INTRODUCTION

Plant-parasitic nematodes cause significant economic losses to a wide variety of crops (Dong and Zhang, 2006). Soybean cyst nematode (SCN) Heterodera glycines Ichinohe causes yield losses in most soybean production areas in the world with an estimated $1.4 billion of damage to soybean production in the US (Wrather et al., 2001a, 2001b). SCN is distributed throughout Japan and the damage caused by SCN is seen mainly in relatively cooler regions from central Honshu to Hokkaido (Aiba, 2001).

Since there is a significant correlation between the initial soil population density of plant-parasitic nematodes and the degree of damage to the host (e.g., Chikaoka, 1983; Ohbayashi, 1989; Koenning, 2000; Back et al., 2006; Gugino et al., 2006), reliable and rapid identification and counts of the casual agents are critically important for successful management of the nematode pests. Mandani et al. (2005) emphasized that precise identification and knowledge about the number of nematodes in field soil are necessary to develop effective integrated pest control and reported the real-time PCR primers specific to the potato cyst nematode Globodera pallida (Stone) and sugar beet cyst nematode H. schachtii Schmidt for such a diagnostic purpose. Real-time PCR primer sets have been also developed for Meloidogyne chitwoodi Golden et al. and M. fallax Karssen (Zijlstra and van Hoof, 2006), the root-lesion nematode Pratylenchus penetrans (Cobb) (Sato et al., 2007), the root-knot nematode M. incognita (Kofoid and White) (Toyota et al., 2008) and potato cyst nematode G. rostochiensis (Wollenweber) (Toyota et al., 2008; Quader et al., 2008). At present, real-time PCR primers specific for H. glycines are not yet reported and thus were newly designed in this study.

Several authors have revealed the usefulness of molecular techniques for characterizing a nematode community structure or quantifying specific nematode species among a variety of soil nematodes (Griffiths et al., 2006; Sato et al., 2007; Toyota et al., 2008; Donn et al., 2008; Okada and Oba, 2008), but firstly nematodes have to be extracted from soil for these experiments. It is reported that the dynamics of plant-parasitic nematodes, such as M. incognita (McSorley et al., 2008; Zasada and Tenuta, 2008) and P. crenatus Loof (Briar et al., 2007), play an important role in estimating their damage in the soil environment, in which the extraction of nematodes is also a prerequisite for the morphological identification of plant-parasitic nematodes. In case of diagnosis of the infestation level by

A novel detection method for the soybean cyst nematode 
Heterodera glycines Ichinohe using soil compaction
and real-time PCR

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The primers SCN44f and SCN124r were designed for the quantitative detection of the soybean cyst nematode (SCN) Heterodera glycines using real-time PCR. A andosol naturally infested with SCN was put into a 100 ml core and compacted to various degrees, i.e. 0.93 to 1.4 g cm⁻³, using a manually-operated compactor. Then, DNA was extracted from the compacted soils as well as non-compacted soil (0.66 g cm⁻³) and used as templates for real-time PCR. Ct values were the lowest at 1.4 g cm⁻³, the maximum physical compaction of the andosol in the compactor, and the difference in the Ct values between compacted and non-compacted soil was four cycles at the largest, suggesting that 16 times more DNA derived from SCN was detected by the compaction. Then, different numbers (10 to 3,000) of SCN eggs were added to 20 g of a non-infested andosol and the soils were compacted to 1.4 g cm⁻³. There was a significant correlation (r² = 0.8615, P < 0.001) between the Ct values and the number of eggs added. These results demonstrated that the present method using a combination of soil compaction and real-time PCR enabled rapid and sensitive quantification of SCN eggs in soil. Nematol. Res. 39 (1), 1-7 (2009).

Key words: diagnosis, DNA extraction, ITS, plant-parasitic nematodes, qPCR.

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SCN in soil, the cysts of SCN must be extracted from soil, using methods such as the Flack method or the Fenwick method, and then the number of eggs in the cysts must be counted under a microscope after destruction of the cysts (Ingham, 1994). These procedures are laborious and time-consuming and this can cause variation in the measurement among analysts.

