A Multiplex Real-Time Polymerase Chain Reaction (TaqMan) Assay for the Simultaneous Detection of *Meloidogyne chitwoodi* and *M. fallax*

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ABSTRACT

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This study describes a multiplex real-time polymerase chain reaction (PCR) approach for the simultaneous detection of *Meloidogyne chitwoodi* and *M. fallax* in a single assay. The approach uses three fluorogenic minor groove binding (MGB) TaqMan probes: one FAM-labeled to detect *M. chitwoodi*, one VIC-labeled to detect *M. fallax*, and one NED-labeled to detect the internal amplification control (IAC) to monitor false negative results. One common primer set is used for the amplification of part of the internal transcribed spacer (ITS) region of *M. chitwoodi* and *M. fallax* and one primer set for the amplification of the IAC. The test enabled detection of *M. chitwoodi* and/or *M. fallax* in DNA samples extracted from batches of juveniles, from single juveniles, and from infected plant material. Compared with current assays to detect *M. chitwoodi* and *M. fallax*, the multiplex real-time PCR offers the following advantages: it is faster because the test can simultaneously detect both quarantine species without the need for post-PCR processing; and it is at least 10 times more sensitive than a comparable regular PCR also targeting the ITS sequence. Inclusion of the IAC facilitates the interpretation of the FAM and VIC cycle threshold (Ct) values and can prevent the scoring of false negative results when FAM, VIC, and NED Ct values are high. The test allows precise quantification when only one of the two species is present in the sample. However, experiments with mixtures of genomic DNA of *M. chitwoodi* and *M. fallax* revealed that the ability of the multiplex real-time PCR assay to detect small quantities of DNA of one species is reduced when large quantities of DNA of the other species are present.

Additional keywords: bulbs, crocus, diagnostics, fluorescence, iris, multi-probe, potato, root-knot nematodes.

Nematodes belonging to the genus *Meloidogyne* cause severe losses on many crops throughout the world. Among the more than 90 species described (4), *Meloidogyne chitwoodi* and *M. fallax* are of particular concern in the more temperate regions. For instance, these nematodes generate external galls and internal spots on potato tubers that reduce both crop quality and yield. In the western part of Europe, *M. chitwoodi* and *M. fallax* have acquired importance as quarantine pests. Identification and early detection of these species is necessary for the design of crop rotation systems; for inspection of harvested crops, starting material, or soil samples; and for research purposes.

Considerable effort has been expended in the development of detection assays for *M. chitwoodi* and *M. fallax*. Protein-based assays (5,13) as well as numerous DNA-based methods (1,10, 11,15–18,20,21) have been described. Most of these DNA-based assays use polymerase chain reaction (PCR) amplification followed by gel electrophoresis to visualize the sizes of the amplified products. To limit the number of detection assays required, some approaches have been developed enabling simultaneous detection of *M. chitwoodi* and *M. fallax* in a single test (10,11, 15,16,18,20,21). However, all of these approaches have specific disadvantages. Some multiplex PCRs (11,18) require a species-specific primer set in the reaction for every species to be detected. This enables the multiplex detection of both *M. chitwoodi* and *M. fallax*, but from our experience (17,19) the sensitivity of such multiplex assays is decreased compared with the simplex assays using only one species-specific primer set. This decreased PCR efficiency probably results from primer interactions (8).

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Alternatively, PCR assays that can detect *M. chitwoodi* and *M. fallax* in a single assay using only two primers are hypothetically more sensitive. But size differences of the amplicons can be too small for routine testing (10,16), and heteroduplex formation can cause confusing DNA banding patterns (16).

This paper describes a multiplex real-time PCR approach based on TaqMan technology (2) for the simultaneous detection of *M. fallax* and *M. chitwoodi* in a single assay. The assay circumvents the problems associated with multiplex tests described above. Moreover, it is faster than current assays because no post-PCR handling steps are required; carryover contamination is reduced because the reaction tubes remain closed throughout the reaction; it enables quantification; and additionally, it also includes an internal amplification control (IAC) that can prevent the scoring of false negative results. The test uses two primer sets (a common one for the two *Meloidogyne* species and one for the IAC) and three minor groove binder (MGB) TaqMan probes (7) (for *M. chitwoodi*, *M. fallax*, and IAC). The specificity, sensitivity, and application of the assay are described.

MATERIALS AND METHODS

Nematodes. The root-knot nematode isolates used in this study (Table 1) were identified based on morphology, and the identifications were verified by species-specific PCR (5,18,19,22). DNA was extracted from juveniles by using the High Pure PCR template preparation kit (Roche, Basel, Switzerland) according to the mammalian tissue protocol. DNA was eluted in 200 µl of water. DNA was extracted from single juveniles by crushing the juvenile in 5 µl of PCR buffer (Roche) with a pipet tip. Genomic DNA of *Globodera pallida* strain GlobPal7 Rookmaker and *G. rostochiensis* strain R01 (Table 1) as well as frozen nematodes of *Caenorhabditis elegans* were provided by the Department of

Nematology, Wageningen University. DNA concentrations were determined by running aliquots on an agarose gel and subsequently comparing the DNA amounts with known amounts of lambda DNA.

Plant material. Potato tubers of cvs. Arinda, Arnova, and Kondor were provided by T. Been, Plant Research International, Wageningen, The Netherlands. Approximately 10 symptomless potato tubers from each of cvs. Arinda and Arnova were harvested from commercial fields in the northern part of The Netherlands. The potato tubers had been grown in clay, a soil type in which *Meloidogyne* sp. generally is not found. Potato tubers of the same cultivars but with symptoms were harvested from a field in Texel naturally infested with *M. chitwoodi*. After harvest, the potato tubers were stored for 4 weeks at 4°C before they were processed.

Bulbs of crocus cv. Geel and iris cvs. Blue Magic and Telstar were obtained from a field in Wintelre naturally infested with *M. chitwoodi* and a field in Smakt naturally infested with *M. fallax*.

