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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

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Abstract

The potato cyst nematode *Globodera pallida* and the beet cyst nematode *Heterodera schachtii* are major nematode pests in world agriculture. Precise identification and knowledge about the number of nematodes in field soil are necessary to develop effective integrated pest control. Here we report the results of the Real-Time PCR assay for the rapid detection and quantification of *G. pallida* and *H. schachtii*. Using species specific primers and SYBR green I dye, we were able to detect a single second stage juvenile of cyst forming nematodes in samples. The specificity of the reaction was confirmed by the lack of amplification of DNAs from other *Heterodera* or *Globodera* species. Validation tests showed a rather high correlation between real numbers of second stage juveniles in a sample and expected numbers detected by Real-Time PCR. Reasons for observed differences in sensitivity and reliability of quantification detection for two species as well as other problems of Real-Time PCR are discussed. The Real-Time PCR assay with SYBR green I dye targeting fragments of the ITS-rDNA provided a sensitive means for the rapid and simultaneous detection and quantification of juveniles of these pests.
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1. Introduction

The potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, are widely distributed in many European and Asian countries as well as in the Americas [5]; they are major nematode pests in potato and sugar beet production, respectively. Their impact on the crop depends largely on their pre-plant density [4,10]. Due to their damaging potential, potato cyst nematodes (*G. pallida* and *G. rostochiensis*) are subject to quarantine regulations in many countries. Cyst forming nematodes are frequently present in mixed populations [5]. Obviously, precise identification of the species and knowledge about

their numbers in soil samples are necessary to initiate control of these nematodes.

The identification and quantification of cyst forming nematodes based on morphology and morphometric characteristics is time consuming, even for an expert. Szalanski et al. [13] and Subbotin et al. [12], however, demonstrated that DNA based methods provide efficient tools for a precise, reliable and rapid identification of cyst nematodes. PCR using species specific primers constitute a major step forward in the development of diagnostic technology, which has successfully been used for sensitive detection of species. Species specific primers inferred from sequence differences in the internal transcribed spacer (ITS) regions of rDNA have been developed for *G. pallida* and *G. rostochiensis* [3,7,15], *H. glycines* [11] and *H. schachtii* [1].

DNA technology provides several methods for quantification of pest organisms in samples [14]. The PCR

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quantification technique measures the nematode number indirectly by assuming that the number of target DNA copies in the sample is proportional to the number of targeted nematodes. Most of the difficulties with this technique arise because only a very small number of the cycles (4–5 out of 40) in a PCR contain useful information. The early cycles have undetectable amounts of the DNA product, whereas the latest cycles, viz. the so-called plateau phase, are almost as uninformative. The quantitative information in a PCR comes from those few cycles where the amount of DNA grows logarithmically from barely above background to the plateau [8]. The real-time technique allows continuous monitoring of the sample during PCR using hybridization probes (TaqMan, molecular beacons, or FRET) or double stranded dyes such as SYBR green I. With this technique, the log-linear region can be easily identified as the fluorescence data appear on the computer screen and then can be compared to the standard.

SYBR green I fluorescent dye has the virtue of being easy to use because (i) it has no sequence specificity, (ii) it can be used to detect any PCR product, and (iii) it binds to any double-stranded DNA. However, this virtue has a drawback, as the dye binds also to any non-specific product including primer-dimers. To overcome this problem, the melting curve analysis can be employed. The products of a PCR reaction are melted by increasing the temperature of the sample. At the melting temperature (T_m) of the product, a sharp reduction is observed in the level of measured fluorescence. Non-specific products tend to melt at a much lower temperature than the longer specific products [8]. Both the shape and position of the DNA melting curve are functions of the GC/AT ratio. The length of amplicons can also be used to differentiate amplification products separated by less than 2 °C in T_m [9].

