**Case study on a modified method to quantify the density of some soil-borne plant-parasitic nematodes in a simpler and less expensive way**

Zejun Cheng1, Sayo Shirai1, Koki Toyota1,* and Karl Ritz2

Quantification of plant-parasitic nematodes (PPN) in soil with real-time PCR is a useful diagnosis to estimate damage to crops. However, previously reported methods involve high consumable and labor costs. The objectives of this study were to combine previously reported methods for soil pretreatment, DNA extraction and real-time PCR to quantify the density of soil-borne PPN in a simpler and less expensive way and to confirm the usefulness of a new simple method. Soils infested with either *Heterodera glycines* (soybean cyst nematode), *Ditylenchus destructor* (potato rot nematode) or *Meloidogyne incognita* (root-knot nematode) were ball-milled. DNA was then extracted with phosphate buffer and purified with a commercially available column. Real-time PCR was conducted to quantify the target nematodes. The cycle threshold (Ct) values obtained by the new method showed highly significant correlations with those by the conventional method for all three species ($R^2 > 0.75$). Significant correlations ($R^2 > 0.987$) were also obtained between the Ct values and the numbers of nematodes inoculated into soils. The DNA extraction from 6 samples by the new simple method required only 1 hr and about $48$ of consumables, while that by the conventional method required 3 hr and about $12$ of consumables. These results demonstrate that the method consisting of ball-milling and simple DNA extraction enables rapid and less expensive quantification of nematodes in soils.


Key words: GEL/PCR, loam, phosphate buffer, purification kit, sandy, silty loam

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1 Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, 2-24-16, Nakacho, Koganei, Tokyo 184-8588, Japan.
2 School of Biosciences, The University of Nottingham, Sutton Bonington Campus, Sutton Bonington, Leicestershire, LE12 5RD, UK.
* Corresponding author, e-mail: kokit@cc.tuat.ac.jp

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INTRODUCTION

Quantification of the density of plant-parasitic nematodes is a useful diagnosis to predict the damage to crops. The Baermann funnel method has been widely and commonly used to quantify nematodes in various samples, including soil and plant tissues (Ingham, 1994). A recent study, however, reported that the Baermann method is not advisable for survey purposes of *Meloidogyne* spp. (Den Nijs and van den Berg, 2013), since only active nematodes are extracted, whereas inactive forms are not. In addition, it is laborious to identify nematodes via microscopic observation following Baermann extraction, as specialist knowledge is required to identify particular species.

To avoid such disadvantages, molecular biology techniques are starting to prevail in nematode identification and quantification procedures. We have developed a real-time PCR method to directly quantify various plant parasitic nematodes in soil, including *Heterodera glycines* Ichinohe, 1952 (Goto et al., 2009); *Globodera rostochiensis* (Wollenweber, 1923); Skarbilovich, 1959 (Toyota et al., 2008); *Meloidogyne incognita* (Kofoid & White, 1919); Chitwood, 1949 (Min et al., 2011); *Meloidogyne graminicola* Golden & Birchfield, 1965, *Heterodera cajani* Koshy, 1967; *Hirschmanniella oryzae* van Breda de Haan, 1902 (Katsuta et al., 2016); *Pratylenchus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, 1941 (Sato et al., 2010); *P. pseudocoffeae* Mizukubo, 1992 and *P. kumamotoensis* Mizukubo, Sugimura & Uesugi, 2007 (Koyama et al., 2016), and *Ditylenchus destructor* Thorne, 1945 (Cheng et al., 2015). Real-time PCR uses a specific primer set for a target nematode species and provides highly reproducible results. Nevertheless, DNA must be extracted from soil and extant DNA extraction methods require various chemicals, consumables, and/or commercial kits, which are not suitable for high-throughput application as they are laborious and involve costly consumables.

