キタネグサレセンチュウ（Pratylenchus penetrans）のReal-time PCRによる検出

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Detection of the root-lesion nematode, Pratylenchus penetrans (Cobb), in a nematode community using real-time PCR

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Pratylenchus penetrans causes damage to different vegetables such as radish, carrot, burdock, lettuce, butterbur, and strawberry (Chikaoka, 1983) and is considered as one of the most important nematode pests for Japanese agriculture. Because it is known that there is a significant correlation between the population density of P. penetrans in soil and degree of damage on the host (Chikaoka, 1983; Ohbayashi, 1989), reliable and rapid identification and counts of the causal agent is critically important for successful management of the nematode pest. Mandani et al. (2005) also emphasized that precise identification and knowledge about the density of nematodes in field soil are necessary to develop effective integrated pest control. However, identification of nematodes based on their morphological characteristics is sometimes difficult, especially for inexperienced nematologists. In addition, to detect a single P. penetrans individual in a sample with an abundant number of nematodes is tedious and time-consuming.

Recently, characteristic 18S rDNA has been used as a biomarker to identify soil nematodes (Foucher and Wilson, 2002; Floyd et al., 2005; Bhadury et al., 2005; Madani et al., 2005; Jones et al., 2006). Specific primers are also reported for differentiating Pratylenchus spp. (Uehara et al., 1998; Waeyenberge et al., 2000) and these are useful to identify P. penetrans if DNA is extracted from individual nematodes as well as to estimate the presence of P. penetrans in a nematode community consisting of a variety of species. However, these primers are not suitable to quantify P. penetrans in a complex community. Thus, the purpose of this study was to develop a rapid and precise method for the detection and quantification of P. penetrans in a nematode community using real-time PCR.

MATERIALS AND METHODS

The ITS1 region from P. penetrans was selected for specific primers to the nematode species and the primer set named NEGf (5'-ATTCGTCCGTGGTTGCTA-3', 21 mer) and NEGr (5'-GCCGAGTGATCCACCGATA AG-3', 21 mer) was designed (expected amplified fragment length is 134 bp). The sequences of the primers were compared with the ITS1 region of P. coffeae obtained from the NCBI database. Because the 3rd and 4th positions from the 3' end of the forward primers were totally different between P. penetrans and P. coffeae, this primer set was considered to amplify DNA only from P. penetrans. Indeed, it was confirmed that the primer set didn't amplify the DNA extracted from a single of P. coffeae (data not shown). The nematodes tested were extracted by the Baermann method from 10 g of soil (wet basis) for 2 days at room temperature (20 to 25°C). The soil was collected from a field in the Kanagawa Prefecture Agriculture Center (Hiratsuka City, Kanagawa, Japan), in which
damage caused by *P. penetrans* on radishes was observed. The presence of *P. penetrans* was confirmed by microscopic observation of the extracted nematodes. The DNA was extracted from about 200 vermiform nematode individuals using a method modified from Sato and Toyota (2006). About 200 μl of a nematode suspension containing about 200 individual nematodes was put into a 2 ml tube with 0.75 g of zirconia beads (0.1 mm in diameter), 0.25 g of glass beads (0.5 mm in diameter) and then 800 μl of lysis buffer (0.5% SDS, 100 mM Tris, 300 mM EDTA, pH 8.0) was added. The nematode suspensions were treated with bead beating at 5,000 rpm for 1 min twice, then 700 μl of the supernatant was transferred to a new 2 ml tube, and 377 μl of 5 M NaCl and 270 μl of 10% CTAB were added. After 10 min incubation at 60°C, 500 μl of chloroform was added and the tube was centrifuged at 12,000 rpm for 20 min. The supernatant was transferred to a new 2 ml tube and 700 ml of lysis buffer was added to precipitate, and the extraction process was repeated. Isopropanol and 5 M ammonium acetate were added to the combined supernatant at 60% and 10% of the combined supernatant volume, respectively, and then glycogen was added for a final concentration of 50 μg ml⁻¹. The resulting mixture was centrifuged at 15,000 rpm for 20 min at 4°C. The precipitated DNA was dissolved in 500 μl distilled water and 300 μl of 20% PEG solution was added. The tube was kept for 15 min at 4°C and centrifuged at 15,000 rpm for 20 min at 4°C and then washed with 70% ethanol. After air drying of the DNA precipitate, 200 μl of TE buffer was added. This solution was considered to contain DNA derived from the equivalent of 5 nematode individuals per μl. DNA extraction was done in triplicates, that is, a total of 600 nematodes (200 × 3) were used.

