Journal of Phytopathology

J Phytopathol **159**:80–84 (2011)
doi: 10.1111/j.1439-0434.2010.01717.x

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A Real-time PCR Assay to Identify *Meloidogyne minor*

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Received July 24, 2009; accepted May 18, 2010

Keywords: identification, *Meloidogyne minor*, real-time PCR, root-knot nematode, TaqMan

Abstract

Meloidogyne minor is a small root-knot nematode that causes yellow patch disease in golf courses and severe quality damage in potatoes. It was described in 2004 and has been detected in The Netherlands, England, Wales, Northern Ireland, Ireland and Belgium. The nematode often appears together with *M. naasi* on grasses. It causes similar symptoms on potato tubers as *M. chitwoodi* and *M. fallax*, which are both quarantine organisms in Europe. An accurate identification method therefore is required. This study describes a real-time PCR assay that enables the identification of *M. minor* after extraction of nematodes from soil or plant samples. Alignments of sequences of rDNA-ITS fragments of *M. minor* and five other *Meloidogyne* species were used to design a forward primer Mminor_f299, a specific primer Mminor_r362 and the specific MGB TaqMan probe P_Mm_MGB321. PCR with this primers and probe results in an amplicon of 64 bp. The analytical specificity of the real-time PCR assay was assessed by assaying it on six populations of *M. minor* and on 10 populations of six other *Meloidogyne* species. Only DNA from *M. minor* gave positive results in this assay. The assay was able to identify *M. minor* using DNA from a single juvenile independent from the DNA extraction method used.

Introduction

Meloidogyne minor was described after it was detected in a potato field in The Netherlands in 2000. The same species had been found before, causing yellow patch disease in golf courses in Wales, England, Northern Ireland and Ireland, but its species name had remained undescribed until the discovery in a potato field (Karssen et al. 2004). Since its description, *M. minor* has been detected in pastures, golf courses, sports fields, dunes and potato fields during surveys organized in England, Ireland, The Netherlands, Wales (Lammers et al. 2006; Turner and Fleming 2005) and Belgium (Viaene et al. 2007). The nematode has a broad host range including several grasses, weeds and economically important crops such as lettuce, tomato, carrot and potato (Karssen et al. 2004; Lammers et al. 2006). On grasses, *M. minor* often appears together with *M. naasi*. On potato tubers, it causes deformation and gives a pimple-like appearance to the potato surface, similar to damage caused by *M. chitwoodi* and *M. fallax*, which are quarantine organisms on potato in the EU and EPPO (Anon 2000). For this reason, *M. minor* is considered a potential alert organism in the EU. There are few studies on pest risk analysis of this species (Lammers et al. 2006; Viaene, pers. comm.), and knowledge of its occurrence in the EU is missing for thorough evaluation of the risk of this species for agriculture. Detection of *Meloidogyne* spp. in soil and plant samples is possible using several extraction methods. However, accurate identification of *M. minor* within the *Meloidogyne* spp. is difficult. The morphological differences with the two *Meloidogyne* species are very subtle, and the nematological expertise that is necessary for identification is not always available. Accurate and reliable methods for identification of *M. minor* are needed to help prevent its spread. They will also be helpful for detection in other habitats and countries during surveys that will reveal more about the natural spread of this newly found species in farming areas.

Isozyme analysis has been used for identification of *M. minor* (Karssen et al. 2004), but this technique relies on the expression of a gene product (Esbenshade and Triantaphyllou 1985) related to a specific stage of the nematode: females. This stage is not found very often in soil samples where second-stage juveniles are more prevalent. Species identification based on molecular methods overcomes limitations of identification based on morphological characters (Hartman and Sasser 1985) and isozyme expression. Moreover, the availability of a molecular identification tool could be very valuable for identifying the species of a single juvenile in soil. Several methods have been described for the

PCR-based identification of *M. chitwoodi, M. fallax, M. hapla* and *M. naasi* (Zijlstra et al. 1995; Petersen et al. 1997; Petersen and Vrain 1997; Zijlstra 1997; Wishart et al. 2002; Zijlstra et al. 2004). A reliable PCR-based identification of a single juvenile of *M. minor* has not been accomplished before.

This article describes the development of a real-time PCR assay, enabling the identification of a single juvenile of *M. minor* using primers and a probe derived from the ITS sequence of *M. minor*.

Materials and Methods

Previously determined rDNA sequences of *M. chitwoodi, M. fallax, M. hapla, M. incognita* (Zijlstra 1997), *M. naasi* (Karssen et al. 2004) and *M. minor* (GenBank Accession No. GU432775) were compared to design *M. minor* specific real-time PCR primers and probe. The alignment of these rDNA sequences was performed using the Megalign module of the Lasergene software (DNASTAR Inc., Madison, WI, USA). Different primers/MGB probe combinations were designed with the PRIMER EXPRESS version 1.5 Software (Applied Biosystems, Foster City, CA, USA). The primers were manufactured by Sigma-Genosys (Haverkill, UK). The MGB TaqMan probe, labelled with 5′-FAM as the reporter dye and with a non-fluorescent 3′-quencher (NFQ), was manufactured by Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands).