Waite et al. (2003) reported a nematode community analysis using DNA directly extracted from 1 g soil. However, it is known that the comparatively low abundance of soil nematodes and their patchy spatial distribution requires extraction from larger soil volumes to achieve a representative sample (Donn et al., 2008). Recently, we found that heavily compacting soil enabled the destruction of SCN cysts in the soil. Therefore, we applied a compaction method to quantify cysts and the eggs in soil in this study. In this method, DNA was extracted from the compacted soil and used as a template for real-time PCR using the specific primers developed in this study. Thus, the purpose of this study was to develop a rapid and sensitive method for the detection and quantification of SCN in soil using a combination of soil compaction and real-time PCR.

MATERIALS AND METHODS

Soils and nematodes used:

A soil (EC 144 µS cm⁻¹, pH (H₂O) 6.8, maximum water holding capacity 0.80 g g⁻¹ dry soil, soil texture: loam, sand : silt : clay = 39% : 22% : 39%) naturally infested with SCN was collected from a farmland in Tokyo in May, 2007 and stored at 15°C. Cysts were extracted from the soil using a modified method of Shinya et al. (2008). Briefly, 50 g of soil was dispersed with running tap water under strong water pressure and passed through a 710-mm-pore diameter sieve onto a 212-mm-pore sieve. Cysts retained under a microscope. The number of cysts was estimated as 64 ± 16 cysts 20 g⁻¹ dry soil (n = 3) and one cyst contained 215 ± 59 eggs (n = 30). In contrast, the number of vermiform SCN in the soil counted by the Baermann method was 11 individuals 20 g⁻¹ dry soil (n = 3).

A soil (EC 187 µS cm⁻¹, pH 7.1, maximum water holding capacity 1.1 g g⁻¹ dry soil, soil texture sandy clay, sand : silt : clay = 65% : 0% : 35%) that had not been planted with soybean for the last 10 years was collected from the Koganei campus of Tokyo University of Agriculture and Technology. The absence of both the juvenile and cyst stages of SCN in the soil was confirmed with the Baermann method and sieving method, and therefore the soil was used as a reference in the compaction experiment described below.

Caenorhabditis elegans (Manpas) was maintained on agar plates inoculated with Escherichia coli (Migula) JM109 based on the method by Hasegawa and Miwa (2004) and used to adjust the number of nematodes in real-time PCR.

Primers:

Specific primers for H. glycines (SCN124f (ITS1 position 44-62, 5'-CTA GCG TTT GCA CCA CCA A-3') - SCN124r (ITS1 position 134-124, 5'-AAT GTT GGG CAG CGT CCA CA-3')) were designed by Takara Bio Inc. (Otsu, Japan) based on the IT51 sequences of H. glycines (accession number AY590280), H. schachtii (AY590282), H. avenue Wollenweber (EF153843) and G. rostochiensis (Wollenweber) (EF153840) (Table 1).

DNA extraction from nematode suspensions:

Different numbers (1, 5, 10, 25, 50, 75, 100, 150, and 200) of SCN eggs were added to 200 µl of sterile distilled water and then mixed stages of vermiform C. elegans were added to adjust the total number to 200 individuals, since it was difficult to extract DNA from suspensions containing only a few individuals. DNA was extracted from the suspensions using the method of Toyota et al. (2008), finally suspended in 200 µl of TE and used as a template in

| Table 1. Comparison of sequences in the positions of the primers used in this study. |
|-----------------------------------|-----------------------------------|
| H. glycines (AY590280)            | CTAGCG-TT-GGCAACCAACCA          |
| H. schachtii (AY590282)           |                                   |
| H. trifolii (AY692354)            |                                   |
| H. elachista (A498391)            |                                   |
| H. avenue (EF153843)              |                                   |
| G. rostochiensis (EF153840)       |                                   |
| Forward primer (SCN44f)           | Reverse primer (SCN124r)         |
| CTAGCG-TT-GGCAACCAACCA            | TGTGGACGCTGCCCAACATT             |
| CAGCACT-CCC-CCC-CCC              |                                   |
| CGGTTTGCA-CA-CA-CA               |                                   |
| ACGTT-CT-CT-CT                   |                                   |
| TCCTTCTG-ACT-AT-CT               |                                   |
| 1 Numbers in parentheses after the species names indicate the accession number. |
| 2 Dots indicate the same base as above. |
| 3 Hyphens indicate deletion of the corresponding base. |
real-time PCR described below after 10 times dilution.