Compared to traditional PCR methods, Real-Time PCR with SYBR green I dye has several advantages. The technique allows a simultaneous faster detection and quantification of target DNA and the automated system overcomes the laborious process of estimating of the quantity of PCR product after gel electrophoresis.

Bates et al. [2] using Real-Time PCR with SYBR green I dye found relative large (4 °C) differences in T_m between specific PCR products of G. rostochiensis and G. pallida amplified using the species specific primers designed by Bulman and Marshall [3]. These differences ensure that melting peaks of these two products can be clearly distinguished in a multiplex reaction. By calculating the ratio of the melting peak height at T_m of each product and comparing it to the standard run under the same conditions, it was even possible to estimate the proportion of each product in the mixture and finally to determine the ratio of juveniles of these two species in the sample [2]. However, this method has not been applied in absolute quantification of these nematodes.

The aim of the present study was to develop a rapid and precise method for the detection and quantification of

the potato and beet cyst nematodes in samples. For the first species we used the species specific primers described by Bulman and Marshall [3]; for the second species we used slightly modified primers from Amiri et al. [1]. The PCRs were run in a ABI Prism 7900 HT sequence detection system with SYBR green I dye.

2. Materials and methods

2.1. Nematode materials

Several populations of G. pallida and H. schachtii collected from fields in Belgium or maintained in cultures at the Agricultural Research Centre, Belgium were used. DNA from several other species of Schachtii group (H. daverti, H. betae, H. ciceri, and H. trifolii) and the genus Globodera (G. rostochiensis, G. artemisiae, G. millifolii, and G. zealandica) were used for verifying the specificity of the species specific primers.

2.2. DNA extraction

The efficacy of DNA extraction methods with proteinase K using either Worm Lysis Buffer (WLB, 10 mM Tris–HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, 0.45% Tween 20) or PCR buffer (Qiagen, Hilden, Germany) was compared using Real-Time PCR. As during the subsequent Real-Time PCR, samples extracted with PCR buffer proved to yield a higher amount of DNA than those extracted with WLB, and so we decided to use PCR buffer in all further experiments.

To obtain a standard curve for both nematode species, 2000 juveniles and eggs of a particular species were transferred to an Eppendorf tube containing 20 µl double distilled water and crushed with a microhomogenizor Vibro Mixer (Zurich, Switzerland) for 1.5 min. Twelve microlitre of proteinase K (600 µg/ml) (Promega Benelux, Leiden, The Netherlands) in 1× PCR buffer (Qiagen, Germany) (Fig. 1) were added to the tubes to make a final volume of 132 µl. The tubes were incubated at 65 °C for 1 h and at 95 °C for 15 min, consecutively. Nematode DNA was then serially diluted two-fold with water. The DNA extraction procedure was conducted from three different samples with 2000 juveniles and eggs for each nematode species. The three series of dilutions were then used in three independent runs of Real-Time PCR reactions (see Section 2.3). DNA extractions from 1, (3 for H. schachtii), 5, 30, or 500 second stage juveniles of each nematode species in ten replicates were used in validation and sensitivity assays.

