線虫溶液を用いた簡易ハイスループットDNA抽出法のリアルタイムPCR法への適用

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[Original Article]

Application of a simple and high-throughput DNA extraction method to real-time PCR quantification of target plant-parasitic nematodes in nematode communities

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Molecular techniques are prevailing in nematode identification and quantification, for which DNA extraction from nematodes is essential. However, the published DNA extraction methods are laborious and often require various expensive consumables and high-end equipment. In order to prepare DNA templates for a high-throughput real-time PCR assay, the present study modified a conventional DNA extraction method of Naklha *et al.* (2010) from nematode suspensions with ordinary lab equipment and achieved results and advantages comparable with two other conventional methods. The results of real-time PCR assays for quantifying *Pratylenchus zeae*, *Tylenchorhynchus leviterminalis* and *Hoplolaimus* sp. using the new protocol were highly correlated with those obtained by morphological counts and comparable to or more sensitive than those obtained by two conventional methods. Although the new protocol took over 100 min for DNA extraction, the manual processing took less than 10 min, *i.e.*, half to one-fourth of the other methods. The running cost was less than half to one-tenth of the other methods. Nematol. Res. 48(1), 1–10 (2018).

Key words: bead-beating, low-cost, nematode suspension, nematode quantification, proteinase K

INTRODUCTION

Plant-parasitic nematodes cause annual crop losses of $125 billion throughout the world (Chitwood, 2003). Such losses are much greater in the developing countries than in the developed countries (Chitwood, 2002). Accurate nematode identification is crucially important to control plant-parasitic nematodes in farmlands and to develop efficient farmland management using chemical, biological, physical and cultural means (Siddiqi, 1997). Morphological identification of nematodes extracted from soil is commonly used to differentiate and count nematodes. Despite its popularity, such morphological identification is time-consuming and sometimes difficult to achieve mainly due to subtle difference among nematode species (Kushida 2013; Pridannikov *et al.*, 2015) and the presence of cryptic species (Archidona-Yuste *et al.*, 2016). This approach also requires trained specialists for accurate nematode identification (McCuiston *et al.*, 2007).

To overcome time consuming and specialist-required procedures in nematode identification with morphological techniques, molecular-based approaches have been developed over the last couple of decades. Among them, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and other PCR-based techniques targeting nematode ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA) are very reliable and have been reported in several major plant-parasitic nematode genera such as *Heterodera*, *Globodera*, *Meloidogyne*, and *Pratylenchus* (Orui, 1996; Mizukubo *et al.*, 1997; Orui, 1997; Orui, 1998; Uehara *et al.*, 1998; Orui and Mizukubo, 1999; Mizukubo *et al.*, 2003). DNA barcoding (Blaxter, 2004) is also very powerful for identifying nematode species as increasing genetic information.

More recently, real-time PCR techniques, which have a double advantage, *i.e.*, quantitative capability and sensitivity, have become available for a wide variety of nematode species, including *Heterodera glycines* (Goto *et al.*, 2009), *H. schachtii* (Madani *et al.*, 2005), *Globodera pallida* (Madani *et al.*, 2005), *G. rostochiensis* (Toyota *et al.*, 2008), *Meloidogyne javanica* (Berry *et al.*, 2008), *M. incognita* (Toyota *et al.*, 2008), *P. neglectus* (Yan *et al.*, 2013), *P. penetrans* (Sato *et al.*, 2007), and *P. zeae* (Berry *et al.*, 2008; Kawanobe *et al.*, 2015). A real time PCR assay uses a specific primer set for the target nematode species and provides a more sensitive result than that from microscopic identification of nematodes

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extracted by the Baermann method (Min *et al.*, 2012). Thus, this technique enables easier and more exact quantification of nematodes.

