Journal of Nematology 42(2):166–172. 2010.
© The Society of Nematologists 2010.

Combining maxRatio analysis with real-time PCR and its potential application for the prediction of *Meloidogyne incognita* in field samples

Yu-long Zhao,[1] Wei-bin Ruan,[1*] Le Yu,[1] Jing-yi Zhang,[1] Jin-miao Fu,[2] Eric B. Shain,[3] Xi-tai Huang,[1*] Jing-guo Wang[4]

Abstract: Diagnosing and quantifying plant-parasitic nematodes is critical for efficient nematode management. Several studies have been performed intending to demonstrate nematode quantification via real-time quantitative PCR. However, most of the studies used dilution of DNA templates to make standard curves, while few studies used samples with different nematode numbers to make the standard curve, resulting in a high standard error. The objective of the present study was to develop a high quality standard curve using samples containing different numbers of the root-knot nematode *Meloidogyne incognita* and evaluate the results of real time qPCR with maxRatio analysis. The results showed that a high quality standard curve was obtained with different nematode numbers using specific primers and cycle threshold (Ct)-PCR (R^2=0.9962, P<0.001, n=9). With the maxRatio analysis, the fractional cycle number (FCN)-PCR cycle curve and adjusted FCN (FCNadj)-PCR cycle curve had similar patterns as those of the Ct-PCR cycle curve. For quantification of nematodes in field soil samples, qPCR estimations with a FCNadj-PCR cycle standard curve was very close to microscope counting of second-stage juveniles (R^2=0.9064, P<0.001, n=10), qPCR estimations with a FCN-PCR cycle standard curve was comparably good (R^2=0.8509, P<0.001, n=10), and the biases with a Ct-PCR cycle standard curve were large (R^2=0.7154, P<0.001, n=10). Moreover, we found that the concentration of Triton X-100 had less of an effect on FCN as compared to Ct, with delta FCN 0.52, and delta Ct 3.94 at 0.8% Triton. The present study suggests, that combined with maxRatio methods, real time qPCR could be a practical approach for quantifying *M. incognita* in field samples.

Key words: Meloidogyne incognita, diagnosis, Real-time qPCR, maxRatio, Cycle threshold, MR, FCN.

Introduction

Plant-parasitic nematodes are some of the most damaging pests in agriculture ecosystems by direct feeding, transmitting viruses, and facilitating bacterial and fungal infections. They cause serious yield losses, estimated at $157 billion annually (Abad et al., 2008). The root-knot nematode (*Meloidogyne* spp.; RKN) is able to infect roots of almost all cultivated plants and disrupt root uptake of water and nutrients resulting in substantial yield loss and poor quality, making it perhaps the most damaging crop pathogen (Trudgill and Blok, 2001). For example, the value of "zero tolerance" for RKN in soil during the establishment of a vineyard was put forward because even one individual per 1000 ml soil can significantly reduce the yield of a susceptible host (Quader et al., 2002). Vegetable cultivation in China has been rapidly increasing, from 3.16 M ha in 1980 to 18.22 M ha in 2007, with over 10% occurring in greenhouses (China Agricultural Yearbook, 1980-2007). RKN has become the most widespread and economically important pathogen for greenhouse vegetable production systems. RKNs were detected in over 95 percent of the greenhouses in Shouguang County, Shandong Province, one of the largest vegetable growing regions in China (Zhao et al, unpublished data).

To control RKNs in greenhouse vegetable production systems, various nematicides have been widely applied. Recently, due to their effects on food safety and potential negative impacts on the environment, toxic nematicides have been phased out (Gan et al., 2000). Thus, the development of alternative approaches and techniques for controlling RKNs is needed.

For efficient control of RKNs, it is important to determine whether these nematodes are present in order to assess the magnitude of potential damage. A significant correlation between the initial population density of plant-parasitic nematodes in soil and the degree of damage to the host has been demonstrated (Koenning, 2000; Niblack, 2005). A diagnostic and predictive nematode assay can provide farmers with treatment guidance, and is a useful method for soybean cyst nematode management in the US (Niblack, et al., 1993). However, a diagnostic and predictive nematode assay is still not widely used for RKN management.

Quantifying second-stage juveniles might be a reasonable way for determining population density of RKNs in fields (McSorley et al., 1994); however, traditional nematode identification methods based on direct observation of morphological characteristics and counting is difficult and time-consuming. In addition, a declining taxonomic skill base is problematic for nematode identification (Coomans, 2002).

