGCAAATAATCGACGCGAAGTTTAAGACGTTTCG	240
<i>G. rostochiensis</i> gDNAC.....	240
<i>G. ellingtonae</i> gDNAC.....	240
<i>H. glycines</i> cDNAC..G...C..AC.....T.....A...A...C.....T...	257
Y45 Com F1		
<i>G. pallida</i> gDNA	GATGCGGGTCAGCAATTGCGAGTTTCGTCACTCGCCACCGAATGGATAAAAAGGGCAGAATT	300
<i>G. rostochiensis</i> gDNAG.....A.....	300
<i>G. ellingtonae</i> gDNAA.....	300
<i>H. glycines</i> cDNA	...G..T.....T...GT..G.....T.....G.....	317
<i>G. pallida</i> gDNA	TGGACTATGCCAGCAAGGTCAAGAACCAGCAGATTGCCAAGGAACTATCGCTTCCTCCCG	360
<i>G. rostochiensis</i> gDNAT.....A.....	360
<i>G. ellingtonae</i> gDNAA.....	360
<i>H. glycines</i> cDNA	...A...G.C...AA.A.....A...A...C..A...G..T...A...G...A...	377
Y45_Com_R1		
<i>G. pallida</i> gDNA	TCAAGCTGCACTGTTCTGA	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> gDNAG.....	464
<i>G. ellingtonae</i> gDNAG.....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-----CAATAAACCCGGCGGATAAATTTA	518
<i>G. rostochiensis</i> gDNA	..T.....A.....G.....	509
<i>G. ellingtonae</i> gDNA	..T.....A.....	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..GTTTCGATATTTCTCTGA	571

Fig. 6. Partial sequences of *Y45F10D.4* gene for *Globodera pallida*, *G. rostochiensis*, *G. ellingtonae*, and *Heterodera glycines*. The sequence of *H. glycines* represents cDNA, whereas the other sequences represent genomic DNA. The position of the primers is indicated by arrows. Hyphens indicate deletion of the corresponding base and the middle dots indicate the same base as *G. pallida*.

sample was crushed in a 2 ml plastic tube with 1 g zirconium dioxide beads (1.5 mm diameter), 350 μ l of RA1 Buffer (Takara Bio Inc.), and 3.5 μ l of 1 M dithiothreitol (FUJIFILM Wako Pure Chemical Co.) using a multi-beads shocker (FastPrep-24™ 5G). Each lysate was centrifuged at $11,000 \times g$ for 2 min and the supernatants were collected. Then, 330 μ l of 70% ethanol was added to each supernatant, and the mixture was applied to a NucleoSpin® RNA Column (Takara Bio Inc.) with a collection tube. After centrifugation ($11,000 \times g$, 2 min), each column was washed using 250 μ l of Buffer RA2 (Takara Bio Inc.) and then 600 μ l and 250 μ l of Buffer RA3 (Takara Bio Inc.). RNA was eluted from each column using 60 μ l of nuclease-free water. Finally, the eluate was diluted 4 times with nuclease-free water.

Since DNase treatment was not performed, the RNA solution contained DNA. Therefore, the species identity of each RNA sample was confirmed using PCR-RFLP of ribosomal RNA internal spacer (ITS) regions (Orui, 1997; Amiri *et al.*, 2002; Subbotin *et al.*, 1999, 2000, 2011). The PCR cocktail contained 20.0 μ l of 2 \times Tks Gflex PCR Buffer (Takara Bio Inc.), 0.8 μ l of Tks Gflex™ DNA polymerase (Takara Bio Inc.), 1.2 μ l each of AB28 and TW81 primers (10 μ M, Joyce *et al.*, 1994), 14.8 μ l of nuclease-free water, and 2.0 μ l of the RNA solution. Thermal cycling was performed under the following conditions: one cycle at 94°C for 1 min, and 35 cycles at 98°C for 10 s, 55°C for 15 s, and 68°C for 40 s, using an Applied Biosystems® SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Inc., MA, USA). The PCR products were digested by *Alu* I, *Hinf* I, or *Rsa* I (Takara Bio Inc.). Reaction cocktails for *Alu* I and *Hinf* I digestion contained 0.5 μ l of the enzyme, 1.0 μ l of 10 \times Buffer, 3.5 μ l of nuclease-free water, and 5.0 μ l of the PCR product. The *Rsa* I digestion cocktail contained 0.5 μ l of the enzyme, 1.0 μ l of 10 \times Buffer, 1.0 μ l of BSA, 2.5 μ l of nuclease-free water, and 5.0 μ l of the PCR

product. Digestion was performed by heating the reaction cocktail at 37°C for 16 h on the above thermal cycler. For *H. glycines*, an additional restriction enzyme *Bme1390 I* (Thermo Fisher Scientific, Inc.) was also used. The reaction cocktail contained 0.5 µl of FastDigest *Bme1390 I*, 1 µl of 10 × FD Buffer, 4.5 µl of nuclease-free water, and 4 µl of PCR product. The cocktail was heated at 37°C for 30 min. The digested products were electrophoresed on a 2% agarose gel in 1 × TAE at 100 V for 30 min. The gel was stained with GelGreenTM (Biotium Inc., CA, USA) and visualized under cyan LED illumination.

The subspecies identity of each *G. tabacum* population was confirmed by PCR-RFLP of CLE peptide coding genes (Alenda *et al.*, 2013; Sakata *et al.*, 2021a). The PCR reaction cocktail was prepared as follows: 0.4 µl of Tks GflexTM DNA Polymerase, 10.0 µl of 2 × Gflex PCR Buffer, 0.6 µl each of primers (10 µM, CAPS CLE1F and CAPS CLE1R, or CAPS CLE5F and CAPS CLE5R; Alenda *et al.*, 2013), 6.4 µl of nuclease-free water, and 2.0 µl of the DNA solution. Thermal cycling was performed on the above thermal cycler under the following conditions: one cycle at 94°C for 1 min, 35 cycles at 98°C for 10 s, 60°C for 15 s, and 68°C for 20 s. PCR products generated by using CAPS CLE1 and CAPS CLE5 primer sets were digested by *BstE II* and *Mbo I* (Takara Bio Inc.), respectively. Reaction cocktails contained 0.5 µl of the enzyme, 1.0 µl of 10 × Buffer, 3.5 µl of nuclease-free water, and 5.0 µl of the PCR product. Digestion was performed by heating the reaction cocktail at 60°C (*BstE II*) or 37°C (*Mbo I*) for 16 h on the above thermal cycler. The digested products were electrophoresed as described before.

To test whether RT-qPCR detected *G. pallida* DNA, a DNA solution extracted from 10 cysts of *G. pallida* from Abashiri (Gp Abashiri) was prepared using a Wizard[®] SV Genomic DNA Purification System (Promega Inc., WI, USA) as described by Sakata *et al.* (2021a). The cysts were put in a 2 ml plastic tube with 10 zirconium oxide beads (2

mm diameter) and homogenized by using a multi-bead shocker (FastPrep-24™ 5G). Then, 280 μ l of Nuclei Lysis Solution, 70 μ l of 0.5 M EDTA, 28 μ l of 20 mg/ml Proteinase K (Nacalai Tesque Inc., Kyoto, Japan), 7 μ l of 4 mg/ml RNase A solution, and 2 μ l of 1 M dithiothreitol were added to the tube. After heating the mixture (55°C for 30 min), 400 μ l of SV Lysis Buffer was added to the mixture, and the tube was vortexed and centrifuged for 2 min at 13,000 \times g. The supernatant was applied to a Wizard® SV Minicolumn Assembly. Subsequent column washing and DNA elution were carried out according to the manufacturer's instructions. The species identity of this DNA sample was confirmed using multiplex PCR, as developed by Sakai *et al.* (2019) using Takara Taq™ HS PCR Kit, UNG plus (Takara Bio Inc.). The reaction cocktail contained 1.0 μ l of 10 \times PCR Buffer for UNG plus, 0.8 μ l of dU plus dNTP Mixture, 0.1 μ l of the *G. rostochiensis*-specific primers (10 μ M, GRmt1SP2f and GRmt1SP2r), 0.3 μ l each of the *G. pallida*-specific primers (10 μ M, GPmt4SP4f and GPmt4SP7r), 0.2 μ l each of the universal primers (10 μ M, 5.8Sf2 and 28Sr), 0.05 μ l Takara Taq HS, 0.1 μ l of UNG, 5.85 μ l of nuclease-free water, and 1.0 μ l of the DNA lysate. Thermal cycling was performed on the above thermal cycler under the following conditions: one cycle at 25°C for 10 min and 95°C for 2 min, 35 cycles at 98°C for 10 s and 68°C for 40 s. The electrophoresis was performed as described before.

3-2-4. Confirmation of species specificity

RT-qPCR was performed on a Mic real-time PCR system using a One Step TB Green® PrimeScript™ PLUS RT-PCR Kit. The reaction cocktail contained 5.0 μ l of 2 \times One Step TB Green RT-PCR Buffer 4, 0.6 μ l of Takara Ex Taq HS Mix, 0.2 μ l PrimeScript PLUS RTase Mix, 0.4 μ l each of Y45_0803_F1 (10 μ M) and Y45_0715_R4

(10 μ M), 2.4 μ l of 7.4% (w/w) polyvinylpyrrolidone (PVP, Merck KGaA, Germany, average Mw ~29,000) solution, and 1.0 μ l of the RNA solution prepared previously. PVP was added to mitigate the effects of the PCR inhibitors (Koonjul *et al.*, 1999; Monpoeho *et al.*, 2000). The cycling conditions were one cycle 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 specificity of the RT-qPCR reaction for each amplified product was confirmed by melting curve analysis, which was carried out as follows: 95°C for 15 s, 60°C for 1 min, and then ramped to 95°C (at 0.3°C s⁻¹ transition rate). Threshold cycles (Ct) were automatically determined using the micPCR software v. 2.8.10. A negative control was prepared using nuclease-free water as the template for each run. The RT-qPCRs were carried out in duplicate, and two independent assays were performed.

To test whether RNA extraction was successful for all nematode populations, an additional endpoint RT-PCR was performed using PrimeScript™ OneStep RT-PCR Kit Ver. 2 (Takara Bio, Inc.). The reaction cocktail contained 0.4 μ l of PrimeScript 1 step Enzyme Mix, 5.0 μ l of 2 \times 1 step Buffer, 0.4 μ l each of Y45_Com_F1 and Y45_Com_R1 (Table 4, 10 μ M each), 2.8 μ l nuclease-free water, and 1.0 μ l of the RNA solution of each species. Thermal cycling was performed under the following conditions: one cycle 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 for 10 s, using the above thermal cycler. A negative control was prepared using nuclease-free water as the template. The PCR products were electrophoresed as described before.

3-2-5. Detection sensitivity and standard curve

I extracted RNA from 1000 eggs of *G. pallida* from Abashiri city (Gp Abashiri)

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

3-3. Results

3-3-1. Design of primer sets

The BLAST search revealed that orthologs of each species were hit (*G. pallida*; base no. 173619–173996 and 174512–174663 in scaffold 77, *G. rostochiensis*; base no. 536749–537126 and 537636–537766 in scaffold 35, *G. ellingtonae*; base no. 328503–328880 and 329340–329468 in scaffold 39). By comparing these sequences with the cDNA sequence of the *Y45F10D.4* gene for *H. glycines*, two introns (approximately 40 and 500 bp, respectively) were identified in each of the *Globodera* sequences (Fig. 6). Then, I designed *G. pallida*-specific primers (Y45_0803_F1 and Y45_0715_R4) and universal primers for *Globodera* spp. and *H. glycines* (Y45_Com_F1 and Y45_Com_R1) (Table 4) to sandwich the approximately 500 bp intron between the forward and reverse primers to prevent amplification of *Y45F10D.4* genomic DNA (Fig. 6). Additionally, the intron divides Y45_Com_R1 into two parts. To prevent primer dimer formation and improve species specificity, the 4th base from the 3' end of Y45_0715_R4 was designated thymine instead of adenine (Table 4).