Taberlet et al. (2012) reported a simple method using saturated phosphate buffer for the isolation of DNA from soil, and Zinger et al. (2016) confirmed that this method is a fast, cheap, and reliable alternative for multi-taxa
surveys of soil (micro)organisms. This method does not include the procedures for cell lysis, however, and thus only extracellular DNA is extracted. We previously reported that ball-milling of soil is the most effective way to disrupt nematode cells, including soil-borne eggs in cysts, and hence improve DNA extraction efficiency (Goto et al., 2010). Consequently, we postulated that a combination of the simple method for extracellular DNA extraction combined with ball-milling of soil to disrupt nematode cells might be an effective method to quantify the density of nematodes in soil. The objective of this study was to combine previously reported methods for soil pretreatment, DNA extraction, and real-time PCR to quantify the density of plant-parasitic nematodes in soils in a simpler and less expensive way, and to confirm the usefulness of this method by comparison with the data obtained by the conventional DNA extraction method.

MATERIALS AND METHODS

Soils, nematodes, and real-time PCR protocols:

Different naturally-infested soils were used to compare DNA extraction efficiency between the conventional method and the simple method, as described below. Soybean cyst nematode (SCN; Heterodera glycines) infested soils (n = 36) were collected from different sites of different soybean fields in Saitama Prefecture in March, 2016 and March 2018 (35°83’ to 35°84’ N latitude, 139°83’ to 84’ E longitude). Root-knot nematode (RKN; Meloidogyne incognita) infested soils (n = 17) were collected from different sites of different cucumber fields in Fukushima Prefecture in October, 2016 (37°58’ N, 140°42’ E). Potato rot nematode (PRN; Ditylenchus destructor) infested soils (n = 23) were collected from different sites of different garlic (Allium sativum L.) fields in Aomori Prefecture in October, 2015 (40°65’ N, 141°34’ E). These 3 species were selected for study because of the degree of damage to crops caused by the nematodes, the availability of the infested soils, and their inherently contrasting life cycles. The soil samples were dried in an oven at 60°C for 1 day, and then the dried soils were homogenized by using a ball-mill as described below.

Three reference soils not infested with the prescribed species were used for making calibration curves. A loam soil (soil texture:sand:silt:clay = 89%:10%/1%, total C content 35.5 mg/g dry soil, total N content 2.9 mg/g dry soil) was collected from a garlic field of the Vegetable Research Institute, Aomori Prefectural Industrial Technology Research Center in October, 2015 (Rokunohe, Aomori; 40°65’ N, 141°34’ E). A sandy soil (sand:silt:clay = 89%:10%/1%, total C content 4.7 mg/g dry soil, total N content 0.4 mg/g dry soil) was collected from a sweet potato field in Tokushima Prefecture in March, 2012 (Ishii, Tokushima; 34°06’ N, 134°44’ E). A silty loam soil (sand:silt:clay = 33%:49%/18%, total C content 41.3 mg/g dry soil, total N content 4.2 mg/g dry soil) was collected from an upland field at Tokyo University of Agriculture and Technology, FS center in October, 2015 (Fuchu, Tokyo; 35°67’ N, 139°47’ E).

Second stage juveniles (J2) of RKN were extracted from infested roots of tomato (Solanum lycopersicum L. c.v. Momotaro) via the Baermann funnel method. Tomato plants were grown in pots containing an infested soil for one month in a Climatron at 27°C. The tomato plants were taken from the pots and root knots obtained. PRN was collected from infested garlic plants by using the Baermann funnel method (Ingham, 1994). The infested garlics were sliced with a knife, put on sieves and nematodes were extracted by 1 day of incubation at room temperature (20–25°C). The resultant suspension was left to settle for several hours at 4°C and the supernatant was discarded. This washing process was repeated three times and then the suspension was used for inoculation. The cysts of SCN were obtained from an infested soil by using the sieving method (Ingham, 1994). In sieving, two kinds of sieves with openings of 710 µm and 212 µm were used to collect eggs.