Molecular methods, which require less specialized skills, could be an alternative to morphological identification of nematodes. Some of the methods tested to date include restriction fragment length polymorphism (Oh et al., 2009), amplified fragment length polymorphism (Fargette et al., 2005), random amplified polymorphism DNA (Fargette et al., 2005), satellite DNA (Carrasco-Ballesteros et al., 2007), and sequence

Received for publication March 12, 2010.
[1]College of Life Sciences, Tianjin 300071, Nankai University.
[2]College of Mathematical Sciences, Tianjin 300071, Nankai University.
[3]Abbott Molecular, Inc. Des Plaines, IL 60018 USA.
[4]College of Resources and Environmental Sciences, China Agricultural University, Beijing 100094, China.
*Corresponding author
E-mail: ruanweinbin2004@hotmail.com, huangxt@nankai.edu.cn
This paper was edited by Melissa Goellner Mitchum.

characterized amplified region-PCR (Adam et al., 2007). Although these methods can differentiate nematode species, they are not suitable for quantification of nematodes. Recently, a less time-consuming PCR-based assay, referred to as real-time PCR (Heid et al., 1996), has shown potential for the specific and rapid detection of *Meloidogyne* spp. (Zijlstra and Van Hoof, 2006; Berry et al., 2008; Toyota et al., 2008). In addition, real-time PCR has been shown to be 10 times more sensitive than standard PCR (Zijlstra and Van Hoof, 2006).

The cycle threshold (Ct) method determines a cycle number based on the point where the fluorescence response extends above the background level to cross a predetermined fluorescence threshold value. The Ct method is an excellent method for quantitative PCR analysis because of the consistency in signal intensity during the exponential growth phase of the PCR. Studies have shown that there is a linear regression between Ct values plotted against the logarithm of the template DNA concentration for potato cyst nematode (Madani et al., 2005, 2008), entomopathogenic nematodes (Torr et al., 2007), and *Meloidogyne* spp. (Toyota et al, 2008). In these studies, a dilution series of DNA template was used to generate a standard curve for assessing the correlation between the mean molecular counts and microscopic estimates. It was hypothesized that the amount of nematode genomic DNA would proportionally increase with the increase in the number of nematode individuals. Berry *et al.* (2008) used samples containing 1, 5, 10, 20, 40 and 80 nematodes to generate standard curves for quantifying *Meloidogyne javanica, Pratylenchus zeae*, and *Xiphinema elongatum* via real-time PCR. There were significant correlations between the Ct value and the number of nematodes for all three nematode species. However, the range of testing points on the standard curve was relatively narrow, ranging from 1 to 80, which might impact the accuracy of the predication when higher numbers of nematodes are present. For quantification of nematodes with real time PCR, a high quality standard curve and relative accuracy of estimation by using the standard curve in comparison to microscope counting using morphological characteristics is of great importance.

A robust quantitative and qualitative real-time PCR analysis called the maxRatio method was developed by Shain and Clemens (2008). The maxRatio method provides a more reliable analysis of the PCR amplification signal resulting in more accurate quantitative analysis of a sample. We hypothesized that maxRatio methods might provide a practical and reliable real time qPCR estimation of RKN density in our field samples comparable to microscopic counting of second-stage juveniles.

The main objectives of the present study were to (1) develop a standard curve with different *Meloidogyne incognita* numbers, (2) utilize the maxRatio method to give robust and reliable analysis for real time quantitative PCR, (3) evaluate the difference in qPCR estimations by cycle threshold (Ct) and maxRatio analysis when compared to microscope counting of second-stage juvenile in field samples, and (4) test the effects of Triton X-100 concentration in the reaction mixture on the changes in Ct and FCN.

Materials and Methods

Soils and Nematodes: *Meloidogyne incognita* was originally isolated from an infested field in Shouguang, Shandong, China and then maintained on tomato Feiguan No.4 (*Lycopersicon esculentum*) in a greenhouse. Eggs of *M. incognita* were collected from the infested roots of tomato plants and hatched in tap water at 25°C. Freshly hatched second-stage juveniles of *M. incognita* were collected within 3 days. *Meloidogyne arenaria* were kindly provided by Professor Hu Xian-Qi,Yunnan Agriculture University, China. The nematodes of *Acrobeloides*, *Apelenchus*, *Chiloplacus*, *Rhabditis*, and *Heterodera* simultaneously present in the field samples and M. *arenaria* were used for confirming the specificity of the primers designed for *M. incognita*. These nematodes, except *M. arenaria*, were extracted from the greenhouse-tomato soil samples using sugar flotation and centrifugation method (Ou et al. 2005).

Genomic DNA Extraction: Total DNA was extracted from nematodes using a modified Triton X-100 method (Zhao, unpublished data). Briefly, for standard curves, 1 ml of sterile water with a known amount of *M. incognita* individuals, or for field soil samples, 1 ml of suspension of nematode mixture was boiled at 100°C until the water was completely evaporated. Then 100 μL 1.0% Triton X-100 lysis buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.45% NP40, 1.0% Triton X-100, and 6 mg/ml Proteinase K) was added to the tube. The mixture was incubated at 65°C for 2 h and then heated at 99°C for 10 min to inactivate the proteinase K and kept at -70°C for future use. Genomic DNA extracted from 9 samples containing a different number of second-stage juveniles of *M. incognita* (1, 2, 5, 10, 20, 50, 100, 200, 1000) were individually used for sensitivity and quantification standard curves. Each sample was run with four replicates. The trial was repeated twice.