TABLE 1. Isolates and sources of nematode species used in this study

Nematode species and code	Origin	Isolate	Source
Meloidogyne incognita			
Ia	Netherlands	Not named	PRI[a]
Ib	Netherlands	Inc568-93	PD[b]
Ik	Egypt Mynia	Mynia	AGERI[c]
Il	Egypt Giza	Giza	AGERI
M. javanica			
Ja	Unknown	Not named	PRI
Jb	Netherlands	C3059	PD
Jd	North Carolina	JNC	AGERI
M. arenaria			
Ah	Netherlands	Xa	PD
M. chitwoodi			
Ca	Netherlands	C3022	PD
Cj	Netherlands	C5273f	PD
Cl	Netherlands	C2960	PD
Co	Netherlands	Horst	AGV[d]
Cbd	Washington	WAMC16, race 2	WSU[e]
Cbf	Oregon	ORMC12, race 1	WSU
Cbh	California	CAMC2, race 3	WSU
Cbu	Netherlands	KBD4	AGV
M. fallax			
Fa	Netherlands	CHB	AGV
Fb	Netherlands	C4571	PD
Fc	Netherlands	C6501	PD
Fd	Netherlands	C6466	PD
M. hapla			
Hh	Netherlands	Not named	AGV
Hi	Netherlands	Sm.Fei92	AGV
Hk	Netherlands	Sl.92	AGV
Han	South Korea	C2346	PD
Hbq	Hungary	C6611	PD
M. minor			
Ma	Netherlands	Not named	PD
M. naasi			
Nc	Netherlands	C6190	PD
Nd	Netherlands	D5133	PD
Ne	Netherlands	Not named	PD
Globodera pallida			
G.pal		GlobPal7 Rookmaker	WU[f]
G. rostochiensis			
G.ros		G.ros R01	WU
Caenorhabditis elegans			
C. el.	Minnesota	N2 Bristol × CB4856 Hawaiian strain	WU

[a] Plant Research International BV, Wageningen, The Netherlands.
[b] Plant Protection Service, Wageningen, The Netherlands.
[c] Agricultural Genetic Engineering Institute, Agricultural Research Centre, Cairo, Egypt.
[d] Research Unit for Arable Farming, Field Production of Vegetables and Multifunctional Agriculture, Lelystad, The Netherlands.
[e] Washington State University, Prosser.
[f] Department of Nematology, Wageningen University, The Netherlands.

Both fields are located in the south part of The Netherlands. The bulbs were provided by the Plant Protection Service, Wageningen, The Netherlands. Because the bulbs had been stored at 4°C for 3 months, their quality was poor.

All infested fields from which potato tubers and bulbs were harvested were identified during surveys by inspection services. The species of root-knot nematode in each field was determined by morphological analysis of the nematodes extracted from soil samples.

DNA extraction from plant material. Two potato tubers without symptoms and four with symptoms (Table 2) were peeled manually with a potato peeler. Each resulting peel was about 2 mm thick and weighed about 30 g. The peels were freeze-dried and subsequently ground and homogenized. From the resulting fine powder of approximately 2.5 to 3.0 g, 40 mg was used for DNA extraction with the Wizard Magnetic DNA Purification System for Food (Promega Benelux b.v., Leiden, The Netherlands). The DNA was dissolved in 50 µl of water.

From one bulb of crocus and four bulbs of iris (Table 2), root segments were removed and placed in liquid nitrogen for 5 min. Subsequently, they were transferred to 2-ml vials containing two stainless steel beads (3.2-mm diameter). The vials were shaken in a Hybaid Ribolyser multiple bead beater (Thermo Electron Corporation, Delft, The Netherlands) at 5,000 rpm for 20 s. Further DNA extraction was done with the Wizard Magnetic DNA Purification System for Food (Promega).

Conventional PCR. To verify that target DNA was suitable for amplification for all nematode species except *C. elegans*, internal transcribed spacer (ITS)-PCR with generic primers was performed as described previously (21). For PCR of *C. elegans*, the primers F4 (5′-CCTGATTGATTCTGTCAGCGCGA-3′) and R78 (5′-CTGCTCTAATGAGCCGTACGCA-3′) were designed, based on the 18S sequence of *C. elegans* (NCBI X03680). The reaction mixture for the *C. elegans* PCR contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 600 nM of each of the primers F4 and R78, 60 µM dNTPs, and 1 unit of *Taq* DNA polymerase (Roche). The cycling parameters were as follows: denaturing step for 4 min at 95°C followed by 35 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s.

M. chitwoodi- and *M. fallax*-specific sequence characterized amplified region (SCAR)-PCR was performed on plant material as described previously (18).

TaqMan probes and primer design for *M. chitwoodi* and *M. fallax*. Two primers for the specific amplification of *M. chitwoodi* and *M. fallax* and two MGB TaqMan probes for detection of *M. chitwoodi* and *M. fallax* were designed within the ITS2 region of the rDNA sequence of *Meloidogyne* spp. (16) (Fig. 1). The primers were FC612ITS (5′-TGTATACTTTATAATTTTTC-TGTTTTG-3′) and RcfTAQ (5′-AAAAAATAAAGCATATTT-GATACAA-3′). The *M. chitwoodi*-specific probe was pMcFAM (5′-TGCAATTTTATTGAATAA-3′), and the *M. fallax*-specific probe was pMfVIC (5′-TACAATTTGTTGAATAAT-3′). To minimize nonspecific detection, the primers and probes were compared with all available relevant sequences using the BLAST database search program (provided online from the National Center for Biotechnology Information [NCBI]). The forward primer was 100% homologous with *Mus musculus* (chromosome 18); the reverse primer was 100% homologous with *Mus musculus* (chromosome 15) and 80% homologous with *C. elegans*. The probe sequence showed no homology with *Mus musculus* or *C. elegans*. Neither organism is normally found in the habitat of root-knot nematodes, with the exception that *C. elegans* can be present in soil. No marked sequence homology between the probe or primer sequences with other organisms was found. The probes and primers were designed with Primer Express version 1.5 software (PE Biosystems, Foster City, CA). The *M. chitwoodi*-specific MGB TaqMan probe (PMcFAM) was 5′ labeled with FAM as the reporter dye. The *M. fallax*-specific MGB TaqMan

probe (PMfVIC) was 5′ labeled with VIC as the reporter dye. The primers were manufactured by Sigma-Genosys (Haverkill, U.K.). The probes were manufactured by Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands). MGB TaqMan probes are always 3′ labeled with a nonfluorescent quencher by Applied Biosystems.

