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Molecular diagnostics of the pigeon pea cyst nematode, *Heterodera cajani* Koshy, 1967, using real-time PCR

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Summary – The pigeon pea cyst nematode, *Heterodera cajani*, is an important nematode pest of pigeon pea that is present in all major growing regions of this crop in India and reported from Pakistan, Egypt and Myanmar. In this study, a new real-time PCR assay for detection of *H. cajani* using a species-specific primer and a TaqMan probe was developed. The primers and a probe were designed to amplify the *COI* gene fragment. The specificity of the primer-probe set was tested in singleplex or multiplex reactions against target and non-target nematodes. In multiplex real-time PCR experiments with the specific and universal primer-probe sets, the signals were simultaneously observed for *COI* and D3 of 28S rRNA target genes. The results showed that the real-time PCR assay with species-specific primer and probe was sensitive enough to detect *H. cajani* DNA extracted from 0.003 egg or second-stage juvenile.

Keywords – *Cajanus cajan*, *COI* gene, D3 expansion segment of 28S rRNA gene, Heteroderidae, quarantine organism.

The pigeon pea cyst nematode, *Heterodera cajani* Koshy, 1967, is an important nematode pest of pigeon pea (*Cajanus cajan* (L.) Millsp) and present in all major growing regions of this crop in India (Sharma, 1998). This nematode is also known to attack sesame. The damage induced by this nematode was also reported from Pakistan, Egypt, and Myanmar (Subbotin *et al*., 2010). *Heterodera cajani* is listed as a potentially harmful organism for several countries and included in the target organism list for 2020 USDA survey in the USA. Development of reliable and sensitive molecular diagnostic methods is important for early detection of this pest and prevention of its distribution.

Heterodera cajani was first molecularly characterised by PCR-ITS-RFLP (Subbotin *et al*., 2000). Sequences of ITS rRNA (Subbotin *et al*., 2001), β-tubulin (Sabo & Ferris, 2004), the D2-D3 expansion segments of 28S rRNA (Subbotin *et al*., 2006) and *COII* (Riepsamen *et al*., 2011) genes were published for the Indian populations of *H. cajani*. The real-time PCR assay with melting curve analysis using a species-specific ITS rRNA gene primer for this species was also developed by Katsuta *et al*. (2016) and tested with Myanmar populations of this species.

This article describes a new real-time PCR assay developed for detection of *H. cajani* using a species-specific primer and a TaqMan probe designed based on the *COI* gene sequence.

Materials and methods

NEMATODE SAMPLES AND DNA EXTRACTION

Cysts of the pigeon pea cyst nematode, *H. cajani*, from India were provided by J. Rowe (Subbotin *et al*., 2000, 2001). Several other cyst nematode species were also included in the tests (Table 1). Cysts were soaked for 10-20 min in double distilled water. One cyst was placed into 20 μl ddH_2O on a glass slide, punctured by a needle under a dissecting microscope and the released second-stage juveniles (J2) and eggs were cut using a stainless steel dental needle under a stereomicroscope. DNA was extracted using a standard protocol with proteinase K as described by Subbotin *et al*. (2000). Fragments of nematodes in water suspension

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Table 1. Species of cyst nematodes used in the present study.

Species	Plant host	Location	Sample code	Collector
Heterodera cajani	*Cajanus cajan*	India	CD2945	J. Rowe
H. avenae	Barley	Turkey	CD2006	I.H. Elekçioğlu
H. fici	*Ficus carica*	Greece, Arta region	CD3222	N. Vovlas
H. filipjevi	Wheat	Turkey	CD1990	I.H. Elekçioğlu
H. glycines	*Glycine max*	USA, North Carolina	CD1450	W. Ye
H. humuli	*Humulus lupulus*	Belgium	CD2877	S.A. Subbotin
H. medicaginis	Unknown	Russia, Rostov region	CD3114	V.N. Chizhov
H. mediterraneae	Wild olive tree	Spain	CD3243	P. Castillo
H. ripae	*Urtica dioica*	Belgium	CD3370	S.A. Subbotin
H. salixophila	*Salix* sp.	Russia, Moscow	CD3321	V.N. Chizhov
H. schachtii	*Beta vulgaris*	Poland	CD3121	P. Castillo
H. trifolii	Unknown	Kyrgyzstan	CD3111	S.A. Subbotin