One SCN egg was mixed with 1,000 individuals of *C. elegans*. DNA was extracted from the mixtures and finally suspended in 1,000 µl of TE and used as a template in real-time PCR.

Soil compaction and DNA extraction from the soil:

A manually-operated compactor (Daiki Rika Kogyo Co., Ltd., Konosu, Japan) shown in Fig. 1 was used to compact soil. Air-dried infested soil was put in a 100-ml soil core (5 cm height) and compacted using the compactor. In this experiment, 38 to 70 g (dry basis) was put into the core and compacted to different degrees to give the same soil height of 2.5 cm. Seven compaction levels were prepared without replicate. DNA was extracted from 0.5 g of the compacted soils in three replicates.

Twenty grams of the reference soil was mixed with different numbers (0, 10, 25, 50, 250, 500, 1,000, 1,500, and 3,000) of SCN eggs, put into a 100-ml soil core and then compacted to 1.4 g cm⁻³. Since 20 g of soil is generally used in triplicate in the Baermann method, a total of 60 g of the infested soil was compacted in three separate compaction treatments. For each infestation level, soil was compacted in one (only in the case of 3,000 eggs) or two replicates. In this experiment, DNA was extracted from a 0.5 g soil sample per replicate.

The compacted soils were mixed well with TE buffer at a ratio of 1:1 (w (fresh basis)/w) using a homogenizer (AUTO CELL MASTER CM-200, AS ONE Corporation, Tokyo, Japan) at 15,000 rpm for 10 min. Then, DNA was extracted from 0.5 g of the mixed soil by the method of Sato et al. (2007), and finally suspended in 100 µl of TE. The DNA extract was used as a template in real-time PCR after 10 times dilution based on Griffiths et al. (2000).

Real-time PCR:

Real-time PCR was performed using a Smart Cycler® II (Cepheid, Takara Bio Inc., Otsu, Japan) in a final volume of 25 µl containing 12.5 µl of SYBR Premix Ex Taq™ (Perfect Real Time, Takara Bio Inc., Otsu, Japan), 5 µM of each primer and 5 µl of template DNA under the manufacturer's recommended conditions (95 °C for 10 sec, (95 °C for 5 sec and 60 °C for 20 sec) × 40 cycles). A negative control was also prepared using distilled water instead of a DNA template.

Statistics:

Significance of correlation coefficient and differences among mean values were analyzed by correlation analysis and ANOVA protected by Fisher’s range test (P < 0.05), respectively, using the software Excel Statistics 2002 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Detection of SCN in nematode mixtures by real-time PCR using DNA extracted from the suspensions:

Ct values in the nematode suspensions containing 1, 5, 10, 25, 50, 75, 100, 150, and 200 eggs of SCN were 30.9 ± 0.2, 29.3 ± 0.6, 28.3 ± 0.2, 27.3 ± 0.5, 26.3 ± 0.3, 25.8 ± 0.5, 24.9 ± 0.5, 24.9 ± 0.5, and 24.0 ± 0.4, respectively (two or three replicates) and there was a highly significant correlation (r² = 0.9648, P < 0.001) between them (Fig. 2). The Ct value in the negative control, in which distilled water was used as a template instead of DNA from a nematode suspension, was 37.1 ± 3.0 (n = 32) and no melting profile was obtained. The Ct value in a nematode suspension with a single egg of SCN and 1,000 individuals of *C. elegans* was 29.8 ± 0.4, while it was more than 40 cycles in the suspension not containing a SCN egg.

Relationship between degree of compaction and Ct value:

The Ct values in the nematode suspensions containing 1, 5, 10, 25, 50, 75, 100, 150, and 200 eggs of SCN were 30.9 ± 0.2, 29.3 ± 0.6, 28.3 ± 0.2, 27.3 ± 0.5, 26.3 ± 0.3, 25.8 ± 0.5, 24.9 ± 0.5, 24.9 ± 0.5, and 24.0 ± 0.4, respectively (two or three replicates) and there was a highly significant correlation (r² = 0.9648, P < 0.001) between them (Fig. 2). The Ct value in the negative control, in which distilled water was used as a template instead of DNA from a nematode suspension, was 37.1 ± 3.0 (n = 32) and no melting profile was obtained. The Ct value in a nematode suspension with a single egg of SCN and 1,000 individuals of *C. elegans* was 29.8 ± 0.4, while it was more than 40 cycles in the suspension not containing a SCN egg.
values between compacted and non-compacted soil was ca. four cycles at the largest.