Molecular-based approaches generally cost much, although from a practical viewpoint, low-cost nematode diagnosis is essential to implement proper nematode management and contribute to improved productivity of farmlands. For this, high-throughput and low-cost nematode diagnosis is in great demand. Sato and Toyota (2006) and Sato *et al.* (2009) reported a DNA extraction method using bead-beating and chloroform for nematode community analysis. Furthermore Okada and Oba (2008) reported a DNA extraction method from nematode suspensions using bead-beating and a commercial DNA purification column kit. These DNA extraction methods have been widely used especially for PCR-denaturing gradient gel electrophoresis (DGGE) analysis of nematode communities (Takemoto *et al.*, 2010; Wada *et al.*, 2011; Bao *et al.*, 2012; Kushida, 2013). The drawbacks for these methods, however, were their lengthy procedures and usage of expensive machines (high-end bead-beater and high-speed centrifuge) and various chemicals, which makes them unsuitable for high-throughput real-time PCR analysis, especially when the number of samples is very large. Nakhla *et al.* (2010) reported a rapid DNA extraction method from nematodes using bead-beating and proteinase K for multiplex real-time PCR assays, although it lacked detailed information such as the volume of beads and the beating speed. This method, however, was applied to only one or two nematode species coexisting in a DNA sample, and thus there was no information on the validity of the method to quantify the target nematodes in complex soil nematode communities. In addition, the method gave relatively high cycle threshold (Ct) values (= low sensitivity), raising questions about its applicability to nematode samples containing a few target nematodes within a community with a large number of non-target nematodes.

This study uses a 3-step approach, which firstly extracts nematodes from soil to avoid PCR inhibitors, secondly extracts DNA from the nematode suspension containing complex nematode species, and finally quantifies target nematodes by using real-time PCR. Among these, we focused on the second step and aimed to develop a simple, low-cost and high-throughput DNA extraction method for complex nematode communities, designed for preparing the templates of a real-time PCR assay.

In this study, we developed a DNA extraction method by modifying the method of Nakhla *et al.* (2010), and tested its validity by two experiments in comparison with two previously reported methods. Firstly, average DNA yields per nematode were compared among the three DNA extraction methods, and their real-time PCR sensitivities were examined. Secondly, calibration curves for three target nematode species were developed using the templates obtained by the newly developed DNA extraction method.

MATERIALS AND METHODS

Nematode samples:

Nematodes were extracted from soil collected from sugarcane fields in Okinawa, Japan with the Baermann funnel method (room temperature, 72 h) (12 replicates for Experiment 1 and 25 replicates for Experiment 2). The nematode suspension in 50 mg/l of polyoxyethylene (20) sorbitan monolaurate was placed on a counting slide, and the nematodes were classified and counted under a stereo-microscope (SZX10, Olympus) based on their morphological characteristics (Japan Plant Protection Association, 2004; Decraemer and Geraert, 2013; Duncan and Moens, 2013). The identities of three plant-parasitic nematode species, *Pratylenchus zeae*, *Tylenchorhynchus leviterminalis*, and *Hoplolaimus* sp., were confirmed by using the previously developed real-time PCR method (Kawanobe *et al.*, 2015). The nematodes were then carefully transferred into a 1.5-ml tube and it was confirmed under a stereo microscope that there were no nematodes left on the slides. In a preliminary study, the soil samples we collected generally contained more than 10 free-living nematode species including *Acrobeles* sp., *Acrobeloides* sp., *Aphelenchoides* sp., *Aphelenchus* sp., *Distolabrellus* sp., *Geomonhystera* sp., *Prismatolaimus* sp., *Rhabditis* sp., *Tylencholaimus* sp. and other Dorylaimida species, in addition to at least one of the plant-parasitic nematode species mentioned above.

DNA extraction:

The nematode suspension in a 1.5-ml tube was divided into three equal parts, each of which was transferred into a new 1.5 ml tube, followed by centrifugation at 6,797×*g* for 2 min. The aliquots then served for DNA extraction by the following three methods, *i.e.*, A after Sato *et al.* (2009), B after Okada and Oba (2008) with minor modifications, and C after Nakhla *et al.* (2010) with several modifications described below (Tables 1 and 2). After centrifugation, the supernatant was carefully removed by a pipette to leave

