Investigating the ecology and evolution of cryptic marine nematode species through quantitative real time PCR of the ribosomal ITS region

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Keywords: cryptic species, qPCR, ITS, absolute quantification, relative quantification

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Running Title: ~~Detection~~ Quantification of cryptic nematode species

~~**Key words:** cryptic species, qPCR, *Rhabditis marina*, ITS, absolute quantification, relative quantification~~

Abstract

The presence of morphologically similar but genetically distinct species has impacted biogeographical and ecological paradigms. ~~This unrecognized diversity should be taken into account when conservation strategies and biodiversity management protocols are formulated.~~ In marine sediments, free-living nematodes form one of the most abundant and diverse faunal groups. Inferring the importance of nematode diversity for ecosystem functioning requires ~~however~~ species level identification, which is hampered by the lack of easily observable diagnostic characters and the presence of cryptic species. New techniques are urgently needed to adequately study the ecology and evolution of cryptic species. The aim of the present study was to evaluate the potential of a quantitative real time PCR (qPCR) assay using the internal transcribed spacer (ITS) region of the ribosomal DNA to detect and quantify cryptic species of the *R. (P.) marina* complex. All primer pairs proved to be highly specific and each primer pair was able to detect a single juvenile in a pool of 100 nematodes. Ct ~~-~~values were significantly different between developmental stages for all species except for PmIII. Despite differences among developmental stages, a strong correlation was observed between the amount of extracted DNA and the number of nematodes present. Relative and absolute quantification estimates were comparable and resulted in strong positive correlations between the qPCR estimate and the actual number of nematodes present in the samples.

The qPCR assay developed here ~~shows~~ provides the ability to quickly identify and quantify cryptic nematode species ~~species enabling~~and will facilitate their study ~~to study the ecology and functioning of cryptic species under controlled~~ in laboratory ~~or~~ and field settings.

Introduction

The discovery of morphologically similar but genetically distinct species throughout the tree of life has substantially increased over the last two decades (Bickford et al. 2007). The presence of cryptic diversity has impacted biogeographical and ecological paradigms, since species with previously wide geographic distributions may actually consist of cryptic species with a much more narrow geographic distribution (Stuart et al. 2006) and so-called 'generalist' species may in fact consist of cryptic complexes of specialist species (Blair et al. 2005). Evidently, this unrecognized diversity should be taken into account when conservation strategies and biodiversity management protocols are being formulated. Furthermore, it remains unclear how such cryptic diversity has evolved: is the morphological similarity the result of morphological stasis or evolutionary convergence? Unfortunately, relatively few studies have addressed the ecological and evolutionary aspects of cryptic speciation.

Cryptic species are found in a wide range of habitats (Bickford et al. 2007) but may be particularly abundant in marine environments (Knowlton 2000). This is because most marine species do not require morphological recognition for mating, but instead rely on chemical cues for mate choice and gamete recognition (Stanhope *et al.* 1992; Palumbi 1994; Lonsdale *et al.* 1998) as well as for ecological interactions (Hay 2009). In marine sediments, free-living nematodes form one of the most abundant and diverse faunal groups, where they reach densities of 10^6 individuals m^{-2} and several tens of species m^{-2} (Heip et al. 1985). They may play a significant role in benthic mineralization processes (Coull 1999), impact microbial communities (De Mesel *et al.* 2004; Moens *et al.* 2005), and transfer carbon and energy to higher trophic levels (Leduc 2009). The use of nematodes as functional indicators relies on their classification into feeding groups or reproductive strategies (Bongers& Bongers 1998), but species belonging to the same feeding guild may also show considerable functional

differences which can be influenced by interspecific interactions (dos Santos et al. 2009). Therefore, inferring the importance of nematode diversity for ecosystem functioning requires species level identification (Yeates 2003). In view of the many species awaiting description (Blaxter 2004) and the discovery of substantial cryptic diversity within marine nematodes (Derycke *et al.* 2005; Derycke *et al.* 2007; Derycke *et al.* 2010), such species level distinction may not be feasible using classical morphology-based identification methods.

The marine nematode *Rhabditis (Pellioditis) marina* is a typical inhabitant of decaying macro algae in the intertidal zone of coasts and estuaries throughout the world (Inglis& Coles 1961). A phylogeographic study across Europe revealed that *R. (P.) marina* comprises at least 10 phylogenetic species (Derycke et al. 2008b) and at least six of them showed morphological differences when using a combination of morphometric characters (Derycke et al. 2008a). Many of these cryptic species have a wide geographic distribution, and frequently occur in sympatry with at least one other species of the complex (Derycke et al. 2008b). In view of the close taxonomic relationship and the very similar morphology of cryptic *R. (P.) marina* species, these sympatric occurrences are at odds with expectations from classical competition theory. Autecological characterization of cryptic species as well as information on interspecific interactions and functional performance are urgently needed, with particular emphasis on sympatrically occurring species, to investigate whether classical ecological concepts of competition also hold for species complexes. Such studies are currently hampered because of the inability to accurately identify and quantify cryptic species.

qPCR has proven to be useful to detect and quantify marine unicellular organisms (Fitzpatrick *et al.*; Creach *et al.* 2006), as well as multicellular parasitic nematodes from animals (MacMillan *et al.* 2006; Pecson *et al.* 2006; Campos-Herrera *et al.* 2010) and plants (Francois *et al.* 2007; Sato *et al.* 2007; Berry *et al.* 2008; Toyota *et al.* 2008; Huang *et al.* 2010; Nakhla

93 *et al.* 2010). Studies in free-living nematodes are hitherto restricted to the detection of few
94 terrestrial genera (Holterman et al. 2008). Here, we developed and tested a quantitative real
95 time PCR (qPCR) assay to detect and quantify four cryptic species of *R. (P.) marina* which
96 occur sympatrically in The Netherlands and which we successfully isolated, cultured and used
97 for competition experiments in the lab (De Meester et al. 2011). The aims of the present study
98 were fourfold: 1/ to detect cryptic species of the *R. (P.) marina* complex using a SYBR Green
99 assay by designing species specific primers for the ribosomal internal transcribed spacer
100 region (ITS), 2/ to determine the detection limit of the assay, 3/ to investigate differences in
101 copy number between developmental stages, and 4/ to assess the reliability of quantification
102 for each species.

Material and methods

Nematodes

Four cryptic species of *Rhabditis (Pellioditis) marina* are found sympatrically in Belgium and the Netherlands (Derycke et al. 2006) and are available from permanent lab cultures maintained on sloppy agar media with unidentified bacteria and *Escherichia coli* as food (Moens& Vincx 1998). These cultures were originally isolated from Paulina salt marsh (The Netherlands, PmI), Texel (The Netherlands, PmII), Woods Hole (USA, PmIII) and Lake Grevelingen (The Netherlands, PmIV).

Primer design

Previously published sequences of the internal transcribed spacer region (ITS1–5.8S–ITS2) of the ten hitherto recorded cryptic species of *R. (P.) marina* and of two congeners *R. (P.) mediterranea* and *R. (P.) ehrenbaumi* were used to develop species specific primers using the ARB software package (Ludwig et al. 2004) as implemented in ARBuntu v 2.0. The alignment contained 42 sequences (GenBank Accession numbers: AM398811–AM398825, AJ867057-AJ867073, AM937041–AM937053). Primers were designed using the following options: no hits to non-target sequences, a minimum of 100 % hits with target sequences, GC percentage between 50-100 and Tm between 30-100 °C. The resulting primer sequences were screened against the ITS alignment by allowing up to six mismatches. Primers with a maximum number of zero non target matches across a wide temperature range were selected, and adjusted so that the mismatches were located at the 3' end of the primer sequence and so that all primers had the same Tm, allowing for simultaneous qPCR of all four species. Primer sequences were then checked against the nematode sequences in Genbank using a Blast search to assess their specificity in silico. From these results, two primer pairs for each

species were tested for specificity and efficiency on a Lightcycler 480 System (Roche Diagnostics), and the best primersets were used for further experiments (Table 1).

Extraction of genomic DNA

DNA from a single specimen was used to test 1/ primer specificity and 2/ differences in ITS copy number between different life stages (juvenile, male, female). Individual nematodes were handpicked from monospecific lab cultures, transferred to sterile distilled water to remove traces of agar and then transferred to a 0.5 ml eppendorf tube containing 20 µl lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % NP 40, 0.45 % Tween20). Tubes were frozen for 10 min at –20 °C, after which 1 µl of proteinase K (10 mg ml^{-1}) was added. Lysis took place in an Eppendorf Mastercycler gradient PCR machine at 65 °C for 1 h followed by 10 min at 95 °C. Finally, the DNA samples were centrifuged for 1 min at maximum speed (14000 r.p.m.), and 1 µl was used as template for qPCR. DNA samples from the six other species of the *Rhabditis (Pellioditis) marina* species complex (PmV-PmX) and from the congeners *Rhabditis (Pellioditis) mediterranea* and *Rhabditis (Pellioditis) ehrenbaumi* were taken from a previous study in which the same DNA extraction procedure was followed (Derycke et al. 2008b).

The DNA used to construct the standard curves, to establish the limit of detection and to assess the accuracy of quantification was prepared using hexadecyltrimethylammoniumbromide (CTAB). Nematodes were rinsed off cultures using 2 x 1 ml S-buffer and centrifuged for 3 min at 3000 rpm. The supernatans was removed, 500 µl of CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris/HCl pH 8.75) was added to the nematode pellet and tubes were frozen at -80 °C for 10 min. Then, enzymatic (6 µl proteinase K 10 mg ml^{-1}) and mechanical (beadbeating)

lysis was performed. DNA was subsequently dissolved in 7.5 M ammonium acetate, followed by precipitation in cold isopropanol. DNA was washed by adding 1 ml washing buffer (76% ethanol and 10 mM ammonium acetate) and dissolved in 20 µl sterile water. The accuracy of the CTAB protocol was investigated using DNA extracts from 1, 5, 10, 50, 100 and 200 nematodes from each species separately and measuring the amount of DNA with the Nanodrop 2000 (Isogen Life Science). A good extraction should provide a positive correlation between the number of nematodes and the amount of DNA extracted. The samples with one nematode were prepared in 6 biological replicates (three females and three males), while all other samples were prepared in three biological replicates. Samples with five nematodes contained two or three males and females, and samples with 10 nematodes contained five females and five males. The remaining three samples (50, 100 and 200 nematodes) were prepared by pipetting from a homogeneous suspension from which five aliquots (100 or 250 µl) had been counted.