TaqMan probe and primers for internal amplification control. IAC primers FPgfp (5′-TGGCCCTGTCCTTTTACCAG-3′) and RPgfp (5′-TTTTCGTTGGGATCTTTCGAA-3′) were derived from Klerks et al. (6). The MGB pIAC TaqMan probe (5′-ACACAATCTGCC-3′) was designed from the DNA sequence of a gfp-coding gene (12) (accession no. P42212) and was 5′ labeled with NED.

Multiplex real-time PCR. Reactions were performed in 30-µl volumes in 0.2-ml optical grade PCR tubes and strips (Biozym, Landgraaf, The Netherlands). The PCR mix was prepared from the qPCR Core Kit (Eurogentec, Seraing, Belgium). The reaction mix contained 200 µM dNTPs with UTP, 5.0 mM $MgCl_2$, 1× reaction buffer containing ROX passive reference, 150 nM of probes pMCFAM and pMfVIC, 1 unit of HotGoldStar DNA polymerase, 0.15 units of AmpErase uracil-DNA glycosylase, 300 nM of each of the primers FC612ITS and RcfTAQ, DNA from samples to be tested, and the ingredients for the IAC (150 nM each of the primers FPgfp and RPgfp, 150 nM of probe pIAC, and 10 fg of IAC DNA, which is a plasmid from strain B6-914 gfp-91 of *Escherichia coli* O157:H7 [6]). In every experiment, a sample with no template DNA, a sample with 3 ng of *M. chitwoodi* DNA, and a sample with 3 ng of *M. fallax* DNA were included. Water was added to give a final volume of 30 µl for each sample. The TaqMan PCR was performed using the ABI 7700 sequence detection system (PE Biosystems). The cycling conditions were as follows: 50°C for 2 min to degrade uracil containing DNA and 95°C for 10 min to inactivate uracil-*N*-glycosidase and to activate *Taq* DNA polymerase followed by 40 cycles at 95°C for 15 s and 56°C for 1 min. The emission was measured at the annealing-extension step. For each PCR reaction, the cycle threshold (Ct) value, which is the number of PCR cycles necessary to increase fluorescence above background, was automatically calculated and analyzed by the ABI prism sequence detection systems software (version 1.9.1, PE Biosystems).

TABLE 2. Detection of *Meloidogyne chitwoodi* and *M. fallax* in potato tubers and flower roots collected from agricultural fields[a]

Plant material	Cultivar	Field location	Symptoms[b]	Nematode morphology[c]	Sequence characterized amplified region-PCR	Ct values		
						FAM *chitwoodi*	VIC *M. fallax*	NED IAC[d]
Potato	Arinda	Northern NL	None	nd	No result	40.0	40.0	31.0
	Arinda	Texel	Just visible	*M. chitwoodi*	No result	32.6	40.0	33.1
	Arnova	Northern NL	None	nd	No result	40.0	40.0	33.0
	Arnova	Texel	Clearly visible	*M. chitwoodi*	*M. chitwoodi*	27.8	40.0	33.5
	Arnova	Texel	Clearly visible	*M. chitwoodi*	*M. chitwoodi*	27.5	40.0	33.3
	Kondor	Texel	Clearly visible	*M. chitwoodi*	No result	32.7	40.0	32.3
Crocus	Geel	Wintelre	Unknown	*M. fallax*	No result	40.0	40.0	39.0
Iris	Blue Magic	Wintelre	Unknown	*M. fallax*	*M. fallax*	40.0	30.3	32.1
	Blue Magic	Smakt	Unknown	*M. chitwoodi*	No result	26.6	40.0	33.5
	Telstar	Wintelre	Unknown	*M. fallax*	No result	40.0	32.2	32.5
	Telstar	Smakt	Unknown	*M. chitwoodi*	No result	40.0	40.0	36.8

[a] Potato tubers from the northern Netherlands (NL) and lacking symptoms were grown in clay, a soil type in which *Meloidogyne* generally is not found. Field locations in Texel, Wintelre, and Smakt were identified as naturally infested with *M. chitwoodi*, *M. fallax*, and *M. chitwoodi*, respectively.

[b] None indicates no spots on the outside; just visible indicates one small spot on the outside; clearly visible indicates several spots and/or galls on the outside; and unknown indicates that symptoms could not be assessed because of the poor quality of bulbs.

[c] Results of the morphological identification of the root-knot nematodes present in the soil from which the plant material was harvested. nd: not determined.

[d] High NED internal amplification control (IAC) cycle threshold (Ct) values indicate poor polymerase chain reaction (PCR) conditions.

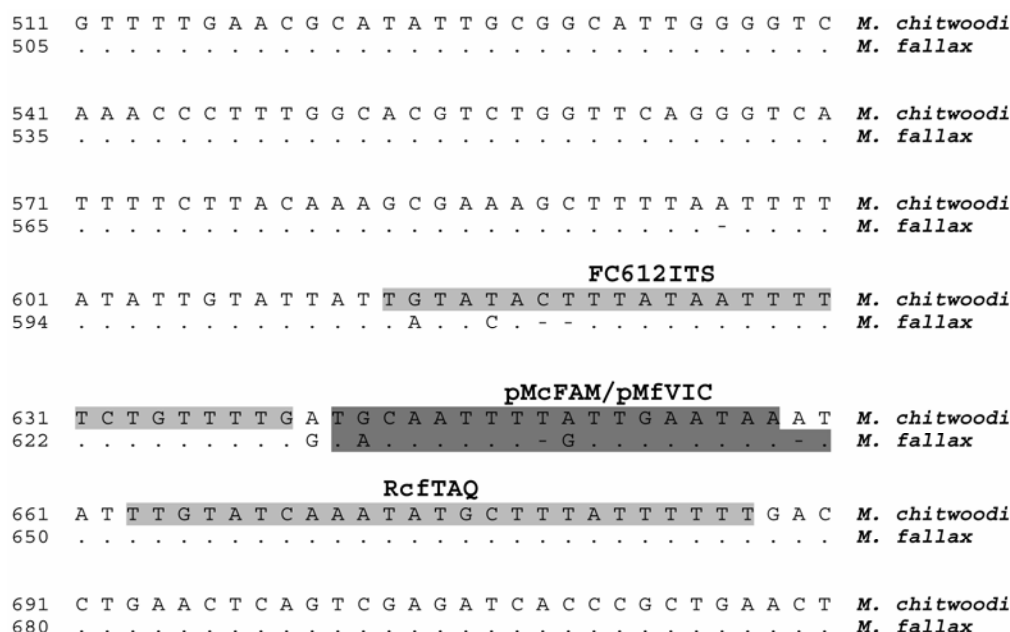

Fig. 1. Part of the alignment of rDNA sequences of *Meloidogyne chitwoodi* and *M. fallax* (16). The locations of primers FC612ITS and RcfTAQ are marked in light gray and the locations for probes pMcFAM and pMfVIC are marked in dark gray.