2.3. Real-Time PCR protocol

Starting from aligned sequences of Globodera spp. and Heterodera (Subbotin et al., unpublished) several primers
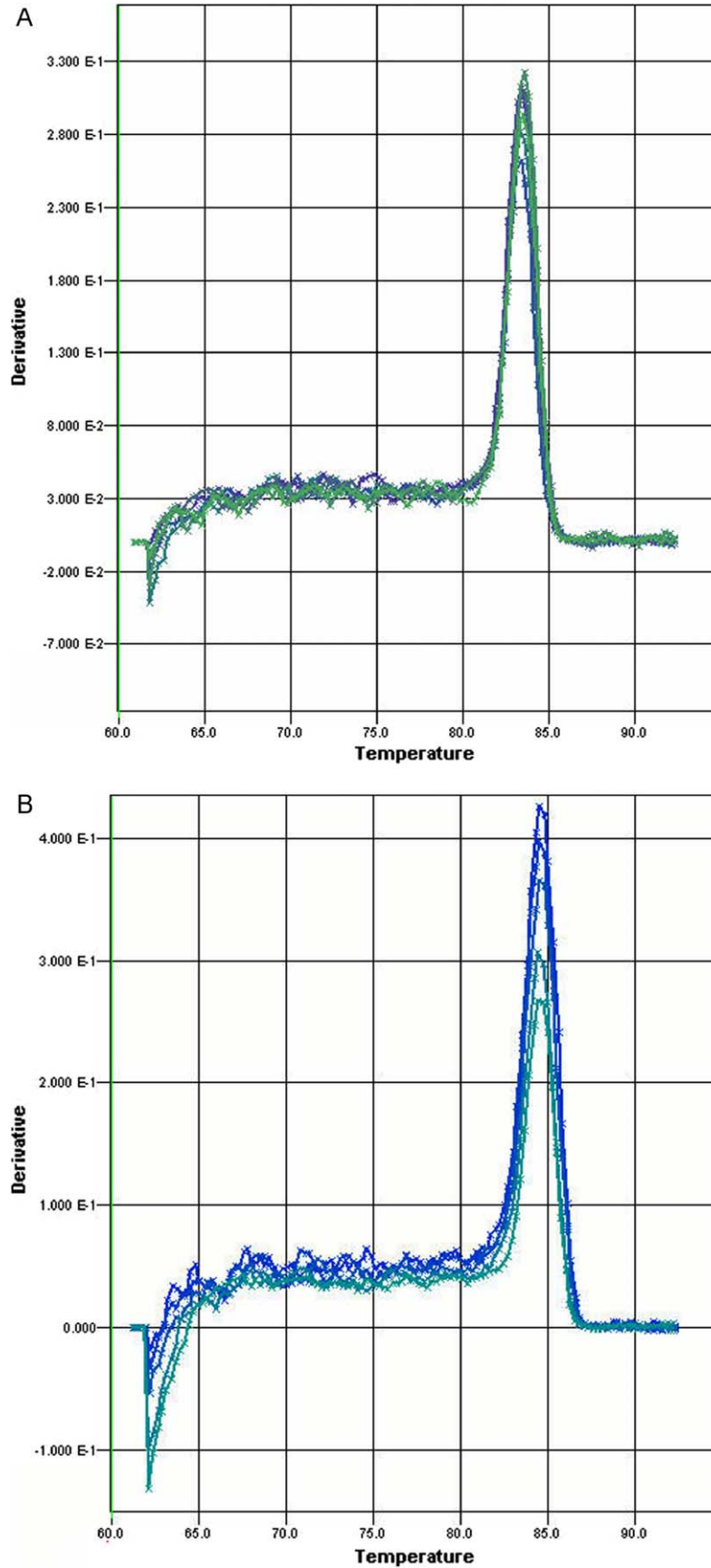
Fig. 1. Melting curves (fluorescence versus temperature) of specific amplicons for (A) *G. pallida* and (B) *H. schachtii*.

for each species that amplified short amplicons were designed using Primer Express Version. 2 (Applied Biosystems, Foster City, CA, USA) and tested with conventional PCR as described by Amiri et al. [1]. Out of five putative primer pairs for *H. schachtii* the following combination was selected because it gave the best result: SH6Mod 5′-CGTGTTCTTACGTTACTTCCA-3′ (modified from Amiri et al., [1]) and SH4 5′-AGCATGCGAAG-GATTGG-3′; for the same reason, the combination PITSp4 5′-ACAACAGCAATCGTCGAG-3′ as described by Bulman and Marshall [3] and Pal3 5′-ATGTTTGGG-CTGGCAC-3′ were chosen for *G. pallida* from a series of four pairs. Subsequently, amplification reactions were performed in real-time with 12 µl 1× SYBR Green I PCR Master Mix (Applied Biosystems), 0.1 µM of each primer, and 4 µl DNA sample in a final volume of 25 µl. Amplification reactions were performed in 96-well Optical Reaction Plates in the automated ABI Prism 7900 HT sequence detection system (Applied Biosystem). The manufacturer's recommended universal thermal protocol was used: 2 min preheating at 50 °C followed by 10 min at 95 °C for initial template denaturation and activation of hot start polymerase; this preparation was followed by 30 cycles of 95 °C for 15 s each, and combined annealing/extension phase of 65 °C for 1 min.