Detection of SCN in soil by real-time PCR using DNA extracted from the soil:

When DNA was extracted from the compacted soils containing different numbers of SCN eggs and used as templates for real-time PCR, there was a significant correlation between the Ct values and the number of eggs added (Fig. 4, $r^2 = 0.8615, P < 0.001$). The Ct values in the soils with 10 eggs 20 g$^{-1}$ soil and without eggs were 31.9 ± 0.8 and 34.8 ± 0.4, respectively, and their difference was significant ($P < 0.05$).

Estimate of SCN egg numbers in soil by a combination of compaction and real-time PCR:

By substituting the Ct values of DNA extracted from the naturally infested soil compacted to 1.4 g cm$^{-3}$ in the equation obtained in Fig. 4, the number of SCN eggs in the soil was estimated as 16,000 ± 1,680 (n = 3) eggs 20 g$^{-1}$ dry soil. As one cyst contained 215 ± 58 eggs, the estimate corresponded to 75 ± 8 cysts 20 g$^{-1}$ dry soil. In contrast, the infested soil contained 64 ± 16 cysts 20 g$^{-1}$ dry soil according to the sieving method, resulting in a value 17% lower than that obtained with the compaction and real-time PCR method.

DISCUSSION

The present study demonstrated that the primers SCN44f and SCN124r sensitively and rapidly detected the soybean cyst nematode (SCN) *Heterodera glycines* in soil by a combination of compaction and real-time PCR. First, sensitivity and quantification of the real-time assay was confirmed using DNA extracted from the nematode suspensions containing different numbers of SCN eggs (Fig. 2). The Ct values were markedly smaller in the nematode suspensions containing one egg of SCN (30.9 ± 0.2) than in distilled water (37.1 ± 3.0, n = 32). The melting profile further supported the specific detection of SCN eggs, since the Tm value was 87°C in the nematode suspensions containing a SCN egg, but not obtained in the distilled water. When one egg of SCN was mixed with 1,000 vermiform *C. elegans* and DNA was extracted from the nematode suspension, the Ct values (29.8 ± 0.4) were detected in the suspension with one egg of SCN, suggesting that this real-time assay was able to detect a single SCN egg among 1,000 non-target nematodes. To confirm specificity of the primers designed in this study, SCN44f and SCN124r were used to amplify the ITS region of *G. rostochiensis, M. incognita* and *P. penetrans*. No amplification was observed from these non-target nematodes (data not shown). Since not only *H. glycines* but also *H. schachtii*...
and H. trifolii Goffart have the same sequences as the specific primers SCN44f and SCN124r (Table 1), caution will be needed in fields where H. schachtii and H. trifolii cysts may be present.

To develop a detection method of SCN directly from soil, soil compaction was applied to destroy the cysts in this study. Firstly, a naturally infested soil was compacted to various degrees from 0.66 (non-compacted control) to 1.4 g cm\(^{-3}\). The results showed smaller Ct values in more compacted soils (Fig. 3), suggesting that soil compaction destroyed the cysts and eggs of SCN and released their DNA to be extracted. Indeed, only 4 ± 0 cysts 20 g\(^{-1}\) were extracted from the most compacted soil compared to 64 ± 16 cysts from the non-compact soil, supporting that most cysts were destroyed by the compaction and the eggs in the cysts may also be damaged.

Next, DNA was extracted from a non-infested soil containing different numbers of SCN eggs following the compaction at a level of 1.4 g cm\(^{-3}\) to make a calibration curve for estimating the number of eggs in a given sample based on the Ct values. There was a highly significant correlation between the Ct values and the number of SCN eggs added (Fig. 4). The Ct value in the soil containing no SCN (34.8 ± 0.4) was significantly (\(P < 0.05\)) larger than the soil containing as few as 10 eggs 20 g\(^{-1}\) of soil (31.9 ± 0.8), indicating that this is the density of the detection limit in this study.