For confirmation of the nematode identification, single nematodes of SCN, RKN, and PRN were handpicked in 20–29 replicates and DNA was extracted according to the method of Iwahori et al. (2000) with minor modifications (Cheng et al., 2015). The DNA extracts were used as templates in real-time PCR after ten-times dilution as described below. The specific primer sets used were for PRN (D. destructor): Ddf (5’-CAC GTC TGA TTC AGG GTC GTA AAT A -3’) and Ddr (5’- AGA AAC ACG TGC TAG GCC AAA G -3’) (Cheng et al., 2015), for RKN (M. incognita): RKNf (5’- GCT GGT TTC AAA AGG CCA CCA -3’) and RKNr (5’- GAG CCT AGT GAT CCA CCG ATA AG -3’) (Toyota et al., 2008), and for SCN (H. glycines): SCNf (5’- GAT GAG GTG TCA AAT AAT AAT AAT AAT AAT -3’) and SCNr (5’- AAT GAT GGG CAG CGT CCA CCA A -3’) (Goto et al., 2009). Real-time PCR was performed in a StepOne Real-Time PCR System (Life Technologies, Carlsbad, California) with a final volume of 10 µl containing 2 µl of template DNA, 0.4 µl of 10 µM primers and 5 µl of Fast SYBR® Green Master Mix (Life Technologies Japan, Tokyo, Japan) under the manufacturer’s recommended conditions (95°C for 10 sec, 95°C for 5 sec, and 60°C for
20 sec), at increasing and decreasing rates of 0.2°C/sec for 45 cycles). A negative control was also included using distilled water instead of a template DNA. Real-time PCR was done once per each DNA extract, since replicate samples showed almost identical values by real-time PCR in our preliminary experiment.

Effect of soil pretreatments:

An infested soil for each plant-parasitic nematode species was selected from the soils described above, and the effects of four different pre-treatments on DNA extraction efficiency were evaluated. DNA was extracted from: [1] fresh soil (neither oven-dried nor ball-milled); [2] soil oven-dried at 60°C for 1 day (not ball-milled); [3] soil oven-dried at 60°C for 1 day and frozen at –80°C for 1 hour (not ball-milled); [4] soil oven-dried at 60°C for 1 day and ball-milled soil. Ball-milling was done using a FastPrep-24 (MP Biomedical, LLC) for 2 min at a speed of 4.5 m/sec. In this treatment, 10 g of dried soil was put into a 15 mL plastic tube with 2 stainless balls (9.5 mm in diameter). Soil was pulverized in duplicate for each PRN- and RKN-infested soil and in triplicate for each SCN-infested soil, and a total of 20 g and 30 g was combined for the former and latter soils, respectively.

Simple DNA extraction method from soil:

DNA was extracted from the pretreated soil samples described above (Table 1) with the method reported by Taberlet et al. (2012). Ten ml of phosphate buffer (Na2HPO4 and NaH2PO4, 0.12 M, pH = 8.0) was added to 5 g of the pretreated soil in duplicate. In this study, to avoid adsorption of DNA to soil (Paulin et al., 2013), 1 mg/g dry soil of salmon sperm DNA was added to each 5 g of soil. Then, the soil and phosphate buffer mixtures were thoroughly mixed in a shaker (TS-10, TAITEC) at 200 rpm for 30 min. After centrifugation for 10 minutes at 8,000 rpm, 50 µl of the supernatants was purified with a GEL/PCR purification kit (Favorgen) according to the manufacturer’s instructions, and DNA was eluted with 50 µl of elution buffer. The DNA extracts were used as templates after 10x dilution in real-time PCR, as described above. All samples were processed in triplicate.

Comparison of Ct values between the conventional method and the simple method:

DNA was extracted in triplicate by using the conventional method (Min et al., 2012) from the same RKN-infested soils, SCN-infested soils, and PRN-infested soils as those extracted by using the simple DNA extraction method described. In brief, soil (0.5 g) was bead-beaten with lysis buffer and DNA was extracted with the hexadecyltrimethylammonium bromide (CTAB) method. Then, the DNA was purified and precipitated with 20% polyethylene glycol 8,000 (PEG) solution. The DNA pellet was washed with 70% ethanol and suspended in 100 µl of TE buffer. Real-time PCR was done as described above by using the DNA templates extracted after ten times dilution. Cycle threshold (Ct) values were compared between the simple DNA extraction method and the conventional method.

Calibration curves:

The reference soils not infested with target nematodes were used to prepare calibration curves. Inoculated nematodes were eggs for SCN and J2 stage for RKN. For PRN, a mixture of males, females, and juveniles was used as inoculum (male:female:juveniles = 6:46:48). The Ct value of SCN eggs was 20.0 ± 0.3 (n = 19), that of RKN J2 was 19.7 ± 0.8 (n = 29), and that of PRN was 20.5 ± 0.5 in male (n = 11), 19.6 ± 0.5 in female (n = 8), 21.0 ± 0.67 in J2 (n = 8).