the designated volumes (A: 200 µl, B: 40 µl, and C: 25 µl of nematode suspension) for each DNA extraction method. Under the method C, a nematode suspension was mixed in a 0.2-ml PCR tube with 5 glass beads (1 mm in diameter, Sigma-Aldrich) and 25 µl of 2× lysis buffer, modified from Black *et al.* (1992), comprising 20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 2% IGEPAL® CA-630 (nonionic detergent, MP Biomedicals), and 200 µg/ml proteinase K (Kanto Chemical Co., Inc.). Two of the tubes were placed into a 15-ml Falcon tube stuffed with cotton at the bottom to hold them tight, and the contents were homogenized with bead beating at 4.0 m/sec for 30 sec using FastPrep 24 (MP Biomedicals). This protocol was designed based on our preliminary experiments, in which we examined bead type (glass and zirconia), bead numbers, bead size, beating time, and the effect of ethanol DNA precipitation after extraction (data not shown). The 0.2 ml tubes were kept at –20°C for 30

Table 1. Required equipment and consumables of the three DNA extraction methods, (A) Sato *et al.* (2009), (B) Okada and Oba (2008) and (C) the modified version of Nakhla *et al.* (2010).

A: Sato *et al.* (2009)	B: Okada and Oba (2008)	C: the modified version of Nakhla *et al.* (2010)
Nematode suspension and DNA extract/sample		
200 µl and 100 µl	40 µl and 500 µl	25 µl and 50 µl
Plastic and bead (prepared in advance)/sample		
2-ml screw cap tube with 0.2 g of ϕ0.1 mm zirconia beads; 1.5 ml tube	2-ml screw cap tube with 0.1 g of ϕ0.1 mm glass and 4 ϕ 1.2 mm zirconium silica beads; 1.5 ml tube	0.2-ml PCR tube with 5 ϕ1 mm glass beads
Chemical and commercial kit/sample		
20 µl of 10xTE (100 mM Tris-HCl, 10 mM EDTA, pH 8.0) 50 µl of skim milk solution (200 mg/ml) 120 µl 3 M NaOAc 200 µl of extraction buffer (5 M NaCl, 0.5M Tris-HCl, 0.5 M EDTA) 500 µl chloroform 8 µl of glycogen (5 mg/ml) 600 µl of isopropanol 500 µl of 70% ethanol Nuclease free water	Wizard SV Genomic DNA Purification System (Promega) 50 µl of skim milk solution (200 mg/ml) 50 µl of EDTA (0.5 M, pH 8.0)	25 µl lysis buffer (final concentration of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1% IGEPAL® CA-630, 100 µg/ml proteinase K)
Special equipment		
High-speed homogenizer (6.5 m/sec); over 20,000 g refrigerated centrifuge at 4℃	High-speed homogenizer (6.5 m/sec); 13,000 g centrifuge; –80℃ freezer	Homogenizer (4.0 m/sec) x 30 sec; thermal cycler

Table 2. Comparative chart of the three DNA extraction methods, (A) Sato *et al.* (2009), (B) Okada and Oba (2008) and (C) the modified version of Nakhla *et al.* (2010).

A: Sato *et al.* (2009)	B: Okada and Oba (2008)	C: the modified version of Nakhla *et al.* (2010)
DNA extraction time (manual process)		
74 min (24 min: 12 steps x 2 min)	72 min (40 min: 20 steps x 2 min)	108 min (8 min: 4 steps x 2 min)
Overall DNA extraction process (machine running time in bold letters)		
1 Nematode suspension into a tube with beads and relevant buffer	1 Nematode suspension into a tube with beads and relevant buffer	1 Nematode suspension into a tube with beads and relevant buffer
2 Homogenization with beads (**3 min**)	2 Freeze at –80℃ (15 m)	2 Homogenization with beads (**0.5 min**)
3 Mix with buffers	3 Homogenization with beads (**2.5 min**)	3 Freeze at –20℃ (**30 min**)
4 Centrifugation (**15 min**) at 4℃	4 Mix with a buffer	4 Enzyme reaction and inactivation (**70 min**)
5 Supernatant into a tube with buffers	5 Centrifugation (**1 min**)	
6 Centrifugation (**15 min**) at 4℃	6 Supernatant into a mini-column	
7 Second supernatant into the tube	7 Centrifugation (**3 min**)	
8 Centrifugation (**15 min**) at 4℃	8-15 Wash the column with solution Centrifugation (**1 min**) Repeat the above two steps 4 times	
9 Ethanol purification		
10 Centrifugation (**2 min**) at 4℃		
11 Air dry	16 Centrifugation (**3 min**)	
12 Dissolvation with nuclease free water (NFW)	17 Set column on a tube with NFW	
	18 Centrifugation (**1 min**)	
	19 Further elution with NFW	
	20 Centrifugation (**3 min**)	
Approx. costs* per1,000 preps (* Plastics, beads, reagents and chemicals only. Machinery, utilities and labor are not included.)		
> *ca.* x2	> *ca.* x10	x1