3-3-2. Confirmation of species specificity

The universal primers, Y45_Com_F1 and Y45_Com_R1, generated approximately 150 bp amplicons from the RNA of all tested nematode populations (Fig. 7). RT-qPCR

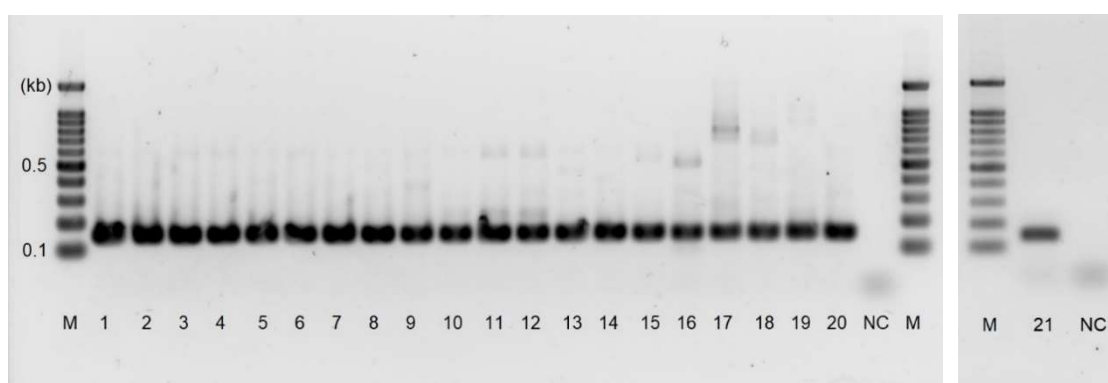


Fig. 7. Endpoint RT-PCR results using the universal primer set (Y45_Com_F1 and Y45_Com_R1). The RNA was extracted from 5 and/or 50 cysts of each nematode population. M: 100 bp DNA ladder (DM2100, SMOBIO Technology, Taiwan); 1: Gp Abashiri; 2: Gp Shari; 3: Gp Chavornay; 4: Gp UK; 5: Gr Kutchan (5 cysts); 6: Gr Shari; 7: Gr Nagasaki; 8: Gr Aomori; 9: Gr Ecosse; 10: Gr UK; 11: Ge 29Y1553; 12: Ge 2Y1141; 13: Gtt Kochi; 14: Gtt Landes; 15: Gts 75140; 16: Ga; 17: Hg; 18: Ht; 19: Hs; 20: Gr Kutchan (50 cysts); 21: He; NC: negative control (nuclease-free water). See Table 3 for code definitions.

using the *G. pallida* specific primer set, Y45_0803_F1 and Y45_0715_R4, yielded an amplicon (367 bp) whose melting temperature (T_m) was 86.3–86.4°C for the RNA of all tested *G. pallida* populations (Table 5, Fig. 8). However, no amplicons or inappropriate amplicons ($< 83^\circ\text{C}$ T_m value) were obtained from the RNA of the other species or DNA of *G. pallida*.

3-3-3. Detection sensitivity and standard curve

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

The dilution series of RNA extracted from 1000 *G. pallida* eggs generated a standard curve (Fig. 9); the relationship between the Ct values (y) and the common logarithm of the number of eggs (x) was $y = -3.635x + 34.157$ ($R^2 = 0.978$, $P < 0.001$, $E = 88.4\%$).

3-4. Discussion

In Chapter 2, I found that the *Y45F10D.4* gene was highly expressed in *G. pallida* eggs. Moreover, in this chapter, I found some sequence differences in the *Y45F10D.4* gene between *G. pallida* and *G. rostochiensis*. Consequently, I designed a primer set for this gene. This primer set amplified all tested *G. pallida* populations without cross-reactivity to other tested species. Additionally, I prepared RNA from 50 cysts of *G. rostochiensis*, based on the assumption that a lot of cysts of this species can be also

Table 5. Species specificity of the primer set, Y45_0803_F1 and Y45_0715_R4

Code	Number of cysts	Nucleic acid type	Ct values
Gp Abashiri	5	RNA	19.5
	10	DNA	nd
Gp Shari	5	RNA	18.6
Gp Chavornay	5	RNA	20.6
Gp UK	5	RNA	19.9
Gr Kutchan	5	RNA	nd
	50	RNA	nd
Gr Shari	5	RNA	nd
Gr Nagasaki	5	RNA	nd
Gr Aomori	5	RNA	nd
Gr Ecosse	5	RNA	nd
Gr UK	5	RNA	nd
Ge 29Y1553	5	RNA	nd
Ge 2Y1141	5	RNA	nd
Gtt Kochi	5	RNA	nd
Gtt Landes	5	RNA	nd
Gts 75140	5	RNA	nd
Ga	5	RNA	nd
Hg	5	RNA	nd
Ht	5	RNA	nd
Hs	5	RNA	nd
He	5	RNA	nd

See Table 3 for code definitions. nd: not detected

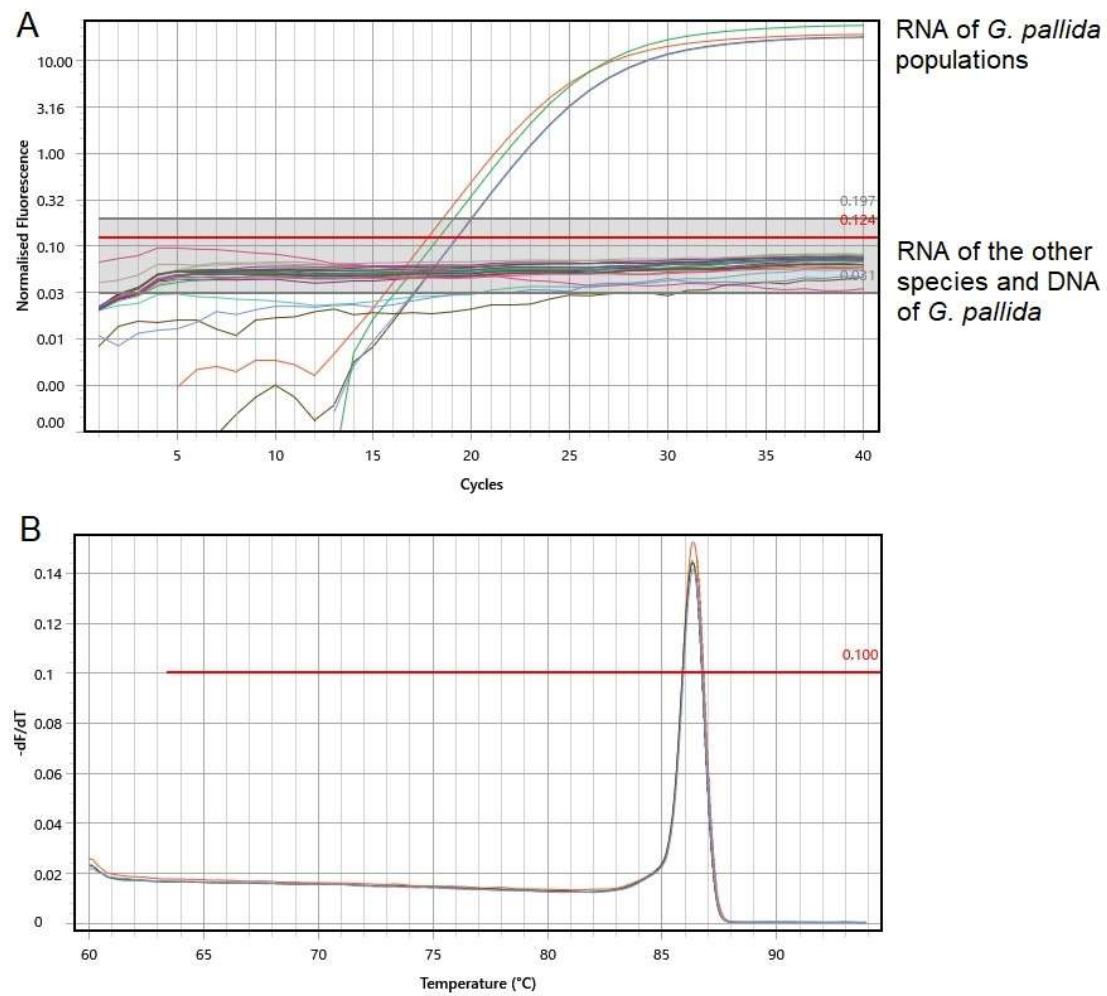


Fig. 8. Results examples of the RT-qPCR using the *G. pallida*-specific primer set (Y45_0803_F1 and Y45_0715_R4). Nucleic acid samples of nematode populations listed in Table 3 were used. A: Amplification plots obtained from nematodes used in this study. B: Melting plots obtained from Gp Abashiri, Gp Shari, Gp Chavornay, and Gp UK.

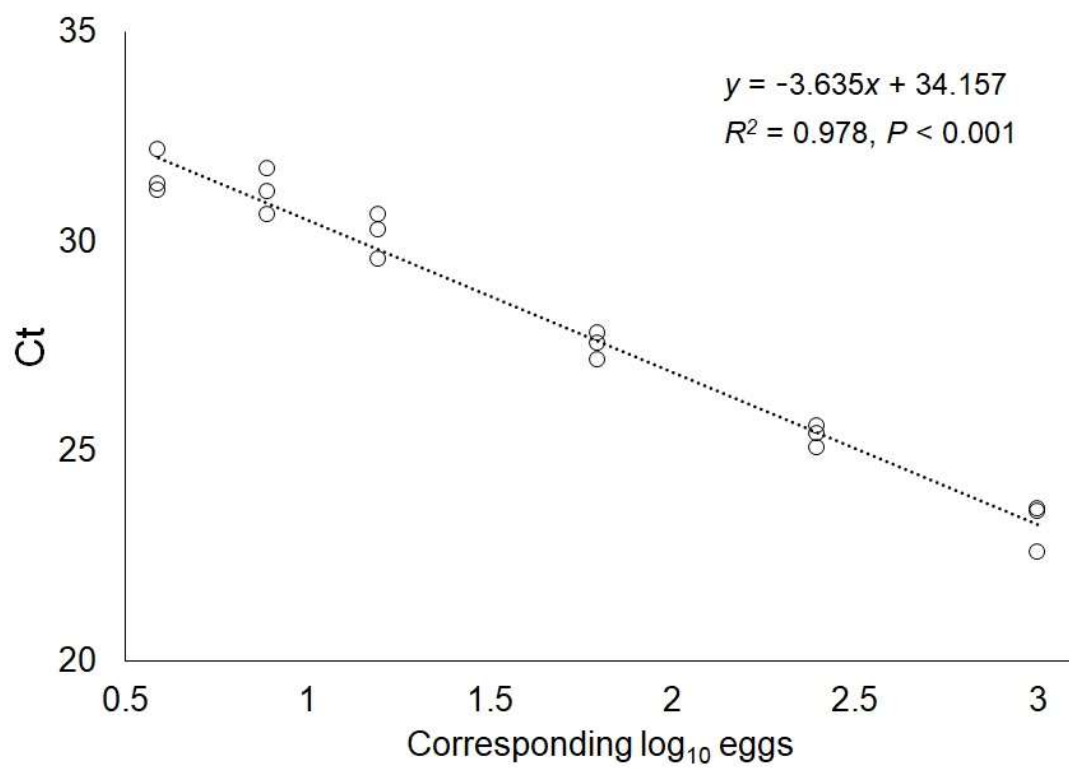


Fig. 9. Standard curve of the RT-qPCR method calculated with the Ct values and corresponding number of eggs.

present in *G. pallida*-infested fields in Japan. However, no appropriate amplicons were obtained for this RNA sample also. These results confirmed the species specificity of this primer set. For *G. pallida*, I used only Japanese and European populations. However, *G. pallida* populations in Algeria, North America, and New Zealand are phylogenetically close to European and Japanese *G. pallida* populations (Skanter *et al.*, 2007; Madani *et al.*, 2010; Tirchi *et al.*, 2016; Ohki *et al.*, 2018; Subbotin *et al.*, 2020). Therefore, this primer set may be applicable to *G. pallida* populations outside of Japan and Europe.

