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SHORT PAPER

Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*

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Abstract

The primers PCN280f and NEPCN398r were designed for the quantitative detection of the potato-cyst nematode *Globodera rostochiensis* using real-time polymerase chain reaction (PCR). One, five, 50, 125 and 250 individuals of the second-stage juveniles (J2) of *G. rostochiensis* were mixed with various stages of vermiform *Caenorhabditis elegans* to make a total of 500 individuals and DNA was extracted from the nematode mixture. There was a significant correlation ($r^2 = 0.9355$, $P < 0.001$) between the threshold cycle values and the number of *G. rostochiensis* added. When nematodes were extracted from soils artificially infested with *G. rostochiensis* to various degrees and real-time PCR was conducted using DNA templates from the nematodes extracted, there was a highly significant correlation in the numbers of *G. rostochiensis* J2 from the real-time PCR method and morphological identification. Real-time PCR sensitively detected a single *G. rostochiensis* J2 out of 1,000 individuals of free-living nematodes. Similarly, real-time PCR primers RKNf and RKNr were designed for the detection of the root-knot nematode *Meloidogyne incognita*. This study demonstrated that the real-time PCR assay for the potato-cyst nematode and the root-knot nematode provides a sensitive and reliable means for the rapid quantification of these vermiform pests.

Key words: detection, diagnostics, plant-parasitic nematode, qPCR.

INTRODUCTION

Plant-parasitic nematodes sometimes cause serious damage to different kinds of crops. It has been accepted for decades that the effective control of plant-parasitic nematodes is dependent on agrochemicals (Hague and Gowen 1987). However, there is a growing concern about the use of agrochemicals. As it is known that there is a significant correlation between the initial population density of plant-parasitic nematodes in soil and the degree of damage to the host (e.g. Back *et al.* 2006; Chikaoka 1983; Gugino *et al.* 2006; Koenning 2000; Ohbayashi 1989), reliable and rapid identification and counts of the causal agent is critically important for the successful management of nematode pests. Madani *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 polymerase chain reaction (PCR) primers for *Globodera pallida* and *Heterodera shachtii*. Recently, the real-time PCR primers were reported for *Meloidogyne chitwoodi* and *Meloidogyne fallax* (Zijlstra and van Hoof 2006) and the root-lesion nematode *Pratylenchus penetrans* (Sato *et al.* 2007). However, real-time PCR primers are not available for other important plant-parasitic nematodes, such as the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. Thus, the purpose of this study was to develop a rapid and precise method for the detection and quantification of *G. rostochiensis* and *M. incognita* from mixed nematode communities using real-time PCR.

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MATERIALS AND METHODS

Soils and nematodes used

A soil naturally infested with the potato-cyst nematode *G. rostochiensis* was collected from a farmland in Hokkaido, Japan. From the soil, second-stage juveniles (J2) and cysts of *G. rostochiensis* were collected using the Baermann method and the double-layer centrifugation technique, respectively, based on our previous report (Sato and Toyota 2006). *Caenorhabditis elegans* was maintained on agar plates inoculated with *Escherichia coli* JM109 and used to adjust the number of nematodes in real-time PCR. Eggs of the root-knot nematode *M. incognita* were collected from the diseased roots of sweet potato grown in a field and individuals of *M. incognita* J2 were collected from the eggs using the Baermann method (20–25°C for 2 days). *Pratylenchus penetrans* J2 was collected from a soil naturally infested with the root-lesion nematode using the Baermann method and was used as a reference in the primer check.

Primers

Primers in real-time PCR should be designed to produce 100–200 bp (Takara Bio, Otsu, Japan). Specific primers designed for *G. rostochiensis* by Bulman and Marshall (1997) produce more than 250 bp of PCR product and those for *M. incognita* designed by Saeki *et al.* (2003) produce more than 500 bp. Therefore, these primers are suitable for specific detection, but not for quantitative detection using real-time PCR. Thus, new specific primers were designed in the ITS1 region for *G. rostochiensis* (PCN280f [5′-GCGTCGTTGAGCGGTTGTT-3′] – PCN398r [5′-CCACGGACGTAGCACACAAG-3′]) and for *M. incognita* (RKNf [5′-GCTGGTGTCTAAGTGTT-GCTGATAC-3′] – RKNr [5′-GAGCCTAGTGATCCAC-CGATAAG-3′]). According to the ITS sequences available in the database National Center for Biotechnology (NCBI), the primer sequences of PCN280f and PCN398r showed a perfect match to *Globodera pallida*, *Globodera tabacum*, *Heterodera hordecalis*, *Heterodera latipons*, *Heterodera ustinovi*, *Heterodera zeae* and those of RKNr and RKNr perfectly matched with *Meloidogyne javanica* and *Meloidogyne arenaria*. The specific primers for *M. incognita* were considered not to amplify *Meloidogyne hapla* because of a one base difference in the second position from the 3′ end of the forward primer. A preliminary experiment showed that the specific primers designed for *G. rostochiensis* (PCN280f and PCN398r) did not react to *M. incognita* J2 and *P. penetrans* J2 and those designed for *M. incognita* (RKNf and RKNr) did not react to *P. penetrans* J2 and *G. rostochiensis* J2.