min, and incubated at 65°C for 1 h for proteinase K reaction, and then at 98°C for 10 min to inactivate the enzyme. The methods A, B, and C finally yielded 100 µl, 500 µl, and 50 µl of DNA extracts, respectively. All DNA extracts thus obtained were frozen at –20°C until further analyses. The procedural time required for each of the three DNA extraction methods was measured for both total time and manual process time as simple accumulation. Further, costs required for 1,000 preparations using each of the three methods were roughly estimated based on running costs, including plastic disposables, reagents and chemicals (not including machinery, utilities, and labor).

Protocol of real-time PCR assays:

Using the DNA templates diluted as indicated in each experiment, real-time PCR assays were performed with a Step One™ Real-Time PCR System (Life Technologies) following Kawanobe *et al.* (2015). A final sample volume of 10 µl contained 5 µl of Fast SYBR Green Master Mix (Life Technologies), 0.4 µM of each primer, 2.2 µl of distilled water, and 2 µl of template DNA. The manufacturer's recommended conditions were used with slight modifications (95°C for 10 sec, 40 cycles of (95°C for 5 sec, and 62°C for 20 sec), and melting curve profiles were generated). A negative control was prepared with distilled water instead of a DNA template. The specific primer sets for *P. zeae*, *T. leviterminalis*, and *Hoplolaimus* sp. were Pzeae, Tlevi2, and Hoplo, respectively, all of which were reported by Kawanobe *et al.* (2015). The specificity, linearity and sensitivity of these primer sets were confirmed using serially diluted DNA extracted from a respective single nematode in Kawanobe *et al.* (2015).

Experiment 1: Average DNA yields per nematode and real-time PCR sensitivity of DNA templates by using each of three methods:

After counting total nematodes, DNA was extracted from one-third of each nematode suspension using each of three methods, in 6 replicates. Each sample for DNA extraction contained a total of 100 to 400 nematodes. DNA concentrations were determined with a Qubit® 3.0 fluorometer (Invitrogen) following the manufacturer's instructions. Average DNA yields per nematode were compared among the three DNA extraction methods.

In addition to the 6 DNA samples shown above, another 6 replicates of DNA samples were extracted from the nematode suspension using each of the three DNA extraction methods. The number of nematodes in each sample (12 replicates in total for each method) was 100 to 400 in total, including 10 to 50 *P. zeae* and 25 to 130 *T. leviterminalis*. *Hoplolaimus* sp. was not tested due to limited numbers (mostly, 0 to 5/sample) of the species in these samples. The DNA extracts were diluted equivalent to 500 µl/sample (extraction methods A and C), 5,000 µl/sample (A and B), and 50,000 µl/sample (all three methods) with distilled water. Since method A showed the highest DNA yield and was the first reported DNA extraction method for nematode community analysis, it was used as the benchmark in this experiment.

Experiment 2: Development of calibration curves using the newly developed DNA extraction protocol:

Each nematode suspension contained *ca.* 100 to 2,000 total nematodes including 1 to 500 of *P. zeae* (25 samples), 1 to 900 of *T. leviterminalis* (22 samples), and 1 to 400 of *Hoplolaimus* sp. (15 samples). DNA was extracted from each suspension following the method C. DNA samples were diluted by 1,000 times (DNA concentration equivalent to 50,000 µl/sample) with distilled water, and used as DNA templates for real-time PCR assays.