DNA is often co-extracted with RNA, and the DNA remaining in the RNA sample may cause false-positive signals derived from dead organisms. However, DNase treatment is time- and labor-intensive. To solve this problem, Leal *et al.* (2013) designed a reverse primer for an exon-exon junction. At first, I tried designing a forward primer that was divided by the approximately 40 bp intron (Fig. 6), but the primer did not have enough species specificity (data not shown). Therefore, I designed a *G. pallida*-specific primer set sandwiching a long (approximately 500 bp) intron. If the primers hybridize with the genomic DNA, the elongation step requires a relatively long time. Under our experimental conditions, where the annealing/extension step was set to 35 s, amplification of *G. pallida* DNA did not occur. However, amplification from DNA may occur with a longer annealing/extension step. In other words, if the annealing/extension step is extended, the primer set may be useful for *G. pallida*-specific detection by conventional DNA-based PCR. In our supplementary experiment, a DNA-based endpoint PCR using the primer set yielded an amplicon (approximately 900 bp) from *G. pallida* DNA but did not from *G. rostochiensis* DNA (Appendix 1).

The RT-qPCR method was sensitive enough to detect RNA corresponding to 3.9 *G. pallida* eggs. Many DNA-based detection methods using the ITS region or

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

Chapter 4. Effect of disinfection with 1,3-dichloropropene on detectability of *Globodera pallida* DNA and RNA

4-1. Introduction

To accurately detect viable nematodes, it is crucial to demonstrate that the method does not detect dead nematodes. In previous studies that developed methods for assessing nematode viability, heat treatment has been used to prepare dead individuals (Ogiga & Estey, 1974; van den Elsen *et al.*, 2012; Leal *et al.*, 2013; Ebrahimi *et al.*, 2015; Mimee *et al.*, 2017; Hajihassani *et al.*, 2018; Pillai & Dandurand, 2019). Also, previous studies demonstrated that RNA was not detected in heat-treated nematodes (Leal *et al.*, 2013; Mimee *et al.*, 2017). On the other hand, van den Elsen *et al.* (2012) reported that the DNA of *G. pallida* was still detectable 55 days after heat treatment, demonstrating that DNA-based methods are not suitable for viable *G. pallida* detection.

In our laboratory's preliminary study, conducted in July and August of 2020 and 2021, soil temperature (10 cm deep, fallow) was measured at 30- or 60 min intervals in a *G. pallida*-infested field. The average daily soil temperature ranged from 15.0 to 27.9°C, and the maximum soil temperature was 31.8°C (unpublished data). Considering that many *G. pallida* eggs were still alive after heating at 40°C for 64 min (Stone & Webley, 1975), it is unlikely that *G. pallida* in Hokkaido's fields died of heat.

In the official control of *G. pallida*, 1,3-dichloropropene (1,3-D) is used. The gassed 1,3-D penetrates the soil, effectively eliminating both hatched juveniles, and eggs contained in cysts. Therefore, there are many cysts containing dead eggs in the field treated with 1,3-D. However, it is unknown whether RNA and DNA can be detected from nematodes died of 1,3-D.

In this chapter, to clarify the detectability of nucleic acids of *G. pallida* died of 1,3-D, I extracted nucleic acids from 1,3-D-treated *G. pallida* and performed the RT-qPCR developed in Chapter 3 and conventional DNA-based PCR methods.

4-2. Materials and methods

4-2-1. RT-qPCR using RNA extracted from 1,3-dichloropropene-treated *Globodera pallida*

I sampled *G. pallida*-infested soil from a field in Shari town, Hokkaido. Approximately 300 cysts and 10,000 eggs in the cysts were found in 100 ml of the soil. I placed 50 ml of the soil in 50 ml glass vials and injected it with 0, 15, or 50 μ l of 1,3-D (mixture of cis- and trans-, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The actual application amount in the field (400 l ha⁻¹) approximately corresponds to 15 μ l (50 ml soil)⁻¹. The vial was tightly capped and incubated at 20–25°C for two weeks. Then, the soil in the vial was degassed by spreading it on a plastic tray in a fume hood for several hours, moistened with approximately 3 ml of distilled water, and incubated at 18°C for 2 weeks in a polyethylene bag. Subsequently, cysts in the soil were separated according to the method described by Sakata *et al.* (2021b). RNA was extracted from half of the cysts and subjected to RT-qPCR as described previously. The other cysts were used in an inoculation test to determine their viability. The cysts were packed in a nylon mesh bag (3 × 3 cm, 100 μ m aperture). The bag was put in a quadrangular 250 ml plastic cup (MH-3, MKTEC Co. Ltd., Toyama, Japan) with a tuber of the potato cultivar ‘Pearl Starch’ (conferring *Hl* gene) and 125 ml of culture soil (0.374 g N, 1.485 g P₂O₅, 0.242 g K₂O, and 0.165 g MgO per kg; Hokusan Co., Ltd., Hokkaido, Japan). A cup without cyst bag was prepared as a control. Each cup was incubated at 18°C in the dark and

watered as required. After 15 weeks, the soil in the cup was dried and the newly formed cysts were isolated and counted using a binocular microscope. Each treatment was replicated three times.

4-2-2. Conventional DNA-based PCR

Detection of DNA in 1,3-D treated *G. pallida* was performed by conventional PCR methods. As mentioned before, since DNase treatment was not performed, the RNA solutions of 1,3-D-treated *G. pallida* contained DNA. Therefore, I used these RNA samples as DNA templates. I performed two methods; one is based on Bulman & Marshall (1997) using ITS region and being used in many studies even recently (Mwangi *et al.*, 2015; Camacho *et al.*, 2017; Mburu *et al.*, 2018; Peng *et al.*, 2022), the other is based on Sakai *et al.* (2019) using small circular mitochondrial DNA and being used in Yokohama Plant Protection Station and JA Kitamirai (Kunneppu, Hokkaido) to identify PCNs. For the first method, the PCR reaction cocktail was prepared as follows: 5.0 μ l of Dream TaqTM Hot Start Green Master Mix (Thermo Fisher Scientific, Inc.), 0.3 μ l each of primers ITS5 and PITSp4 (10 μ M each, Bulman & Marshall, 1997), 1.0 μ l of the RNA solution, and 3.4 μ l of nuclease-free water. Thermal cycling was performed using an Applied Biosystems[®] SimpliAmpTM Thermal Cycler under the following cycling conditions: one cycle at 94°C for 2 min, and 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A negative control was prepared using nuclease-free water as the template. For the second method, the PCR reaction was performed according to Sakai *et al.* (2019) (see Chapter 3). Visualization of the PCR products was performed as described in Chapter 3.

4-3. Results

4-3-1. RT-qPCR using RNA extracted from 1,3-dichloropropene-treated *Globodera pallida*

When RNA was extracted from *G. pallida* treated with 0 or 15 μ l of 1,3-D, amplification was successful for all replicates (Table 6). The Ct values of untreated *G. pallida* were lower than those of *G. pallida* treated with 15 μ l of 1,3-D. T_m values of appropriate amplicons ranged from 86.3 to 86.5°C. No appropriate amplicon was obtained from RNA extracted from *G. pallida* treated with 50 μ l of 1,3-D. In the inoculation test, *G. pallida* treated with 0 or 15 μ l of 1,3-D produced new cysts (Table 6). In contrast, *G. pallida* treated with 50 μ l of 1,3-D did not reproduce.

4-3-2. Conventional DNA-based PCR

Both the first and second PCR methods yielded *G. pallida*-specific amplicons (approximately 250 and 300 bp, respectively) from all tested samples (Fig. 10). The band luminance of *G. pallida* treated with 50 μ l of 1,3-D was lower than that treated with 0 or 15 μ l of 1,3-D. The negative control did not generate amplicons for both PCR assays.

4-4. Discussion

I showed that *G. pallida*, treated with 50 μ l of 1,3-D, did not reproduce, and its RNA was not detected by RT-qPCR. This result demonstrated that the RT-qPCR method did not detect *G. pallida* died of 1,3-D. In our experiment, RNA extraction was performed 2 weeks after 1,3-D treatment lasting for 2 weeks. Thus, the mRNA of the *G. pallida* *Y45F10D.4* gene appears to be undetectable 2 to 4 weeks after death. A previous study showed that mRNA of the *Bursaphelenchus xylophilus* *Hsp70* gene was detectable

Table 6. Effect of 1,3-dichloropropene (1,3-D) treatment of infested soil on the detectability by the RT-qPCR method and evaluation of viability of *Globodera pallida* cysts by the inoculation test

Quantity of 1,3-D (μ l) added to 50 ml soil	RT-qPCR (Ct values) ¹	Inoculation test (The number of newly formed cysts) ^{1, 2}
0	21.5 \pm 0.4	167.3 \pm 27.7
15	29.1 \pm 0.5	1.7 \pm 0.9
50	nd	0
Without cysts	-	0

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

¹ Data represent mean \pm SE (n = 3). nd: not detected. -: not tested

² Cysts were isolated from the entire soil in the cups and counted using a binocular microscope.

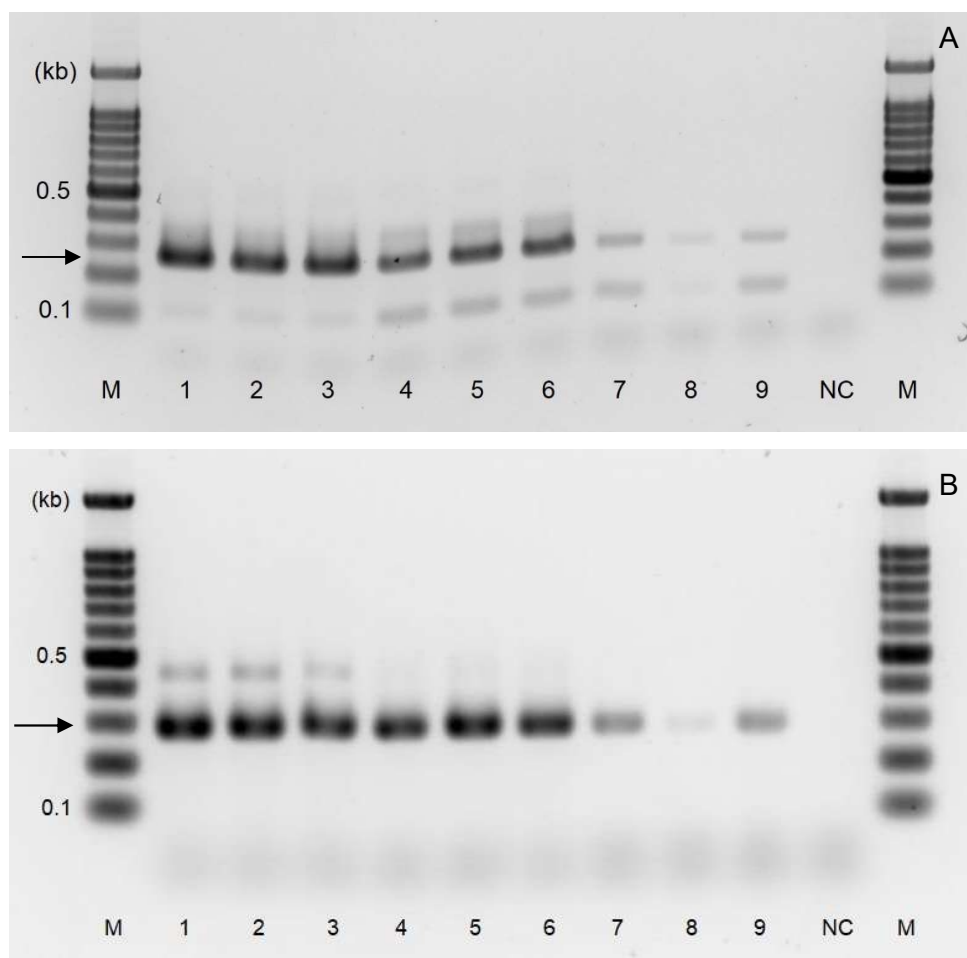


Fig. 10. Amplification results of endpoint PCR (A: ITS region; B: small circular mitochondrial DNA) using RNA of 1,3-dichloropropene (1,3-D) treated *Globodera pallida*. Since these RNA samples were not treated with DNase, they were used as DNA templates. M: 100 bp DNA ladder; 1–3: *G. pallida* treated with 0 μ l of 1,3-D; 4–6: *G. pallida* treated with 15 μ l of 1,3-D; 7–9: *G. pallida* treated with 50 μ l of 1,3-D; NC: negative control (nuclease-free water). Arrows represent the positions of *G. pallida*-specific amplicons.

after 11 days of heat treatment, but was undetectable after 14 days (Leal *et al.*, 2013). Our study yielded similar data to those of a previous study on the durability of nematode mRNA after death. *G. pallida* treated with 15 μ l of 1,3-D produced several cysts and generated appropriate amplicons with Ct values of approximately 30. This result suggested that a small number of individuals survived after the 1,3-D treatment, and such viable individuals were detected by the RT-qPCR.