RESULTS

Real-time PCR specificity. To determine whether primers FC612ITS and RcfTAQ would amplify *C. elegans* DNA (RcfTAQ has 80% homology with *C. elegans*), both primers were used in a PCR with DNA extracted from *C. elegans*. No amplification products were visible, whereas PCR with nematode primers F4 and R78 resulted in the expected 97-bp DNA fragment, indicating that the DNA was suitable for PCR (data not shown).

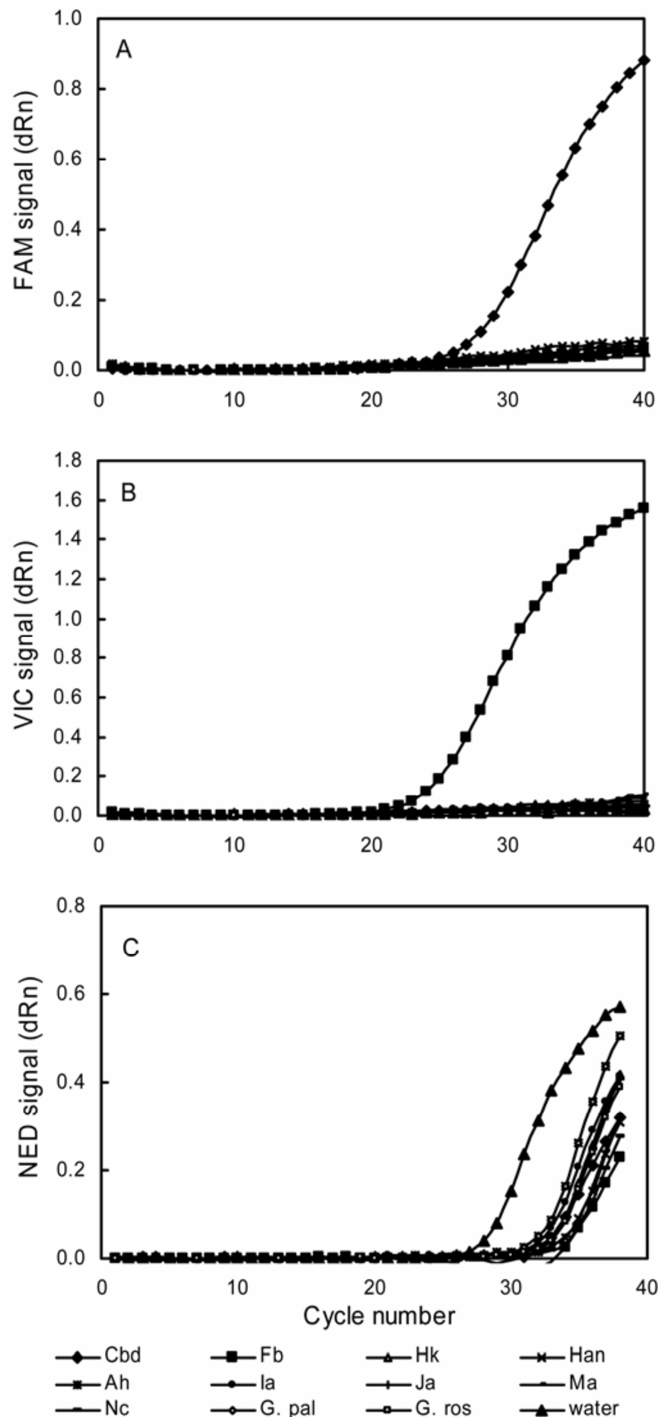

Fig. 2. Specificity of the multiplex real-time polymerase chain reaction (PCR) for *Meloidogyne chitwoodi* and *M. fallax*. Typical amplification plots using 3 ng of genomic DNA of eight *Meloidogyne* species and *Globodera pallida* and *G. rostochiensis*. Quantification of **A,** the FAM signal (showing specificity for *M. chitwoodi*); **B,** the VIC signal (showing specificity for *M. fallax*); and **C,** the NED signal (showing that the test conditions allowed PCR for all reactions). Isolate codes are explained in Table 1. dRn = normalized fluorescence.

Subsequently, the specificity of the multiplex real-time PCR was tested on 3 ng of genomic DNA from the isolates listed in Table 1. All isolates were tested once. FAM fluorescence was detected only when the assay contained DNA of *M. chitwoodi* (Fig. 2A), and VIC was detected only when the assay contained DNA of *M. fallax* (Fig. 2B). FAM amplification patterns were the same among the different *M. chitwoodi* isolates tested, and VIC amplification patterns were the same among the different *M. fallax* isolates tested. No FAM or VIC signals were obtained when template DNA was from *M. hapla*, *M. naasi*, *M. minor*, *M. incognita*, *M. javanica*, *M. arenaria*, *G. pallida*, *G. rostochiensis*, or *C. elegans* (Fig. 2A and 2B), whereas ITS-PCR or 18S-PCR performed on the same DNA samples resulted in amplification of the expected amplicons in all cases (data not shown). The NED signal of the IAC was observed in every reaction, but the NED Ct value of the water sample was lower than those of the DNA samples (Fig. 2C).

Sensitivity and detection range of the multiplex real-time PCR. To determine the sensitivities and the detection range of the multiplex real-time PCR, 10-fold dilution series of genomic DNA from *M. chitwoodi* or *M. fallax* were prepared. Real-time PCR was performed three times with genomic DNA amounts ranging from 100 fg to 1 ng per reaction. For *M. chitwoodi* genomic DNA, the response was linear (Fig. 3A) with a slope of –3.566, an R^2 of 0.9979, and a *y* intercept of 31.45. The response was also linear for *M. fallax* DNA (Fig. 3B) with a slope of –3.600, an R^2 of 0.9967, and a *y* intercept of 31.32.