To estimate the potential for other nematodes to be counted as *G. rostochiensis* in real-time PCR assay, nematodes were extracted from an agricultural soil in Kanagawa prefecture, which is considered not to contain *G. rostochiensis*, using the Baermann method, and real-time PCR was done to detect *G. rostochiensis*, using 200, 500 and 1,000 individual nematodes in triplicate as described below.

DNA extraction from nematodes

The DNA was extracted from approximately 200–1,000 individual nematodes using the modified method of Sato and Toyota (2006). Three hundred microliters of a nematode suspension was put into a 2-mL tube with 0.2 g of zirconia beads (0.1 mm in diameter) and then 30 μL of 10× TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) buffer (pH 8.0) was added. The nematode suspension was treated with bead beading at 2,770 g for 90 sec two times (Bead-smash 12; Wakenyaku, Kyoto, Japan) and the tube was placed on ice. Fifty microliters of skim-milk solution (200 mg mL^{-1}) was added to the tube, along with 30 μL of 3 mol L^{-1} sodium acetate, 200 μL of extraction buffer (5 mol L^{-1} NaCl, 0.5 mol L^{-1} Tris-HCl [pH 8.0], and 0.5 mol L^{-1} ethylenediaminetetraacetic acid [pH 8.0]) and 500 μL of chloroform and mixed well. After centrifugation for 15 min at 4°C at 20,350 g (3740; Kubota, Osaka, Japan), 400 μL of the supernatant was transferred to a new tube with 5 μL of glycogen solution (5 mg mL^{-1}), 80 μL of 3 mol L^{-1} sodium acetate and 600 μL of isopropanol. Four hundred microliters of distilled water was added to the tube, mixed well and then centrifuged for 15 min at 4°C at 20,350 g. Four hundred microliters of this supernatant was combined with the first 400 μL of the supernatant. The DNA was precipitated by centrifugation for 15 min at 4°C at 20,350 g, washed with 70% ethanol once and air-dried. Finally, the DNA was dissolved in TE buffer to make a concentration of one individual nematode per 1 μL.

Real-time PCR

Real-time PCR was carried out using a Smart Cycler II (Cepheid; Takara Bio) in a final volume of 25 μL containing 12.5 μL of SYBR Premix Ex TaqTM (Perfect Real Time; Takara Bio), 5 μmol L^{-1} of each primer and 5 μL of template DNA using the manufacturer's recommended conditions (95°C for 10 s, [95°C for 5 s and 60°C for 30 s] × 45 cycles). A single real-time PCR was done in each DNA extract and means from three to six replicates of DNA extract were calculated as threshold cycle number (*Ct*) values. A negative control was also prepared in triplicate using distilled water instead of a DNA template.

Application of real-time PCR

Autoclaved Koganei soil (475 g non-infested soil [dry basis]) was put into a 1-L pot, mixed with 5% (w/w) of soil artificially infested with *G. rostochiensis* and 50 g

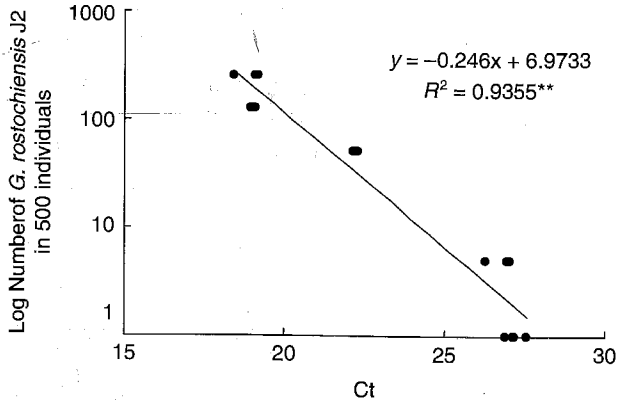

Figure 1 Real-time polymerase chain reaction assay for 500 individuals in nematode mixtures containing different numbers of *Globodera rostochiensis* J2 and various stages of *Caenorhabditis elegans*. *Ct*, threshold cycle value. $^{**}P < 0.01$.