In contrast, conventional DNA-based PCR methods detected DNA from *G. pallida* treated with 50 μ l of 1,3-D, however, no viable individual was detected after this treatment. Therefore, DNA of the *G. pallida* was still detectable 2 to 4 weeks after death, and DNA-based PCR methods are not useful for viable *G. pallida* detection after 1,3-D use.

The band luminance of *G. pallida* treated with 50 μ l of 1,3-D was relatively low compared with that treated with 0 or 15 μ l of 1,3-D. This suggested that DNA was also degraded to a certain degree after 50 μ l of 1,3-D treatment. The first PCR based on Bulman & Marshall (1997) generated approximately 100 bp bands. They were thought to be non-specific bands. The second PCR based on Sakai *et al.* (2019) generated approximately 450 bp bands. They were generated by universal primers for cyst nematodes contained in the PCR cocktail of this method.

To date, the effects of fumigants on the detectability of nematode nucleic acids have not been assessed well. A previous study showed that RNA of *G. rostochiensis* was not detected after methyl bromide treatment (Mimee *et al.*, 2017). However, the methyl bromide treatment was performed by immersing cysts in methyl bromide solution, which differs from the original usage of fumigants. In this study, I injected 1,3-D into *G. pallida*-infested soil and evaporated it in the soil. This is the first study to test the

detectability of dead nematode's nucleic acids by using nematodes treated with fumigants under conditions akin to fields.

Chapter 5. Comparison of detection sensitivity of *Globodera pallida* between the RT-qPCR and the cup test

5-1. Introduction

In Japan, a bioassay, named the ‘cup test’, has been used to assess *G. pallida* viability by Plant Protection Station (Narabu, 2019). As stated in Chapter 1, this is a kind of inoculation test; a tuber of potato (*HI* conferred variety) is planted in a plastic cup, and after 60 to 70 days, cysts and females visible on the roots are counted through the wall and bottom of the cup. It is relatively easy to perform but takes a long time. In addition, cysts formed inside the cup are not detected, meaning some viable *G. pallida* individuals may be overlooked.

As demonstrated in Chapters 3 and 4, the RT-qPCR method is suitable for viable *G. pallida* detection and does not take a long time. However, to replace the cup test with the RT-qPCR method, it is required to show that the detection sensitivity of the RT-qPCR is similar to or higher than that of the cup test.

In this chapter, I compared the viable *G. pallida* detection sensitivity of the RT-qPCR method to that of the cup test using soil samples collected from Abashiri city, Shari town, and Kiyosato town.

5-2. Materials and methods

A total of 24 soil samples from 10 fields in Abashiri city (A01–A10), 12 fields in Shari town (S01–S12), and 2 fields in Kiyosato town (K01 and K02) were prepared. Fields A07–A09, S01–S12, K01, and K02 were infested with *G. pallida* and the other fields were applied with 1,3-D and cultivated with *S. peruvianum*. For the cup test, a

tuber of potato cultivar ‘Kita-akari’ (conferring *Hl* gene) was planted in a 430 ml plastic cup (Matsuyoshi & Co., Ltd., Tokyo, Japan) with 200 ml of each soil sample. Four holes (approximately 4 mm in diameter) were created in the cover for watering. The cover was attached to a cup with gummed tape. Each cup was incubated at 18°C in the dark and watered, as required. After 60–70 days, the cysts visible on the roots through the transparent wall and the bottom of the cup were counted. Five replicates of each soil sample were prepared (1000 ml each).

For RT-qPCR, cysts were extracted from 200 ml of each soil sample. The number of cysts contained in each soil sample were counted to analyze its effect on the detection sensitivity. RNA was extracted from the cysts as described above; however, I used EconoSpin® RNA Mini Spin Columns (Epoch Life Science Inc., TX, USA) instead of NucleoSpin® RNA Columns to reduce the cost of RNA extraction. This change in the RNA extraction method did not affect the Ct values in our supplementary experiment (Appendix 2). Five replicates of each soil sample were prepared (1000 ml each). RT-qPCRs were performed once in duplicate for each sample. Nuclease-free water and RNA from five cysts of *Gp* Abashiri (the same sample used in the species specificity test) were used as controls for each run.

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

microscope. The test was performed once for each soil sample.

5-3. Results

Each soil sample contained 24–634 cysts per 200 ml (Table 7). In all RT-qPCR trials, appropriate amplicons were obtained from five cysts of Gp Abashiri (average Ct value was 20.7), whereas none were obtained from nuclease-free water. Newly formed cysts were detected in soil samples A07, S02, S03, S05, S06, S08, S09, S10, and K01 using the cup test (Table 7). In addition, appropriate amplicons were obtained from the RNA extracted from the cysts contained in these soil samples. The cup test did not detect newly formed cysts from soil samples A08, A09, S04, S11, and K02, whereas RT-qPCR yielded appropriate amplicons from the RNA of cysts contained in these soil samples. No newly formed cysts were detected in the other soil samples, and no appropriate amplicons were obtained. T_m values of appropriate amplicons ranged from 86.1 to 86.9°C.

The additional inoculation test found new cysts in pots inoculated with cysts isolated from soil samples A08, A09, S04, S11, and K02 (Table 7).

5-4. Discussion

The RT-qPCR method yielded appropriate amplicons from all samples in which newly formed cysts were detected using the cup test. The cup test did not detect newly formed cysts in the soil samples A08, A09, S04, S11, and K02. However, the RT-qPCR obtained appropriate amplicons from the cysts contained in these soil samples. The additional pot test showed that cysts from these soil samples produced new cysts, which is clear evidence that these soil samples contained viable *G. pallida*. These results

Table 7. Comparison of detection sensitivity of *Globodera pallida*

Soil sample	The number of cysts ^{1, 2}	Status of <i>G. pallida</i> infection ³	RT-qPCR		Cup test		Additional pot test
			Ct values ²	No. positives/ No. replicates	The number of cysts ²	No. positives/ No. replicates	The number of cysts ⁴
A01	166 ± 11	–	nd	0/5	0	0/5	-
A02	87 ± 3	–	nd	0/5	0	0/5	-
A03	95 ± 5	–	nd	0/5	0	0/5	-
A04	105 ± 10	–	nd	0/5	0	0/5	-
A05	111 ± 6	–	nd	0/5	0	0/5	-
A06	24 ± 2	–	nd	0/5	0	0/5	-
A07	43 ± 2	+	27.2 ± 1.0	4/5	9.6 ± 2.5	5/5	-
A08	252 ± 7	+	30.3 ± 0.9	5/5	0	0/5	50
A09	124 ± 15	+	32.3	1/5	0	0/5	48
A10	53 ± 4	–	nd	0/5	0	0/5	-
S01	270 ± 10	+	nd	0/5	0	0/5	-
S02	391 ± 13	+	33.5 ± 0.4	5/5	6.8 ± 1.2	5/5	-
S03	159 ± 9	+	32.2 ± 0.2	4/5	6.0 ± 1.5	5/5	-
S04	202 ± 6	+	31.1 ± 1.0	5/5	0	0/5	46
S05	213 ± 9	+	31.3 ± 1.0	5/5	0.8 ± 0.5	2/5	-

S06	634 ± 14	+	31.6 ± 0.7	5/5	4.4 ± 1.6	4/5	-
S07	159 ± 7	+	nd	0/5	0	0/5	-
S08	74 ± 2	+	25.2 ± 0.8	5/5	18.4 ± 4.7	5/5	-
S09	204 ± 18	+	23.2 ± 0.7	5/5	63.6 ± 14.5	5/5	-
S10	385 ± 13	+	25.5 ± 0.7	5/5	50.2 ± 7.5	5/5	-
S11	41 ± 2	+	28.4	1/5	0	0/5	3
S12	174 ± 17	+	nd	0/5	0	0/5	-
K01	106 ± 24	+	28.1 ± 0.8	4/5	1.6 ± 0.7	3/5	-
K02	236 ± 57	+	31.2	1/5	0	0/5	12

¹ Per 200 ml each of soil sample

² Data represent mean ± SE. nd: not detected

³ —: Fields applied with 1,3-dichloropropene and cultivated with *Solanum peruvianum*. +: *G. pallida* infested fields

⁴ Cysts were isolated from the entire soil in the pots and counted using a binocular microscope. -: not tested

showed that the sensitivity of the RT-qPCR method was higher than that of the cup test. In the cup test, inspectors check only the cysts visible through the wall and bottom of the cup. Therefore, in soil samples A08, A09, S04, S11, and K02, it is possible that some individuals formed new cysts inside the cup, which were not visible through walls. Furthermore, the additional pot test was performed by gathering cysts in one place (i.e., mesh bag) and inoculating them. Therefore, mating might have occurred more easily during these tests. Although fields S01, S07, and S12 were infested with *G. pallida*, viable individuals were not detected. This could be caused by a low population density and the patchy distribution of *G. pallida* in these fields, and the samples I collected may not have contained a detectable number of viable individuals.

Soil samples A08, S04, and K02 contained more than 200 cysts, which are presumably *G. rostochiensis* or dead *G. pallida*, per 200 ml. Cysts contain polyphenols (Clarke, 1968), which can inhibit PCR reactions (Koonjul *et al.*, 1999). Therefore, I assumed that too many cysts may decrease the detection sensitivity. However, the RT-qPCR method yielded appropriate amplicons from these samples, while the cup test failed to detect viable *G. pallida* in them. These results indicate that the RT-qPCR can detect viable *G. pallida* at such low densities that they were undetectable by the cup test, even in the presence of a lot of non-target cysts and/or cysts containing no viable eggs.

To show the validity of nematode detection methods, it is important to validate the methods using field soil samples. Beniers *et al.* (2014) and Mimee *et al.* (2017) also validated their RT-qPCR methods for the detection of viable PCNs using field soil samples. However, Beniers *et al.* (2014) used only four soil samples, which seems not to be enough for appropriate validation. Moreover, since all the soil samples were collected from fields that have not been applied with fumigants, the effects of fumigation

on RNA detectability were unclear. Mimee *et al.* (2017) used 24 soil samples, but this study did not perform conventional methods, such as microscopic inspection, hatching tests, and inoculation tests, using the same soil samples. Also, they did not declare whether the soil samples have been applied with fumigants. In this chapter, I used 24 soil samples, some of which have been applied with 1,3-D, and showed that the detection sensitivity of the RT-qPCR method was higher than that of the cup test. This study using enough and appropriate field samples properly validated the RT-qPCR method for detecting viable *G. pallida*.