Real-time PCR was performed using a Smart Cycler® II (Cepheid) in a final volume of 25 μl containing 12.5 μl of SYBR® Premix Ex Taq™ (Perfect Real Time, Takara), 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 30 sec) × 45 cycles). A single real-time PCR was done in each DNA extract and means of triplicate DNA extracts were calculated as Ct (the threshold cycle number) values. A negative control was also prepared in triplicate using distilled water instead of a DNA template.

In the first experiment, the relationship between DNA concentration and Ct values was estimated. DNA was extracted from 100 individuals of *P. penetrans* containing mixed developmental stages (juveniles, males and females) in duplicates, diluted to 1/10, 1/20, 1/40, and 1/100 and then used as templates for real-time PCR. The PCR was done in duplicates for each DNA template and the mean values were used for regression analysis.

In the second experiment, 1, 5, 25, 50, or 100 early stage *P. penetrans* (mostly comprised of J2) juveniles were picked up with a needle from the nematodes extracted from soil and added to a water suspension containing free-living nematodes to adjust the total number of nematodes used for DNA extraction to 200 individuals. The free-living nematodes were extracted from a compost (a mixture of municipal bio-waste and paper). The DNA extracted from these nematode suspensions was used for real-time PCR and it was also used for denaturing gradient gel electrophoresis (DGGE) analyses to confirm the usefulness of real-time PCR in terms of quantification. DNA was extracted in three, five, three, two, or two replicates for nematode samples containing 1, 5, 25, 50, or 100 early stage juveniles of *P. penetrans*, respectively, and real-time PCR was singly done for each DNA extract. PCR amplification for DGGE was performed using the same template DNA in a 25 μl volume con-
taining 5 μl template DNA, 5 μl of 5 × PrimeSTAR™ buffer (Mg²⁺ plus; Takara), 200 μM each dNTP, 0.5 μM each primer (SSU18A and SSU9R+GC clamp; Oba et al., 2006) and 0.24 U of PrimeSTAR™ HS DNA polymerase (Takara). The temperature program was as follows: a denaturing step at 98°C for 3 min, followed by 30 cycles of 98°C for 10 sec, 54°C for 15 sec, 72°C for 40 sec, and final extension step of 72°C for 10 min. DGGE was performed using a Bio-Rad Dcode™ mutation analysis system (Bio-Rad). Electrophoresis was done using a 6% (w/v) polyacrylamide gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8) under 75 volts at 60°C for 16 hr. The polyacrylamide gels were made with parallel denaturing gradients 15-50%, 100% denaturant contains 7 M urea and 40% formamide. Duplicate DNA extracts with lower Ct values among two to five replicates were used for DGGE analysis. For a negative control, DNA was extracted in duplicates from 200 individual nematodes only from the compost and used as a template.

In the third experiment, the detection limit in the presence of abundant numbers of nematodes was examined for a single P. penetrans J2. The number of the free-living nematodes extracted from the compost, which contained no P. penetrans, was adjusted to 200, 500 or 800 individuals and then a single P. penetrans was added into the nematode suspension. DNA was extracted in triplicates from these nematode suspensions and real-time PCR was done singly for each DNA extract. Data analyses were done using Excel Statistic 2002 for Windows® (SSRI).

