1 **Investigating the ecology and evolution of cryptic marine nematode species through**
2 **quantitative real time PCR of the ribosomal ITS region**
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11 **Keywords:** cryptic species, qPCR, ITS, absolute quantification, relative quantification
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19 **Running Title:** Detection-Quantification of cryptic nematode species
20 ~~**Key words:** cryptic species, qPCR, *Rhabditis marina*, ITS, absolute quantification, relative~~
21 ~~**quantification**~~
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24 Abstract

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

45 **Introduction**

46 The discovery of morphologically similar but genetically distinct species throughout the tree
 47 of life has substantially increased over the last two decades (Bickford et al. 2007). The
 48 presence of cryptic diversity has impacted biogeographical and ecological paradigms, since
 49 species with previously wide geographic distributions may actually consist of cryptic species
 50 with a much more narrow geographic distribution (Stuart et al. 2006) and so-called
 51 ‘generalist’ species may in fact consist of cryptic complexes of specialist species (Blair et al.
 52 2005). Evidently, this unrecognized diversity should be taken into account when conservation
 53 strategies and biodiversity management protocols are being formulated. Furthermore, it
 54 remains unclear how such cryptic diversity has evolved: is the morphological similarity the
 55 result of morphological stasis or evolutionary convergence? Unfortunately, relatively few
 56 studies have addressed the ecological and evolutionary aspects of cryptic speciation.
 57 Cryptic species are found in a wide range of habitats (Bickford et al. 2007) but may be
 58 particularly abundant in marine environments (Knowlton 2000). This is because most marine
 59 species do not require morphological recognition for mating, but instead rely on chemical
 60 cues for mate choice and gamete recognition (Stanhope *et al.* 1992; Palumbi 1994; Lonsdale
 61 *et al.* 1998) as well as for ecological interactions (Hay 2009). In marine sediments, free-living
 62 nematodes form one of the most abundant and diverse faunal groups, where they reach
 63 densities of 10^6 individuals m^{-2} and several tens of species m^{-2} (Heip et al. 1985). They may
 64 play a significant role in benthic mineralization processes (Coull 1999), impact microbial
 65 communities (De Mesel *et al.* 2004; Moens *et al.* 2005), and transfer carbon and energy to
 66 higher trophic levels (Leduc 2009). The use of nematodes as functional indicators relies on
 67 their classification into feeding groups or reproductive strategies (Bongers & Bongers 1998),
 68 but species belonging to the same feeding guild may also show considerable functional

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

75 The marine nematode *Rhabditis (Pellioditis) marina* is a typical inhabitant of decaying macro
76 algae in the intertidal zone of coasts and estuaries throughout the world (Inglis & Coles 1961).
77 A phylogeographic study across Europe revealed that *R. (P.) marina* comprises at least 10
78 phylogenetic species (Derycke et al. 2008b) and at least six of them showed morphological
79 differences when using a combination of morphometric characters (Derycke et al. 2008a).

80 Many of these cryptic species have a wide geographic distribution, and frequently occur in
81 sympatry with at least one other species of the complex (Derycke et al. 2008b). In view of the
82 close taxonomic relationship and the very similar morphology of cryptic *R. (P.) marina*
83 species, these sympatric occurrences are at odds with expectations from classical competition
84 theory. Autecological characterization of cryptic species as well as information on
85 interspecific interactions and functional performance are urgently needed, with particular
86 emphasis on sympatrically occurring species, to investigate whether classical ecological
87 concepts of competition also hold for species complexes. Such studies are currently hampered
88 because of the inability to accurately identify and quantify cryptic species.

89 qPCR has proven to be useful to detect and quantify marine unicellular organisms (Fitzpatrick
90 *et al.*; Creach *et al.* 2006), as well as multicellular parasitic nematodes from animals
91 (MacMillan *et al.* 2006; Pecson *et al.* 2006; Campos-Herrera *et al.* 2010) and plants (Francois
92 *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 (Pellioiditis) 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 T_m 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 T_m, 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 μ l lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl₂, 0.45 % NP 40, 0.45 % Tween20). Tubes were frozen for 10 min at -20 °C, after which 1 μ l 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 μ l 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 μ l 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 μ l 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 μ l 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 μ l) 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 μ l reaction volume on 96-well plates using 10 μ l LightCycler 480 SYBR Green I Master 2X solution, 3 μ l PCR-grade water, 6 μ l of each primer (end concentration of 1 μ M for PmI and PmIII, 500 nM for PmII and 200 nM for PmIV) and 1 μ l of template DNA. For quantitative analyses, 384-well plates were used and the qPCR mixtures were prepared for 10 μ l 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 (Pellioiditis) 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 (10², 10¹, 10⁰, 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

To investigate the detection limit of the qPCR assay, a single juvenile of one species was added to 99 nematodes containing equal numbers of the three other species, in three biological replicates. DNA extraction was performed using the CTAB protocol. For this experiment, three technical replicates were made.