Statistical analysis:

The statistical difference was analyzed by one-way ANOVA. When ANOVA was significant, *post-hoc* pairwise comparisons were conducted using Tukey's test. Pearson's correlation coefficient analysis and simple regression were used to assess the relationships between nematode density ($\log_2$) and Ct value. Analysis of covariance (ANCOVA) was used to test the difference between the two regression slopes and intercepts following Ichihara (1990). Amplification efficiency (AE) of each primer set was determined by $AE = 10^{(-1/slope)} - 1$ following Adams (2006). The slope used for the AE calculation was based on the regression analysis between nematode density ($\log_{10}$) and Ct value. Statistical analyses were conducted with Microsoft Excel and its add-in software Statcel (3rd ed., OMS).

RESULTS

Experiment 1: DNA yields and real-time PCR sensitivity:

The average amount of DNA per nematode extracted by the method A (Sato *et al.*, 2009) was 1.2 ng/nematode, which was significantly more than that by the methods B (Okada and Oba, 2008; 0.3 ng/nematode; $P < 0.01$) and C (modified Nakhla *et al.*, 2010; 0.6 ng/nematode; $P < 0.05$) (Fig. 1).

Simple regression analysis in *P. zeae* ($\log_2$) at the

dilution of 500 µl/sample showed that the method C resulted in –1.31 of the slope, relatively approximate to –1 (the theoretical value) and a lower intercept (= higher sensitivity) compared with those of the method A (Table 3). The slopes and the intercepts were different ($P = 0.075$ and $P < 0.01$, respectively) between the methods A and C (Table 3). At the dilution of 50,000 µl/sample, all of the three methods were comparable, but the regression line of the method C remained the lowest among the three methods, and showed an improved approximation slope toward –1 at the dilution of 50,000 µl/sample from that of the 500 µl/sample (Table 3). The slopes and the intercepts of the method C at the dilutions of 500 µl and 50,000 µl/sample did not show any significant difference (Table 3).

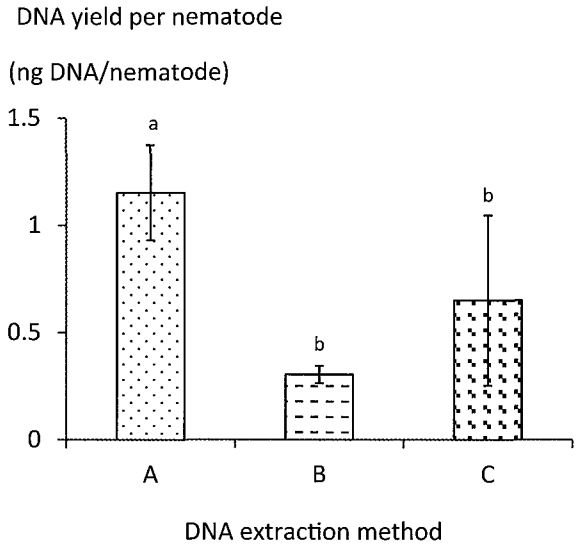

Fig. 1. Average DNA yield (ng DNA/nematode) extracted from nematode communities containing from 100 to 400 nematodes that naturally infested soils collected from Okinawa. The DNA extraction methods used were A: Sato *et al.*, 2009; B: Okada and Oba, 2008; and C: the modified version of Nakhla *et al.*, 2010. Different letters for each parameter indicate significant difference ($P < 0.05$).

Similar results for the slopes and the intercepts were observed in *T. leviterminalis* experiments (Table 3). The slope at 500 µl/sample using the method C was –0.89, relatively approximate to –1 compared to that of the method A (no statistical significance), while the intercept using the method C was significantly ($P < 0.05$) lower than that of the method A. At the dilution of 50,000 µl/sample, the regression line of the method C stayed at the lowest among the three methods. The regression lines in the method C showed an improved approximation slope toward –1 at the dilution of 50,000 µl/sample from that of the 500 µl/sample. While the slopes of the method C at the dilutions of 500 µl and 50,000 µl/sample were not significantly different, the intercepts were significantly ($P < 0.05$) higher in the 50,000 µl/sample than in the 500 µl/sample. Our preliminary study also showed that the dilution of 1,000 times (the final volume of 50,000 µl/sample) was the most stable in the real-time PCR assays compared with the other dilutions from 2 times (100 µl/sample) to 10,000 times (500,000 µl/sample) (data not shown).