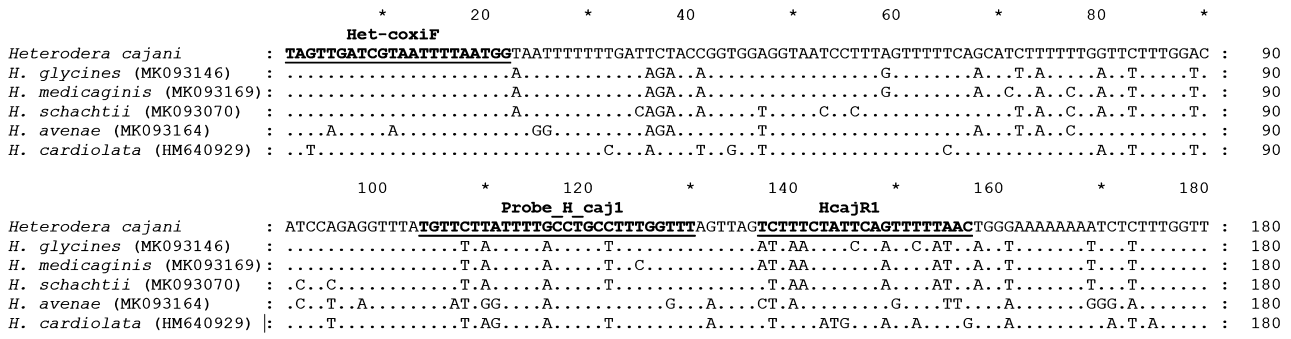

Fig. 1. The fragment of *COI* gene sequence alignment of cyst nematodes with indications of primer and probe positions.

were transferred into a 0.2 ml Eppendorf tube, 3 μl proteinase K (600 μg ml^{-1}) (Promega) and 2 μl 10× PCR buffer (*Taq* PCR Core Kit, Qiagen) were added to each tube. The tubes were incubated at 65°C (1 h) and 95°C (15 min) consecutively. After incubation, the tubes were centrifuged and kept at −20°C until use. This stock DNA extract obtained from 100 J2 (in 25 μl total volume) was used to make a series of dilution: 1:5, 1:25, 1:125, 1:625 and 1:3125. DNA concentration was measured with a NanoVue spectrophotometer (GE Healthcare).

Conventional PCR with species-specific primer

The *COI* gene sequences for *H. cajani* and several species of cyst nematodes from the *Schachtii* and other groups were obtained from unpublished studies (Subbotin *et al*., unpubl.) and GenBank. Several species-specific primers for *H. cajani* were designed using the sequence alignment of the *COI* gene for cyst nematodes (Fig. 1) and then tested. The PCR mixture of *Taq* PCR Core Kit (Qiagen) was prepared as described by Subbotin *et al*. (2000). The PCR amplification profile consisted of 4 min at 94°C, 30 cycles of 1 min at 94°C, 45 s at 57°C, and 45 s at 72°C, followed by a final step of 7 min at 72°C. From 2-5 μl of the PCR products were run on a 1% TAE buffered agarose gel, stained and photographed. All cyst nematode samples were used to test the specificity of PCR with new designed species-specific primers.

Real-time PCR with species-specific primer-probe set

Specific and universal *COI* gene primers developed and tested successfully in conventional PCR were used in these assays. TaqMan probe for *H. cajani* was designed using the sequence alignment of the *COI* gene and labelled with fluorescent FAM reporter dye. Universal primers for nematodes and the TaqMan probe labelled