ITS copy number differences between developmental stages

Differences in qPCR signal between developmental stages may blur an adequate quantification of nematode samples. Such differences may be caused by a different efficiency of DNA extraction between species or stages, or, more likely, by differences in cell number between (un)fertilized females, males and juveniles. Therefore, gDNA from a single juvenile, male and female (five biological replicates, two technical replicates) was extracted from each of the four species (PmI-PmIV), and used for qPCR with species specific primers.

Relative quantification

Relative quantification was evaluated using the strategy outlined in Mommer *et al.* (2008) in two series of experiments. In a first series of experiments, we prepared 18 artificial nematode mixtures in three replicates with different amounts of specimens from each species but keeping a total of 100 nematodes per mixture. The number of nematodes for each species ranged between 0 and 85 (Table 2). For numbers above 20, nematode suspensions were prepared by pipetting from a homogeneous solution from which five aliquots (100 or 250 µl) had been counted. ~~Such~~ The suspensions ~~suspensions~~ were prepared by washing nematodes with S-buffer from agar dishes, and contained a mixture of males, females and juveniles. The average number of nematodes counted in five aliquots from this suspension was then used to 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 $10 \text{ ng } \mu\text{l}^{-1}$ 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 $\text{ng}/\mu\text{l}$. 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

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

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 ($Ct = 25.69 \pm 0.13$, whereas $Ct = 10.45 \pm 0.03$ for PmI) and for PmX with primer pair PmII ($Ct = 33.34 \pm 0.07$, whereas $Ct = 14.22 \pm 0.08$ 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 $20.69 (\pm 0.52)$, $24.00 (\pm 5.25)$, $20.02 (\pm 0.26)$ and $20.71 (\pm 0.11)$ 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/ μ l 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 43).

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 54). 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 C_t -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 65). 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 standard curves 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 76).

Discussion

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

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

distantly related species would be amplified. We therefore feel that the primers tested here may also be applicable for detecting the cryptic *R. (P.) marina* species in field samples.

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

contain a large number of eggs in the uterus and often reproduce through ovovivipary, while PmIII females contain only few eggs in the uterus and rapidly deposit their eggs in the culture medium. This can explain the lack of difference in Ct value between developmental stages in PmIII, as well as the low Ct values of PmI and PmIV females. This shows the importance of optimization for each species separately, no matter how closely related they might be.

Despite these differences in cell numbers, a strong positive correlation was observed for each species between the amount of extracted DNA and the number of nematodes present in the sample, indicating that quantification is possible. We have substantially tested and validated the quantification accuracy of the qPCR assay. Our results demonstrate that it is essential to use reference samples and standard curves that have been generated in the same way as the experimental treatments to obtain accurate quantification estimates. When the experimental samples were obtained by pipetting or by handpicking, quite different estimates were obtained. The difference between pipetted and handpicked experiments was especially prominent for species PmI and PmIV, suggesting that these differences were related to differences in Ct values between developmental stages. We therefore expected to find more accurate estimates in the experiments with only juveniles than in those with only adults. This was clearly the case in our test with PmI and PmIII adults and juveniles (Fig 54). While a strong positive correlation was observed for PmI and PmIII juveniles with the qPCR estimate, no significant correlation was observed for the adults. It is possible to separate juveniles from adults from a practical perspective: adults of all four Pm species are larger than 1 mm (Fonseca et al. 2008), while juveniles are substantially smaller; therefore, sieving the samples across different mesh sizes can separate adults from juveniles.

The qPCR assay significantly differentiates between low and high numbers of 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.