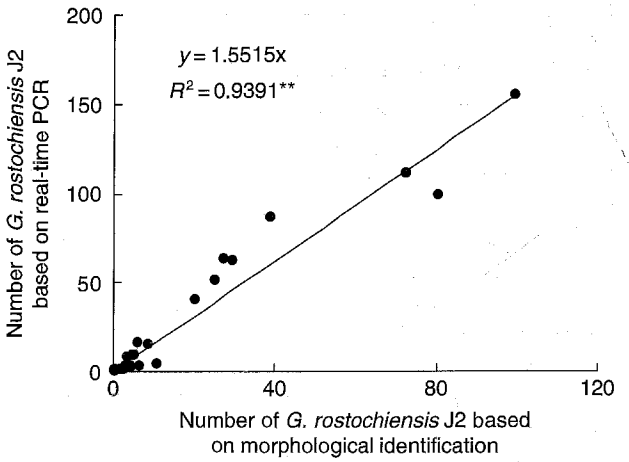

Figure 2 Relationship between the numbers of *Globodera rostochiensis* J2 estimated from morphological identification and from real-time polymerase chain reaction (PCR). $^{**}P < 0.01$.

(dry basis) of OC (Okara and coffee residue) compost was added (pH (H_2O) (1:5), 6.0; electrical conductivity (1:10), 0.35 S m^{-1}; water content, 353 g kg^{-1} fresh weight; ash content, 70.7 g kg^{-1}; total N, 55 g kg^{-1}; P_2O_5, 12 g kg^{-1}; K_2O, 16 g kg^{-1}; CaO, 3.9 g kg^{-1}; MgO, 2.9 g kg^{-1}; Na_2O, 0.2 g kg^{-1}). The soil–compost mixture was adjusted so that the moisture content was 60% of the maximum water-holding capacity and incubated at 20°C (day : night = 16:8 h). Four replicate pots were prepared. After 3 weeks, nematodes were extracted using the Baermann method in triplicate per pot and the DNA was extracted based on the method described above from nematode suspensions in triplicate per pot.

Statistics

The significance of correlation coefficients and differences among mean values were analyzed using correlation analysis and ANOVA protected by Fisher's range test ($P < 0.05$), respectively, using Excel statistics 2002 software (Social Survey Research Information, Tokyo, Japan).

RESULTS AND DISCUSSION

Although the *Ct* value (±standard deviation [SD]) in distilled water was 36.8 ± 0.6, the values in nematode suspensions of 500 individuals containing 1, 5, 50, 125 and 250 individuals of *G. rostochiensis* J2 were 27.2 ± 0.3, 26.8 ± 0.4, 22.2 ± 0.1, 19.0 ± 0.1 and 18.9 ± 0.4, respectively (Fig. 1). There was a highly significant correlation ($r^2 = 0.9355$, $P < 0.01$) between the *Ct* values and the number of *G. rostochiensis* J2. A single J2 of *G. rostochiensis* was mixed with 500 and 1,000 individuals of various stages of vermiform *C. elegans* and DNA was extracted from the mixtures. Although the *Ct* value of 200 individuals of *C. elegans* was 34.4 ± 0.4, the values in 500 and 1,000 individuals of *C. elegans* containing a single J2 of *G. rostochiensis* were 27.2 ± 0.3 and 27.6 ± 0.6, respectively, and the difference in the *Ct* values between the presence and absence of a single *G. rostochiensis* was significant ($P < 0.05$). This sensitive detection was further supported by the melting temperatures for amplicons, 86.4 ± 0.1°C, in nematode samples containing an individual of *G. rostochiensis* J2 compared with the temperatures, 82.9 ± 0.2°C, in nematode samples consisting of only *C. elegans*. The *Ct* values of 200, 500 and 1,000 individuals of nematode communities in a soil were 42.6 ± 1.2, 36.1 ± 0.7 and 34.7 ± 1.9, respectively. The *Ct* values tended to be lower with higher numbers of nematodes, but were markedly greater in samples containing a single J2 of *G. rostochiensis* (27.2–27.6). Thus, it was considered that nematodes other than *G. rostochiensis* may not be counted as *G. rostochiensis* in this real-time PCR assay.