Real time quantitative PCR (qPCR)

qPCR was performed using the Lightcycler 480 System and the Lightcycler 480 SYBR Green I master kit (Roche Diagnostics). Following optimization of primer concentrations and cycling conditions, the qPCR mixture was prepared for a 20 µl reaction volume on 96-well plates using 10 µl LightCycler 480 SYBR Green I Master 2X solution, 3 µl PCR-grade water, 6 µl of each primer (end concentration of 1 µM for PmI and PmIII, 500 nM for PmII and 200 nM for PmIV) and 1 µl of template DNA. For quantitative analyses, 384-well plates were used and the qPCR mixtures were prepared for 10 µl volumes in the same concentrations as for the 96-well plates. All experiments always entailed a negative control (NTC, no template control) and two technical replicates. The thermal cycling protocol comprised an initial

denaturation for 10 min at 95 °C followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 20 s at 60 °C and extension for 20 s at 72 °C. A melting curve analysis was performed using a temperature range of 65 to 97 °C and an increase of 0.6 °C s^{-1} to confirm that only the specific products were amplified and primer dimers were absent. Finally, the samples were cooled down to 40 °C for 10 s.

Detection of cryptic species: primer specificity and efficiency

Primer specificity was assessed by running each of the four primer pairs with DNA from all cryptic species of the *Rhabditis (Pellioditis) marina* species complex and from two congeners *R.(P.) mediterranea* and *R. (P.) ehrenbaumi*. Next, a 10-fold serial dilution of an approximately 100 ng μl^{-1} gDNA extract from a single species (10^{2}, 10^{1}, 10^{0}, 10^{-1}, 10^{-2} ng μl^{-1}) was made to investigate the accuracy of the qPCR amplification across a range of DNA concentrations. Ct values (the cycle at which the fluorescence level raises above the background noise), standard curves and PCR amplification efficiencies (E) were calculated using the second derivative maximum method as implemented in the Lightcycler 480 Software (Roche Diagnostics). Ideally, E should be in the range of 1.8 – 2.2 (Schmittgen& Livak 2008). Next, serial dilutions of a 100 ng μl^{-1} gDNA mixture were prepared containing DNA from all four species in equal amounts (~25 ng μl^{-1}) or containing 100 ng μl^{-1} of one species and 10 ng μl^{-1} of the three other species. This allowed investigating whether primer efficiencies were affected by the presence of non-target DNA. All serial dilutions were prepared from three biological replicates.

Limit of detection of the assay

198 To investigate the detection limit of the qPCR assay, a single juvenile of one species was
199 added to 99 nematodes containing equal numbers of the three other species, in three
200 biological replicates. DNA extraction was performed using the CTAB protocol. For this
201 experiment, three technical replicates were made.
202
203 ITS copy number differences between developmental stages
204 Differences in qPCR signal between developmental stages may blur an adequate
205 quantification of nematode samples. Such differences may be caused by a different efficiency
206 of DNA extraction between species or stages, or, more likely, by differences in cell number
207 between (un)fertilized females, males and juveniles. Therefore, gDNA from a single juvenile,
208 male and female (five biological replicates, two technical replicates) was extracted from each
209 of the four species (PmI-PmIV), and used for qPCR with species specific primers.
210
211 Relative quantification
212 Relative quantification was evaluated using the strategy outlined in Mommer *et al.*
213 (2008) in two series of experiments. In a first series of experiments, we prepared 18 artificial
214 nematode mixtures in three replicates with different amounts of specimens from each species
215 but keeping a total of 100 nematodes per mixture. The number of nematodes for each species
216 ranged between 0 and 85 (Table 2). For numbers above 20, nematode suspensions were
217 prepared by pipetting from a homogeneous solution from which five aliquots (100 or 250 µl)
218 had been counted. ~~Such~~ The suspension~~suspensions~~ was~~ere~~ prepared by washing nematodes
219 with S-buffer from agar dishes, and contained a mixture of males, females and juveniles. The
220 average number of nematodes counted in five aliquots from this suspension was then used to
221 calculate the volume of the solution that was needed to obtain 25-85 nematodes. Nematode

numbers below 20 were manually picked one by one ~~manually~~, and contained adult nematodes only. A reference sample was made by pipetting 25 specimens of each species in one tube. This was repeated 40 times, and gDNA of all samples was extracted using the CTAB protocol, measured with the ND2000 Nanodrop and diluted to a 10 ng μl^{-1} solution to avoid inhibition by too high amounts of DNA. Ct values were averaged across duplicates and biological replicates (40 for the standard, three for the experiments).

In a second series of experiments, we prepared eight artificial nematode mixtures in three replicates each, with different numbers of three species (PmI, PmIII and PmIV), and always keeping a total of 33 nematodes. PmII was not included because of poor culture results at that time. In the first four mixtures, only adult nematodes were handpicked, while in the next four only juveniles were included. Reference samples were prepared by handpicking and pooling 11 adult or 11 juvenile nematodes from each of the three species. Adult reference samples were prepared in 10 replicates, juvenile reference samples in 5 replicates (variation in Ct values of juveniles was considerably lower than in adults (see results)).

Absolute quantification

Absolute quantification was evaluated using the strategy described in Campos-Herrera *et al.* (2010). For the first 18 experiments, a standard curve was constructed using three biological replicates of 100 nematodes. The DNA extracts of the three replicates were pooled and diluted so that the total DNA concentration was between 100 and 200 ng/µl. A tenfold serial dilution was made for each species by testing several starting concentrations. The most accurate standard curves (E around 2, error less than 0.02) were obtained with the highest DNA concentration corresponding to 10 (PmI and PmII), 20 (PmIV) or 40 (PmIII) nematodes. The serial dilution was loaded in duplicate for each species, and then used to

construct a standard curve from which our 'unknown' experimental samples could be quantified. These 'unknown' samples were brought to a DNA concentration of 10 ng/µl, to avoid inhibition effects when too much DNA template is present. The estimated number of nematodes was then corrected for the dilution factor.

Data analysis

All statistical analyses were performed with the Statistica 7 software (Statsoft 2004). Data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) to infer whether parametric or nonparametric statistics were required.

Detection of cryptic species: primer specificity and efficiency

The reliability of the CTAB DNA extraction method was investigated with a parametric 'Pearsons r' correlation coefficient by plotting the amount of DNA measured with the Nanodrop against the number of nematodes (1, 5, 10, 50, 100, 200). Differences in primer efficiencies of single and multi species DNA samples, and between the four species were investigated by factorial ANOVA with species and DNA mixtures as main effects. The posthoc Tukey HSD test was performed to investigate pairwise differences between species and mixtures.

ITS copy number differences between developmental stages

Differences in Ct values between males, females and juveniles within species were investigated by a one-way ANOVA. The Tukey HSD posthoc test was performed to investigate pairwise differences between developmental stages.

Relative quantification

Differences in Ct values among the 18 treatments for relative quantification were calculated using the $\Delta\Delta C_T$ method (Livak& Schmittgen 2001) adjusted according to Mommer et al. (Mommer et al. 2008). Because variances were not homogeneous for PmII, PmIII and PmIV (even after log (x+1) transformation), a non-parametric Spearman Rank correlation was performed to evaluate the relationship between the proportion of nematodes present in the sample and proportion of nematodes estimated by the qPCR assay. Subsequently, we used ANOVA (PmI) and Kruskal-Wallis ANOVA (PmII, PmIII and PmIV) to investigate whether significant differences between the estimated number of nematodes within each species occurred when they were grouped according to the actual number of nematodes present in the sample. In other words, all replicates with the same number of nematodes for a particular species were pooled irrespective of their experimental treatment. Tukey HSD for unequal sample size (PmI) or two-tailed multiple comparisons (PmII, PmIII and PmIV) were done to search for significant differences between percentage of nematodes.

For the second series of experiments, parametric correlation analyses were performed between the estimates of PmI and PmIII adults, and PmIII juveniles and the actual number of nematodes present in the sample. ANOVA and the Tukey HSD post hoc test were performed on PmIII juveniles to look for differences between qPCR estimates. Nonparametric correlation and Kruskal-Wallis ANOVA were performed to analyse estimates of PmI juveniles because variances of PmI were not homogeneous, even after log transformation.

Absolute quantification

The accuracy of absolute quantification was evaluated by two correlation analyses: 1/ the log of the amount of DNA from the serial dilutions was plotted against Ct values to investigate

294 the reliability of the qPCR assay, and 2/ the number of nematodes estimated from qPCR was
295 plotted against the actual number of nematodes inoculated into the experiments to infer the
296 reliability of absolute quantification. The former correlation was evaluated through the
297 parametric 'Pearsons r' correlation coefficient. Since the data for the latter correlation analysis
298 were not normally distributed, a non parametric Spearman Rank correlation was performed.
299 To investigate whether significant differences could be observed between qPCR estimates
300 when grouped according to the actual number of nematodes inoculated, ANOVA (for PmI
301 and PmIV) or Kruskal-Wallis ANOVA (PmII and PmIII) was performed. Tukey HSD for
302 unequal sample size (for PmI and PmIV) or two-tailed multiple comparisons (for PmII and
303 PmIII) were used for posthoc pairwise comparisons.
304

Results

Detection of cryptic species: primer specificity and efficiency

The amount of DNA measured with the Nanodrop was positively correlated with the number of nematodes from which the DNA was extracted (Spearman Rank r = 0.96, 0.98, 0.74 and 0.88 for PmI, PmII, PmIII and PmIV respectively, $p < 0.001$), indicating that the DNA extraction method was highly reliable. All four primer pairs proved to be highly specific: no cross amplification within the *R. (P.) marina* species complex or with the congeners *R. (P.) mediterranea* and *R. (P.) ehrenbaumi* was observed for primer pairs PmIII and PmIV. A late Ct call was observed for *R. (P.) mediterranea* with primer pair PmI (Ct = 25.69 ± 0.13, whereas Ct = 10.45 ± 0.03 for PmI) and for PmX with primer pair PmII (Ct = 33.34 ± 0.07, whereas Ct = 14.22 ± 0.08 for PmII). Since the fluorescence level of these signals was low and did not reach the plateau phase of the amplification curve, these false positive results were easily detected. Melting curve analysis showed a single peak for each of the four primer pairs, indicating the absence of aspecific products or primer dimers. Optimization of the primer concentration yielded accurate primer efficiencies in the range of 1.8 - 2.1 which were unaffected by the presence of non target DNA ($F_{2,24} = 1.99$, $p = 0.158$).

Detection limit of the assay

Each primer pair was able to detect a single juvenile in a pool of 100 nematodes. Average Ct-values were 20.69 (± 0.52), 24.00 (± 5.25), 20.02 (± 0.26) and 20.71 (± 0.11) for PmI, PmII, PmIII and PmIV respectively.

ITS copy number differences between developmental stages

Ct -values were significantly different between developmental stages for PmI ($F_{2, 12} = 49.3$, $p < 0.0001$), PmII ($F_{2, 11} = 45.9$, $p < 0.0001$) and PmIV ($F_{2, 12} = 100.9$, $p < 0.0001$), but not for PmIII ($F_{2, 12} = 1.4$, $p = 0.28$) (Fig 1). Posthoc comparisons showed that Ct values were significantly different between females, males and juveniles of PmI and PmIV (all $p < 0.001$, except for females and males of PmI, where $p = 0.02$). For PmII, juveniles had significantly higher Ct values than males and females ($p < 0.0001$), while no significant differences were observed between Ct values of males and females. The highest variation in Ct values was observed between females of the four species, while males and juveniles yielded similar Ct values between species (Fig 1).