Multiplex detection of mixtures of *M. chitwoodi* and *M. fallax*. To test the multiplex real-time PCR when *M. chitwoodi* and *M. fallax* have to be detected simultaneously, mixtures of genomic DNA of *M. chitwoodi* and *M. fallax* were prepared with ratios of *M. chitwoodi*/*M. fallax* DNA quantity ranging from 1:10,000 to 10,000:1. The mixtures contained the following quantities of *M. chitwoodi/M. fallax* DNA: 100 fg/1 ng, 1 pg/100 pg, 10 pg/10 pg, 100 pg/1 pg, and 1 ng/100 fg. When mixtures contained 10 pg or more of *M. chitwoodi* DNA and 10 pg or less of *M. fallax* DNA, FAM Ct values for detection of *M. chitwoodi* were unaffected by the presence of the *M. fallax* DNA (Fig. 4A). But when mixtures contained less than 10 pg of *M. chitwoodi* DNA and more than 10 pg of *M. fallax* DNA, FAM Ct values were increased by the presence of *M. fallax* DNA (Fig. 4A). The same relationship occurred with detection of *M. fallax* in mixtures, i.e., VIC Ct values increased when the concentration of *M. fallax* DNA was substantially less than the concentration of *M. chitwoodi* DNA (Fig. 4B). In the presence of 1 ng of *M. fallax* DNA, 100 fg of *M. chitwoodi* genomic DNA could not be detected reliably because the Ct value (FAM) was 39.5 (Fig. 4A). The same relationship was seen for the detection of *M. fallax* when 100 fg of *M. fallax* DNA was mixed with 1 ng of *M. chitwoodi* DNA (Fig. 4B). In all other combinations, both species were detected (Fig. 4A and 4B).

The ability of the multiplex real-time PCR to detect 100 fg of *M. chitwoodi* or *M. fallax* DNA in the presence of different quantities of DNA of the other species was assessed. Increasing quantities of *M. chitwoodi* DNA reduced detection of 100 fg of *M. fallax* DNA, but 100 fg of *M. fallax* DNA did not affect detection of the *M. chitwoodi* DNA (Fig. 5A); the same relationship was evident with a constant 100 fg of *M. chitwoodi* DNA and different quantities of *M. fallax* DNA (Fig. 5B).

Detection of single juveniles. The multiplex real-time PCR was performed with DNA extracted from single juveniles of *M. chitwoodi* ($n = 3$). The FAM Ct value was 27.3 ± 0.6. The NED Ct value was 32.9 ± 0.6.

Detection in plant material. The multiplex real-time PCR was performed on DNA (2 µl) extracted from potato tubers from fields infested with *M. chitwoodi* or from noninfested fields. Ct values of 40 indicated that *M. fallax* was not detected in any of the potato fields and that neither *M. fallax* nor *M. chitwoodi* was detected in the two potato fields that were expected to be non-

infested (Table 2). The four samples from tubers with symptoms had FAM Ct values of ≤32.7 (Table 2), indicating that they contained *M. chitwoodi*. NED Ct values confirmed that the assay conditions allowed PCR for all samples. To compare the multiplex real-time PCR with a conventional PCR, the same samples were used for *M. chitwoodi* SCAR-PCRs. SCAR-PCR products were only observed with the two Arnova samples from tubers with clear symptoms (Table 2).

To determine whether the multiplex real-time PCR detects *M. chitwoodi* and/or *M. fallax* in plant material of poor quality and to demonstrate the value of the IAC, DNA (2 µl per sample) extracted from decaying root samples of iris and crocus, originating from fields containing *M. chitwoodi* or *M. fallax*, were subjected to the multiplex real-time PCR. Data from the multiplex real-time PCR and morphology were consistent, i.e., multiplex real-time PCR detected *M. fallax* in two samples from fields known to contain *M. fallax* based on juvenile morphology and detected *M. chitwoodi* in one sample from a field known to contain *M. chitwoodi* based on juvenile morphology (Table 2). In two samples from infested fields, the multiplex real-time PCR did not detect *M. chitwoodi* or *M. fallax*, but the high NED Ct values for these samples (Table 2) indicated that conditions did not support

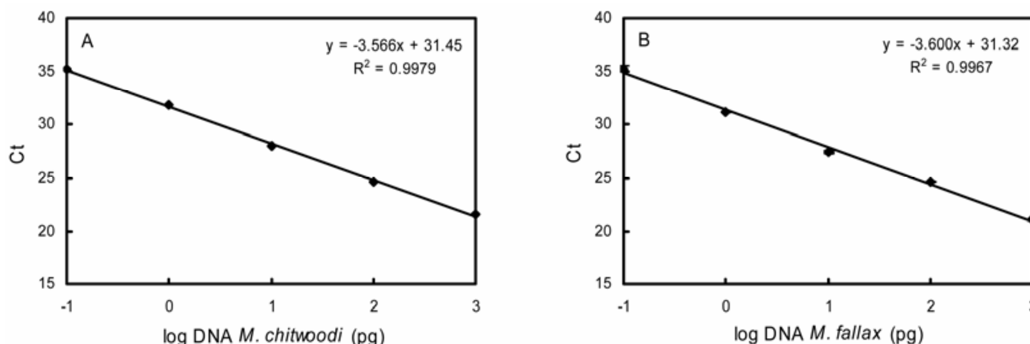

Fig. 3. Standard curves showing the relationship between cycle threshold (Ct) and quantity of genomic DNA of *Meloidogyne chitwoodi* and *M. fallax* using the multiplex real-time polymerase chain reaction. **A,** FAM Ct values plotted against the quantity (log base 10) of *M. chitwoodi* DNA. **B,** VIC Ct values plotted against the quantity of *M. fallax* DNA.