The Sequence Detection Software was used to generate the amplification curves for each reaction. The threshold cycle number (*Ct*) was also calculated with SDS and an automatic setting of the baseline. To differentiate species amplicons from non-specific products, a disassociation curve was generated after each reaction. DNA melting curve analysis of the resulted amplicon was performed at the end of amplification by cooling the samples at 1.6 °C/s (ramp 100%) to 60 °C and then increasing the temperature to 95 °C at 0.03 °C/s (ramp 2%). The fluorescence emission was measured every 7 s (each data point collection in 0.2 °C/s) over 20 min. For comparison and estimation of PCR efficacy, all samples amplified in this study were analysed on agarose gel electrophoresis. Control samples without DNA template were included in each experiment with Real-Time PCR.

3. Results and discussion

3.1. Conventional PCR with specific primers

PCR performed with the primer combination SH6Mod and SH4 produced only the single expected fragment of 116 kb for all studied *H. schachtii* populations. No PCR products were obtained in the negative control lacking DNA template and with DNA of other *Schachtii* group species and other tested nematodes. PCR with the primer combination PITSp4 and Pal3 yielded a single fragment of 114 kb for all studied *G. pallida* populations, as was predicted by sequence analysis. No PCR products were obtained in the negative control lacking DNA template and with DNA of other *Globodera* species and other tested nematodes.

3.2. Real-Time PCR and melting curve analysis

The results obtained in Real-Time PCR with SYBR green I confirmed the results obtained in conventional PCR. Melting curve analysis revealed the presence of a single pick only. Meting temperatures for amplicons were 84.6 ± 0.1 and 84.1 ± 0.1 for *H. schachtii* and *G. pallida*, respectively. Melting profiles for the amplicons are given in Fig. 1. The concentration of template DNA in the samples did not influence the melting temperature of the amplicon (data not shown). The PCR products obtained in real-time revealed on agarose gels only a single band and the absence of primer-dimers, which could disturb the accuracy of detection and quantification (Fig. 2).

The results of runs of the three serial dilutions of DNA samples of *G. pallida* showed a highly significant ($R^2=0.98$–0.99) negative correlation between the *Ct* value and nematode number over the range studied, with little variation between the replicated samples and runs (Fig. 3). The standard deviation (SD) of *Ct* values of three different sample preparations and of different runs of the same samples ranged from 0.01 to 0.13. These results demonstrate that amplifications using Real-Time PCR were highly reproducible within replicates of the same sample preparation but also between different sample
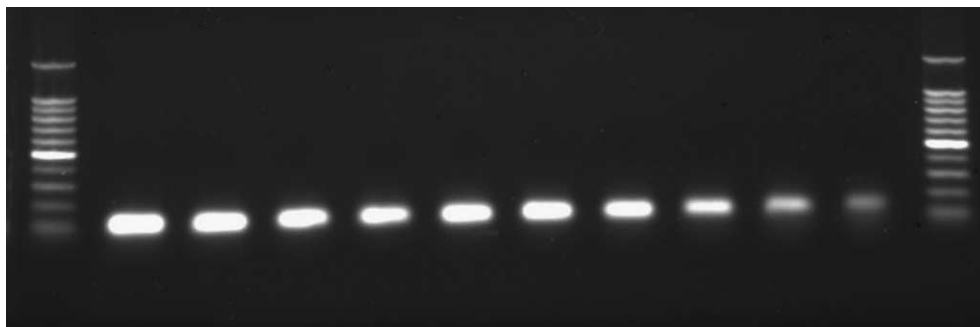