Relative quantification

The amount of DNA extracted from 100 nematodes from the first 18 experiments yielded an average of 597.1 ng/µl DNA. A significant positive correlation between the qPCR estimate and the actual number of nematodes was observed for all four species (Spearman Rank order correlation $r = 0.70$, $r = 0.82$, $r = 0.73$ and $r = 0.68$, for PmI, PmII, PmIII and PmIV respectively, all $p < 0.05$). For some experiments, an overestimation of the proportion of nematodes was, however, obtained (Fig 2). This was especially true for experiments 1, 2, 3, 5, 6 and 7 for PmIV, which contained 16 or 20 handpicked adult nematodes. In contrast, when 16 nematodes (experiment 13) or 25 nematodes (experiment 15) of PmIV were added by pipetting (and thus containing a mixture of juveniles and adults), the estimate was very similar to the actual number of nematodes added to the samples (Fig 2). Correlation coefficients with only pipetted experiments were high for PmII and PmIV (Spearman Rank order correlation $r = 0.52$, $r = 0.94$, $r = 0.67$ and $r = 0.95$, for PmI, PmII, PmIII and PmIV respectively, all $p < 0.05$). ~~For PmI and PmIII, correlations increased considerably when three~~

~~and six outliers were removed (Fig 3; r = 0.85 and r = 0.86 for PmI and PmIII, respectively, p < 0.05).~~ ANOVA and Kruskal-Wallis ANOVA highlighted significant differences between the estimated number of all four nematode species when grouped according to the actual percentage of nematodes present in the sample (PmI: $F_{6,20} = 11.35$, $p < 0.0001$; PmII: $H_{7,25} = 22.04$, $p = 0.003$; PmIII: $H_{6,27} = 21.3$, $p = 0.002$; PmIV: $H_{6,26} = 23.02$, $p = 0.0008$). Pairwise comparisons revealed that these differences were caused between the treatments with no nematodes (0 %) and treatments with the highest proportion of a nematode species (70 and 85 % for PmII, 85% for PmIII, 50 and 85 % for PmIV), but the parametric Tukey HSD test revealed more significant differences for PmI (Fig ~~4~~3).

In a second series of eight experiments, we investigated whether using only adults or only juveniles would result in more accurate qPCR estimates of PmI and PmIII nematodes. Juveniles resulted in more accurate estimates than adults (Fig ~~5~~4). No significant product moment correlation coefficients were observed for the experiments with adults. In contrast, strong correlations were observed for PmI ($r = 0.89$, $p < 0.05$) and for PmIII ($r = 0.89$, $p < 0.05$) juveniles. Kruskal-Wallis and ANOVA yielded significant differences among groups (PmI: $H_{3,11} = 8.20$, $p = 0.042$; PmIII: $F_{3,8} = 43.04$, $p < 0.001$).

Absolute quantification

The log transformed DNA concentrations from the gDNA dilution series (single and multi species extracts) were significantly negatively correlated with Ct -values for all species over the entire DNA concentration range (Pearsons r ranging between -0.94 and -0.99, $p < 0.0001$). In general, the qPCR estimate was very close to the actual number of nematodes present in the experiments, especially for PmII and PmIII (Fig ~~6~~5). For PmI and PmIV, there was a strong overestimation for those experiments where nematodes were manually added

(experiments 3, 7 and 8 for PmI, and experiments 1, 2, 3, 5, 6 and 7 for PmIV). Since standardcurves were generated from pipetted nematodes, the reliability of quantification for all species was further investigated using only the experiments for which individuals were pipetted (in bold in Table 2). For all four species, a significant positive correlation was observed between the actual number of nematodes added and the estimated number by the qPCR. Product-moment correlation coefficients were 0.625 (p=0.002), 0.864 (p=0.000), 0.808 (p=0.000) and 0.909 (p=0.000) for PmI, PmII, PmIII and PmIV, respectively. ANOVA and Kruskal-Wallis ANOVA indicated significant differences between the estimated number of nematodes when they were grouped according to the actual number of nematodes present in the sample for all species (PmI: $F_{6, 17} = 3.89$, $p = 0.01$; PmII: $H_{6,23} = 19.36$, $p = 0.004$; PmIII: $H_{6,24} = 19.94$, $p = 0.003$; PmIV: $F_{5, 18} = 20.54$, $p < 0.0001$) (Fig ~~7~~6).

389 **Discussion**

390 Although the increased use of molecular tools has provided new insights in a wide
391 range of biological and evolutionary disciplines, surprisingly few molecular techniques have
392 been used two answer ecological questions (Johnson et al. 2009). One key question in
393 ecology concerns the regulation of ecosystem functioning by biodiversity (Hillebrand&
394 Matthiessen 2009). Understanding the relationship between biodiversity and ecosystem
395 functioning requires the identification and quantification of diversity units (generally
396 considered to be species) as well as knowledge on the functional role of the species in the
397 ecosystem. In the case of cryptic species, such information can only be achieved when
398 molecular tools are developed to identify and quantify each of the species in the species
399 complex.

400 The present study has demonstrated the reliability and sensitivity of qPCR to detect
401 cryptic species of a marine nematode species complex. The accurate detection of any species
402 through qPCR requires the use of specific primers which in turn relies on the adequate
403 resolution of the DNA region under study. For nematodes, the 18S rDNA (MacMillan et al.
404 2006) and the internal transcribed spacer region (ITS) (e.g. Pecson *et al.* 2006; Sato *et al.*
405 2007; Toyota *et al.* 2008; Campos-Herrera *et al.* 2010; Nakhla *et al.* 2010) have most
406 frequently been used. Here, we chose the ITS region because it showed the best level of
407 variation to develop species specific primers when compared to the COI and D2D3 sequence
408 databases obtained from previous studies of this species complex (Derycke *et al.* 2008a;
409 Derycke *et al.* 2008b; Fonseca *et al.* 2008). Our results demonstrate that the four cryptic
410 *Rhabditis* species can be reliably identified from their closest relatives in experimental
411 samples. In view of the high interspecific variability of the ITS region, it is unlikely that more

412 distantly related species would be amplified. We therefore feel that the primers tested here
413 may also be applicable for detecting the cryptic *R. (P.) marina* species in field samples.
414 Next to the high specificity, the assay proved to be highly sensitive since a single
415 juvenile was detected in a pool of 100 nematodes. This high sensitivity is in agreement with
416 results from parasitic nematode species (MacMillan *et al.* 2006; Pecson *et al.* 2006; Sato *et al.*
417 2007; Huang *et al.* 2010), and enables an accurate determination of presence/ absence of the
418 cryptic *R. (P.) marina* species. The high sensitivity of the qPCR assay is further demonstrated
419 in the significant difference in Ct values between a single juvenile, male or female. Juveniles
420 of the rhabditid model organism *Caenorhabditis elegans* contain approximately half the
421 number of cells of adult males and females (Sulston& Horvitz 1977; Sulston *et al.* 1983).
422 Since the embryonic development and cell number in juveniles is very similar between *C.*
423 *elegans* and *R. (P.) marina* (Houthoofd et al. 2003), the observed difference in Ct value
424 between adults and juveniles was not surprising, and has also been observed in the root-lesion
425 nematode *Pratylenchus penetrans* (Sato *et al.* 2007; Huang *et al.* 2010). Low variation in Ct
426 values between juveniles and between males of the four species was observed, while the ITS
427 copy number between females proved to be highly different between the four species. This
428 high variation can be explained by a different number of eggs present in the uterus of adult
429 females. Within the *R. (P.) marina* species complex, and even within one and the same
430 cryptic species, mode of reproduction may vary from oviparous to ovoviviparous. The
431 number of cells in a developing egg increases from one (the zygote) to 638 (the J1 stage)
432 (Houthoofd et al. 2003), and this entire range may be present within the uterus of a single
433 gravid female. Obviously, females containing different numbers of eggs in different stages of
434 embryological development will yield different Ct values. Observations on cultures of the
435 four cryptic *R. (P.) marina* species show that females of species PmI and PmIV typically

436 contain a large number of eggs in the uterus and often reproduce through ovovivipary, while
437 PmIII females contain only few eggs in the uterus and rapidly deposit their eggs in the culture
438 medium. This can explain the lack of difference in Ct value between developmental stages in
439 PmIII, as well as the low Ct values of PmI and PmIV females. This shows the importance of
440 optimization for each species separately, no matter how closely related they might be.
441 Despite these differences in cell numbers, a strong positive correlation was observed
442 for each species between the amount of extracted DNA and the number of nematodes present
443 in the sample, indicating that quantification is possible. We have substantially tested and
444 validated the quantification accuracy of the qPCR assay. Our results demonstrate that it is
445 essential to use reference samples and standard curves that have been generated in the same
446 way as the experimental treatments to obtain accurate quantification estimates. When the
447 experimental samples were obtained by pipetting or by handpicking, quite different estimates
448 were obtained. The difference between pipetted and handpicked experiments was especially
449 prominent for species PmI and PmIV, suggesting that these differences were related to
450 differences in Ct values between developmental stages. We therefore expected to find more
451 accurate estimates in the experiments with only juveniles than in those with only adults. This
452 was clearly the case in our test with PmI and PmIII adults and juveniles (Fig ~~5~~4). While a
453 strong positive correlation was observed for PmI and PmIII juveniles with the qPCR estimate,
454 no significant correlation was observed for the adults. It is possible to separate juveniles from
455 adults from a practical perspective: adults of all four Pm species are larger than 1 mm
456 (Fonseca et al. 2008), while juveniles are substantially smaller; therefore, sieving the samples
457 across different mesh sizes can separate adults from juveniles.
458 The qPCR assay significantly differentiates between low and high numbers of
459 nematodes of each of the four cryptic species in our artificially generated test samples. We

used a total of 100 nematodes for each of the experiments tested here, but in real samples total nematode abundances are much higher and differ widely among samples. It is therefore likely that significant~~, and~~ differences in abundances ~~among species~~between natural samples ~~are likely to be larger~~will be more pronounced than the differences between our experiments. Moreover, significant differences were observed within the juvenile only experiments containing between two and 30 specimens of a particular species (Table 2). ~~As in other qPCR assays (Mommer et al. 2008), individual estimates sometimes show a relatively large deviation from the mean. The variation among biological replicates may partially be caused by the way our test samples were prepared. It is possible that some nematodes were stuck to the needle and thus were not transferred perfectly to the tubes. It is therefore likely that real samples will show less variation. Both methods of quantification yielded quite similar results. Relative quantification yielded slightly higher correlation coefficients than the absolute quantification and the preparation of a good standard curve requires more optimization than preparing the reference samples for relative quantification. The choice of quantification method for future experiments will greatly depend on the experimental setup: if laboratory cultures are present of each nematode, it is quite easy to generate sufficient reference samples and relative quantification may be better suited.~~ Based on our results, the best strategy to quantify real samples may well be achieved by first counting the total number of nematodes in the sample. From this, the volume to obtain 100 nematodes can be determined, and this suspension is then subjected to DNA-extraction. To avoid any inhibition by excess of DNA, the DNA sample should be diluted to ca 10 ng μl^{-1} before qPCR amplification with each of the species specific primers. For absolute quantification, the abundance of each species is then calculated by correcting the qPCR estimate with the dilution factor applied to get the 10 ng μl^{-1} and by converting this number to the total amount of nematodes that was present in the

sample. In addition, higher precision may be achieved by separating juveniles from adults by first sieving the sample, and then quantify both fractions separately. For the four species used here, males and females can easily be distinguished under a binocular. In case stage-specific patterns would be of interest, females, males and juveniles can be separated under a binocular by handpicking, and each of the three fractions can then be quantified separately. This approach will inevitably come at a cost of efficiency, since there is no easy way other than manual sorting to rapidly separate males from females.