Each soil (5 g oven-dried) was inoculated in 3 to 6 replicates with different numbers of PRN (sandy, loam, silty loam: 10, 100, 1,000, 5,000), SCN eggs (sandy, loam, silty loam: 10, 40, 200, 1,000), and RKN J2 (sandy: 40, 200, 1,000; loam: 30, 150, 750; silty loam: 20, 100, 500). Immediately after inoculation, the soil samples were oven-dried for 24 h at 60°C and then homogenized with the ball mill for 2 min. DNA extraction and real-time PCR was done as described above by using the DNA templates extracted after ten times dilution. Cycle threshold (Ct) values were compared between the simple DNA extraction method and the conventional method.

Table 1. Effect of different pretreatments of soil on the Ct values obtained with the simple method from soils infested with Heterodera glycines (SCN), Ditylenchus destructor (PRN), or Meloidogyne incognita (RKN) (mean values ± SD, n = 3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>60°C oven-dry&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Frozen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ball-milling&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SCN</th>
<th>PRN&lt;sup&gt;b&lt;/sup&gt;</th>
<th>RKN&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>35.7 ± 0.8</td>
<td>un</td>
<td>27.3 ± 0.2</td>
</tr>
<tr>
<td>Dry</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>37.1 ± 1.2</td>
<td>un</td>
<td>30.3 ± 0.8</td>
</tr>
<tr>
<td>Dry and frozen</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>37.0 ± 0.9</td>
<td>un</td>
<td>31.8 ± 0.6</td>
</tr>
<tr>
<td>Ball-milling</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>28.5 ± 1.0</td>
<td>31.8 ± 0.2</td>
<td>24.8 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>+: with treatment, –: without treatment
<sup>b</sup>un; undetermined (less than the detection limit)
PCR were carried out by using the simple method, described above.

Statistical analysis:
The data obtained by the conventional method and the simple method developed in this study were subjected to one-way analysis of variance, and linear regression analysis was used for the calibration curves. All the analyses were conducted by using the statistical analysis software Excel statistics 2010 for Windows ver. 1.13.

RESULTS

Effect of soil pre-treatment on Ct values obtained with the simple method:
Fresh, dry, and dry and frozen soils showed notably large Ct values or undetermined values for all the nematode species, indicating low DNA extraction efficiency for the pre-treatments of soils (Table 1). Ball-milled soils, however, showed significantly lower Ct values \((P < 0.01)\), indicating that ball-milling was effective for improving quantification of nematode densities in soil. In particular, Ct values were not obtained from dried or dry and frozen PRN-infested soils, but were obtained from ball-milled soils.

Comparison of Ct values between the simple method and the conventional method:
Nematode densities were measured in different naturally-infested soils, and the Ct values were compared between the two methods (Fig. 1). The simple DNA extraction method showed highly significant correlations \((P < 0.001 \text{ in all cases})\) with the conventional method for each nematode species (Fig. 1).

Calibration curves:
There were highly significant \((P < 0.001)\) negative correlations between the Ct values and the log-transformed number of nematodes \((x)\) for all 3 species in all 3 types of soil (Fig. 2). Comparison of the Ct values at the same inoculum number of nematodes among the loam, silty loam, and sandy soils showed that the sandy soil gave the lowest Ct values among all three soils, indicating the highest DNA extraction efficiency was in the sandy soil.

DISCUSSION

The present study confirms the efficacy and reliability of a simple DNA extraction method to quantify plant-parasitic nematodes in soil. A fast, cheap, and reliable DNA extraction method was originally reported by Taberlet et al. (2012), and this method was applied to soils infested with different plant-parasitic nematodes in this study. Taberlet's method resulted in large Ct values, i.e., very low DNA extraction efficiency, even where the method was combined with freeze-thawing, which is an effective way to disrupt microbial cells (Tsai and Olson, 1991). According to the report by Taberlet et al. (2012), their method extracts only extracellular DNA and not intracellular forms, suggestive of low concentrations of extracellular nematode DNA in soil and little disruption of nematode cells by freeze-thawing. When DNA was extracted with the Taberlet method from ball-milled soils, however, the Ct values became notably low, and this tendency was observed not only in root-knot nematodes and potato rot nematodes but also in cyst