Chapter 6. General discussion and conclusion

In Japan, the official control of *G. pallida* has been implemented since 2016, with the goal of eradicating this species. In infested fields, both the application of 1,3-D and the cultivation of *S. peruvianum* have been implemented. In the context of the official control of *G. pallida* in Japan, when viable individuals are not detected in a field, the control measures are terminated there. Therefore, the detection of viable *G. pallida* is important for appropriate management of the species. However, the conventional cup test takes a relatively long time (more than two months) to assess *G. pallida* viability and may lead overlooking of some viable individuals. In addition, microscopic inspection, staining, hatching test, and trehalose method do not discriminate species and some of them are time- and labor-intensive. Conventional DNA-based PCR methods are not suitable for viable nematode detection, since they detect dead nematodes. DNA-based PCR methods along with PMA treatment seem to be labor intensive. While probe-based RT-qPCR is rapid and can differentiate between species, probes are expensive and probe-based qPCRs may have low detection sensitivity. Therefore, I developed an intercalator-based RT-qPCR method to detect viable *G. pallida*.

In Chapter 2, I evaluated expression levels of 10 housekeeping genes (*act-1*, *Ama-1*, *APC1*, *BUB3*, *cdc-42*, *elf4A*, *GR*, *Mce1*, *TUBG2*, and *Y45F10D.4*) in *G. pallida* eggs by RT-qPCR. As a result, *Y45F10D.4* was the gene with the lowest Ct value. This suggested that *Y45F10D.4* was highly expressed in *G. pallida* eggs and that this gene is suitable as a target gene for viable *G. pallida* detection.

In Chapter 3, I developed a primer set specific to *G. pallida*. This primer set successfully amplified the *Y45F10D.4* gene fragment for all tested *G. pallida* without

amplifying that for other species. This study used all *Globodera* species occurring in Japan. Therefore, the RT-qPCR method has enough species-specificity for nematodes at least in Japan.

It should be noted that cyst nematode *Y45F10D.4* gene sequences have been hardly clarified. Furthermore, within cyst nematodes, only *G. pallida* (GenBank accession no. GCA_000724045, GCA_020449905, and GCA_023343765), *G. rostochiensis* (GCA_018350315, GCA_018350325, and GCA_900079975), *G. ellingtonae* (GCA_001723225), *H. glycines* (GCA_000150805, GCA_004148225, and GCA_015680885), *H. schachtii* (GCA_019095935, GCA_020449115, and GCA_023374025), and *H. carotae* (GCA_024500135) have their complete genome sequences available as of 2023. Consequently, it is difficult to examine the species-specificity of the primer set through *in silico* analysis. Although *H. avenae*, *H. humuli*, *H. koreana*, *H. latipons*, *H. sojae*, and *Cactodera cacti* have been reported in Japan (Momota, 2004; Sekimoto *et al.*, 2017; Sakai & Kushida, 2019), these species were not utilized in this study due to lack of recent reports of their occurrences in agricultural fields. Verification using these species will provide more confirmatory data regarding the effectiveness of this method. In particular, another *Globodera* species, *G. mexicana* has been found in Mexico (Thiéry *et al.*, 1997; Grenier *et al.*, 2002). *G. mexicana* can develop on tomatoes and *Solanum nigrum* but can not on potato (Thiéry *et al.*, 1997). In addition, to our best knowledge, the occurrence of this species has not been reported outside Mexico. Therefore, the occurrence risk of *G. mexicana* in potato fields in Japan seems to be low. However, this species is the most closely related species to *G. pallida* (Bossis & Mugniéry, 1993; Subbotin *et al.*, 2020). To date, DNA sequences of *G. mexicana* are hardly available except for rRNA gene, several mitochondrial genes, and

several genes coding for cell wall degrading enzymes. Thus, verification using *G. mexicana* is also required.

DNA is often co-extracted with RNA and the DNA remaining in the RNA sample may cause false-positive signals derived from dead organisms. In this study, the *G. pallida*-specific primer pair was designed to sandwich a long (approximately 500 bp) intron to prevent amplification from DNA. This enabled us to omit the DNase treatment during RNA extraction, resulting in simplification and cost reduction for RNA extraction. It is noted that optimal annealing/extension time is important to prevent amplification of *G. pallida* DNA.

In Chapter 4, I tested whether DNA and RNA can be detected from 1,3-D treated *G. pallida*. In the official control of *G. pallida*, 1,3-D was often used, therefore, there should be many *G. pallida* eggs died of 1,3-D in fields where the control measures have been perfected. However, no study has tested the effect of 1,3-D on the detectability of nematode DNA and RNA. This study showed that *G. pallida* RNA was not detected after being treated with an enough amount of 1,3-D, suggesting that the RT-qPCR method is effective to assess *G. pallida* viability after 1,3-D use. In contrast, DNA was detected from 1,3-D treated *G. pallida*, suggesting that the conventional DNA-based PCR methods underestimate the control efficacy.

This finding can be a caution not only for nematode diagnosis but also for nematode community study. Nematode community structure profiles can be used as indicators of the status of soil systems (Bongers & Ferris, 1999; Porazinska *et al.*, 1999). To analyze nematode communities in fields, metagenomic analysis using DNA samples directly extracted from soil (Davey *et al.*, 2021; Kawanobe *et al.*, 2021) is effective because the method can detect nematodes in immobile states such as eggs. However, our results

suggested that if the fields have been applied with nematicides, it is likely to detect dead nematode DNA, as van den Elsen *et al.* (2012) reported. Nevertheless, analysis of nematode population recovery after nematicide treatment may provide insight into the indirect effects of the nematicide treatments on soil systems. RNA-based nematode community studies may produce a good understanding of the nematode community in nematicide-treated fields.

The RT-qPCR method detected RNA corresponding to a minimum of 3.9 *G. pallida* eggs (Chapter 3). Although the sensitivity is higher than that of a previous RT-qPCR method developed by Beniers *et al.* (2014), it was lower than that of conventional DNA-based PCR methods (Nakhla *et al.*, 2010; Sakai *et al.*, 2019; Kushida and Sakai, 2022). However, in Chapter 5, I conducted the RT-qPCR and the cup test using 24 soil samples and showed that the sensitivity of the RT-qPCR method was higher than that of the cup test. Therefore, the detection sensitivity of the RT-qPCR method appears to be high enough to replace the cup test. This method consists of cyst extraction, RNA extraction and RT-qPCR and requires only approximately 1 week, even considering the time required to dry soil samples to isolate cysts. Therefore, this method saves significantly more time than the cup test.

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

tried to extract RNA from *G. pallida* cysts with floating debris, appropriate amplicons were hardly obtained by the RT-qPCR method (data not shown). Novel techniques may be required for effective RNA extraction with floating debris.

In fields that have been perfected the control measures, it is recommended to cultivate *G. pallida*-resistant potato cultivars to prevent the reoccurrence of this species. In Japan, two *G. pallida*-resistant potato cultivars, ‘Furia’ and ‘Kitasuzuka’ are available as of 2023 (NARO, 2020, 2022). These cultivars prevent *G. pallida* multiplication by 80 to 90% (unpublished data). However, several controlled inoculation experiments revealed that continuous use of *G. pallida*-resistant cultivar can result in resistance breaking (Phillips & Blok, 2008; Fournet *et al.*, 2013; Varypatakis *et al.*, 2019). In addition, occurrences of resistance-breaking *G. pallida* populations in potato fields (not experimental condition) have been reported in Germany (Niere *et al.*, 2014). The authors assumed that one cause of the phenomenon might be the continuous cultivation of *G. pallida*-resistant potato cultivars. Therefore, even after the termination of the official control, it is important to monitor the presence/absence of viable *G. pallida* to prevent the occurrences of resistance-breaking populations in Japan. The RT-qPCR method would be helpful not only for the official control but also long-term management of *G. pallida*.

In conclusion, this study developed an intercalator-based RT-qPCR method to detect viable *G. pallida* rapidly and reliably. The method is expected to contribute to appropriate *G. pallida* management as an alternative of the lengthy conventional cup test.

Acknowledgements

I would like to express my sincere gratitude to Prof. Koki Toyota for the continuous support of my Ph.D. study. I also thank Dr. Atsuhiko Kushida (NARO), Dr. Takashi Narabu (NARO), Dr. Taketo Uehara (NARO), Mr. Kenji Ito (NARO), Dr. Hiromichi Sakai (NARO), Prof. Hideaki Iwahori (Ryukoku University), and Prof. R. N. Perry (University of Hertfordshire) for their technical advice. I also thank Mr. Takashi Iseki of Yokohama Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries (PPS, MAFF) for advice regarding the cup test. I am grateful to Dr. F. G. W. Jones (Rothamsted Research), Dr. E. Grenier (INRAE), Dr. T. Prior (FERA), Dr. I. Zasada (USDA), Dr. H. Sakai (NARO), and Dr. H. Okada (NARO) for providing the nematodes. This research was supported by the Research Program for Development of Innovative Technology Grants (Grant No. JPJ007097, Project No. 01024C) from the Project of the Bio-oriented Technology Research Advancement Institution, NARO.

References

- Alenda, C., Gallot-Legrand, A., Fouville, D. & Grenier, E. (2013). Sequence polymorphism of nematode effectors highlights molecular differences among the subspecies of the tobacco cyst nematode complex. *Physiological and Molecular Plant Pathology* 84, 107-114. DOI: 10.1016/j.pmpp.2013.08.004
- Alum, A., Sbair, B., Asaad, H., Rubino, J. R., & Ijaz, M. K. (2012). ECC–RT-PCR: a new method to determine the viability and infectivity of *Giardia* cysts. *International Journal of Infectious Diseases* 16, 350-353. DOI: 10.1016/j.ijid.2012.01.004
- Amiri, S., Subbotin, S.A. & Moens, M. (2002). Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* 108, 497-506. DOI: 10.1023/A:1019974101225
- Beniers, J.E., Been, T.H., Mendes, O., van Gent-Pelzer, M.P. & van der Lee, T.A. (2014). Quantification of viable eggs of the potato cyst nematodes (*Globodera* spp.) using either trehalose or RNA-specific real-time PCR. *Nematology* 16, 1219-1232. DOI: 10.1163/15685411-00002848
- Bongers, T. & Ferris, H. (1999). Nematode community structure as a bioindicator in environmental monitoring. *Trends in Ecology & Evolution* 14, 224-228. DOI: 10.1016/S0169-5347(98)01583-3
- Bossis, M. & Mugniéry, D. (1993). Specific status of six *Globodera* parasites of solanaceous plants studied by means of two-dimensional gel electrophoresis with a comparison of gel patterns by a computed system. *Fundamental and Applied Nematology* 16, 47-56.
- Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I. & Villegas, E.N.