Both methods of quantification yielded quite similar results. Relative quantification yielded slightly higher correlation coefficients than the absolute quantification and the preparation of a good standard curve for absolute quantification requires more optimization than preparing the reference samples for relative quantification. However, absolute quantification is often preferred in ecological studies and environmental monitoring. Overestimation of the abundance of one species will not automatically result in the underestimation of other species, since the abundance is determined based on the standard curves for each species separately. Choosing the mode of quantification and the precision of the estimates will greatly depend on the research question at hand. When the primary objective is to monitor abundances of cryptic species over time in function of the presence or absence of other cryptic species, then a relative quantification of the total nematode community may be sufficient. When the objective is however to look at fine scale responses of cryptic species to environmental variables, then life-stage dependent estimates through an absolute quantification may be more relevant.

The next step now is to design controlled laboratory experiments with single and multi species treatments under different abiotic conditions to investigate the ecology and functioning of cryptic marine species and their importance in maintaining the functioning of

ecosystems without the need for the laborious sorting and specimen-by-specimen analysis of high numbers of individuals.

Conclusions

The qPCR assay developed here shows the ability to quickly identify and quantify cryptic, closely related nematode species. This is of special interest, since cryptic species prevail in the marine environment (Knowlton 1993). They remain, however, difficult to study using traditional morphological identification tools because of a general lack of easily observable diagnostic characters. Very often, such diagnostic characters are completely lacking in juvenile stages. The qPCR method described and validated here offers a way to study the ecology and functioning of cryptic species in a way that was not possible before.

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652

Data accessibility

DNA sequences used to develop the primers: GenBank Accession numbers: AM398811–AM398825, AJ867057-AJ867073, AM937041–AM937053.

Raw data (Ct values of standard replicates and experimental samples for each of the four species- Experiments 1-18), with calculation of relative quantification: supplementary file S1.

Raw data (Ct values and concentration of experimental samples – Experiments 1 -18), with calculation of absolute quantification: supplementary file S2.

Raw data (Ct values of standard replicates and experimental samples for juveniles and adults – Experiments 19-26), with calculation of relative quantification: supplementary file S3.

Acknowledgements

SD acknowledges Paul Mooijman and Hans Helder for introducing their qPCR protocol for detection of soil nematodes. Raquel Campos-Herrera is gratefully acknowledged for providing valuable guidelines and information during optimization of the qPCR protocol. This research was financially supported by the Flemish Fund for Scientific Research (F.W.O) through the project 3G040407 and the research grant 1507709, and by Ghent University through the BOF project B/07778/02. S.D. acknowledges a postdoctoral fellowship from the F.W.O.

672 **Figure Legends**

673

674 **Figure 1:** Scatterplot of Ct values of developmental stages of four cryptic species of
675 *Rhabditis (Pellioditis) marina*. (F) females, (M) males and (J) juveniles. Vertical bars denote
676 95% confidence intervals.

677

678 **Figure 2:** Relative quantification of the four *Rhabditis (Pellioditis) marina* species in the first
679 18 experiments. Experiments along the X-axis are plotted according to increasing percentage
680 of nematodes that were put in the experiments. Numbers along the X-axis correspond to
681 experiments as mentioned in Table 2. Black bars indicate the average qPCR estimate of three
682 biological and two technical replicates, white bars represent the actual percentage of
683 nematodes present in the experiments. Vertical bars denote the standard deviation of all
684 replicates.

685

686 ~~**Figure 3:** Scatterplot of actual versus estimated percentage of nematodes for the four~~
687 ~~*Rhabditis (Pellioditis) marina* species. Only experiments that have been pipetted are included.~~
688 ~~Black coloured labels are outliers which were removed to calculate Pearson's correlation.~~
689 ~~Dashed lines indicate 95 % confidence intervals.~~

690

691 **Figure 43:** Box plots of actual versus estimated percentage of the four *Rhabditis* species
692 using relative quantification. Only experiments with pipetted animals were included. The
693 mean was taken from three to nine replicates. Letters indicate significant differences as shown
694 by the Tukey HSD test for unequal sample size (PmI) or two tailed multiple comparisons

(PmII, PmIII and PmIV). When letters are shared or absent, no significant differences were observed.

Figure ~~5~~4: Relative quantification of adults and juveniles of three *Rhabditis (Pellioditis) marina* species. Experiments along the X-axis are plotted according to increasing number of nematodes that were put in the experiments. Numbers along the X-axis correspond to experiments as mentioned in Table 2. Black bars indicate the average qPCR estimate of three biological and two technical replicates, white bars represent the actual number of nematodes present in the experiments. Vertical bars denote the standard deviation of all replicates.

Figure ~~6~~5: Absolute quantification of the four *Rhabditis (Pellioditis) marina* species in the first 18 experiments. Experiments along the X-axis are plotted according to increasing number of nematodes that were put in the experiments. Numbers along the X-axis correspond to experiments as mentioned in Table 2. Black bars indicate the average qPCR estimate of three biological and two technical replicates, white bars represent the actual number of nematodes present in the experiments. Vertical bars denote the standard deviation of all replicates.

Figure ~~7~~6: Box plots of actual versus estimated number of four *Rhabditis* species using absolute quantification. Only pipetted experiments are included. The mean was taken from three to nine replicates. Letters indicate significant differences as shown by the Tukey HSD test for unequal sample size (PmI and PmIV) or two tailed multiple comparisons (PmII and PmIII). When letters are shared or absent, no significant differences were observed.

719 **Tables**

720 Table 1: *Rhabditis (Pellioditis) marina* species specific primers for qPCR. Target: the target
721 species for which the primers were designed (PmI, PmII, PmIII or PmIV). Tm: melting
722 temperature of the primer. GC: percentage GC of the primers..

Target	Primer sequence (5´ → 3´)	Amplicon length	Tm (°C)	GC (%)
PmI	F: CGCTGACCTTCACTGGAATTTT	135	53	45.45
	R: CCGACTCCGGTTCAACTCA		53	57.89
PmII	F: GATCATCGCTGACCTTGG	294	50	55.56
	R: CGCACCATGTTGCCATGA		50	55.56
PmIII	F: AGCGGGGTGAAAGCCCA	410	52	64.71
	R: CTGAACTAGAATGGGTACATTCA		52	39.13
PmIV	F: CGATGGATGGTTTTCGCG	134	50	55.56
	R: GTGTATTGACGCTGTCCGTT		52	50