RESULTS AND DISCUSSION

The primer set NEGf and NEGr successfully amplified the target fragment, 134 bp, from DNA extracted from a nematode suspension containing P. penetrans (data not shown). Ct values of 1/10, 1/20, 1/40, and 1/100-diluted DNA samples were 20.1 ± 0.3 (Mean ± SD), 20.9 ± 0.2, 21.8 ± 0.2, and 23.1 ± 0.2, respectively, and the relationship between the Ct values and dilution rates of P. penetrans DNA showed highly significant linearity ($R^2 = 0.9806$, $P < 0.001$) (Fig. 1).

The Ct value of the negative control was 34.5 ± 1.2, while that of 200 nematode individuals containing a single P. penetrans was 27.6 ± 0.5, suggesting that this real-time PCR successfully detected a single P. penetrans from 200 nematodes (Fig. 2). Ct values of the nematode

![Fig. 1. The relationship between the Ct values and dilution rates of P. penetrans DNA. A: PCR growth curve. Values indicate DNA dilution ratios, N.C. indicates negative control. B: Regression of Ct to dilution rate obtained in the real-time PCR assay for P. penetrans (***, $P < 0.01$, x: the dilution rate of P. penetrans DNA, y: Ct).](image-url)
suspensions containing 5, 25 or 100 juveniles of *P. penetrans* decreased to 25.5 ± 0.6, 24.8 ± 0.4 and 23.3 ± 0.3, respectively. There was a significant regression of the Ct values to the number of *P. penetrans* in 200 nematodes ($R^2 = 0.8471$, $P<0.001$). The results of PCR-DGGE using the same templates as the real-time PCR showed that the *P. penetrans*-derived band was not detected in the PCR-DGGE in the case of 200 individual nematodes containing a single *P. penetrans* (Fig. 3). According to Gelsomino et al. (1999), specific communities constituting more than 0.5 to 1.5% of the total bacterial community were detectable in PCR-DGGE analysis, suggesting that detection of a single *P. penetrans* in a community containing 200 individual nematodes is difficult in DGGE analysis, while real-time PCR is effective to detect such a low ratio of

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Fig. 2. Real-time PCR assay for 200 nematodes containing 1, 5, 25 or 100 *P. penetrans* juveniles. Solid line in the graph indicates regression line (**P < 0.01, x: the number of *P. penetrans* juveniles, y: Ct) and broken lines indicate the 95% confidence range.

Fig. 3. PCR-DGGE analysis of 200 nematodes containing 1, 25 or 50 juveniles of *P. penetrans*. ‘Std’ lanes are reference lanes of clones of soil fauna including mainly nematodes. Bands A to F in the Std lanes were generated from (A) *Laaptgastar* sp., (B) *Acrobeloides apiculatus*, (C) *Aphelenchus* sp., (D) *Pratylenchus penetrans*, (E) *Aporcelaimellus obtusicaudatus* and (F) *Prismatolaimus intermedius*. 
a specific nematode.

Higher variations were detected in DNA samples extracted from different numbers of *P. penetrans* (Fig. 2) than in simply diluted DNA samples (Fig. 1). Two possible reasons should be considered. One might be the variation in the extraction and purification of DNA from nematode suspensions. When the DNA purification step using PEG was omitted, variation in Ct was higher, suggesting that certain inhibitors are contained in the DNA extracts (data not shown). Another possibility might be related to the developmental stages of *P. penetrans*. In a preliminary experiment in which Ct values were compared among a male and a female and a small juvenile of *P. penetrans*, the Ct values of the male (22.7 ± 0.7) and female (23.0 ± 0.3) were significantly lower (*P* < 0.05) than those of the small juvenile (25.1 ± 0.4).

Ct values of a single *P. penetrans* in the presence of 200, 500 or 800 free-living nematodes were almost the same (29.6 ± 2.1 for 200, 29.6 ± 4.5 for 500 and 29.7 ± 1.2 for 800 individual nematodes), while the negative control was 36.8 ± 1.0 (data not shown). The Ct values of a single *P. penetrans* in the presence of 200, 500 or 800 individuals of free-living nematodes were significantly lower than the negative control. Thus, it was considered that the real-time PCR assay can detect a single *P. penetrans* even in the presence of 800 individual nematodes.

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**LITERATURE CITED**


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