- (2009). *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. *Applied and Environmental Microbiology* 75, 6856-6863. DOI: 10.1128/AEM.00540-09
- Bulman, S.R. & Marshall, J.W. (1997). Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25, 123-129. DOI: 10.1080/01140671.1997.95139987
- Byrne, J.T., Maher, N.J. & Jones, P.W. (2001). Comparative responses of *Globodera rostochiensis* and *G. pallida* to hatching chemicals. *Journal of Nematology* 33, 195-202.
- Camacho, M.J., Nóbrega, F., Lima, A., Mota, M. & Inácio, M.L. (2017). Morphological and molecular identification of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* in Portuguese potato fields. *Nematology* 19, 883-889. DOI: 10.1163/15685411-00003094
- Christoforou, M., Pantelides, I.S., Kanetis, L., Ioannou, N. & Tsaltas, D. (2014). Rapid detection and quantification of viable potato cyst nematodes using qPCR in combination with propidium monoazide. *Plant Pathology* 63, 1185-1192. DOI: 10.1111/ppa.12193
- Clarke, A.J. (1968). The chemical composition of the cyst wall of the potato cyst-nematode, *Heterodera rostochiensis*. *Biochemical Journal*, 108, 221-224. DOI: 10.1042/bj1080221
- Clarke, A.J. & Perry, R.N. (1977). Hatching of cyst-nematodes. *Nematologica* 23, 350-368. DOI: 10.1163/187529277X00075
- Clarke, A.J., Perry, R.N. & Hennessy, J. (1978). Osmotic stress and hatching of

- Globodera rostochiensis*. *Nematologica* 24, 384-392. DOI: 10.1163/187529278X00506
- Dandurand, L.M. & Knudsen, G.R. (2016). Effect of the trap crop *Solanum sisymbriifolium* and two biocontrol fungi on reproduction of the potato cyst nematode, *Globodera pallida*. *Annals of Applied Biology* 169, 180-189. DOI: 10.1111/aab.12295
- Dandurand, L.M., Zasada, I.A. & LaMondia, J.A. (2019). Effect of the trap crop, *Solanum sisymbriifolium*, on *Globodera pallida*, *Globodera tabacum*, and *Globodera ellingtonae*. *Journal of Nematology* 51, 1-11. DOI: 0.21307/jofnem-2019-030
- Davey, M.L., Utaaker, K.S. & Fossey, F. (2021). Characterizing parasitic nematode faunas in faeces and soil using DNA metabarcoding. *Parasites & Vectors* 14, 1-13. DOI: 10.1186/s13071-021-04935-8
- Devine, K.J., Byrne, J., Maher, N. & Jones, P.W. (1996). Resolution of natural hatching factors for golden potato cyst nematode, *Globodera rostochiensis*. *Annals of Applied Biology* 129, 323-334. DOI: 10.1111/j.1744-7348.1996.tb05755.x
- Devine, K. J., & Jones, P. W. (2001). Effects of hatching factors on potato cyst nematode hatch and in-egg mortality in soil and in vitro. *Nematology* 3, 65-74. DOI: 10.1163/156854101300106900
- Dheda, K., Huggett, J. F., Bustin, S. A., Johnson, M. A., Rook, G., & Zumla, A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 37, 112-119. DOI: 10.2144/04371RR03
- Ebrahimi, N., Viaene, N. & Moens, M. (2015). Optimizing trehalose-based quantification of live eggs in potato cyst nematodes (*Globodera rostochiensis* and

- G. pallida*). *Plant Disease* 99, 947-953. DOI: 10.1094/PDIS-09-14-0940-RE
- Eigner, J., Boedtke, H. & Michaels, G. (1961). The thermal degradation of nucleic acids. *Biochimica et Biophysica Acta* 51, 165-168. DOI: 10.1016/0006-3002(61)91028-9
- Ellis, P.R. (1968). Resistance to the potato cyst-nematode, *Heterodera rostochiensis*, in the plant genus *Lycopersicon*. *Annals of Applied Biology* 61, 151-160. DOI: 10.1111/j.1744-7348.1968.tb04518.x
- Ellis, P.R. & Smith, J.W.M. (1971). Inheritance of resistance to potato cyst eelworm (*Heterodera rostochiensis* Woll.) in the genus *Lycopersicon*. *Euphytica* 20, 93-101. DOI: 10.1007/BF00146779
- EPPO (2017). PM 7/40 (4) *Globodera rostochiensis* and *Globodera pallida*. *EPPO Bulletin* 47, 174-197. DOI: 10.1111/epp.12391
- EPPO (2022). EPPO global database. <https://gd.eppo.int/>. Accessed 20 December 2022.
- Evans, K., Franco, J. & De Scurrah, M.M. (1975). Distribution of species of potato cyst-nematodes in South America. *Nematologica* 21, 365-369. DOI: 10.1163/187529275X00103
- Evans, K. & Stone, A.R. (1977). A review of the distribution and biology of the potato cyst-nematodes *Globodera rostochiensis* and *G. pallida*. *PANS* 23, 178-189. DOI: 10.1080/09670877709412426
- Expósito-Rodríguez, M., Borges, A.A., Borges-Pérez, A. & Pérez, J.A. (2008). Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC plant biology* 8, 1-12. DOI: 10.1186/1471-2229-8-131
- Fenwick, D.W. (1949). Investigations on the emergence of larvae from cysts of the potato-root eelworm *Heterodera rostochiensis*. I. Technique and variability. *Journal of Helminthology* 23, 157-170. DOI: 10.1017/S0022149X0003248X

- Fournet, S., Kerlan, M.C., Renault, L., Dantec, J.P., Rouaux, C. & Montarry, J. (2013). Selection of nematodes by resistant plants has implications for local adaptation and cross-virulence. *Plant Pathology* 62, 184-193. DOI: 10.1111/j.1365-3059.2012.02617.x
- Graham, C.W. (1966). Potato root eelworm and tomato rootstocks. *Plant Pathology* 15, 76-85. DOI: 10.1111/j.1365-3059.1966.tb02801.x
- Greco, N. (1981). Hatching of *Heterodera carotae* and *H. avenae*. *Nematologica* 27, 366-371. DOI: 10.1163/187529281X00377
- Grenier, E., Blok, V.C., Jones, J.T., Fouville, D. & Mugniéry, D. (2002). Identification of gene expression differences between *Globodera pallida* and *G. 'mexicana'* by suppression subtractive hybridization. *Molecular Plant Pathology* 3, 217-226. DOI: 10.1046/j.1364-3703.2002.00111.x
- Hajihassani, A. & Dandurand, L.M. (2018). An improved technique for sorting developmental stages and assessing egg viability of *Globodera pallida* using high-throughput complex object parametric analyzer and sorter. *Plant Disease* 102, 2001-2008. DOI: 10.1094/PDIS-09-17-1428-RE
- Handoo, Z.A. & Subbotin S.A. (2018). Taxonomy, identification and principal species. In: Perry, R.N., Moens, M. & Jones, J.T. (Eds). *Cyst nematodes*. Wallingford, UK, CAB International, pp. 365-398.
- Handoo, Z.A., Carta, L.K., Skantar, A.M. & Chitwood, D.J. (2012). Description of *Globodera ellingtonae* n. sp. (Nematoda: Heteroderidae) from Oregon. *Journal of Nematology* 44, 40-57.
- Hesling, J.J. & Ellis, P.R. (1972). The pathogenicity and increase of *Heterodera rostochiensis* on tomato cultivars, self-rooted or grafted on to rootstocks. *Annals of*

- Applied Biology* 71, 251-261. DOI: 10.1111/j.1744-7348.1972.tb05089.x
- Hu, R., Fan, C., Li, H., Zhang, Q., & Fu, Y. F. (2009). Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. *BMC molecular biology* 10, 1-12. DOI: 10.1186/1471-2199-10-93
- Iseki, T. & Sekimoto, S. (2012). Quarantine pests to alert invasion into Japan (3) Nematodes. *Plant Protection* 66, 191-194. (in Japanese)
- Ito, K., Onodera, K. & Narabu, T. (2020). Measures for the emergency control of white potato cyst nematode, *Globodera pallida*. *Hokunou* 87, 281-289. (in Japanese)
- Janssen, R., Bakker, J. & Gommers, F.J. (1987). Circumventing the diapause of potato cyst nematodes. *Netherlands Journal of Plant Pathology* 93, 107-113. DOI: 10.1007/BF02000561
- Jones, L.M., Eves-van den Akker, S., van-Oosten Hawle, P., Atkinson, H.J. & Urwin, P.E. (2018). Duplication of *hsp-110* is implicated in differential success of *Globodera* species under climate change. *Molecular Biology and Evolution* 35, 2401-2413. DOI: 10.1093/molbev/msy132
- Joyce, S.A., Reid, A., Driver, F. & Curran, J. (1994). Application of polymerase chain reaction (PCR) methods to identification of entomopathogenic nematodes. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.P. (Eds). *COST 812 Biotechnology: genetics of entomopathogenic nematode-bacterium complexes*. Proceedings of Symposium & workshop, St. Patrick's College, Maynooth, Co. Kildare, Ireland, Luxembourg, European Commission, DG XII, pp. 178-187.
- Kawanobe, M., Toyota, K. & Ritz, K. (2021). Development and application of a DNA metabarcoding method for comprehensive analysis of soil nematode communities. *Applied Soil Ecology* 166, 103974. DOI: 10.1016/j.apsoil.2021.103974

- Koonjul, P.K., Brandt, W.F., Lindsey, G.G. & Farrant, J.M. (1999). Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Research* 27, 915-916. DOI: 10.1093/nar/27.3.915
- Kort, J., Ross, H., Rumpfenhorst, H.J. & Stone, A.R. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes *Globodera rostochiensis* and *G. pallida*. *Nematologica* 23, 333-339. DOI: 10.1163/187529277X00057
- Kushida, A. & Momota, Y. (2005). Multiplication of Japanese populations of the potato cyst nematode, *Globodera rostochiensis*, on *H1* resistant potato cultivar. *Nematological Research* 35, 87-90. (In Japanese) DOI: 10.3725/jjn1993.35.2_87
- Kushida, A. & Sakai, H. (2022). Development of a simple method for simultaneous detection and differentiation of *Globodera pallida* and *G. rostochiensis*. *Journal of General Plant Pathology* 88, 251-258. DOI: 10.1007/s10327-022-01065-6
- LaMondia J.A. (1995). Hatch and reproduction of *Globodera tabacum* in response to tobacco, tomato, or black nightshade. *Journal of Nematology* 27, 382-386
- Lax, P., Dueñas, J.C.R., Franco-Ponce, J., Gardenal, C.N. & Doucet, M.E. (2014). Morphology and DNA sequence data reveal the presence of *Globodera ellingtonae* in the Andean region. *Contributions to Zoology* 83, 227-243. DOI: 10.1163/18759866-08304002
- Leal, I., Foord, B., Allen, E., Campion, C., Rott, M. & Green, M. (2013). Development of two reverse transcription-PCR methods to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood. *Forest Pathology* 43, 104-114. DOI: 10.1111/efp.12003

- Lin, B., Tao, Y., Wang, H., Liao, J. & Zhuo, K. (2020). Duplex real-time quantitative PCR for simultaneous detection and quantification of *Pratylenchus neglectus* and *P. thornei*. *European journal of plant pathology*, 157, 185-196. DOI: 10.1007/s10658-020-01999-7
- MacMillan, K., Blok, V., Young, I., Crawford, J. & Wilson, M.J. (2006). Quantification of the slug parasitic nematode *Phasmarhabditis hermaphrodita* from soil samples using real time qPCR. *International Journal for Parasitology* 36, 1453-1461. DOI: 10.1016/j.ijpara.2006.08.005
- Madani, M., Subbotin, S.A., Ward, L.J., Li, X. & De Boer, S.H. (2010). Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome *b* genes. *Canadian Journal of Plant Pathology* 32, 252-263. DOI: 10.1080/07060661003740033
- MAFF (2022a). Quarantine Pest List (Annexed Table 1 of the Ordinance for Enforcement of the Plant Protection Act). https://www.maff.go.jp/pps/j/law/houki/shorei/E_Annexed_Table1_from_20210428.html Accessed 26 December 2022.
- MAFF (2022b). *Globodera rostochiensis* occurrence area in Japan. https://www.maff.go.jp/pps/j/law/houki/yoko/attach/pdf/yoko_119_html_119-4.pdf. Accessed 2 December 2022.
- MAFF (2022c). Information about *G. pallida*. https://www.maff.go.jp/j/syouan/syokubo/keneki/k_kokunai/gp/gp.html. Accessed 18 September 2022.
- Markou, A.N., Smilkou, S., Tsaroucha, E. & Lianidou, E. (2021). The effect of genomic

- DNA contamination on the detection of circulating long non-coding RNAs: The Paradigm of MALAT1. *Diagnostics* 11, 1160. DOI: 10.3390/diagnostics11071160
- Mburu, H. & Cortada, L. (2018). First report of potato cyst nematode *Globodera pallida* infecting potato (*Solanum tuberosum*) in Kenya. *Plant Disease* 102, 1671. DOI: 10.1094/PDIS-11-17-1777-PDN
- Mimee, B., Soufiane, B., Dauphinais, N. & Bélair, G. (2017). A qRT-PCR method to evaluate viability of potato cyst nematode (*Globodera* spp.). *Canadian Journal of Plant Pathology* 39, 503-513. DOI: 10.1080/07060661.2017.1382574
- Min, Y.Y., Toyota, K., Goto, K., Sato, E., Mizuguchi, S., Abe, N., Nakano, A. & Sawada, E. (2011). Development of a direct quantitative detection method for *Meloidogyne incognita* in sandy soils and its application to sweet potato cultivated fields in Tokushima prefecture, Japan. *Nematology* 13, 95-102. DOI: 10.1163/138855410X504916
- Minnis, S.T., Haydock, P.P.J. & Evans, K. (2004). Control of potato cyst nematodes and economic benefits of application of 1,3-dichloropropene and granular nematicides. *Annals of Applied Biology* 145, 145-156. DOI: 10.1111/j.1744-7348.2004.tb00370.x
- Monpoeho, S., Dehee, A., Mignotte, B., Schwartzbrod, L., Marechal, V., Nicolas, J.C., Billaudel, S. & Ferre, V. (2000). Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *Biotechniques* 29, 88-93. DOI: 10.2144/00291st03
- Momota, Y. (2004). Identification of cyst nematodes in Japan. In: Japan Plant Protection Association (Ed). *Identification of nematode species*. Tokyo, Japan, Japan Plant Protection Association, pp. 23-28.