723

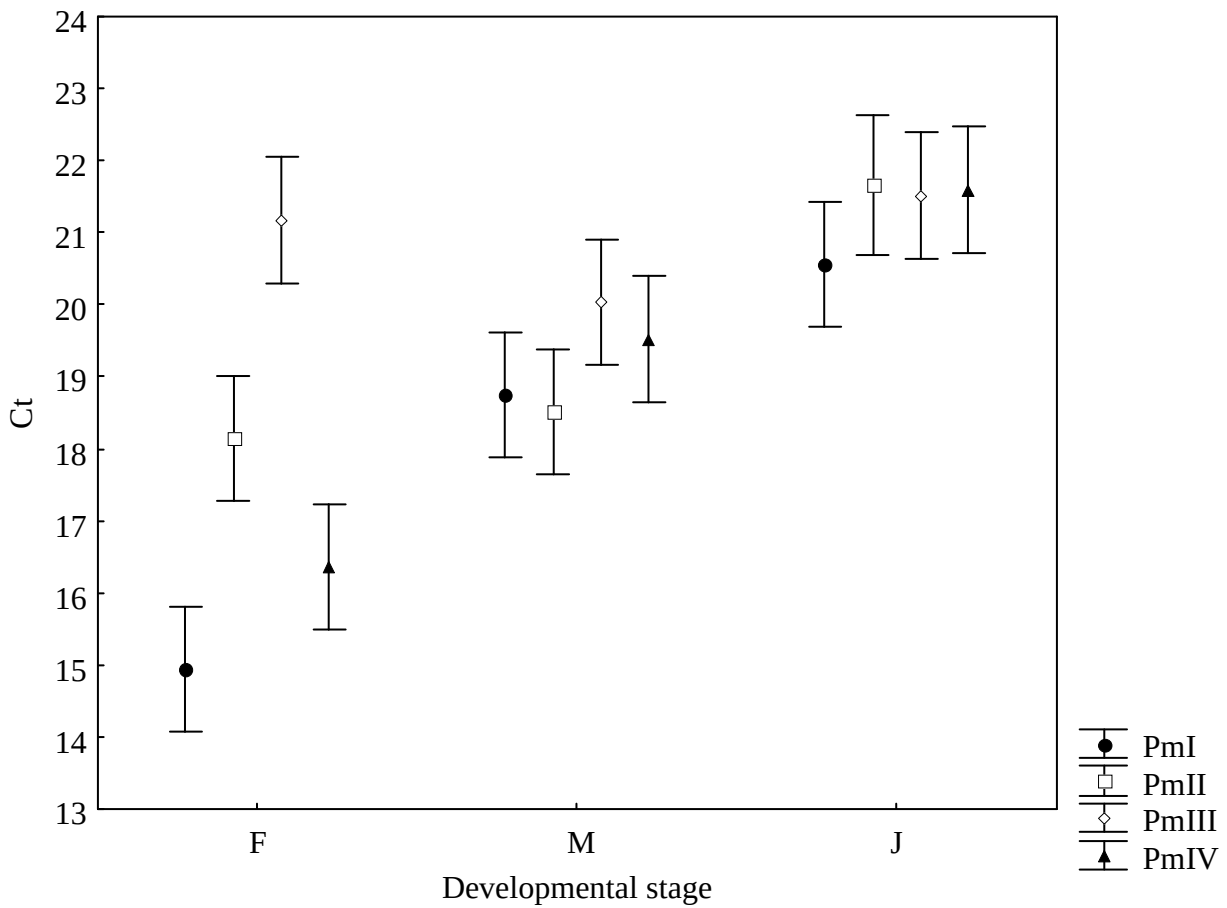
724

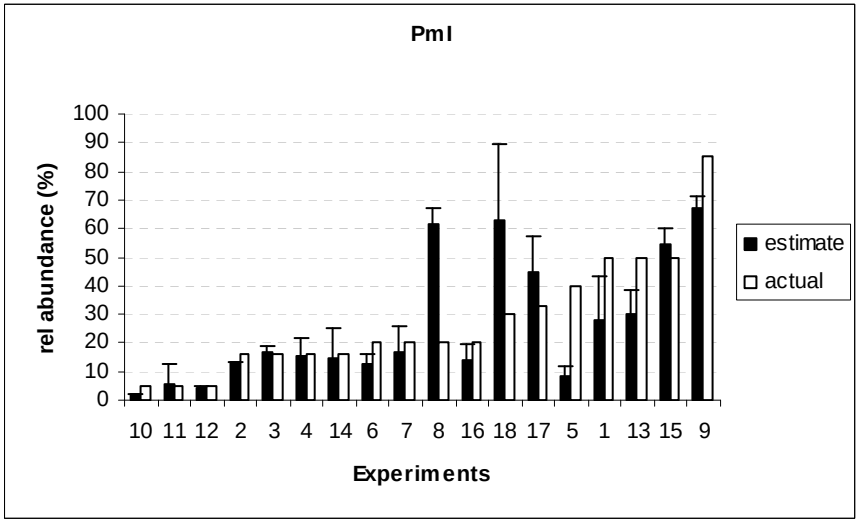
725

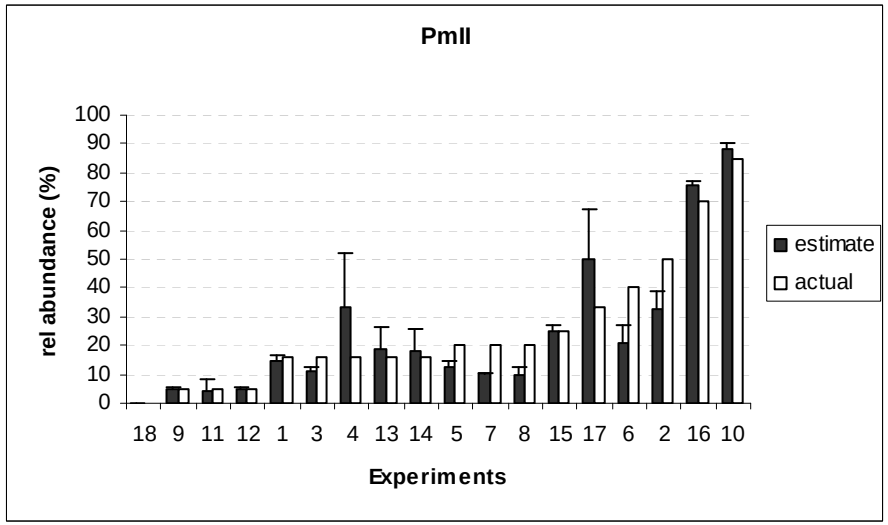
726 Table 2: Nematode composition of 26 artificial experiments with different numbers of PmI,
727 PmII, PmIII and PmIV. Nematodes that were pipetted are indicated in bold. Numbers
728 not bold were handpicked. For experiments 19 – 26, only adults or only juveniles were
729 handpicked, while for the first 18 experiments a mixture of adults and juveniles was
730 used.
731

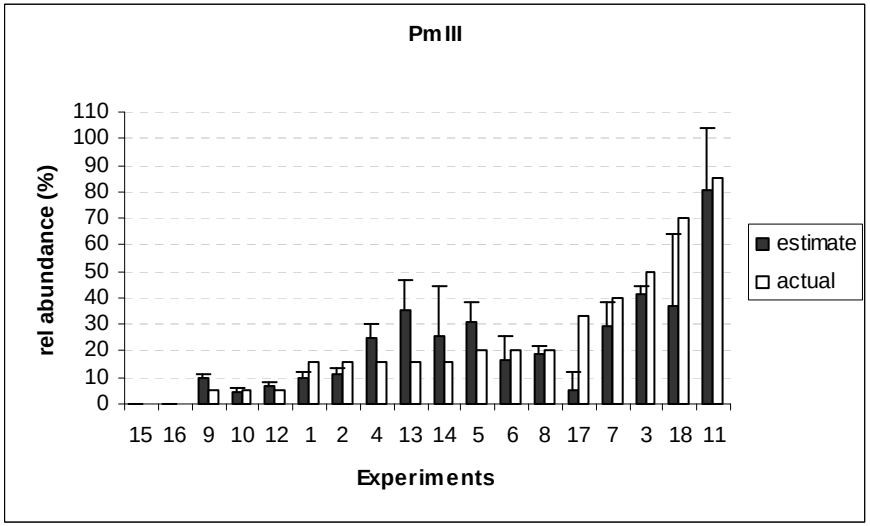
Experiment	# PmI	# PmII	# PmIII	# PmIV	developmental stage
1	**50**	16	16	16	mix
2	16	**50**	16	16	mix
3	16	16	**50**	16	mix
4	16	16	16	**50**	mix
5	**40**	20	20	20	mix
6	20	**40**	20	20	mix
7	20	20	**40**	20	mix
8	20	20	20	**40**	mix
9	**85**	5	5	5	mix
10	5	**85**	5	5	mix
11	5	5	**85**	5	mix
12	5	5	5	**85**	mix
13	**50**	**16**	**16**	**16**	mix
14	**16**	**16**	**16**	**50**	mix
15	**50**	**25**	**0**	**25**	mix
16	**20**	**70**	**0**	**10**	mix
17	**33**	**33**	**33**	**0**	mix
18	**30**	**0**	**70**	**0**	mix
19	16	0	10	5	adults
20	5	0	21	5	adults
21	26	0	4	2	adults
22	2	0	28	2	adults
23	16	0	10	5	juveniles
24	5	0	21	5	juveniles
25	26	0	4	2	juveniles
26	2	0	28	2	juveniles

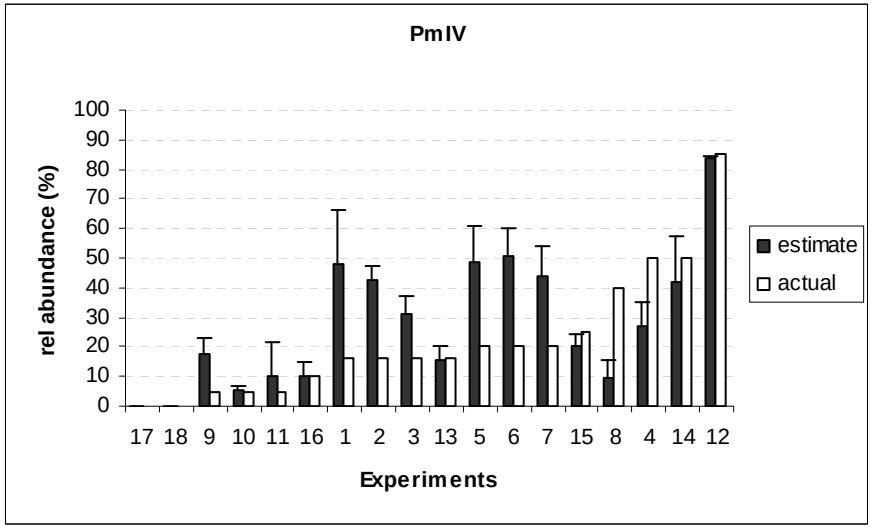
732
733

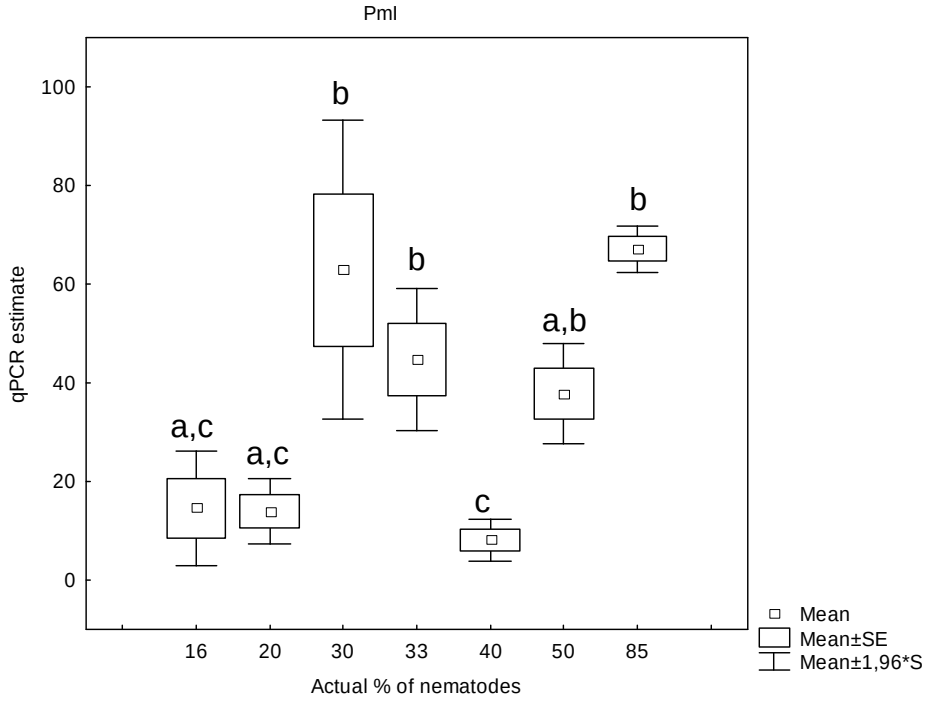

24
23
22
21
20
19
18
17
16
15
14
13
Ct
F
M
J
Developmental stage
PmI
PmII
PmIII
PmIV

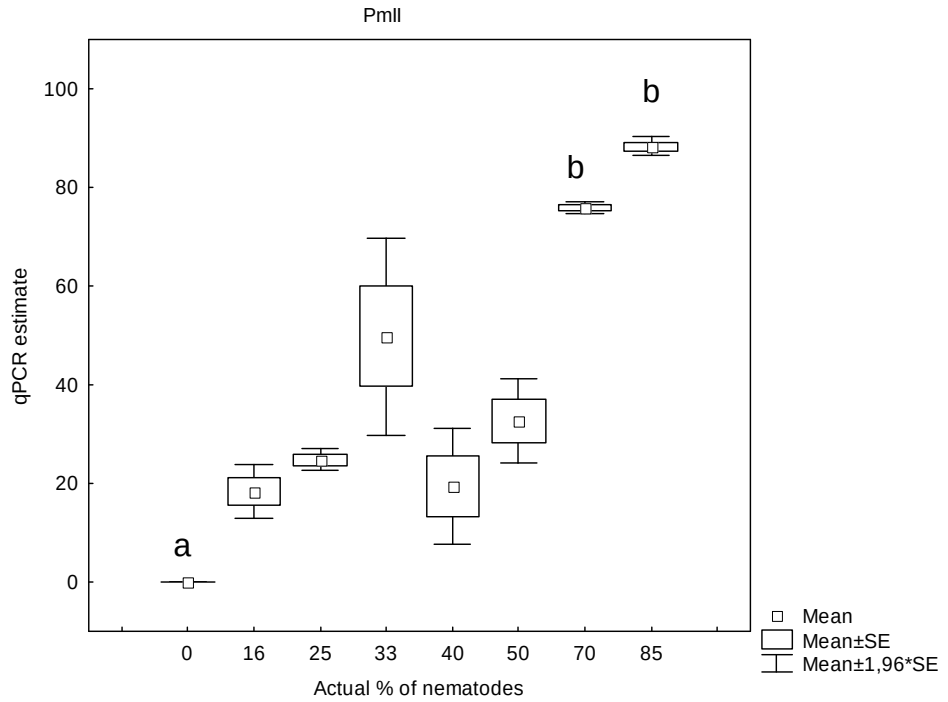

PmI
rel abundance (%)
100
90
80
70
60
50
40
30
20
10
0
10 11 12 2 3 4 14 6 7 8 16 18 17 5 1 13 15 9
Experiments
estimate
actual