The calibration curve in Fig. 2, obtained based on the suspensions containing different numbers of SCN eggs, was incorporated into Fig. 4, which shows the calibration curve based on the soils containing different numbers of SCN eggs. When the Ct values in the soil containing 1,500 eggs 20 g\(^{-1}\) soil were compared with that in the suspension containing an equivalent number of SCN eggs to the soil, the former was 28.6 and the latter 27.3 and the difference was not significant. However, with smaller numbers of SCN eggs, the difference in the Ct values of DNA templates containing the same numbers of SCN eggs from soil and suspension became larger (Fig. 4). This result suggested that the number of SCN was underestimated in such soils with lower densities of SCN, compared to the nematode suspension. It was considered that a larger part of DNA from SCN eggs might bind to soil particles and/or humic substances and become unextractable at lower egg densities. Khanna and Stotzky (1992) reported that when DNA adsorbed to clay particles, more bound at lower DNA concentrations. These findings also suggest that the degree of inhibition in the PCR reaction or binding of DNA to soil particles might differ depending on the soil type. Therefore, we need to improve the DNA extraction method in order to detect SCN more precisely from soil containing low SCN population densities and evaluate differences in the calibration curves among different soils in the next step.

The Ct values in the naturally infested soil were ca. four times smaller with the compaction treatment (25.5 ± 0.2) than with no treatment (29.5 ± 0.3) (Fig. 3). This result suggested that 16 times more SCN was detected by the compaction treatment. The result that the Ct values (29.5 ± 0.3) in the non-compaction treatment were significantly smaller than that in soil without SCN (34.8 ± 0.4) suggested that some SCN were destroyed and their DNA was released even without compaction treatment. In the non-compacted treatment, a bead-beating method, known as the most efficient method to extract DNA from soil (Kresk and Wellington, 1999; Burgmann et al., 2001), was used to destroy SCN in soil and thus a part of SCN cysts may be destroyed by bead-beating. Therefore, the conventional method could be used to detect SCN in soil, but it was proved that the efficiency was greatly improved by soil compaction. In addition, most of the conventional methods use soil samples of less than 10 g, raising a question of representativeness of such small amounts of soil (Opel-Keller et al., 2008). In the compaction method, 60 g of soil is used to get a representative sample, similar to that used in the Baermann method and therefore this method has merit in complementing the heterogeneous distribution of nematodes over the conventional methods.

The calibration curve obtained in Fig. 3 was applied to estimate the number of SCN in the naturally infested soil, in terms of the egg equivalent, and the density was estimated as 16,000 ± 1,680 (n = 3) eggs 20 g\(^{-1}\) dry soil, 17% higher than the sieving method, which was estimated as 13,800 eggs 20 g\(^{-1}\) dry soil, based on 64 ± 16 (n = 3) cysts 20 g\(^{-1}\) dry soil and 215 eggs cyst\(^{-1}\). Although it was possible that vermiform SCN were also more efficiently destroyed by the compaction treatments, their contribution was considered to be small since the number of vermiform SCN was much smaller (11 individuals 20 g\(^{-1}\) dry soil) than that of eggs. It was considered that the overestimate observed in this study may be caused by the extraction efficiency of the sieving methods, since a number of eggs are lost during the sieving process.

In the sieving method, a lot of time is required to sieve soil, collect cysts and count the number of cysts and eggs. In addition, to select only viable cysts is laborious and sometimes difficult and thus it can cause variation among analysts. In contrast, the present method only requires 30 min for compaction, two hours for DNA extraction and 40 min for real-time PCR and thus a total of four hr per four
samples and is considered less biased because no sieving procedure is used. The present study demonstrated a rapid and sensitive detection method for cysts of SCN in soil using real-time PCR and soil compaction that does not require laborious extraction methods of cysts. However, our primer set may also detect *H. schachtii* and *H. trifolii* with the same efficiency and thus caution is required when these cyst nematodes are also present in the soil. We reported the quantification method using real-time PCR for root-knot nematode (*Meloidogyne incognita*: Toyota et al., 2008), root-lesion nematode (*Pratylenchus penetrans*: Sato et al., 2007) and potato cyst nematode (*Globodera rostochiensis*: Toyota et al., 2008). Therefore, quantification of these plant-parasitic nematodes using a combination of soil compaction and real-time PCR may be a powerful tool for rapid and sensitive detection in soil.

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**LITERATURE CITED**


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