The number of *G. rostochiensis* J2 was estimated based on morphological identification and real-time PCR using the same nematode suspensions collected from soils differently infested with the nematode. There was a significant correlation (Fig. 2; $r^2 = 0.9391$, $P < 0.01$, $n = 26$) between the number of *G. rostochiensis* J2 estimated from the equation in Fig. 1 in the real-time PCR assay and the number based on morphological identification, although the slope was 1.6 rather than 1. One of the possible reasons for this is a difference in the body size of *G. rostochiensis* J2. Although nematodes with a larger size showed lower *Ct* values (Sato *et al.* 2007), we regard a nematode, irrespective of the body size. Another possible reason is experimental error because the correlation equation in Fig. 1 is based on log-transformed

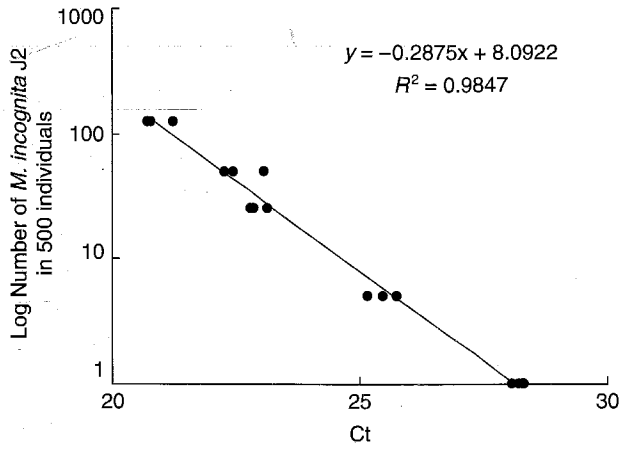

Figure 3 Real-time polymerase chain reaction assay for 500 individuals in nematode mixtures containing different numbers of *Meloidogyne incognita* J2 and various stages of *Caenorhabditis elegans*. *Ct*, threshold cycle value.

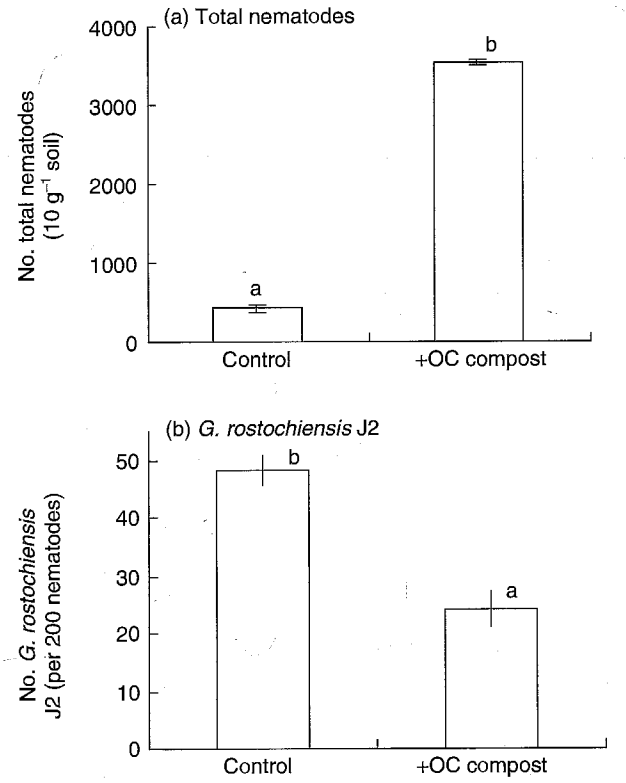

Figure 4 Effect of okara/coffee extraction residue compost on the numbers of (a) total nematodes and (b) *Globodera rostochiensis* J2 estimated from real-time polymerase chain reaction. Data are the means of four replicates (±standard deviation). Data with different letters are significantly different ($P < 0.05$).

data on both the x-axis and the y-axis. Therefore, the equation may better describe the difference in orders of magnitude, but may not be sensitive enough for smaller differences. However, the exact reason for the discrepancy between the estimates using real-time PCR and morphological identification remains to be identified.

When the specific primers for *M. incognita* were applied to a total of 500 individuals containing different numbers of *M. incognita* J2 and *C. elegans*, the *Ct* value in the control (no *M. incognita*) was 34.5 ± 2.8 and the value in nematode suspensions with a single *M. incognita* J2 was 28.2 ± 0. 1, and this difference was significant ($P < 0.05$). There was a highly significant correlation ($r^2 = 0.9847$, $P < 0.01$) between the *Ct* values and the number of *M. incognita* J2 (Fig. 3).

The total number of nematodes was greatly increased by the addition of OC compost (Fig. 4) and the majority of nematodes were free-living based on their morphologies. The number of *G. rostochiensis* J2 estimated from a real-time PCR assay showed a significant decrease with OC compost when the number was expressed as the number of *G. rostochiensis* J2 per 200 individuals. In the morphological identification, to find a single *G. rostochiensis* from a large number of nematodes, like those in the OC-compost treatment (more than 3,000 individuals per 10 g), is tedious work, but real-time PCR was able to detect a single *G. rostochiensis* J2 from 1,000 nematodes. Therefore, quantification of *G. rostochiensis* or *M. incognita* using real-time PCR may be a powerful tool for samples containing a large number of nematodes.

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