- Mulvey, R.H. (1958). Parthenogenesis in a cyst-forming nematode, *Heterodera trifolii* (Nematoda: Heteroderidae). *Canadian Journal of Zoology* 36, 91-93. DOI: 10.1139/z58-009
- Mwangi, J.M., Kariuki, G.M., Waceke, J.W. & Grundler, F.M. (2015). First report of *Globodera rostochiensis* infesting potatoes in Kenya. *New Disease Reports* 31, 18. DOI: 10.5197/j.2044-0588.2015.031.018
- Nakhla, M.K., Owens, K.J., Li, W., Wei, G., Skantar, A.M. & Levy, L. (2010). Multiplex real-time PCR assays for the identification of the potato cyst and tobacco cyst nematodes. *Plant Disease* 94, 959-965. DOI: 10.1094/PDIS-94-8-0959
- Narabu, T., Ohki, T., Onodera, K., Fujimoto, T., Itou, K. & Maoka, T. (2016). First report of the pale potato cyst nematode, *Globodera pallida*, on potato in Japan. *Plant Disease* 100, 1794.
- Narabu, T. (2019). Recent occurrence of pale potato cyst nematode, *Globodera pallida* in Japan, and the effect of control measures to eradicate it. *Plant Protection* 73, 439-443. (in Japanese)
- NARO (2020). Furia, a *Globodera pallida*-resistant potato cultivar. *NARO Technical Report* 7, 34-35. (in Japanese)
- NARO (2022). Kitasuzuka. <https://www.naro.go.jp/collab/breed/0100/0106/155459.html> (Accessed 22 December 2022)
- Niere B, Krüssel S, Osmers K. (2014) Auftreten einer außergewöhnlich virulenten Population der Kartoffelzystennematoden. *Journal für Kulturpflanzen*. 66, 426-427.
- Nocker, A., Cheung, C.Y. & Camper, A.K. (2006). Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective

- removal of DNA from dead cells. *Journal of Microbiological Methods* 67, 310-320.
DOI: 10.1016/j.mimet.2006.04.015
- Ogiga, I.R. & Estey, R.H. (1974). The use of Meldola Blue and Nile Blue a, for distinguishing dead from living nematodes. *Nematologica* 20, 271-276. DOI: 10.1163/187529274X00302
- Ohki, T., Narabu, T., Kushida, A., Onodera, K., Fujimoto, T., Itou, K., & Maoka, T. (2018). Molecular characterization of *Globodera pallida* found in Japan using ribosomal DNA and mitochondrial cytochrome *b* gene sequences. *Journal of General Plant Pathology* 84, 230-236. DOI: 10.1007/s10327-018-0776-5
- Okada, T. (1971). The hatching responses of the soybean cyst nematode, *Heterodera glycines* Ichinohe (Tylenchida: Heteroderidae). *Applied Entomology and Zoology* 6, 91-93. DOI: 10.1303/aez.6.91
- Orui, Y. (1997). Discrimination of *Globodera rostochiensis* and four *Heterodera* species (Nematoda: Heteroderidae) by PCR-RFLP analysis. *Nematological Research* 27, 67-75. (in Japanese with English summary) DOI: 10.3725/jjn1993.27.2_67
- Palomares-Rius, J.E., Hedley, P.E., Cock, P.J., Morris, J.A., Jones, J.T., Vovlas, N. & Blok, V. (2012). Comparison of transcript profiles in different life stages of the nematode *Globodera pallida* under different host potato genotypes. *Molecular Plant Pathology* 13, 1120-1134. DOI: 10.1111/J.1364-3703.2012.00821.X
- Palomares-Rius, J.E., Hedley, P., Cock, P.J., Morris, J.A., Jones, J.T. & Blok, V.C. (2016). Gene expression changes in diapause or quiescent potato cyst nematode, *Globodera pallida*, eggs after hydration or exposure to tomato root diffusate. *PeerJ* 4, e1654. DOI: 10.7717/peerj.1654
- Peetz, A.B., Baker, H.V. & Zasada, I.A. (2019). Further elucidation of the host range of

- Globodera ellingtonae*. *Nematropica* 49, 12-17
- Peng, D., Liu, H., Peng, H., Jiang, R., Li, Y. Q., Wang, X., Ge, J., Zhao, S., Feng, X. & Feng, M. (2022). First detection of the potato cyst nematode (*Globodera rostochiensis*) in a major potato production region of China. *Plant Disease* 107, 233. DOI: 10.1094/PDIS-06-21-1263-PDN
- Perry, R.N. (1989). Dormancy and hatching of nematode eggs. *Parasitology today* 5, 377-383. DOI: 10.1016/0169-4758(89)90299-8
- Phillips, M.S. & Blok, V.C. (2008). Selection for reproductive ability in *Globodera pallida* populations in relation to quantitative resistance from *Solanum vernei* and *S. tuberosum* ssp. *andigena* CPC2802. *Plant Pathology* 57, 573-580. DOI: 10.1111/j.1365-3059.2007.01771.x
- Pickup J., Roberts, A.M. & den Nijs, L.J. (2018). Quarantine, distribution patterns and sampling. In: Perry, R.N., Moens, M. & Jones, J.T. (Eds). *Cyst nematodes*. Wallingford, UK, CAB International, pp. 128-153.
- Pillai, S.S. & Dandurand, L.M. (2019). Evaluation of fluorescent stains for viability assessment of the potato cyst nematodes *Globodera pallida* and *G. ellingtonae*. *Advances in Bioscience and Biotechnology* 10, 244-258. DOI: 10.4236/abb.2019.108019
- Pillai, S.S & Dandurand, L.M. (2021). Potato cyst nematode egg viability assessment and preparasitic juvenile screening using a large particle flow cytometer and sorter. *Phytopathology* 111, 713-719. DOI: 10.1094/PHYTO-06-20-0255-R
- Porazinska, D.L., Duncan, L.W., McSorley, R. & Graham, J.H. (1999). Nematode communities as indicators of status and processes of a soil ecosystem influenced by agricultural management practices. *Applied Soil Ecology* 13, 69-86. DOI:

10.1016/S0929-1393(99)00018-9

- R Development Core Team (2022) R: A Language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>. (Accessed 12 December 2022)
- Rawsthorne, D. & Brodie, B.B. (1986). Relationship between root growth of potato, root diffusate production, and hatching of *Globodera rostochiensis*. *Journal of Nematology* 18, 379-384.
- Reid, A., Evans, F., Mulholland, V., Cole, Y. & Pickup, J. (2015). High-throughput diagnosis of potato cyst nematodes in soil samples. In: Lacomme, C. (Ed). *Plant pathology: techniques and protocols, methods in molecular biology*. New York, US, Humana Press, pp. 137-148.
- Rice, S.L., Leadbeater, B.S.C. & Stone, A.R. (1985). Changes in cell structure in roots of resistant potatoes parasitized by potato cyst-nematodes. I. Potatoes with resistance gene *H1* derived from *Solanum tuberosum* ssp. *andigena*. *Physiological Plant Pathology* 27, 219-234. DOI: 10.1016/0048-4059(85)90069-4
- Sabeh, M., Duceppe, M.O., St-Arnaud, M. & Mimee, B. (2018). Transcriptome-wide selection of a reliable set of reference genes for gene expression studies in potato cyst nematodes (*Globodera* spp.). *PLoS One* 13, e0193840. DOI: 10.1371/journal.pone.0193840
- Sakai, H., & Kushida, A. (2019). Molecular confirmation of the occurrence of *Heterodera sojae* in Japan. *Nematological Research* 49, 29-33. DOI: 10.3725/jjn.49.29
- Sakai, H., Kushida, A. & Narabu, T. (2019). Identification of the potato cyst nematodes based on two-step multiplex endpoint PCR with the dUTP/UNG system for carry-

- over prevention. *Nematological Research* 49, 19-27. DOI: 10.3725/jjn.49.19
- Sakata, I., Sakai, H. & Kushida, A. (2021a). Subspecies identification of the Japanese population of *Globodera tabacum*. *Nematological Research* 51, 37-40. DOI: 10.3725/jjn.51.37
- Sakata, I., Kushida, A. & Tanino, K. (2021b). The hatching-stimulation activity of solanoeclipin A toward the eggs of *Globodera* (Tylenchida: Heteroderidae) species. *Applied Entomology and Zoology* 56, 51-57. DOI: 10.1007/s13355-020-00707-5
- Salazar, A. & Ritter, E. (1993). Effects of daylength during cyst formation, storage time and temperature of cysts on the in vitro hatching of *Globodera rostochiensis* and *G. pallida*. *Fundamental and applied nematology* 16, 567-572.
- Sasser, J.N. & Freckman, D.W. (1987). A world perspective of Nematology: the role of Society. In: Veech, J.A. & Dickson, D.W. (Eds). *Vistas on nematology : a commemoration of the twenty-fifth anniversary of the Society of Nematologists*. US, Society of Nematologists, pp. 7-14.
- Sekimoto, S., Uehara, T. & Mizukubo, T. (2016). Geographical distribution of *Heterodera trifolii* in eastern Japan. *Nematological Research* 46, 1-8. DOI: 10.3725/jjn.46.1
- Sekimoto, S., Uehara, T. & Mizukubo, T. (2017). Morphological and molecular characterisation of *Heterodera koreana* (Vovlas, Lamberti & Choo, 1992) Mundo-Ocampo, Troccoli, Subbotin, Del Cid, Baldwin & Inserra, 2008 (Nematoda: Heteroderidae) from bamboo in Japan. *Nematology* 19, 333-350. DOI: 10.1163/15685411-00003052
- Sekimoto, S., Hisai, J. & Iwahori, H. (2019). First report of the sugar beet cyst nematode, *Heterodera schachtii*, on *Brassica* sp. in Japan. *Plant Disease* 103, 1433-1433

DOI: 10.1094/PDIS-09-18-1541-PDN

- Shimomoto, M., Nakaishi, K. & Fukui, Y. (2000). Occurrence and control of tobacco cyst nematode in Kochi Prefecture. *Proceeding of the Association for Plant Protection of Shikoku* 35, 60. (in Japanese)
- Singh, S.K., Hodda, M. & Ash, G.J. (2013). Plant-parasitic nematodes of potential phytosanitary importance, their main hosts and reported yield losses. *Eppo Bulletin* 43, 334-374. DOI: 10.1111/epp.12050
- Skantar, A.M., Handoo, Z.A., Carta, L.K. & Chitwood, D.J. (2007). Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. *Journal of Nematology* 39, 133-144.
- Skarbilovich, T.S. (1959). On the structure of the systematics of nematode order Tylenchida Thorne, 1949. *Acta Parasitologica Polonica* 7, 117-132.
- Stone, A.R. (1973). *Heterodera pallida* n. sp. (Nematoda: Heteroderidae), a second species of potato cyst nematode. *Nematologica* 18, 591-606.
- Stone, L.E.W. & Wesley, D. (1975). The effect of heat on the hatch of potato cyst eelworms. *Plant Pathology* 24, 74-76. DOI: 10.1111/j.1365-3059.1975.tb01866.x
- Subbotin, S.A., Halford, P.D. & Perry, R.N. (1999). Identification of populations of potato cyst nematodes from Russia using protein electrophoresis, rDNA-RFLPs and RAPDs. *Russian Journal of Nematology* 7, 57-64.
- Subbotin, S.A., Waeyenberge, L. & Moens, M. (2000). Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. *Nematology* 2, 153-164. DOI: 10.1163/156854100509042
- Subbotin, S.A., Vera, I.C.D.P., Mundo-Ocampo, M. & Baldwin, J.G. (2011). Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes