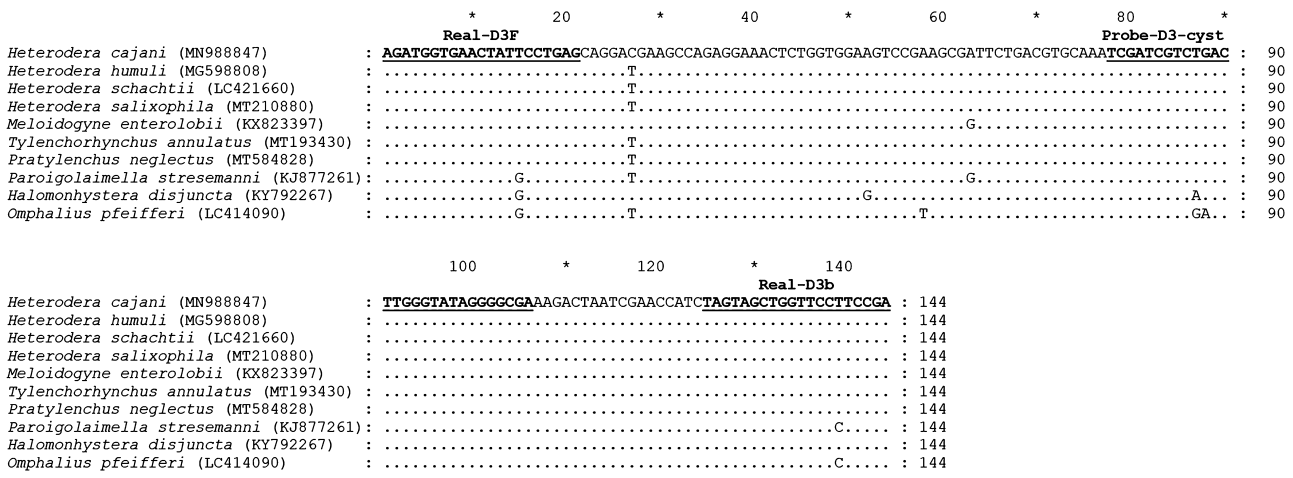

Fig. 2. The fragment of 28S rRNA gene sequence alignment of nematodes and the archaegastropod mollusc *Omphalius pfeifferi*, with indications of primer and probe positions.

Table 2. Species-specific primer and probe sets used and developed in the present study for conventional and real-time PCR diagnostics of *Heterodera cajani*.

Species	Primer and probe set ($5' \rightarrow 3'$)	Expected amplicon length
Heterodera cajani	Het-coxiF – TAG TTG ATC GTA ATT TTA ATG G HcajR1 – GTT AAA AAC TGA ATA GAA AGA Probe_H_caj1 – TGT TCT TAT TTT GCC TGC CTT TGG TTT	157 bp
Nematodes and other organisms	Real-D3F – AGA TGG TGA ACT ATT CCT GAG Real-D3b – TCG GAA GGA ACC AGC TAC TA Probe-D3-cyst – CGA TCG TCT GAC TTG GGT ATA GGG GCG A	144 bp

with JOE reporter dye were designed using the sequence alignment of the D3 of 28S rRNA gene sequences (Fig. 2; Table 2). The universal nematode primer-probe set was used in multiplex reactions to confirm that nematode DNA was successfully extracted from a sample. The BlastN search showed that this primer set can amplify the D3 of 28S rRNA gene of other organisms, including molluscs.

The 20 μl PCR reaction mixture contained 10 μl of 2× SensiFast Probe Lo-ROX master mix kit (Thermo Fisher Scientific), 1 μl each of 200 μM forward primer, reverse primer and 0.4 μl probe, 5.6 μl water and 2 μl of DNA template. Tests with singleplex (target: *H. cajani COI* gene) and multiplex (targets: *H. cajani COI* and D3 rRNA genes) Real-time PCR were performed. The two-step thermal cycling program was as follows: denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing and extension at 60°C for 60 s in Quant Studio 7 Flex (Applied Biosystems). Amplification curves were visualised and analysed by plotting fluorescence (ΔRn: normalisation of the reporter dye fluorescence) against the cycle number. Standard curves were constructed from real-time PCR performed with serial 5-fold dilutions of DNA template prepared from 100 eggs and J2 (DNA concentration = 45.5 ng μl^{-1}). Standard linear regressions ($y = a + bx$) of the log concentration of the template copies (x) *vs* the mean Ct values (y) were obtained. Several nematode samples (Table 1) were used to determine the specificity of assays. All reactions were performed at least three times and a negative control (distilled water) was used in each experiment. Diluted purified *COI* PCR product of *H. cajani* obtained with Het-coxiF and Het-coxiR (5′-CCT AAA ACA TAA TGA AAA TGW GC-3′) primers was also used as positive control in some experiments.

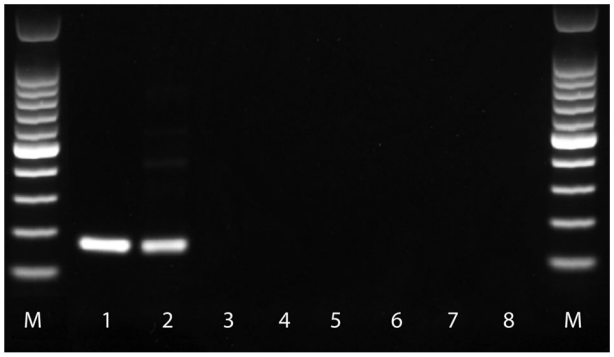

Fig. 3. Agarose gel with amplicons obtained from PCR with the universal cyst nematode Het-coxiF primer and the species-specific HcajR1 primer used for diagnostics of *Heterodera cajani*. Lanes: M = 100 bp DNA ladder (Promega); 1, 2 = *H. cajani*; 3 = *H. fici*; 4, 5 = *H. ripae*; 6, 7 = *H. salixophila*; 8 = negative control (no DNA).