Primers: The primers were designed according to the target DNA sequence of *M. incognita* (NCBI FJ534516.1) including the internal transcribed spacer 1 (ITS1) region, between ribosomal DNA 18S and 5.8S. To ensure the primers' specificity, they were blasted with NCBI (The National Center for Biotechnology Information) (Fig. 1A, B). The universal primer was MIF: 5'-TGGAA ACCAATCTAATCGCAGT-3' and the specific primer was MIR: 5'-CACTTAAGAGGCTCATTAAGTCTTAG-3'. Based on the general principle of primer design, the first three sites at the 3'-end of the primer are crucial for

```
A:
  Consensus -TT--TTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 174
  incognita -TTACTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 153
  hispanica -TTACTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 95
      minor GTTTTTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 158
      hapla -TTACTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 155
     fallax GTTTTTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 156
   arenaria -TTACTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 153
   javanica -TTACTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 132
      naasi GTTTTTTTGATGGAAACCAATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAAC 169
B:
  Consensus GGA-----------------------------TAAGAC-TAATGAGCCTCTTAAGTGA 406
  incognita GGA----------------------------CTAAGACTTAATGAGCCTCTTAAGTGA 344
  hispanica GGACT-------C-TT-T----AAT-GAGTT-TAAGACTTAATGAGCCTCTTAAGTGA 299
      minor GGATT-----ATCGTTAT----AATCG-----TAAGACTTAATGAGCCTCTTAAGTGA 370
      hapla GGAC--------C-TT------AATCGGGTT-TAAGACTTAATGAGCCTCTTAAGTGA 355
     fallax GGATT-----CTTTTTAT----AA--GAATCGTAAGACTTAATGAGCCTCTTAAGTGA 361
   arenaria GGACT-------C-T-------AAT-GAGTT-TAAGACCTAATGAGCCTCTTAAGTGA 353
   javanica GGAC--------C-TT------AATCGGGTT-TAAGACTTAATGAGCCTCTTAAGTGA 332
      naasi GGATTTTTTTCTCTTTATGGAGAAT--AATCGTAAGACTTAATGAGCCTCTTAAGTGA 392
```

FIG. 1. Nucleotide sequences and location of MIF (A) and MIR (B) in partial ITS1 gene (NCBI FJ534516.1).

specificity. If any discrepancy is present in this area, the target gene will not be amplified successfully via PCR. An alignment of the ITS1 sequences of eight species of the genus *Meloidogyne* was used to design an *M. incognita*-specific reverse primer (Fig.1B). Moreover, *M. arenaria* was employed to test the specificity of the primers in the present study. Because there are more than 80 species in the genus *Meloidogyne*, the specificity of the primers to all other untested species might not be exclusive.

Real-Time Quantitative PCR (qPCR): The real-time PCR reaction was performed with SYBR Green Perfect mix (Qiagen, China), and the signals were detected on an iQ5 (Bio-Rad), as described in the manufacturer's instructions. The reaction contained 12.5 μl SYBR Green, 10 mM of each specific primer, 5 μl DNA template, and sterile water to final volume of 25 μl. The reaction was performed with an initial step of 95°C for 15 min and then 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final step at 72°C for 10 min. A negative control with three replicates was prepared using distilled water instead of a DNA template. The real time PCR data was automatically provided by the iQ5. The products from the real-time PCR reactions were separated by electrophoresis in 1% TAE agarose gel and were visualized with UV transilluminator (UVP, Cambridge, UK) after staining with ethidium bromide.

MaxRatio Analysis: The maxRatio analysis for real-time PCR was based on Shain and Clemens (2008). Briefly, the equation: $Ratio_n = (Signal_n/Signal_{n-1})-1$ was applied to the present study, where $Signal_n$ shows real-time PCR fluorescence response at cycle n and $Signal_{n-1}$ represents the fluorescence data at cycle n-1 for a specific sample. The ratio curve has a well-defined peak the height of which is a relative measure of the fluorescence signal growth throughout the PCR reaction that is obtained by transformation calculation. The magnitude of the ratio curve at the maximum is defined as the MR (maxRatio) value. The position of the ratio curve at which the maximum occurs is defined as the FCN value or fractional cycle number. Based on the correlated behavior between the FCN and MR response values for each samples, an adjusted FCN value (FCNadj) that uses the MR value to compensate for the FCN determination was calculated. In the present study, the FCNadj is defined as $FCNadj = FCN + Log_2 (MR)$.

Effects of Triton X-100: Different concentrations of Triton X-100 (0, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.8%) were included in the reaction mixture of real-time PCR amplification system. The template DNA of *M. incognita* was pretreated using a DNA Purification Kit (Promega) to remove the Triton X-100 in the lysis buffer for nematode genomic DNA extraction. The other components in the reaction mixture and the program of real-time PCR were the same as that mentioned above.