The method B did not show any results different

Table 3. Comparison of the regression lines (slopes and intercepts) for the relationship between the number (Log_2) of nematodes (*Pratylenchus zeae* and *Tylenchorhynchus leviterminalis*) and Ct value by using the three different DNA extraction methods at different dilution levels.

Species	Volume/ sample	Method[a]	Regression line[b]		
			slope	intercept	*P* for slope
P. zeae	500 µl	A	–2.51	41.98	< 0.001
		C	–1.31	32.63*	< 0.01
	5,000 µl	A	–2.27	43.39	< 0.01
		B	–1.21	37.93	< 0.01
	50,000 µl	A	–0.54	36.79	< 0.05
		B	–1.22	40.89*	< 0.01
		C	–0.98	36.70	< 0.10
T. leviterminalis	500 µl	A	–1.98	34.4	< 0.01
		C	–0.89	25.87*	< 0.01
	5,000 µl	A	–1.95	36.68	< 0.05
		B	–0.86	30.31	< 0.01
	50,000 µl	A	–0.85	31.70	< 0.001
		B	–0.91	34.06	< 0.05
		C	–0.99	31.24	< 0.01

a: DNA extraction methods, A: Sato *et al.* (2009); B: Okada and Oba (2008); and C: the modified version of Nakhla *et al.* (2010). b: Simple regression line was used to assess the relationships between nematode density (log_2) and Ct value under the real-time PCR assay. Asterisk indicates significant difference of the regression line from the method A (ANCOVA, $P < 0.05$).

from thouse of method A for the slope of *P. zeae* at the dilution of 5,000 µl/sample, but lower ($P = 0.075$) for the intercept (Table 3). At the dilution of 50,000 µl/sample, the intercept using the method B for *P. zeae* was significantly ($P < 0.05$) higher than that of the method A, while the slope did not differ significantly (Table 3). As for *T. leviterminalis* at the dilutions of 5,000 µl and 50,000 µl/sample, no significant difference was observed between the methods of A and B (Table 3).

Experiment 2: Calibration curves using the DNA extraction method C:

The results of simple regression analyses for differently diluted DNA samples of *P. zeae*, *T. leviterminalis* and *Hoplolaimus* sp. showed the slopes of –1.05, –1.14, and –1.10, respectively, ($P < 0.001$; Fig. 2-i, ii, and iii), which were close to –1 (the theoretical value). Amplification efficiencies of primer sets, Pzeae, Tlevi2 and Hoplo were 93%, 84%, and 87%, respectively.

Simple procedural and time comparisons:

The manual process for method C was completed within 10 min while those for methods A and B took longer than 20 min and 40 min, respectively (Table 2). The required running costs for methods A and B are twice and 10 times higher, respectively, than that for method C, (our quick back-of-the-envelope calculation, Table 2).

DISCUSSION

In the present study, we developed a DNA extraction method from soil nematodes, the method C, by newly modifying the one originally described by Nakhla *et al.* (2010). The average DNA yield per nematode in method C was approximately half of that in the method A, described by Sato *et al.* (2009), and almost double of that in the method B, described by Okada and Oba (2008) (Fig. 1). In contrast, the results of real-time PCR assays showed that the regression lines of method C for both primer sets of Pzeae (for quantification of *P. zeae*) and Tlevi2 (for *T. leviterminalis*) lay below those of method A for the dilutions of 500 µl and 50,000 µl/sample, indicating a higher PCR amplification efficiency in method C, in spite of a lower DNA yield compared to that of method A. The regression lines of method B at the dilution of 5,000 µl/sample lay below those of method A. At the dilution of 50,000 µl/sample, however, the results were opposite. These inconsistent results (higher DNA yields but lower real-time PCR amplification efficiency) may suggest possible effects of PCR inhibitors that remained after DNA purification in method A. They might also suggest a lower efficiency in the breakup of nematode cuticles and in DNA extraction or any damage in the extracted DNA in method A. Budding *et al.* (2014) discussed the possibility of bead-beating damaging DNA when the amount of target microorganisms (bacteria)