References

- Berry SD, Fargette M, Spaull VW, Morand S, 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.
- Bickford D, Lohman DJ, Sodhi NS, *et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**, 148-155.
- Blair CP, Abrahamson WG, Jackman JA, Tyrrell L (2005) Cryptic speciation and host-race formation in a purportedly generalist tumbling flower beetle. *Evolution* **59**, 304-316.
- Blaxter ML (2004) The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **359**, 669-679.
- Bongers T, Bongers M (1998) Functional diversity of nematodes. *Applied Soil Ecology* **10**, 239-251.
- Campos-Herrera R, Johnson EG, El-Borai RJ, *et al.* (2010) Long-term stability of entomopathogenic nematode spatial patterns in soil as measured by sentinel insects and real-time PCR assays. *Annals of Applied Biology*, 1-14.
- Coull BC (1999) Role of meiofauna in estuarine soft-bottom habitats. *Australian Journal of Ecology* **24**, 327-343.
- Creach V, Ernst A, Sabbe K, *et al.* (2006) Using quantitative PCR to determine the distribution of a semicryptic benthic diatom, *Navicula phyllepta* (Bacillariophyceae). *Journal of Phycology* **42**, 1142-1154.
- De Meester N, Derycke S, Bonte D, Moens T (2011) Salinity effects on the coexistence of cryptic species: A case study on marine nematodes. *Marine Biology* **in press**.
- De Mesel I, Derycke S, Moens T, *et al.* (2004) Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. *Environmental Microbiology* **6**, 733-744.
- Derycke S, Backeljau T, Vlaeminck C, *et al.* (2006) Seasonal dynamics of population genetic structure in cryptic taxa of the *Pellioditis marina* complex (Nematoda : Rhabditida). *Genetica* **128**, 307-321.
- Derycke S, Backeljau T, Vlaeminck C, *et al.* (2007) Spatiotemporal analysis of population genetic structure in *Geomonhystera disjuncta* (Nematoda, Monhysteridae) reveals high levels of molecular diversity. *Marine Biology* **151**, 1799-1812.
- Derycke S, De Ley P, De Ley IT, *et al.* (2010) Linking DNA sequences to morphology: cryptic diversity and population genetic structure in the marine nematode *Thoracostoma trachygaster* (Nematoda, Leptosomatidae). *Zoologica Scripta* **39**, 276-289.
- Derycke S, Fonseca G, Vierstraete A, *et al.* (2008a) Disentangling taxonomy within the *Rhabditis (Pellioditis) marina* (Nematoda, Rhabditidae) species complex using molecular and morphological tools. *Zoological journal of the Linnean Society* **152**, 1-15.
- Derycke S, Remerie T, Backeljau T, *et al.* (2008b) Phylogeography of the *Rhabditis (Pellioditis) marina* species complex: evidence for long-distance dispersal, and for range expansions and restricted gene flow in the northeast Atlantic. *Molecular Ecology* **17**, 3306-3322.
- Derycke S, Remerie T, Vierstraete A, *et al.* (2005) Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Marine Ecology-Progress Series* **300**, 91-103.

- dos Santos GAP, Derycke S, Genevois VGF, *et al.* (2009) Interactions among bacterial-feeding nematode species at different levels of food availability. *Marine Biology* **156**, 629-640.
- Fitzpatrick E, Caron DA, Schnetzer A Development and environmental application of a genus-specific quantitative PCR approach for *Pseudo-nitzschia* species. *Marine Biology* **157**, 1161-1169.
- Fonseca G, Derycke S, Moens T (2008) Integrative taxonomy in two free-living nematode species complexes. *Biological Journal of the Linnean Society* **94**, 737-753.
- Francois C, Castagnone C, Boonham N, *et al.* (2007) Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus*. *Molecular Plant Pathology* **8**, 803-809.
- Hay ME (2009) Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science* **1**, 193-212.
- Heip C, Vincx M, Vranken G (1985) The ecology of marine nematodes. *Oceanography and Marine Biology: an annual review* **23**, 399-489.
- Hillebrand H, Matthiessen B (2009) Biodiversity in a complex world: consolidation and progress in functional biodiversity research. *Ecology Letters* **12**, 1405-1419.
- Holterman M, Rybarczyk K, Van den Elsen S, *et al.* (2008) A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats. *Molecular Ecology Resources* **8**, 23-34.
- Houthoofd W, Jacobsen K, Mertens C, *et al.* (2003) Embryonic cell lineage of the marine nematode *Pellioditis marina*. *Developmental Biology* **258**, 57-69.
- Huang L, Ye JR, Wu XQ, *et al.* (2010) Detection of the pine wood nematode using a real-time PCR assay to target the DNA topoisomerase I gene. *European Journal of Plant Pathology* **127**, 89-98.
- Inglis W, Coles J (1961) The species of *Rhabditis* (Nematoda) found in rotting seaweed on British beaches. *Bulletin of the British Museum of Natural History (Zoology)* **7**, 320-333.
- Johnson JB, Peat SM, Adams BJ (2009) Where's the ecology in molecular ecology? *Oikos* **118**, 1601-1609.
- Knowlton N (1993) Sibling species in the sea. *Annual Review of Ecology and Systematics* **24**, 189-216.
- Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia* **420**, 73-90.
- Leduc D (2009) Description of *Oncholaimus moanae* sp nov (Nematoda: Oncholaimidae), with notes on feeding ecology based on isotopic and fatty acid composition. *Journal of the Marine Biological Association of the United Kingdom* **89**, 337-344.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**, 402-408.
- Lonsdale DJ, Frey MA, Snell TW (1998) The role of chemical signals in copepod reproduction. *Journal of Marine Systems* **15**, 1-12.
- Ludwig W, Strunk O, Westram R, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Research* **32**, 1363-1371.
- MacMillan K, Blok V, Young I, Crawford J, Wilson MJ (2006) Quantification of the slug parasitic nematode *Phasmarhabditis hermaphrodita* from soil samples using real time qPCR. *International Journal for Parasitology* **36**, 1453-1461.