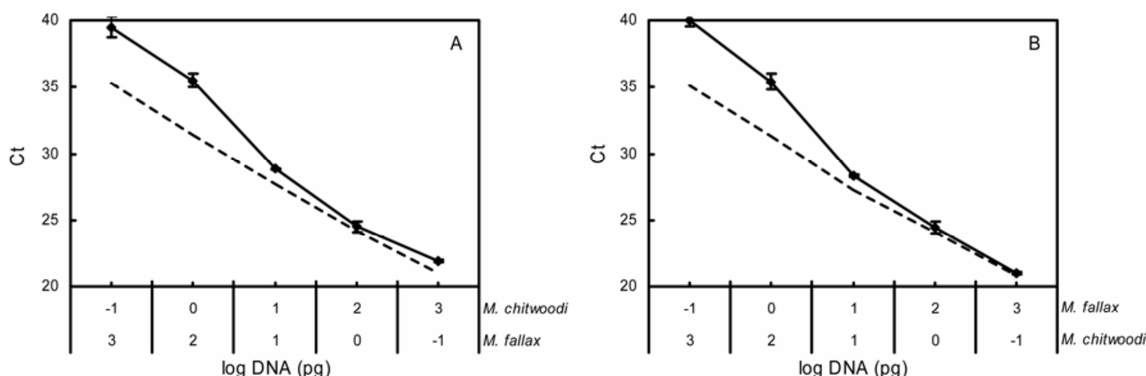

Fig. 4. Sensitivity of the multiplex real-time polymerase chain reaction when samples contain DNA of both *Meloidogyne chitwoodi* and *M. fallax*. **A,** FAM cycle threshold (Ct) values plotted against the quantities of *M. chitwoodi* and *M. fallax* DNA (log base 10). The solid line indicates the FAM signal for samples containing DNA of both species and the dashed line indicates the FAM signal for samples containing DNA of *M. chitwoodi* alone. **B,** VIC Ct values plotted against the quantities of *M. fallax* and *M. chitwoodi* DNA. The solid line indicates the VIC signal for samples containing DNA of both species and the dashed line indicates the VIC signal for samples containing DNA of *M. fallax* alone. The dashed lines in **A** and **B** represent the standard curves of Figure 3A and B, respectively.

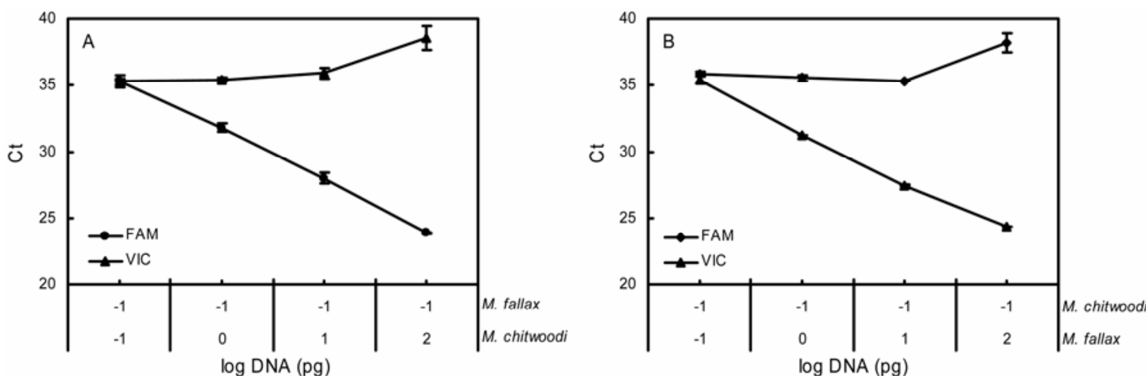

Fig. 5. Detection of a small and constant quantity of DNA from one *Meloidogyne* species as affected by different quantities of DNA from the other *Meloidogyne* species. **A,** FAM (*M. chitwoodi*) and VIC (*M. fallax*) cycle threshold (Ct) values plotted against different quantities of *M. chitwoodi* DNA in a background of 100 fg of *M. fallax* DNA (log base 10); **B,** VIC (*M. fallax*) and FAM (*M. chitwoodi*) Ct values plotted against different quantities of *M. fallax* DNA in a background of 100 fg of *M. chitwoodi* DNA. The regression equation and R^2 describe FAM in **A** and VIC in **B**.

PCR. SCAR-PCR detected no *M. chitwoodi* and detected *M. fallax* in only one of the root samples (Table 2).

DISCUSSION

This study has resulted in the development of a multiplex real-time PCR assay for *M. chitwoodi* and *M. fallax*. The assay positively identified *M. chitwoodi* and *M. fallax* in DNA extracted from several isolates of *M. chitwoodi* and *M. fallax* and in infected plant material. No FAM (*M. chitwoodi*) or VIC (*M. fallax*) signals were obtained when the test was performed on samples containing different nematode species, whereas the NED signal of the IAC was observed in every reaction, indicating that the test conditions allowed PCR. Moreover, all samples, including those from nematodes other than *M. chitwoodi* and *M. fallax*, enabled ITS-PCR or 18S-PCR, indicating that each reaction contained sufficient and suitable nematode DNA for PCR.

With respect to sensitivity, it was essential to design one single set of primers amplifying both *M. chitwoodi* and *M. fallax*, combined with two specific TaqMan probes for each of the organisms. The reason for using one common primer set is that a greater number of primer sets increases the possibility that primer combinations will reduce the sensitivity of the assay (8).

Even though the forward primer sequence has two mismatches with the *M. fallax* primer annealing site, the detection efficiency of *M. fallax* using the multiplex real-time assay was comparable to that of *M. chitwoodi*. The standard curves for detection of *M. chitwoodi* and *M. fallax* were similar. Consequently, Ct values were similar when the same quantities of *M. chitwoodi* or *M. fallax* were tested.

The ITS region was chosen as the target sequence because of its multicopy character, which enhances the sensitivity of the test. The disadvantage of using this region, however, is that it shows little variation between *M. chitwoodi* and *M. fallax* and it contains a high AT percentage. The MGB technology has enabled successful design of probes. DNA probes with conjugated MGB groups form extremely stable duplexes with single-stranded A/T-rich DNA targets, and allow reduction in the probe's length (7). In comparison with unmodified DNA probes, MGB probes have a higher melting temperature for the same length and an increased specificity. The use of a nonfluorescent quencher with the MGB probes gives very low fluorescent background.