Fig. 2. Gel analysis of amplicons obtained for *H. schachtii* in serial dilution (1:1–1:1024). DNA size marker—100 bp DNA ladder (Promega).
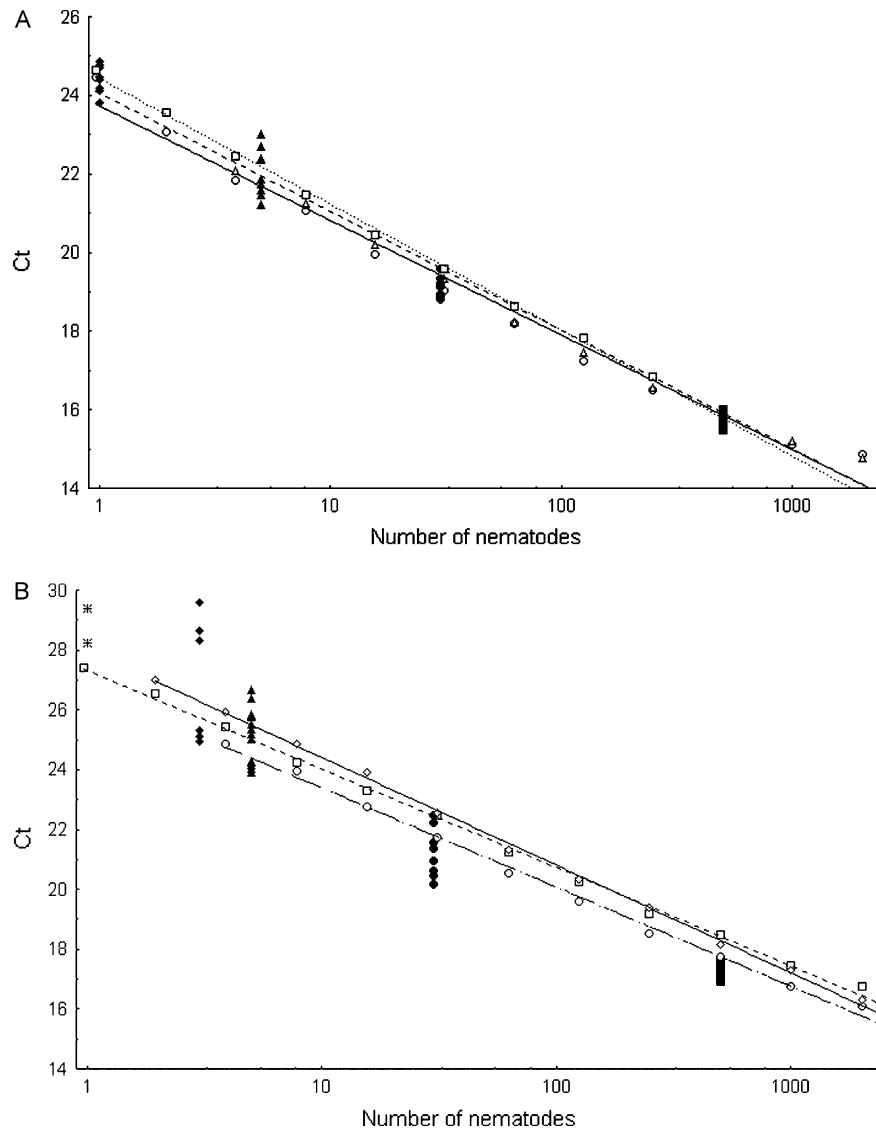
Fig. 3. Sensitivity, detection range, and specificity of the Real-Time PCR assay for *G. pallida* and *H. schachtii*. The reproducibility of the assay was determined by testing a dilution series of three independent DNA extractions. Black figures represent the results of the validation test with known numbers of second stage juveniles [1, 3 (for *H. schachtii*) 5, 30, or 500]. (A) *G. pallida*. (○) $y = -0.842x + 13.461$, $R^2 = 0.9912$, (△) $y = -0.810x + 13.505$, $R^2 = 0.9879$, (□) $y = -0.9815x + 15.721$, $R^2 = 0.9987$. (B) *H. schachtii*. (□) $y = -0.990x + 15.456$, $R^2 = 0.9979$; (△) $y = -0.9953x + 14.748$, $R^2 = 0.9952$; (○) $y = -1.0825x + 15.046$, $R^2 = 0.9983$.