- (Nematoda: Heteroderidae) as inferred from analysis of ITS-rDNA. *Nematology* 13, 805-824. DOI: 10.1163/138855410X552661
- Subbotin, S.A., Franco, J., Knoetze, R., Roubtsova, T.V., Bostock, R.M. & del Prado Vera, I.C. (2020). DNA barcoding, phylogeny and phylogeography of the cyst nematode species from the genus *Globodera* (Tylenchida: Heteroderidae). *Nematology* 22, 269-297. DOI: 10.1163/15685411-00003305
- Tefft, P.M. & Bone, L.W. (1985). Plant-induced hatching of eggs of the soybean cyst nematode *Heterodera glycines*. *Journal of Nematology* 17, 275-279.
- Thiéry, M., Fouville, D. & Mugniery, D. (1997). Intra-and interspecific variability in *Globodera*, parasites of Solanaceous plants, revealed by Random Amplified Polymorphic DNA (RAPD) and correlation with biological features. *Fundamental and Applied Nematology* 20, 495-504.
- Tirchi, N., Troccoli, A., Fanelli, E., Mokabli, A., Mouhouche, F. & De Luca, F. (2016). Morphological and molecular identification of potato and cereal cyst nematode isolates from Algeria and their phylogenetic relationships with other populations from distant their geographical areas. *European Journal of Plant Pathology* 146, 861-880. DOI: 10.1007/s10658-016-0965-z
- Trudgill, D.L. (1986). Yield losses caused by potato cyst nematodes: a review of the current position in Britain and prospects for improvements. *Annals of Applied Biology* 108, 181-198. DOI: 10.1111/j.1744-7348.1986.tb01979.x
- Trudgill, D.L., Evans, K. & Parrott, D.M. (1975a). Effects of potato cyst nematodes on potato plants. I. Effects in a trial with irrigation and fumigation on the growth and nitrogen and potassium contents of a resistant and a susceptible variety. *Nematologica* 21, 169-182. DOI: 10.1163/187529275X00536

- Trudgill, D.L., Evans, K. & Parrott, D.M. (1975b). Effects of potato cyst nematodes on potato plants. II. Effects on haulm size, concentration of nutrients in haulm tissue and tuber yield of a nematode resistant and a nematode susceptible potato variety. *Nematologica* 21, 183-191. DOI: 10.1163/187529275X00545
- Trudgill, D.L., Phillips, M.S. & Elliott, M.J. (2014). Dynamics and management of the white potato cyst nematode *Globodera pallida* in commercial potato crops. *Annals of Applied Biology* 164, 18-34. DOI: 10.1111/aab.12085
- Turner, S. & Evans, K. (1998). The origins, global distribution and biology of potato cyst nematodes (*Globodera rostochiensis* (Woll.) and *Globodera pallida* Stone). In: Marks, R.J. & Brodie, B.B. (Eds). *Potato cyst nematodes, biology, distribution and control*. Wallingford, UK, CAB International, pp. 7-26.
- Valdes, Y., Viaene, N., Blok, V., Palomares-Rius, J.E. & Moens, M. (2012). Changes in the pre-parasitic developmental stage of *Globodera rostochiensis* in response to green manures. *Nematology* 14, 925-932. DOI:10.1163/156854112X635869
- van den Elsen, S., Ave, M., Schoenmakers, N., Landeweert, R., Bakker, J. & Helder, J. (2012). A rapid, sensitive, and cost-efficient assay to estimate viability of potato cyst nematodes. *Phytopathology* 102, 140-146. DOI: 10.1094/PHYTO-02-11-0051
- Varypatakis, K., Jones, J.T. & Blok, V.C. (2019). Screening of populations of *Globodera pallida* selected for increased virulence on several potato varieties. *Nematology* 21, 995-998. DOI: 10.1163/15685411-00003283
- Whitehead, A.G., Fraser, J.E. & French, E.M. (1979). Control of potato cyst-nematode, *Globodera pallida*, on tomatoes grown under glass, by applying steam or chemical nematicides to the soil. *Annals of Applied Biology* 92, 275-278. DOI: 10.1111/j.1744-7348.1979.tb03874.x

- Whitehead, A.G., Nichols, A.J.F. & Senior, J.C. (1994). The control of potato pale cyst-nematode (*Globodera pallida*) by chemical and cultural methods in different soils. *The Journal of Agricultural Science* 123, 207-218. DOI: 10.1017/S0021859600068477
- Wollenweber, H. (1923) Krankheiten und Beschädigung der Kartoffel. *Arbeiten Forschungs Institut für Kartoffel Berlin* 7, 1-56.
- Wood, C., Kenyon, D.M. & Cooper, J.M. (2017). Allyl isothiocyanate shows promise as a naturally produced suppressant of the potato cyst nematode, *Globodera pallida*, in biofumigation systems. *Nematology* 19, 389-402. DOI: 10.1163/15685411-00003054
- Yamada, E., Takakura, S. & Tezuka, H. (1972). On the occurrence of the potato cyst nematode, *Heterodera rostochiensis* Wollenweber in Hokkaido, Japan. *Japanese Journal of Nematology* 2, 12-15. DOI: 10.14855/jjn1972.2.12
- Yaron, S. & Matthews, K.R. (2002). A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157: H7: investigation of specific target genes. *Journal of applied microbiology* 92, 633-640. DOI: 10.1046/j.1365-2672.2002.01563.x
- Zasada, I.A., Ingham, R.E., Baker, H. & Phillips, W.S. (2019). Impact of *Globodera ellingtonae* on yield of potato (*Solanum tuberosum*). *Journal of Nematology* 51. 1-10. DOI: 10.21307/jofnem-2019-073

Appendix 1: DNA-based PCR using Y45_0803_F1 and Y45_0715_R4

DNA was extracted from 10 cysts of Gp Abashiri and Gr Kutchan by the method of Sakata *et al.* (2021a) (see Chapter 3). The PCR reaction cocktail contained 0.4 μ l of Tks GflexTM DNA Polymerase, 10.0 μ l of 2 \times Gflex PCR Buffer, 0.6 μ l of Y45_0803_F1 (10 μ M) and Y45_0715_R4 (10 μ M), 2.0 μ l of the DNA solution, and 6.4 μ l of nuclease-free water. Thermal cycling was performed under the following conditions: one cycle at 94°C for 1 min, and 35 cycles at 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s, using an Applied Biosystems[®] SimpliAmpTM Thermal Cycler. A negative control was prepared using nuclease-free water as the template. The PCR products were electrophoresed as described in Chapter 3.

The PCR obtained an approximately 900 bp amplicon from Gp Abashiri. In contrast, no amplicon was obtained from Gr Kutchan and negative control (Fig. 11). Therefore, at least, the primer set can be used to discriminate *G. pallida* and *G. rosotochiensis* by DNA-based PCR.

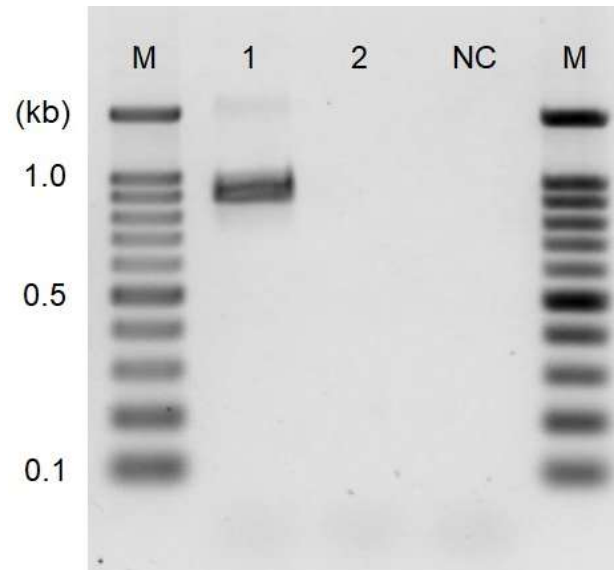


Fig. 11. Amplification results of endpoint PCR using Y45_0803_F1 and Y45_0715_R4. The DNA was extracted from 10 cysts of Gp Abashiri and Gr Kutchan. M: 100 bp DNA ladder; 1: Gp Abashiri; 2: Gr Kutchan; NC: negative control (nuclease-free water). See Table 3 for code definitions.

Appendix 2: Effect of RNA extraction methods on Ct values

RNA was extracted from 5 cysts of Gp Abashiri using NucleoSpin® RNA or EconoSpin® RNA. RT-qPCRs were performed as well as the species-specificity test. Five replicates of each sample were used. The effect of RNA extraction methods on Ct values was analyzed using one-way analysis of variants (ANOVA). The R software v. 4.1.3 (R Development Core Team, 2022) was used for statistical analysis.

As a result, the RNA extraction methods did not affect the Ct values (Fig. 12, $df = 1$, $F = 0.24$, $P = 0.637$). Therefore, I determined that there was no difference in detection sensitivity using either RNA extraction method.

In Japan, reagents for RNA extraction used in this study were individually available, whereas NucleoSpin® RNA Columns were not and can only be purchased as part of a kit (NucleoSpin® RNA). This causes an increase in RNA extraction cost and an accumulation of unnecessary reagents used for DNase treatment. However, I showed that EconoSpin® RNA Mini Spin Columns, which are individually available, replaced NucleoSpin® RNA Columns without a change in detection sensitivity. This enables to the preparation of all necessary consumables and reagents for RNA extraction individually, resulting in cost-cutting and prevention of unnecessary reagent accumulation.

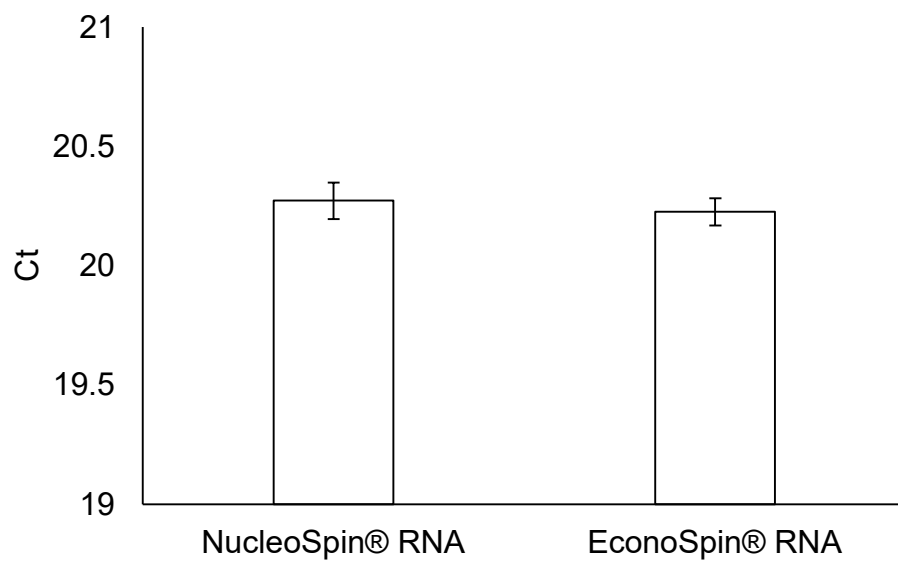


Fig. 12. Effect of RNA extraction methods on Ct values (mean \pm SE, n = 5). The RNA was extracted from 5 cysts of Gp Abashiri using NucleoSpin® RNA or EconoSpin® RNA.