Every assay in this study included a negative water control, positive *M. chitwoodi* and *M. fallax* DNA controls, and an IAC. The water control allows detection of contamination of the PCR mixture with *M. chitwoodi* and/or *M. fallax* DNA, and the positive DNA controls allow the verification of suitable test conditions. The IAC allows the detection of PCR inhibition (14) via an NED Ct shift when comparing the negative control with other samples. The negative water controls produced NED Ct values that were lower than those of samples containing nematode DNA, indicating that the DNA samples contained some inhibitors. A more striking effect caused by co-extracted PCR inhibiting agents was seen when plant material was tested. It was anticipated that the DNA extracted from the roots of the decaying bulbs would contain inhibiting agents. This assumption was confirmed when NED Ct values of 39.0 and 36.8 were obtained in the assays of crocus cv. Geel and iris cv. Telstar. Because of these high NED Ct values, the FAM and VIC Ct values of these assays were considered unreliable, preventing the scoring of false negative results. This illustrates the value of the IAC. The alternative for detecting inhibition in samples is the inclusion of positive *M. chitwoodi* and *M. fallax* control DNA both in the sample (with inhibitors) and in water (without inhibitors), but this is much more laborious and sometimes there is just not enough sample DNA available for twice as many reactions.

NED Ct values tended to increase with higher template amounts of *M. chitwoodi* and/or *M. fallax* (data not shown). For instance, NED Ct values obtained with 100 fg of *M. chitwoodi* and/or *M. fallax* template DNA averaged 31.8, whereas reactions with 1 pg, 10 pg, 100 pg, and 1 ng of template DNA averaged NED Ct values of 32.0, 32.4, 33.4, and 33.8, respectively. This phenomenon can be explained by an accumulation of amplification products during later PCR cycles that inhibits *Taq* DNA polymerase (3). More *M. chitwoodi* and/or *M. fallax* amplicons will be made with higher target amounts (which is confirmed by the lower FAM and VIC Ct values in these cases), resulting in less amplification of the IAC than would have been the case when fewer amplicons of *M. chitwoodi* and/or *M. fallax* were produced. But since the FAM and VIC Ct values in these cases were low, there is no doubt that the PCR worked.

Whereas high NED Ct values combined with high VIC and FAM values indicate that no diagnosis can be made, low NED Ct values do not guarantee that the VIC and FAM values can always be relied on. One could imagine that a sample contains target nematodes, but before or during DNA extraction the nematode DNA is damaged. This could result in FAM and VIC Ct values of 40 and a low NED Ct value, and one could incorrectly conclude that the PCR worked and the nematodes were absent. In this hypothetical situation, however, no molecular assay would detect the nematodes. Moreover, when a series of similar samples is being tested, and in some samples *M. chitwoodi* and/or *M. fallax* is detected, one can assume that the DNA extraction procedure used for this material enabled the successful extraction of nematode DNA suitable for PCR. But when the samples tested are not similar (if for instance they were obtained from different soil types or plant species), then this assumption cannot be made. If it is known that the sample contained nematodes, a general nematode PCR, such as the ITS-PCR used in this study, could be used to test whether any nematode DNA was present in the extracted DNA. In the current study, this was done in the experiment in which different nematode species were tested with the real-time TaqMan assay (Fig. 2).

A potential disadvantage of using the IAC is the presence of an additional primer set, which may reduce the sensitivity of real-time PCR (3,6). However, small quantities (100 fg) of *M. chitwoodi* and *M. fallax* genomic DNA can still easily be detected. Because the haploid genome size of a juvenile root-knot nematode is 50 fg (9) and a nematode consists of approximately 1,000 cells, each containing a diploid genome, the DNA content of a juvenile root-knot nematode would be approximately 100 pg. It follows that the multiplex real-time PCR could detect a small portion (approximately 0.001) of the DNA present in a single root-knot nematode provided that amplification conditions were optimal and DNA extraction was 100% efficient.

DNA extraction from single juveniles was apparently less than 100% efficient in our study. Consider that the mean FAM Ct value of 27.3 obtained for one juvenile represents 14 pg of *M. chitwoodi* DNA according to the standard curve in Figure 3A. Although this quantity is only about 14% of the DNA in a juvenile, the estimate seems reasonable given the method used to crush single juveniles.

To compare the sensitivity of the multiplex real-time PCR with that of a regular PCR also targeting the ITS sequence of *M. chitwoodi* and *M. fallax*, template amounts varying from 100 fg to 1 ng of DNA were subjected to regular PCR using the primers CF-ITS and HCFI-28S and PCR conditions as described previously (16). Although amplicons originating from 10 pg or more of DNA were always visible on the agarose gel and those originating from 1 pg were occasionally visible, amplicons originating from less than 1 pg of DNA were never visible (data not shown). In contrast, the multiplex real-time PCR described here easily detected DNA quantities of 100 fg. This indicates that the multiplex real-time PCR is at least 10 times more sensitive than a standard PCR.

Interestingly, the experiments with mixtures of genomic DNA from the two species revealed that the sensitivity of the multiplex real-time PCR depends on the relative and or absolute quantities

of DNA from each species. Thus, the ability of the assay to detect small quantities of DNA of one species was reduced when large quantities of DNA from the other species were present. The sensitivity of the assay, however, was less affected when DNA quantities were similar. The following hypothesis could explain this phenomenon. PCR results in the exponential accumulation of a specific target fragment until a plateau phase is reached where no additional amplicons are produced, presumably because of reannealing of specific product at high concentrations or inhibition of DNA polymerase by the amplification products (3). In our case, one primer set is used to target two similar fragment sequences. Amplification efficiency of both target fragments can be considered similar since there will be hardly any differences in primary and secondary structures given the high sequence homology and the short lengths of the target sequences. The copy number of the target sequences was also similar in our situation (21). Moreover, the standard curves (Fig. 3) indeed show that the detection efficiency using the multiplex real-time assay was similar for *M. fallax* and *M. chitwoodi*. When both target sequences are present in almost equal amounts and amplification efficiency of both target fragments is similar, multiplex real-time PCR will produce similar amounts of cleaved TaqMan probe from amplicons of both target sequences and similar Ct values. However, these Ct values will be slightly higher (theoretically 1 Ct value) compared with the situation when the same amount of genomic DNA of only one species is added to the reaction tube. In the first situation, only half of the PCR products will be produced per species after a certain number of cycles compared with the situation where only one species is present, resulting in half the amount of cleaved TaqMan probes per species. An additional cycle is needed to reach the same fluorescence level. The FAM and VIC Ct values measured with 10 pg of genomic DNA of *M. chitwoodi* and *M. fallax* were indeed higher than with 10 pg of genomic DNA of each species alone (Fig. 4). One should be aware of this phenomenon when trying to precisely quantify the species. Precise quantification of both species would require two independent real-time PCRs, each with a species specific set of primers, because co-amplification of any other DNA sequence in the sample will reduce the accuracy of the quantification.