Results and discussion

Conventional PCR with species-specific primer

Species-specific primers were designed for *H. cajani* based on differences in the *COI* gene sequences. Several primer sets were tested and one species-specific primer set, which gave the best amplification, was selected for this study (Table 2). The BlastN search showed that this primer set can only amplify the *COI* gene of *H. cajani*. The combination of the universal cyst nematode Het-coxiF primer with the species-specific HcajR1 primer for *H. cajani* yielded a single PCR product of 157 bp (Fig. 3) from samples containing DNA of this species. No amplification was observed in samples of non-target nematodes and in the negative control (without DNA).

Real-time PCR with species-specific primer and probes

The specificity of primer-probe set was tested in singleplex and multiplex reactions against *H. cajani* and non-target nematodes. The species-specific primer-probe set reacted when tested against *H. cajani* only (Fig. 4A). In a multiplex PCR experiment with the specific and universal primer-probe sets (probes with FAM and JOE reporter dyes, respectively), signals were simultaneously observed for both target genes (Fig. 4B). Amplification plots were observed for both target genes performed with serial five-fold dilutions of DNA template (Fig. 4C, D). In the reaction volume, samples containing approximately 8, 1.6, 0.32, 0.06, 0.01 and 0.003 eggs and J2 exhibited Ct values proportional to the log-transformed numbers of nematodes, illustrating that the relative numbers of *H. cajani* eggs and juveniles could be quantified. Standard curves were constructed from real-time PCR performed with serial five-fold dilutions of DNA template are given in Figure 5. There were highly significant linear correlations ($R^2 = 0.99$) between the Ct values and nematode DNA concentration. The results showed that the real-time PCR with species-specific primer and probe was able to detect *H. cajani* DNA extracted from 0.003 egg or J2 juvenile and placed in a reaction tube (1:3125 dilution of the stock sample).

Thus, in this study we developed an efficient method for detection and quantification of eggs and juveniles of *H. cajani*. The real-time PCR assay was sensitive enough to detect DNA extracted from a single specimen of the target species within an impure sample. The method described here might have some advantages over a real-time PCR assay using melting curve analysis as already developed by Katsuta *et al*. (2016) for this species. It has been shown in many studies (see Mackay, 2004) that TaqMan-based Real-Time detection has higher specificity, sensitivity, and reproducibility over dsDNA binding dye-based detection.

Because of quarantine restrictions, in this phase of this project we were not able to obtain soil field samples from crops infected with *H. cajani* and validate our method in practice, or conduct comparative research using both methods in the laboratory. The pigeon pea cyst nematode is listed as a quarantine organism in a number of countries and the availability of several detection methods gives diagnostics laboratories more opportunities to establish reliable identification of this pest.