PmII
rel abundance (%)
100
90
80
70
60
50
40
30
20
10
0
18 9 11 12 1 3 4 13 14 5 7 8 15 17 6 2 16 10
Experiments
estimate
actual


PmIII
rel abundance (%)
110
100
90
80
70
60
50
40
30
20
10
0
15 16 9 10 12 1 2 4 13 14 5 6 8 17 7 3 18 11
Experiments
estimate
actual


PmIV
rel abundance (%)
100
90
80
70
60
50
40
30
20
10
0
17 18 9 10 11 16 1 2 3 13 5 6 7 15 8 4 14 12
Experiments
estimate
actual


PmI
qPCR estimate
100
80
60
40
20
0
a,c
a,c
b
b
c
a,b
b
16
20
30
33
40
50
85
Actual % of nematodes
Mean
Mean±SE
Mean±1,96*S


PmII
qPCR estimate
100
80
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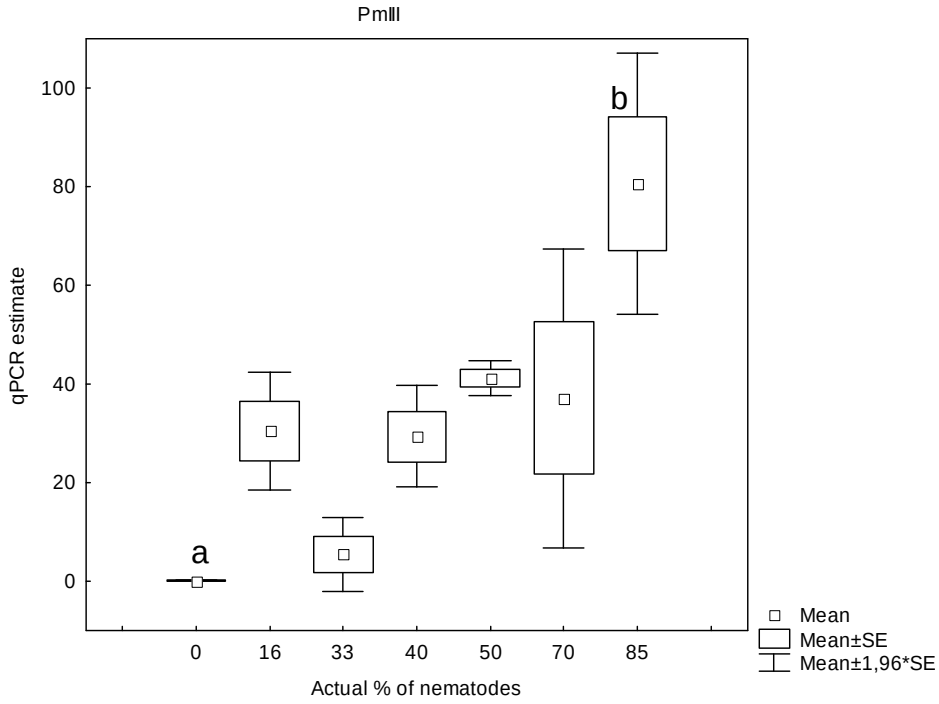
40
20
0
a
b
b
b
0
16
25
33
40
50
70
85
Actual % of nematodes
Mean
Mean±SE
Mean±1,96*SE


PmIII
qPCR estimate
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40
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b
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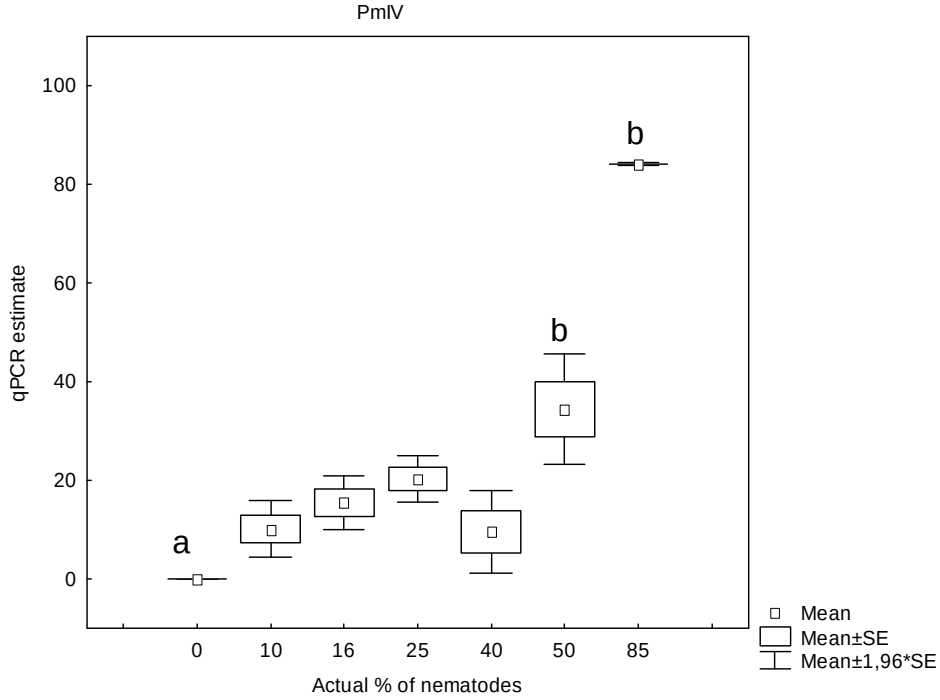
70
85
Actual % of nematodes
Mean
Mean±SE
Mean±1,96*SE


PmIV
qPCR estimate
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80
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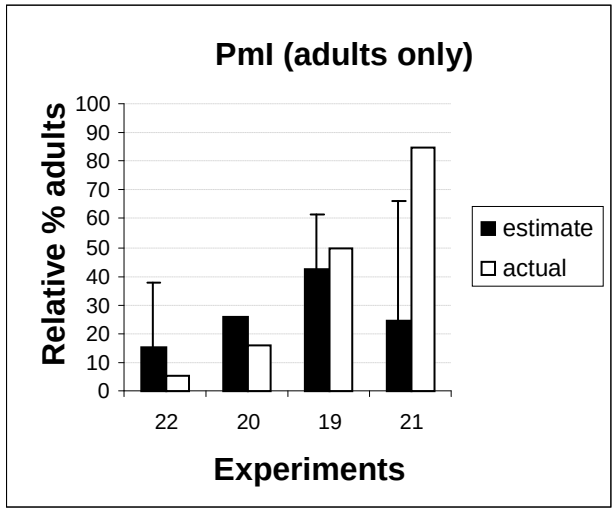
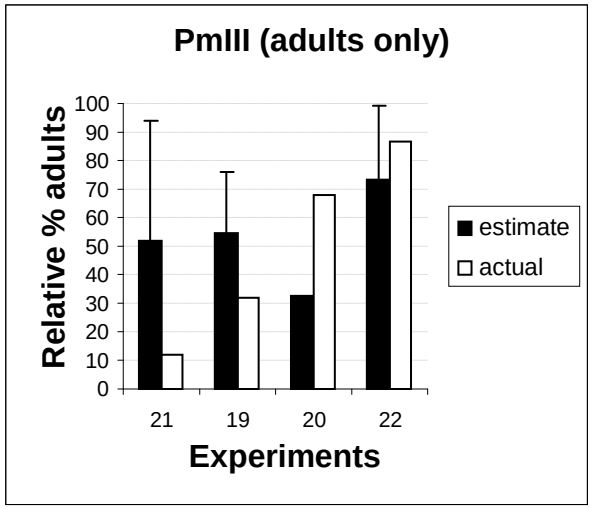
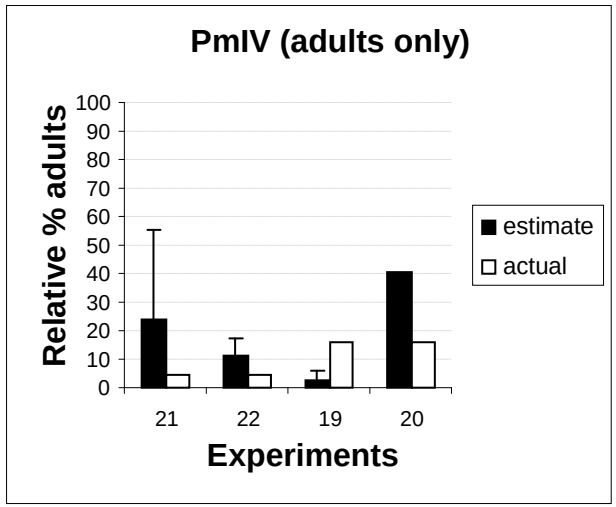
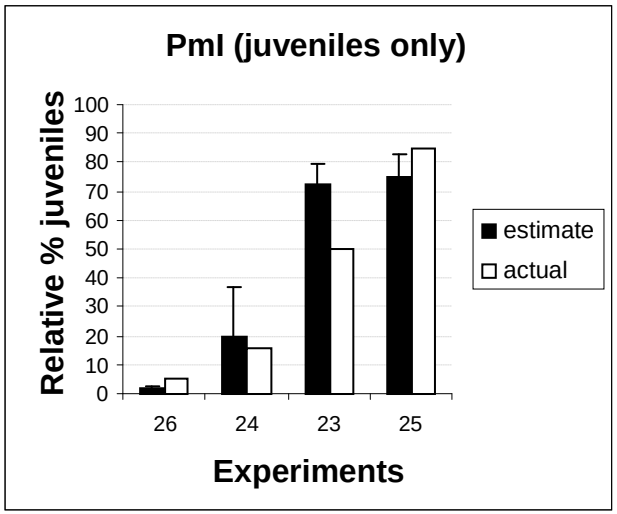
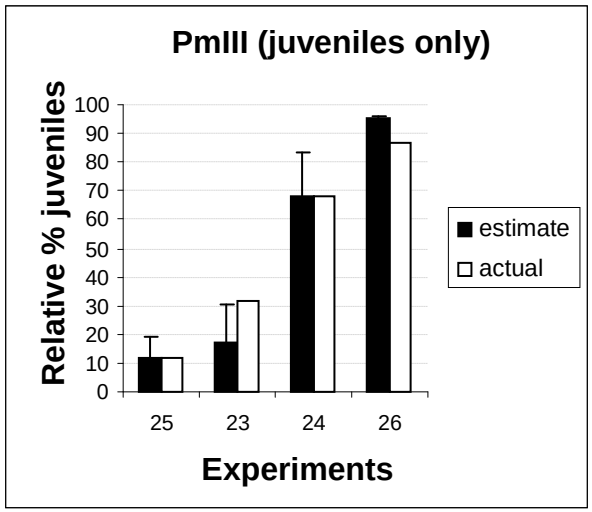
0
a
b
b
0
10
16
25
40
50
85
Actual % of nematodes
Mean
Mean±SE
Mean±1,96*SE