Moreover, when the initial ratio of the two target sequences is high, the initial exponential nature of sequence amplification by PCR results in an even bigger ratio: the more abundant target sequence will be more quickly amplified in such a way that the plateau phase will be reached preventing the less abundant target from being amplified to detectable levels. Despite this phenomenon, the multiplex real-time PCR for *M. chitwoodi* and *M. fallax* is still useful for determining the presence of one or both of these quarantine organisms. The test is sensitive enough to always detect one of the two species when 100 fg of one of the species is present in the assay and when PCR is not too inhibited by co-extracted components (14).

Some PCR inhibition occurred with DNA extracted from pure nematode cultures, and more inhibition can be expected when field samples are tested. Our preliminary tests with field samples indicated that the multiplex real-time PCR can detect *M. chitwoodi* and *M. fallax* in infected potato tubers and flower bulbs. Results from the multiplex real-time PCR never conflicted with results from established methods of nematode identification, and the assay appeared to be more sensitive than a conventional PCR-based SCAR analysis.

Our study did not determine the detection limits of the multiplex real-time PCR when using plant material. Additional experiments are required to determine the number of nematodes (adults, juveniles, or eggs of single or mixed species) that can be detected per quantity of plant material and how detection in plant material is affected by plant cultivar and plant condition.

Although much research must be done before the multiplex real-time PCR can be reliably used for field samples, our results are encouraging. Whether the assay will enable quantification when both nematode species are present in a field sample is unclear. But even if not used for quantification, the multiplex real-time PCR would still be very valuable for qualitative determination of *M. chitwoodi* and *M. fallax* presence and absence.

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LITERATURE CITED

1. Castagnone-Sereno, P., Leroy, F., Bongiovanni, C., Zijlstra, C., and Abad, P. 1999. Specific diagnosis of two root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, with satellite DNA probes. Phytopathology 89:380-384.
2. Holland, P. M., Abramson, R. D., Watson, R., and Gelfand, D. H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5′→3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. USA 88:7276-7280.
3. Kainz, P. 2000. The PCR plateau phase—Towards an understanding of its limitations. Biochim. Biophys. Acta 1494:23-27.
4. Karssen, G. 2000. The plant-parasitic nematode genus *Meloidogyne* Göldi, 1892 (Tylenchida) in Europe. Brill Academic Publishers, Leiden, The Netherlands.
5. Karssen, G., Bolk, R. J., Van Aelst, A. C., Van den Beld, I., Kox, L. F. F., Korthals, G., Molendijk, L., Zijlstra, C., Van Hoof, R., and Cook, R. 2004. Description of *Meloidogyne minor* n. sp. (Nematoda: Meloidogynidae), a root-knot nematode associated with yellow patch disease in golf courses. Nematology 6:59-72.
6. Klerks, M. M., Zijlstra, C., and Van Bruggen, A. H. C. 2004. Comparison of TaqMan PCR methods for detection of *Salmonella enterica* and *Escherichia coli* O157:H7, and introduction of a general internal amplification control. J. Microbiol. Methods 59:337-349.
7. Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B., and Hedgpeth, J. 2000. 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28:655-661.
8. Landegren, U., and Nilsson, M. 1997. Locked on target: Strategies for future gene diagnostics. Ann. Med. 29:585-590.
9. Pablo, E. C., and Triantaphyllou, A. C. 1989. DNA complexity of the root-knot nematode (*Meloidogyne* spp.) genome. J. Nematol. 21:260-263.
10. Petersen, D. J., and Vrain, T. C. 1996. Rapid identification of *Meloidogyne chitwoodi*, *M. hapla*, and *M. fallax* using PCR primers to amplify their ribosomal intergenic spacer. Fund. Appl. Nematol. 19:601-605.
11. Petersen, D. J., Zijlstra, C., Wishart, J., Blok, V., and Vrain, T. C. 1997. Specific probes efficiently distinguish root-knot nematodes species using signature sequences in the ribosomal intergenic spacer. Fund. Appl. Nematol. 20:619-626.
12. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111:229-233.
13. Tastet, C., Val, F., Lesage, M., Renault, L., Marche, L., Bossis, M., and Mugniery, D. 2001. Application of a putative fatty-acid binding protein to discriminate serologically the two European quarantine root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, from other *Meloidogyne* species. Eur. J. Plant Pathol. 107:821-832.
14. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.
15. Wishart, J., Philips, M. S., and Blok, V. C. 2002. Ribosomal intergenic spacer: A polymerase chain reaction diagnostic for *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla*. Phytopathology 92:884-892.
16. Zijlstra, C. 1997. A fast PCR assay to identify *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax*, and to sensitively differentiate them from

each other and from *M. incognita* in mixtures. Fund. Appl. Nematol. 20:505-511.
17. Zijlstra, C. 2000. Reliable identification of the quarantine root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* by PCR-based techniques. OEPP/EPPO 30:575-579.
18. Zijlstra, C. 2000. Identification of *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* based on SCAR-PCR: A powerful way of enabling reliable identification of populations or individuals that share common traits. Eur. J. Plant Pathol. 106:283-290.
19. Zijlstra, C., Donkers-Venne, T. H. M., and Fargette, M. 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based PCR assays. Nematology 2:847-853.
20. Zijlstra, C., Lever, A. E. M., Uenk, B. J., and Van Silfhout, C. H. 1995. Differences between ITS regions of isolates of the root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. Phytopathology 85:1231-1237.
21. Zijlstra, C., Uenk, B. J., and Van Silfhout, C. H. 1997. A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. Fund. Appl. Nematol. 20:59-63.
22. Zijlstra, C., Van Hoof, R. A., and Donkers-Venne, D. 2004. A PCR test to detect the cereal root-knot nematode *Meloidogyne naasi*. Eur. J. Plant Pathol. 110:855-860.