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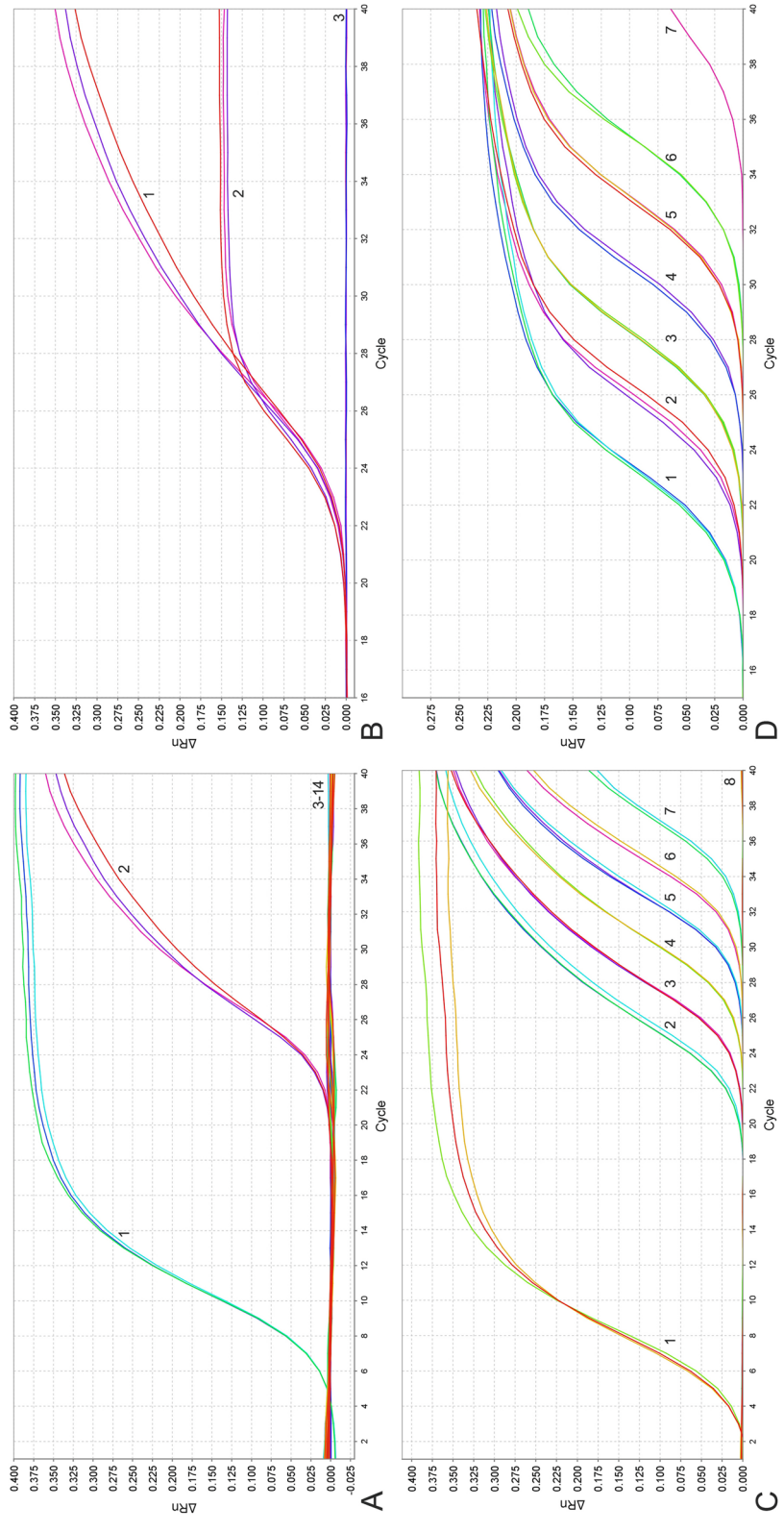

Fig. 4. A. Real-time PCR result with amplification plots. A: Detection of *Heterodera cajani* with species-specific primers and probe. Lanes: 1: diluted *COI* PCR product of *H. cajani* (as positive control); 2: DNA sample extracted from eggs and second-stage juveniles (J2) of *H. cajani*; 3-13: DNA samples extracted from non-target nematodes listed in Table 1; 14: negative control without DNA; B: Detection of *H. cajani* in multiplex real-time PCR reaction with species-specific primer-probe set targeting the *COI* gene (1), with universal nematode primer-probe set targeting the D3 of the 28S rRNA gene (2) and negative control (3); C: Detection of *H. cajani* in DNA serial dilutions with species-specific primer-probe set targeting the *COI* gene. Amplification curve: 1: diluted *COI* PCR product of *H. cajani* (positive control); 2: *ca* 8 eggs and J2; 3: *ca* 1.6 eggs and J2; 4: *ca* 0.32 eggs and J2; 5: *ca* 0.06 eggs and J2; 6: *ca* 0.01 eggs and J2; 7: *ca* 0.003 eggs and J2 per tube; 8: negative control; D: Detection of *H. cajani* in DNA serial dilutions with universal primer-probe set targeting the D3 of the 28S rRNA gene. Amplification curve: 1: *ca* 8 eggs and J2; 2: *ca* 1.6 eggs and J2; 3: *ca* 0.32 eggs and J2; 4: *ca* 0.06 eggs and J2; 5: *ca* 0.01 eggs and J2; 6: *ca* 0.003 eggs and J2 per tube; 7: negative control.

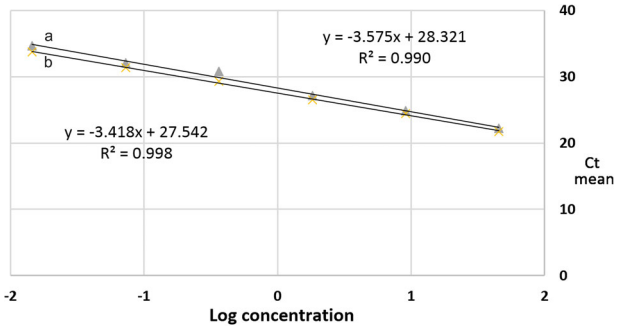

Fig. 5. Standard curves calculated with the log starting quantity and threshold cycle of the five-fold serially diluted DNA from *Heterodera cajani* with a species-specific primer-probe set targeting the *COI* gene (a) and universal nematode primer-probe set targeting the D3 of the 28S rRNA gene (b).

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