preparations. Similar results were obtained for *H. schachtii*, for which a highly significant negative correlation between the *Ct* value and the nematode number was also found (Fig. 3). The SD of *Ct* between runs of the same sample varied from 0.01 to 0.15, whereas among the three DNA samples it was higher than that for the experiments with *G. pallida* and reached 0.77. The Real-Time PCR assay revealed a signal in the sample dilution, which corresponded to single second stage juvenile in sample. The gel analysis data confirmed that the observed fluorescence signal was due to the presence of target DNA, the amount of which decreased with increased dilution as expected (data not shown).

3.3. Detection limit of the assay and determination of the second stage juveniles in sample

To demonstrate the preciseness in quantification of cyst nematode juveniles with the above determined standard curves, DNA extracted from different precise numbers of juveniles was run. The results of these experiments are presented in Fig. 3. The validation test showed a rather high match between the numbers of *G. pallida* second stage juveniles present in the sample and the expected numbers detected by Real-Time PCR. Positive results were obtained from all samples containing juveniles, and thus the sensitivity of this method can be considered 100%.

The sensitivity of *H. schachtii* detection was lower than 100% for samples containing less than 5 juveniles. A single and three second stage juveniles were detected in only 20 and 60% of the samples, respectively. For both species, the preciseness of the quantification decreased with decreasing nematode numbers in the samples.

In this paper, we demonstrate the usefulness of Real-Time PCR with SYBR Green I dye using a combination of continuous fluorescence monitoring of PCR with melting curve analysis for the rapid specific detection and quantification of two cyst nematode species. The assay itself is more rapid than conventional specific cyst nematode PCR assays previously described and excludes the time consuming post-PCR agarose gel electrophoresis.

The quantification of nematodes depends on the efficiency of DNA extraction methods, which should be consistent over all experiments. In our study we used a mechanical technique for tissue homogenization, which resulted in equal DNA amounts in several replicates. Recently, the method based on the chemical lysis of nematodes using NaOH was proposed by Floyd et al. [6]. Due to the complete destruction of nematode tissues, it can increase the DNA efficiency, reduce the time for DNA preparation and exclude the most critical step of mechanical homogenization.

The specific primers amplified similarly sized PCR products for both species. However, variation in detection and quantification of these two species was observed during our study. More PCR product was amplified from the same number of the second stage juveniles for *G. pallida* than for *H. schachtii* by these species specific primers due to differences in the amount of template rDNA. Because RFLP and sequence analysis of the ITS region revealed very high level of similarity of *H. schachtii* with closely related species of the *Schachtii* group [1,12], it has been shown that only one haplotype containing the specific signature for *H. schachtii* can be used for diagnostics of this species. The SH6 primer for conventional PCR [1] and SH6Mod primer for Real-Time PCR (present study) was designed to amplify only one of the ITS haplotypes of *H. schachtii* [1], where as PITSp4 amplifies all ITS haplotypes of *G. pallida* [3], i.e. more ITS copies. Although we concluded that the haplotype amplified by SH6Mod primer was present in all studied *H. schachtii* populations [1], it is still not clear whether it is distributed in the same ratio among all populations of this species. The quantification procedure might be disrupted by a non-consistent distribution of this haplotype within populations of this species. For such populations, experiments constructing the standard curve should be done.

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