Quantifying Nematodes from Field Soil Samples Using Real Time qPCR: Six *M. incognita* samples containing 1, 5, 10, 50, 100, 200 individuals respectively, were prepared for making standard curve using the procedure mentioned above. Correlations between real-time qPCR estimation using Ct, FCN, FCNadj cycle numbers and microscopic estimation of second-stage juveniles using standard quantification were conducted with ten field soil samples.

For the field sample assay, nematodes were obtained from ten natural greenhouse-tomato soil samples from Shouguang County, Shandong Province, China using a sugar flotation and centrifugation method (Ou et al. 2005). Here, the microscopic counting and real-time quantitative PCR analysis of the same ten samples were performed by two persons, respectively.

Statistical Analysis: The regression analysis between Ct, FCN, and FCNadj cycle numbers and Log_{10} of nematode number were performed. Also, the regressions between microscopic counting and real-time qPCR estimation by Ct, FCN and FCNadj were analyzed. T-test was undertaken for analyzing the differences in the delta Ct and delta FCN at each concentration of added Triton X-100 using SPSS17.0, and the treatment without Triton X-100 addition was regarded as the control. The delta Ct and delta FCN at 0% Triton X-100 was referred as 0. Differences among means were significant at $P<0.05$. Data were shown as means ± SE.

RESULTS

Specificity of the Primers: Second-stage juveniles (J2) were hatched from egg masses collected from RKN-infected pepper (*Capsicum annuum* L.) plants cultured under greenhouse conditions. The ITS1 sequence was amplified, sequenced and blasted to GenBank, to confirm that the tested RKNs were *Meloidogyne incognita*. PCR performed with the primers MIF and MIR (Fig. 1) produced only the single expected fragment of 237bp for all studied *M. incognita* individuals (Fig.2A). No PCR products were obtained in the negative (water) control and with template of *M. arenaria* and other soil nematode

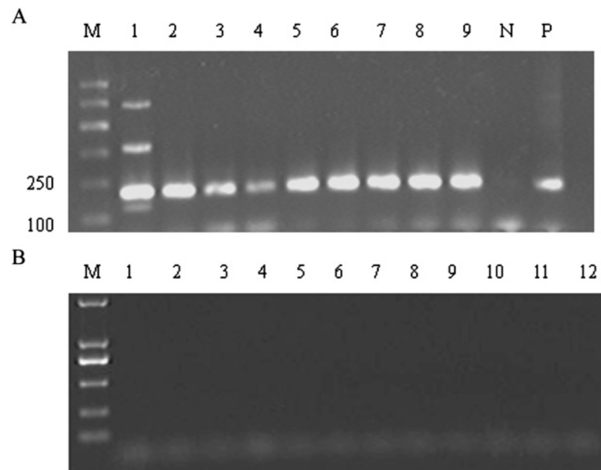

Fig. 2. Specificity of the primers for *M. incognita*. A: 1-9, *Meloidogyne incognita*; N, sterile water as negative control; P, purified PCR product of internal transcribed spacer 1 of *M. incognita* as positive control;B: Lane 1-8, *M. arenaria*; Lane 9, *Acrobeloides* sp.; Lane 10, *Aphelenchus* sp.; Lane 11, *Chiloplacus* sp.; Lane 12, *Heterodera* sp.; Lane M, Marker DL 2000.

species tested (Fig. 2B), suggesting that the pair of primers were specific to *M. incognita*.

Quantitative Data Analysis Using maxRatio and Ct: With the maxRatio assay, the ratio transformation curves were generated. The non-reactive action was clearly recognized from the curve shape. And mostly curves of samples containing different nematode numbers were distinguishable, although those curves were not easily to be judged with traditional fluorescence-cycle number analysis (Fig. 3A, B). Meanwhile, Figure 3C showed the characteristic MR–FCN plot using MR and corresponding FCN values. The only confirmed non-reactive action was the negative control using sterile water instead of DNA template in Fig. 3C.

Standard curve preparation: There were significant negative correlations between Ct value and the Log_{10} nematode number, including 9 samples ranging from 1 to 1000 J2 individuals (Fig. 4) (R^2=0.9962, P<0.001, n=9). A similar linear correlation was observed between FCN and the Log_{10} nematode number (R^2=0.9819, P<0.001, n=9), as well as between FCNadj and Log_{10} nematode number (R^2=0.9858, P<0.001, n=9).

Effects of Triton X-100 delta Ct and delta FCN: To evaluate differences in the response to Trition X-100 between Ct value and FCN values derived from maxRatio analysis during real-time PCR, different concentrations of Triton X-100 (0, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.8%) were included in the reaction mixture of real-time PCR amplification system. With an increase in the Triton X-100 concentration from 0.05% to 0.8%, Ct value was increased by 3.59, or 16.18%, from 22.19 to 25.78 whereas the increase of FCN value was only 0.52, from 15.42 to 15.94. In general, the changes in Ct values were greater than FCN values, particularly at 0.8% Triton X-100 (P<0.01) (Fig. 5).