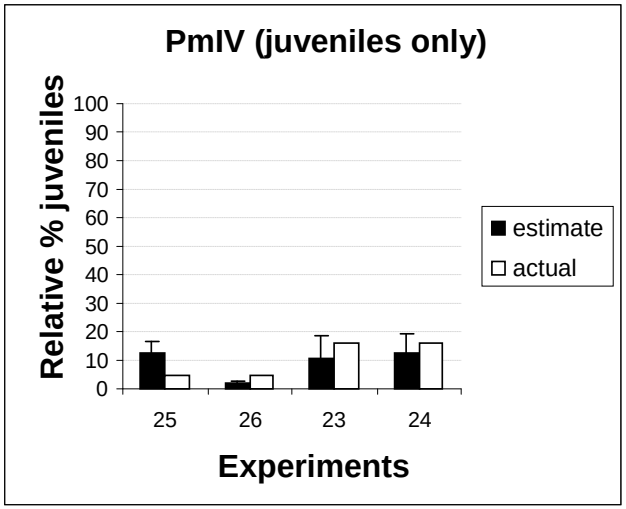

PmI (adults only)
Relative % adults
100
90
80
70
60
50
40
30
20
10
0
22
20
19
21
Experiments
estimate
actual

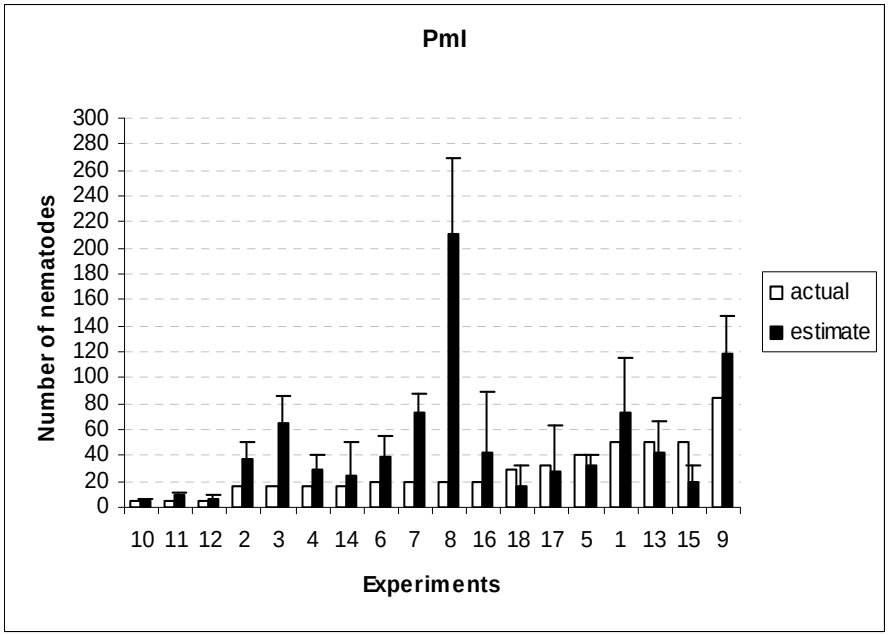

PmIII (adults only)
Relative % adults
100
90
80
70
60
50
40
30
20
10
0
21
19
20
22
Experiments
estimate
actual

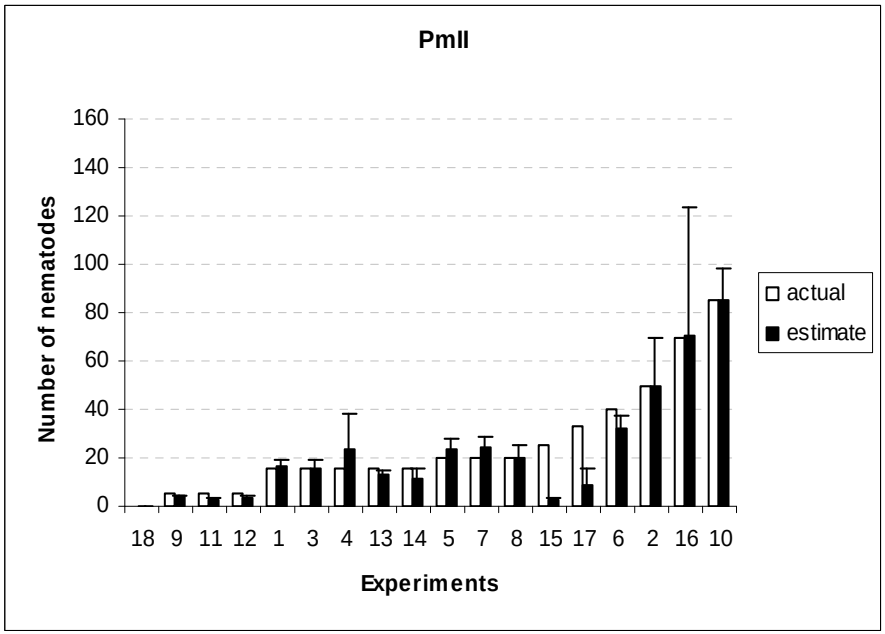

PmIV (adults only)
Relative % adults
100
90
80
70
60
50
40
30
20
10
0
21
22
19
20
Experiments
estimate
actual

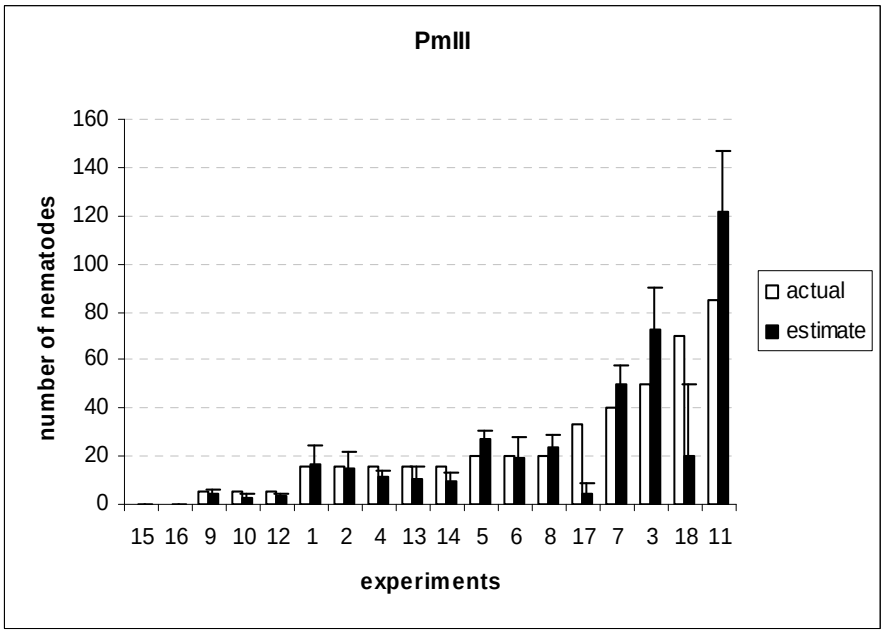

PmI (juveniles only)
Relative % juveniles
100
90
80
70
60
50
40
30
20
10
0
26
24
23
25
Experiments
estimate
actual

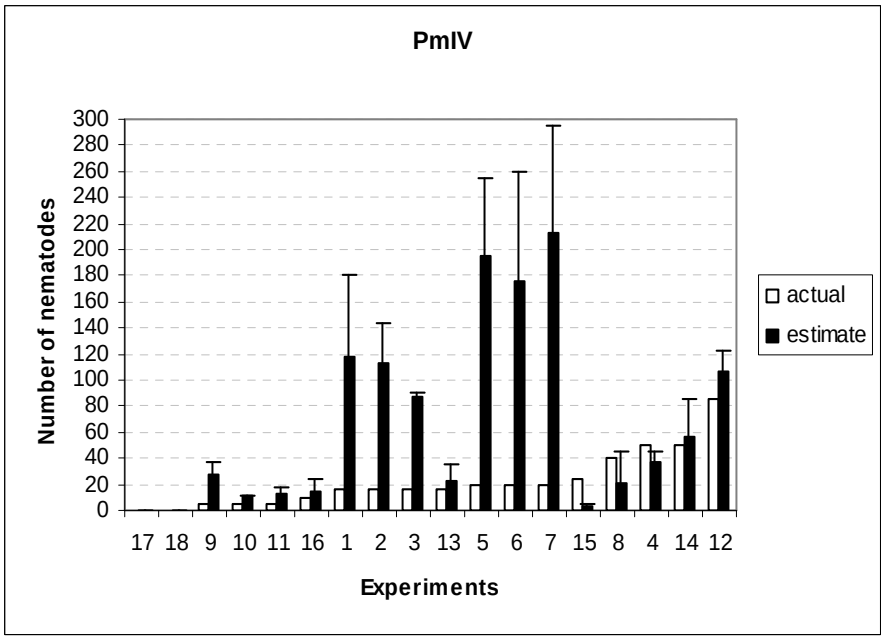

PmIII (juveniles only)
Relative % juveniles
100
90
80
70
60
50
40
30
20
10
0
25
23
24
26
Experiments
estimate
actual