- Moens T, dos Santos GAP, Thompson F, *et al.* (2005) Do nematode mucus secretions affect bacterial growth? *Aquatic Microbial Ecology* **40**, 77-83.
- Moens T, Vincx M (1998) On the cultivation of free-living marine and estuarine nematodes. *Helgolander Meeresuntersuchungen* **52**, 115-139.
- Mommer L, Wagemaker CAM, De Kroon H, Ouborg NJ (2008) Unravelling below-ground plant distributions: a real-time polymerase chain reaction method for quantifying species proportions in mixed root samples. *Molecular Ecology Resources* **8**, 947-953.
- Nakhla MK, Owens KJ, Li WB, *et al.* (2010) Multiplex real-time PCR assays for the identification of the potato cyst and tobacco cyst nematodes. *Plant Disease* **94**, 959-965.
- Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* **25**, 547-572.
- Pecson BM, Barrios JA, Johnson DR, Nelson KL (2006) A real-time PCR method for quantifying viable *Ascaris* eggs using the first internally transcribed spacer region of ribosomal DNA. *Applied and Environmental Microbiology* **72**, 7864-7872.
- Sato E, Min YY, Shirakashi T, Wada S, Toyota K (2007) Detection of the root-lesion nematode, *Partylenchus penetrans* (Cobb), in a nematode community using real-time PCR. *Japanese Journal of Nematology* **37**, 87-92.
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C-T method. *Nature Protocols* **3**, 1101-1108.
- Stanhope MJ, Connelly MM, Hartwick B (1992) Evolution of a crustacean chemical communication channel - Behavioral and ecological genetic evidence for a habitat-modified, race-specific pheromone *Journal of Chemical Ecology* **18**, 1871-1887.
- Statsoft I (2004) STATISTICA (Data analysis software system), version 7. www.statsoft.com.
- Stuart BL, Inger RF, Voris HK (2006) High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. *Biology Letters* **2**, 470-474.
- Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. *Developmental Biology* **56**, 110-156.
- Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans* *Developmental Biology* **100**, 64-119.
- Toyota K, Shirakashi T, Sato E, Wada S, Min YY (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.
- Yeates GW (2003) Nematodes as soil indicators: functional and biodiversity aspects. *Biology and Fertility of Soils* **37**, 199-210.

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.

Figure Legends

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

Figure 2: Relative quantification of the four *Rhabditis (Pellioditis) marina* species in the first 18 experiments. Experiments along the X-axis are plotted according to increasing percentage 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 percentage of nematodes present in the experiments. Vertical bars denote the standard deviation of all replicates.

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

Figure 43: Box plots of actual versus estimated percentage of the four *Rhabditis* species using relative quantification. Only experiments with pipetted animals were 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) or two tailed multiple comparisons

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

Figure 54: 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 65: 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 76: 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.

Tables

Table 1: *Rhabditis (Pellioditis) marina* species specific primers for qPCR. Target: the target species for which the primers were designed (PmI, PmII, PmIII or PmIV). Tm: melting 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: CGATGGATGGTTTTTCGCG	134	50	55.56
	R: GTGTATTGACGCTGTCCGTT		52	50

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

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

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: CGATGGATGGTTTTTCGCG	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