Quantification of M. incognita in field soil samples with real-time qPCR: Three high quality standard curves were made (Fig. 6A). Although the results of the amount of *M. incognita* J2 from natural field samples by real time qPCR estimation, Ct, FCN, or FCNadj were significantly correlated to that of microscopic counting, the slope and R^2 value of correlation equation were different among Ct, FCN, or FCNadj, with the low R^2 =0.7154 with Ct analysis, and high R^2 value with FCNadj of 0.9064 (Fig. 6B).

Discussion

With a modified nematode genomic DNA extraction protocol, we created high quality standard curves with low standard error (Fig.4). Further, with the maxRatio method (Shain and Clemens 2008), the reactive/non-reactive determinations as well as discrimination of normal versus inhibited responses in the real-time PCR for *Meloidogyne incognita* quantitative analysis were found by MR values and Ratio-cycle curves. In line with our hypothesis, real-time qPCR estimation with the maxRatio method (FCN and FCNadj) had a higher correlation with microscopic counting than that with Ct analysis (Fig.6B), suggesting that FCN and FCNadj would provide more accurate prediction for quantifying nematodes in field samples than Ct analysis alone.

Using this method, the difference in the Ct value between one and two nematodes can be significantly distinguished from the standard curve (Fig. 4) suggesting that real-time PCR is sensitive for nematode quantitative analysis. Although nematode numbers were used to make the calibration curve in the present study, the coefficient of determination was as high as those studies using dilutions of DNA templates (Madani et al., 2005; Torr et al., 2007; Madani et al., 2008). However, the nematode sample with 1000 individuals was not linearly quantitative with all the other samples for Ct, FCN, or FCNadj, especially for FCN. This suggests that the maximum nematode number in the standard curve should be less than 1000 for obtaining a good quality of standard. The same volume of lysis solution for 1 individual was also applied to 1000 individuals, and too much protein or lipids in the solution containing 1000 individuals may have negatively affected the result of the real-time PCR. Thus further optimization of the assay might improve the dynamic range.

The lower change in FCN values indicated that the effect of other components in the reaction mixture had less of an effect on the result of real time PCR when compared to the changes in Ct value (Fig.5). Considering that same amount of DNA template was used, the lower variation in the results should give more reliable and consistent interpretation in the case of the presence of other unknown factors negatively affecting the real-time PCR process.

The estimation of nematode number using Ct values of the quantitative PCR was more varied in comparison