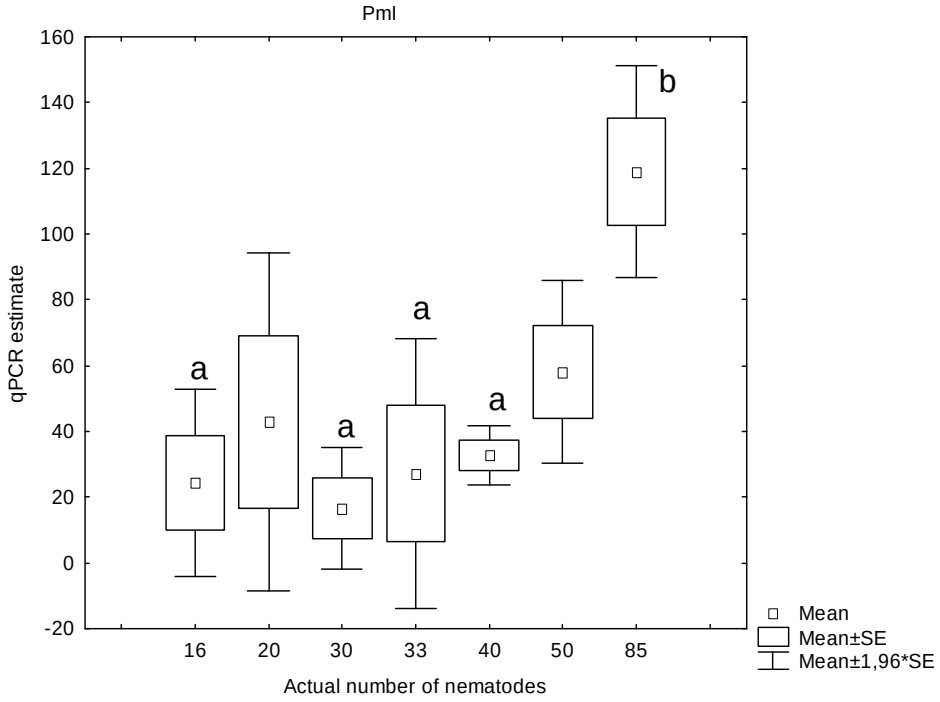

PmIV (juveniles only)
Relative % juveniles
100
90
80
70
60
50
40
30
20
10
0
25
26
23
24
Experiments
estimate
actual

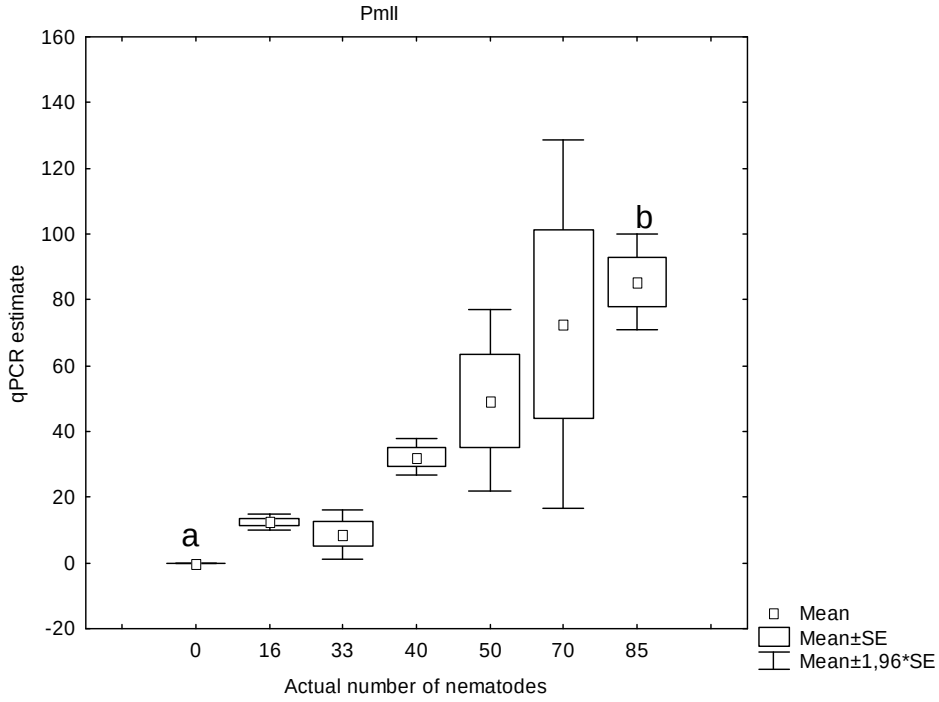

PmI
Number of nematodes
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
10 11 12 2 3 4 14 6 7 8 16 18 17 5 1 13 15 9
Experiments
actual
estimate

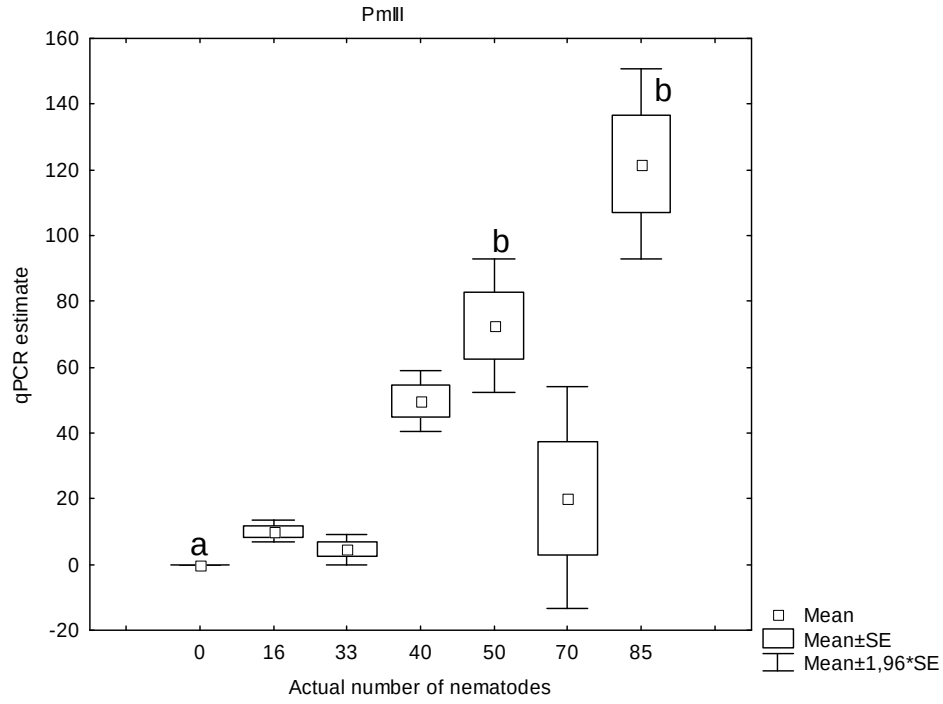

PmII
Number of nematodes
0 20 40 60 80 100 120 140 160
18 9 11 12 1 3 4 13 14 5 7 8 15 17 6 2 16 10
Experiments
actual
estimate

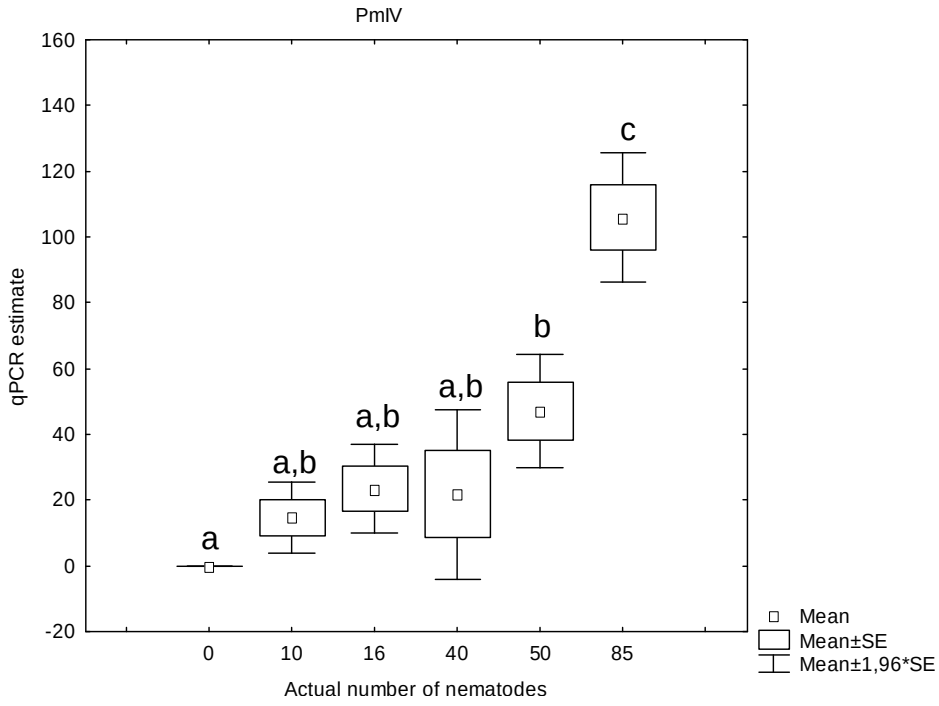

PmIII
number of nematodes
0 20 40 60 80 100 120 140 160
15 16 9 10 12 1 2 4 13 14 5 6 8 17 7 3 18 11
experiments
actual
estimate


PmIV
Number of nematodes
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
17 18 9 10 11 16 1 2 3 13 5 6 7 15 8 4 14 12
Experiments
actual
estimate


PmI
qPCR estimate
Actual number of nematodes
16
20
30
33
40
50
85
a
a
a
a
b
Mean
Mean±SE
Mean±1,96*SE


PmII
qPCR estimate
Actual number of nematodes
0
16
33
40
50
70
85
a
b
Mean
Mean±SE
Mean±1,96*SE


PmIII
qPCR estimate
Actual number of nematodes
0
16
33
40
50
70
85
a
b
Mean
Mean±SE
Mean±1,96*SE


PmIV
qPCR estimate
Actual number of nematodes
0
10
16
40
50
85
a
a,b
a,b
a,b
b
c
Mean
Mean±SE
Mean±1,96*SE

Target	Primer sequence (5′ → 3′)	Amplicon length	Tm (°C)	GC (%)
PmI	F: CGCTGACCTTCACTGGAATTTT	135	53	45.45
	R: CCGACTCCGGTTCAACTCA		53	57.89
PmII	F: GATCATCGCTGACCTTGG	294	50	55.56
	R: CGCACCATGTTGCCATGA		50	55.56
PmIII	F: AGCGGGGTGAAAGCCCA	410	52	64.71
	R: CTGAACTAGAATGGGTACATTCA		52	39.13
PmIV	F: CGATGGATGGTTTTCGCG	134	50	55.56
	R: GTGTATTGACGCTGTCCGTT		52	50

Experiment	# PmI	# PmII	# PmIII	# PmIV	developmental stage
1	**50**	16	16	16	mix
2	16	**50**	16	16	mix
3	16	16	**50**	16	mix
4	16	16	16	**50**	mix
5	**40**	20	20	20	mix
6	20	**40**	20	20	mix
7	20	20	**40**	20	mix
8	20	20	20	**40**	mix
9	**85**	5	5	5	mix
10	5	**85**	5	5	mix
11	5	5	**85**	5	mix
12	5	5	5	**85**	mix
13	**50**	**16**	**16**	**16**	mix
14	**16**	**16**	**16**	**50**	mix
15	**50**	**25**	**0**	**25**	mix
16	**20**	**70**	**0**	**10**	mix
17	**33**	**33**	**33**	**0**	mix
18	**30**	**0**	**70**	**0**	mix
19	16	0	10	5	adults
20	5	0	21	5	adults
21	26	0	4	2	adults
22	2	0	28	2	adults
23	16	0	10	5	juveniles
24	5	0	21	5	juveniles
25	26	0	4	2	juveniles
26	2	0	28	2	juveniles