\[
y = 0.948x + 4.92
\]
\[
R^2 = 0.900***, n = 36
\]

\[
y = 0.837x + 8.71
\]
\[
R^2 = 0.914***, n = 19
\]

\[
y = 0.627x + 14.3
\]
\[
R^2 = 0.750***, n = 17
\]
nematodes, suggesting that ball-milling disrupts nematode cells in soil, including cysts, and helps nematode DNA to be released into the phosphate buffer.

DNA was extracted from different infested soils by using the conventional method and the present simple method consisting of ball-milling and the Taberlet’s method. The Ct values obtained with the simple method showed highly significant correlations ($r^2 > 0.91, P < 0.001$) with those performed by the conventional method in SCN- and PRN-infested soils. In RKN-infested soils, though the former needs only similar reliability to the conventional method, even simple method quantifies nematode densities in soil with results showing that PCR amplification is inhibited by higher clay and organic matter contents in soil (Zhou et al., 1996). The loam and silt loam soils showed almost identical results in SCN, and similar results in PRN and RKN, suggesting that soil texture ranging from loam to silt loam might show similar DNA extraction efficiencies. The present simple method enables the estimation of SCN, PRN or RKN densities in a target soil by applying the calibration curve of a soil type that is close to the target soil. For greater precision, a bespoke calibration curve should be created for any particular soil.

Another merit of the present simple DNA extraction method is in the amount of soil used for DNA extraction. The conventional method extracts DNA from 0.5 g of soil. According to Ranjard et al. (2003), amounts of soil greater than 1.0 g are required to obtain robust and reproducible fingerprinting analysis of fungal communities, although small amounts of soil less than 1.0 g are sufficient for microbial (bacterial) communities. This result suggests that amounts of soil greater than 1.0 g are required to evaluate organisms present at relatively lower densities, such as nematodes. To minimize such heterogeneity in nematode distribution in soil, DNA is extracted from 0.5 g of soil that is taken from 20 g of pulverized soil, as we described previously (e.g. Min et al., 2012). Since the present method extracts DNA from 5.0 g of pulverized soil, more robust and reproducible nematode quantification is achieved. Indeed, Ct values between duplicate or triplicate DNA samples deriving from single soil samples differed by 2.7 to 0 in the conventional method, but the differences ranged from 1.1 to 0 in the present simple method, suggesting that more reproducible results are obtained in the present simple method.

A disadvantage of the present simple DNA extraction method is the detection limit. The conventional method quantifies nematode densities as low as 4 to 5 individuals (20 g soil$^{-1}$; Min et al., 2012). In contrast, the detection
limits via the simple method were 40 individuals/20 g of
dry soil for PRN in all three soils and 40 individuals/20 g 
of dry soil for SCN in silty loam and sandy soils (Fig. 2). 
In RKN, 40 individuals/20 g of dry soil were not detected 
in some of the replicates in all three soils (data not 
shown), indicating that the detection limits were greater 
than 40 individuals/20 g of dry soil. In contrast, Ito et al. 
(2017) reported that an economic threshold causing a 
10% yield loss in green soybean production was 81 eggs 
of SCN/20 g dry soil. Mwaura et al. (2015) reported that 
the potato tuber weight was decreased in the soils 
containing more than 57 PRN/20 g dry soil. Watanabe et 
al. (2013) reported the yields of eggplant were low in the 
soils containing more than 128 J2 of RKN/20 g of dry 
soil. Since the economic thresholds are greater than the 
detection limits in the present simple method, this shows 
that it is applicable to nematode diagnosis in green 
soybean fields infested with SCN, in potato fields with 
PRN, and in eggplant fields with RKN.

In terms of costs, the conventional method requires 
more steps (taking 3 h for 6 samples) and consumables 
(about $2/sample at 2017 prices) than the simple method 
(1 h for 6 samples and about $0.8 consumables).

In conclusion, the present simple method will enable 
high-throughput analysis for the nematode diagnosis of 
soils infested with soil-borne plant parasitic nematodes.

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