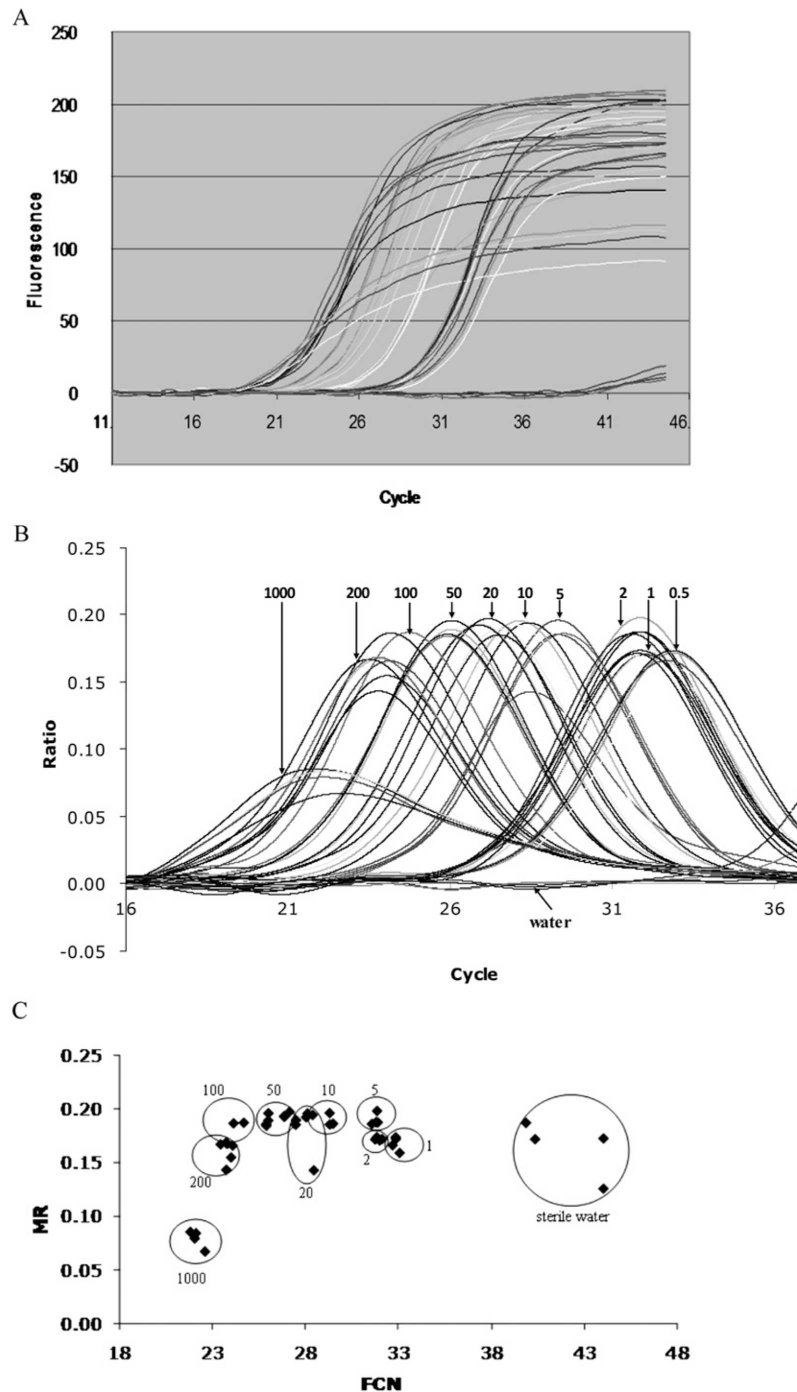

FIG. 3. Analysis by maxRatio of real-time *M. incognita* assay amplification plots. Nematode numbers ranging from 1 to 1000 were tested in four replicates. (A) Amplification plots of *M. incognita* fluorescence versus cycle number. (B) Corresponding plots after applying the ratio transformation. (C) Plot of MR versus FCN values.

with the microscopic counting based on morphological characters. Berry et al. (2008) also mentioned the underestimation of nematode numbers by means of Ct value analysis with a slope of 0.78, R^2 = 0.83. The same was demonstrated in another study where a slope of 0.7381 and R^2 = 0.639 was calculated (Stirling et al., 2004). Similarly, an underestimation of nematode numbers by 45% was observed in the present study, where the slope was 0.5521, R^2 = 0.7154. In contrast, using Ct value analysis an overestimation was also shown in another study where the slope was 1.515, R^2 = 0.9391 (Toyota et al., 2008) and it was also reported that a slight overestimation of nematode numbers occurred when compared with the taxonomic counts (Torr et al. 2007). The variation of slope values ranging from 0.73 to 2.53 between qPCR based on Ct and microscopic estimation in this and other studies demonstrates that qPCR based on Ct is not suitable for nematode estimation even if the

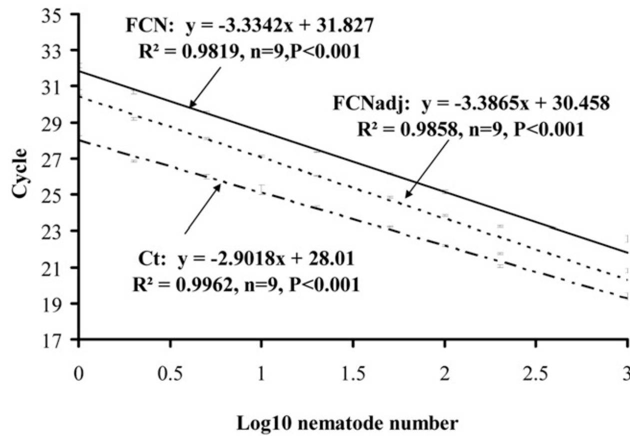

FIG. 4. Standard curves showing the relationship between cycle threshold (Ct) and Log_{10} nematode number, between FCN and Log_{10} nematode number, and between FCNadj and Log_{10} nematode number. Nematode numbers consisted of 1, 2, 5, 10, 20, 50, 100, 200 or 1000 *Meloidogyne incognita* individuals per tube. Data were shown as Means ± SE (n=4).

curve had a significantly high correlation (Fig. 6). In the present study, for real-time qPCR estimation, the slope was 0.8759 and R^2 was 0.8509 in FCN estimation and slope = 0.9558, R^2 = 0.9064 in FCNadj estimation when compared to a slope = 0.5521, R^2 = 0.7154 using Ct estimation, indicating that the data estimated by using FCN and FCNadj in quantitative PCR had a better match to microscopic counting.

Several factors may partially contribute to the underestimation or overestimation of nematode numbers. Toyota et al. (2008) suggested the a log-transformed correlation equation might not be sensitive enough for smaller differences and cause experimental error. With the calibration curve of the present study, samples differing by a single nematode could be distinguished, indicating that the log-transformed data may not be the

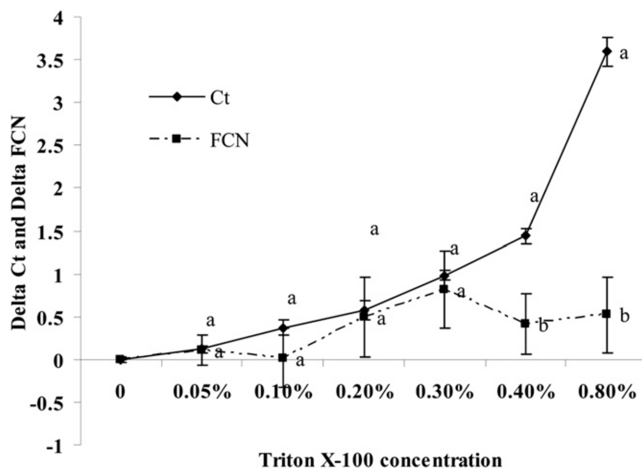

FIG. 5. Effects of Triton X-100 on the changes in Ct and FCN. The purified DNA was used here. Data were shown as means ± SE (n=4). Means within the same Triton X-100 concentration followed with different letters were significantly different according to the t-test (P<0.05).