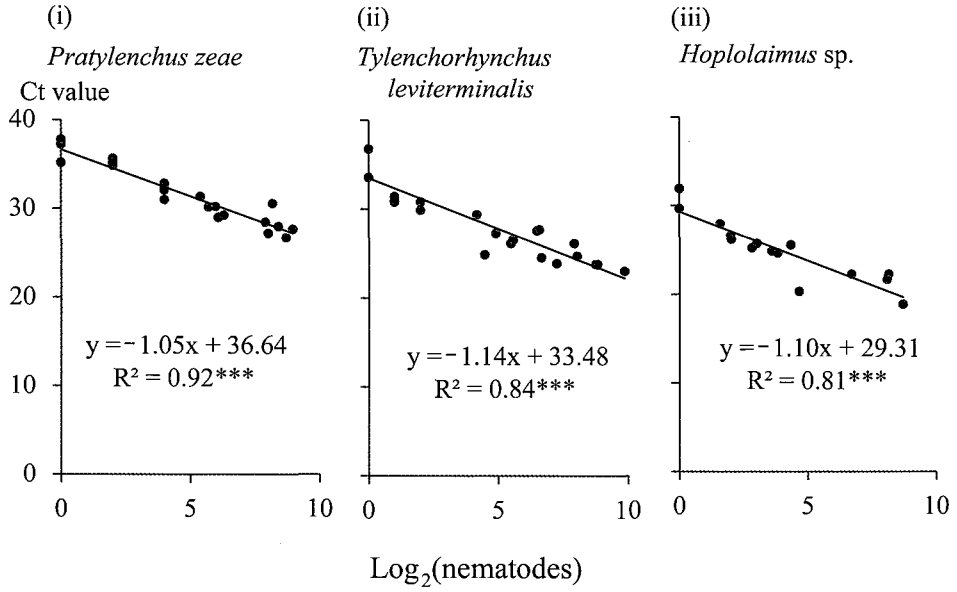

Fig. 2. Relationship in the DNA extraction method C: the modified version of Nakhla *et al.*, 2010 between Ct values and the numbers (Log_2) of each target nematode (50,000 µl/sample) of (i) *Pratylenchus zeae*, (ii) *Tylenchorhynchus leviterminalis*, and (iii) *Hoplolaimus* sp. (***: $P < 0.001$).

was low. Our results suggested that high-speed and long-time beating with high-density beads of smaller diameter, such as zirconia, might have damaged DNA under the methods A and B. The method C employed slow-speed beating and low-density glass beads, and, therefore, DNA in the nematode cells kept more intact and was more easily dispersed in the lysate in the method C than in the other two methods, although the nematode body, itself, was damaged. Indeed, Zhou *et al.* (1996) reported that the conventional 'freezing and thawing' approach, which was also used in the method C, increased DNA yields possibly owing to more disruption of microbial cells. In addition, proteinase K used in the method C might efficiently degrade proteins and inactivate nuclease. Through these procedures, the DNA extracts obtained by the method C might be more sensitive in real-time PCR assays than those obtained by the other two methods.

At the dilution of 50,000 µl/sample, no statistically significant difference was observed in the slope between the methods A and C. Yet, the slopes of the standard curves in the *P. zeae* and *T. leviterminalis* experiments using the method C were equivalent to –0.98 and –0.99, respectively, virtually the same as the theoretically most efficient slope (–1), whereas those using the method A were –0.54 and –0.85. In the method C, the difference in intercepts between the dilutions of 500 µl/sample and those of the 50,000 µl/sample was *ca.* 4.1 for *P. zeae* and *ca.* 5.3 for *T. leviterminalis*, which was smaller than the theoretical difference (6.6) by 2.5 and 1.3, respectively (Table 3). This suggests that the sensitivity of DNA extracts obtained under the method C in real-time PCR assays might be much greater at the dilution of 50,000 µl/sample than at the dilutions of 500 µl/sample. This suggestion is supported by the finding that higher dilutions of crude DNA templates generally avoid the influence of inhibitors (Miller *et al.*, 1999; Wang *et al.*, 2017). Recently, Braun-Kiewnick *et al.* (2016) applied a DNA extraction method which was similar to the method C, but without beads, in the preparation of template DNA for real-time PCR assays. In our preliminary study, however, a DNA extraction method with 5 beads showed more sensitive results (lower Ct values by *ca.* 4, non-statistical comparison) on real-time PCR assays than without beads, suggesting that a treatment using beads itself is useful for the preparation of DNA templates for real-time PCR assays.