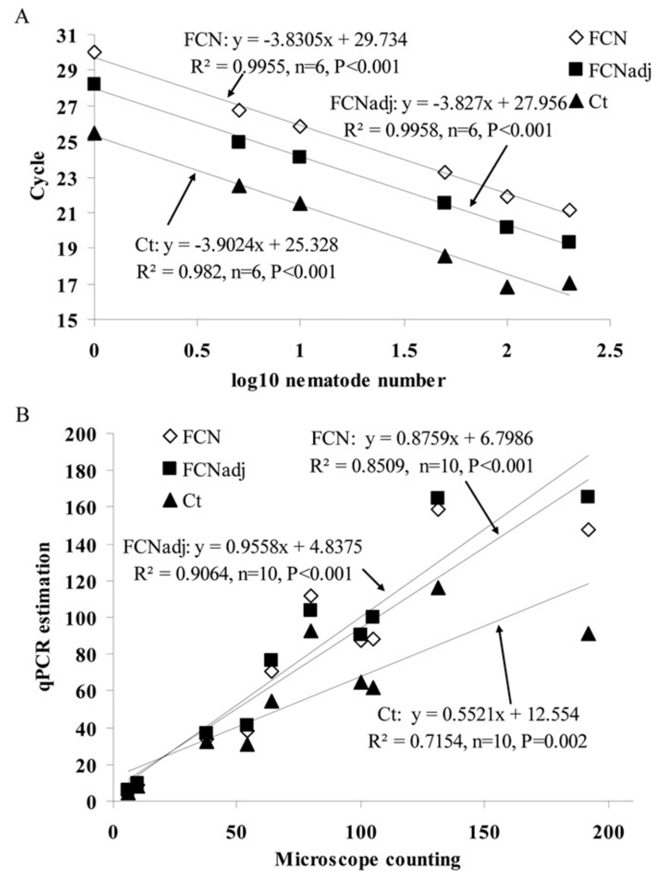

FIG. 6. Standard curve (A) and the correlations between microscope counting and the real-time quantitative PCR estimation by the corresponding Ct, FCN or FCNadj curves for the identical field samples (B).

factor causing biased estimation. Humic compounds are the most commonly reported group of inhibitors in environmental samples and appear to have deleterious effects on several reaction components and their interactions (Wilson, 1997). In the present study, although nematodes extracted from soil by the sugar flotation-centrifugation method were washed with tap water, some tiny residues in the nematode suspensions could be clearly observed, and may have contributed to the underestimation in our study. Other nematode components such as protein, carbohydrate and lipid in the reaction mixture might negatively impact nematode estimation. Certainly, discrepancy between the microscopic estimation and the real number of *M. incognita* could take place in the present study since it is very difficult to distinguish *M. incognita* from other nematode species in the same genus under the microscope and could lead to the variation of slope. The closer the slope value was to 1, the more reliable the estimation by real time quantitative PCR was found to be.

Real time qPCR offers an alternative assay to the time-consuming traditional method of morphological counting and would provide more reliable information. In the present study, we made a high quality standard curve using the modified Triton X-100 method for

nematode genomic DNA extraction (Zhao, unpublished data). However, in light of the fact that underestimation or overestimation will greatly constrain the practical application of a nematode service assay via real-time PCR, the factors and mechanisms causing underestimation or overestimation of nematode in the real-time PCR quantitative analysis deserves further study. With further investigation, a standardized protocol using real time qPCR for nematode analysis could be developed. The advantages of a real time PCR assay, such as timesaving and low cost, could then be explored. Here we demonstrate that real-time quantitative analysis combined with robust maxRatio analysis, could be a practical, economic, and reliable tool as a routine service assay for *M. incognita* as well as other nematode species in the future.

Acknowledgments. This work was jointly supported by the National Nature Science Foundation of China (30870415), and National High-Tech Research and Development Program (863) of China (2006AA10Z423).

Literature Cited

Abad, P., Gouzy, J., Aury, J. M., Castagnone-Sereno, P., Danchin, E. G. J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V. C., Caillaud, M. C., Coutinho, P. M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J. V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T. R., Markov, G. V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Segurens, B., Steinbach, D., Tytgat, T., Ugarte, E., van Ghelder, C., Veronico, P., Baum, T. J., Blaxter, M., Bleve-Zacheo, T., Davis, E. L., Ewbank, J. J., Favery, B., Grenier, E., Henrissat, B., Jones, J. T., Laudet, V., Maule, A. G., Quesneville, H., Rosso, M. N., Schiex, T., Smant, G., Weissenbach, J., and Wincker, P. 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nature Biotechnology 26:909–915.

Adam, M. A. M., Phillips, M. S., and Blok, V. C. 2007. Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). Plant Pathology 56:190–197.

Berry, S. D., Fargette, M., Spaull, V. W., Morand, S., and Cadet, P. 2008. Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. Molecular and Cellular Probes 22:168–176.

Carrasco-Ballesteros, S., Castillo, P., Adams, B. J., and Perez-Artes, E. 2007. Identification of *Pratylenchus thornei*, the cereal and legume root-lesion nematode, based on SCAR-PCR and satellite DNA. European Journal of Plant Pathology 118:115–125.

Coomans, A. 2002. Present status and future of nematode systematics. Nematology 4:573–582.

Fargette, M., Lollier, V., Phillips, M., Blok, V., and Frutos, R. 2005. AFLP analysis of the genetic diversity of *Meloidogyne chitwoodi* and *M. fallax*, major agricultural pests. Comptes Rendus Biologies 328:455–462.

Gan, J. Y., Becker, J. O., Ernst, F. F., Hutchinson, C., Knuteson, J. A., and Yates, S. R. 2000. Surface application of ammonium thiosulfate fertilizer to reduce volatilization of 1,3-dichloropropene from soil. Pest Management Science 56:264–270.

Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. 1996. Real time quantitative PCR. Genome Research 6:986–994.

Koenning, S. R. 2000. Density-dependent yield of *Heterodera glycines*-resistant and -susceptible cultivars. Journal of Nematology 32:502–507.

Madani, M., Subbotin, S. A., and Moens, M. 2005. Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. Molecular and Cellular Probes 19:81–86.

Madani, M., Ward, L. J., and De Boer, S. H. 2008. Multiplex real-time polymerase chain reaction for identifying potato cyst nematodes, *Globodera pallida* and *Globodera rostochiensis*, and the tobacco cyst nematode, *Globodera tabacum*. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie 30:554–564.

McSorley, R., Dickson, D. W., de Brito, J. A., Hewlett, T. E., and Frederick, J. J. 1994. Effects of Tropical Rotation Crops on *Meloidogyne arenaria* Population Densities and Vegetable Yields in Microplots. Journal of Nematology 26:175–181.

Niblack, T. L. 2005. Soybean cyst nematode management reconsidered. Plant Disease 89:1020–1026.

Niblack, T. L., Heinz, R. D., Smith, G. S., and Donald, P. A. 1993. Distribution, density, and diversity of *Heterodera glycines* in Missouri. Journal of Nematology 25:880–886.

Oh, H. K., Bae, C. H., Il Kim, M., Wan, X., Oh, S. H., Han, Y. S., Lee, H. B., and Kim, I. 2009. Molecular Biological Diagnosis of *Meloidogyne* Species Occurring in Korea. Plant Pathology Journal 25:247–255.

Ou, W., Liang, W. J., Jiang, Y., Li, Q., and Wen, D. Z. 2005. Vertical distribution of soil nematodes under different land use types in an aquic brown soil. Pedobiologia 49:139–148.

Quader, M., Riley, I. T., and Walker, G. E. 2002. Damage threshold of *Meloidogyne incognita* for the establishment of grapevines. International Journal of Nematology 12:125–130.

Shain, E. B., and Clemens, J. M. 2008. A new method for robust quantitative and qualitative analysis of real-time PCR. Nucleic Acids Research 36:e91.

Stirling, G. R., Griffin, D., Ophel-Keller, K., McKay, A., Hartley, D., Curran, J., Stirling, A. M., Monsour, C., Winch, J., and Hardie, B. 2004. Combining an initial risk assessment process with DNA assays to improve prediction of soilborne diseases caused by root-knot nematode (*Meloidogyne* spp.) and *Fusarium oxysporum* f. sp lycopersici in the Queensland tomato industry. Australasian Plant Pathology 33:285–293.

Torr, P., Spiridonov, S. E., Heritage, S., and Wilson, M. J. 2007. Habitat associations of two entomopathogenic nematodes: a quantitative study using real-time quantitative polymerase chain reactions. Journal of Animal Ecology 76:238–245.

Toyota, K., Shirakashi, T., Sato, E., Wada, S., and Min, Y. Y. 2008. Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. Soil Science and Plant Nutrition 54:72–76.

Trudgill, D. L., and Blok, V. C. 2001. Apomictic, polyphagous root-knot nematodes: Exceptionally successful and damaging biotrophic root pathogens. Annual Review of Phytopathology 39:53–77.

Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Applied Environmental Microbiology 63:3741–3751.

Zijlstra, C., and Van Hoof, R. A. 2006. A multiplex olyreal-time pmerase chain reaction (TaqMan) assay for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax*. Phytopathology 96:1255–1262.