Furthermore, this study confirmed the validity of this newly modified DNA extraction protocol for the samples of nematode communities compristing a variety of species (more than 10 species), instead of a few species in a DNA sample described by Nakhla *et al.* (2010). The protocol shown in this study might generate even better results than that of Nakhla *et al.* (2010), although a precise comparison might not be feasible. The Ct value for *G. rostochiensis* in Nakhla *et al.* (2010) was found to be around 30 by using bead-beating and proteinase K, while our previous study (Toyota *et al.*, 2008) using the method A gave a Ct value of around 27 for *G. rostochiensis*. Therefore, the method given in Toyota *et al.* (2008) should be 8 times more sensitive in real-time PCR assays than that in Nakhla *et al.* (2010), even without adjusting the concentration of DNA templates: the templates used in Toyota *et al.* (2008) were 2–4 times more diluted than those in Nakhla *et al.* (2010). Although we need to examine all factors including not only DNA extraction methods but also real-time PCR protocols for precise comparisons, the above quick analysis suggests that the method C used in the present study, including the real-time PCR protocol, could be 8 times more sensitive than that in Nakhla *et al.* (2010).

In this study, real-time PCR assays were performed by using DNA samples extracted from 100 to 2,000 nematodes in total containing 1 to over 400 of each target nematode species. The assay with primer sets for *P. zeae*, *T. leviterminalis*, and *Hoplolaimus* sp. succeeded in proper quantification, and even a single nematode of these 3 nematode species was properly detected even in a 2 µl DNA template of 50,000 µl/sample. Further analyses might be essential to prove the real-time PCR sensitivity with different primers and target nematodes, since the copy number of target DNA sequences can vary significantly among different nematode species. The results in the real-time PCR sensitivities were consistent with those of Koyama *et al.* (2016), who also detected the target nematodes, three *Pratylenchus* species, from 2 µl DNA templates of 50,000 µl to 100,000 µl/sample derived from nematode individuals, suggesting the appropriateness of our protocol for DNA extraction.

This study provides a low-cost, less-laborious, and high-throughput protocol for generally time sensitive DNA extraction. Most of the process time required for our method is for freezing in an ordinary refrigerator, and activating and inactivating the enzyme in a low-end thermal cycler. These procedures do not require continuous monitoring by a researcher. As the manual process takes a relatively long time for the methods A and B, these methods are not appropriate for high-throughput assays. Furthermore, the running cost required for each of the methods A and B is much higher

than that of the method C. This is mainly because the methods A and B use a variety of chemicals, a commercial kit, and expensive beads and tubes. Considering the costs of the high-end equipment required for the methods A and B, the cost advantage of the method C may be much larger.

In conclusion, the method C, which was newly modified in this study generated DNA extracts from nematode suspensions that were more sensitive as templates for real-time PCR assay than the other two conventional methods A and B. This method should also be suitable for high-throughput real-time PCR assays.

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英文報文（原著論文・短報・研究資料）の和文摘要

線虫定量において遺伝子解析手法が広く使われるようになった。遺伝子解析では線虫からの遺伝子抽出が必須であるが、これまで一般に使われてきたDNA抽出法は手間がかかり、また様々な高額機器や消耗品を必要とする。本研究では、線虫溶液からのDNA抽出法を、Naklhaら（2010）をもとに、大量サンプルを、通常の機器や消耗品のみを使って、リアルタイムPCR法による線虫遺伝子の定量に適用できるように改変した。リアルタイムPCR法によるモロコシネグサレセンチュウなどの植物寄生線虫種の定量試験に本手法を適用したところ、検鏡法で頭数を確認した結果と高い相関を得た。本手法はDNA抽出にかかる時間は100分程度と既報の手法と大きな違いはないが、手作業時間は10分以下と、既報の半分から四分の一程度に簡素化された。また、ランニングコストも既報の手法より半分から十分の一まで減らすことができた。