Table 1
Isolates and sources of *Meloidogyne* species used in this study with annotation of the applied DNA extraction method

Species	Isolate code	Source[a]
M. chitwoodi	CAMC2[b]	WSU
M. chitwoodi	E5087[b]	PD
M. chitwoodi	E5092[b]	PD
M. fallax	CHB[b]	AGV
M. hapla	n.a.[b]	AGV
M. hapla	D8218[b]	PD
M. incognita	n.a.[b]	PRI
M. minor	D4826(NL)[b,c,d]	PD
M. minor	WVR.P5(BE)[e]	ILVO
M. minor	H.8A (BE)[b,c]	ILVO
M. minor	E4259 (NL)[b]	PD
M. minor	E4662 (NL)[b]	PD
M. minor	n.a. (Ireland)[b]	PD
M. naasi	C6190[b]	PD
M. naasi	D7949[b]	PD
M. ulmi	D8236[b]	ISZA

[a]Sources of isolates. WSU = Washington State University, Prosser, USA, PD = Plant Protection Service, Wageningen, The Netherlands; AGV, Research Unit for Arable Farming, Field Production of Vegetables and Multifunctional Agriculture, Lelystad, The Netherlands, PRI = Plant Research International B.V., Wageningen, the Netherland, ILVO = Institute for Agricultural and Fisheries Research, Plant, Crop Protection, Merelbeke, Belgium, ISZA = Instituto Sperimentale per la Zoologia Agraria, Firenze, Italy.
[b]DNA isolation method 1.
[c]DNA isolation method 2.
[d]DNA isolation method 3.
[e]DNA isolation method 4.

DNA was isolated from seven different *Meloidogyne* species, including six *M. minor* isolates (Table 1) using four different methods. In method 1, pure DNA was extracted from 1000 to 10 000 juveniles with the High Pure PCR template preparation kit (Roche, Almere, The Netherlands). In method 2, DNA from single juveniles was extracted using the WLB-method (Yu et al. 2003). In this method, a single juvenile was added to 3 μl of worm lysis buffer (10 mM Tris-HCl pH8, 50 mM KCl, 1.5 mM $MgCl_2$, 1.9 mM DDT, 0.45% Tween 20 and 0.1 mg/ml proteinase K) and incubated for 1 h at 65°C and 10 min at 95°C. The DNA extract was diluted 10 times before use. In method 3, a single juvenile was isolated on a glass slide using a dissecting microscope and destroyed by crushing it with a pipette tip (Harris et al. 1990). The destroyed nematode was then transferred into 5 μl of highly purified water to be used completely in the real-time PCR assay. Method 4 is a combination of methods 2 and 3: a single juvenile was isolated on a glass slide and destroyed in a drop of highly purified water (40 μl) by cutting it with a sterile scalpel knife. Eight microlitres of water holding the pieces of nematodes was than transferred to an Eppendorf tube already containing 12 μl of worm lysis buffer. The mixture was frozen at −80°C for at least 10 min, subsequently incubated for 1 h at 65°C and 10 min at 95°C and finally centrifuged (1 min at 21 000 ***g*** in a microcentrifuge). The DNA extract was diluted 10 times before use.

Two microlitres of DNA, isolated with method 1, or 5 μl of DNA extract from single juveniles (isolated with methods 2–4) was amplified in 30 μl volumes in 0.2 ml optical grade PCR tubes (Biozym, Landgraaf, The Netherlands). PCR master mix was prepared with reagents from the qPCR Core Kit (Eurogentec Nederland B.V, Maastricht, the Netherlands). The PCR mix contained 200 μM of each dNTP (with dUTP), 5 mM $MgCl_2$, 1X reaction buffer containing ROX passive reference dye, 300 nM of probe P_Mm_MGB321, 0.75 U of HotGoldStar DNA polymerase, 0.15 U of AmpErase uracil-*N*-glycosylase (Applied Biosystems) and 300 nM of each of the primers Mminor_f299 and Mminor_r362. Annealing temperature had been optimized as well as primer and probe concentration (concentrations varying from 50 to 900 nM have been assayed). Real-time PCR was performed in a ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA) using the following conditions: 50°C for 2 min to degrade uracil-containing DNA and 95°C for 10 min to inactivate uracil-*N*-glycosylase, followed by 40 cycles containing a denaturation step at 95°C for 15 s and an annealing step at 58°C for 1 min. The emission was measured at the annealing-extension step. The Ct value for each PCR, being the number of PCR cycles necessary to increase the fluorescence above background, was automatically calculated and analyzed by the ABI PRISM sequence detection system software (version 1.9.1).

To be sure that the samples extracted are containing DNA that allowed PCR, they were assayed in a generic PCR with the primers F194 and R195 as described by Ferris et al. (1993).

The analytical sensitivity (detection limit) of the assay was assayed in triplicate with a dilution series of DNA isolated from *M. minor* isolate D4826 (method 1) with amounts ranging from 1 ng to 1 pg per reaction and with DNA from single juveniles (isolates D4826, WVR.P5, H.8A, methods 2–4). The dilution series was made by preparing a DNA solution with a concentration of 0.5 ng/μl. This concentration was measured spectrophotometrically (NanoDrop products, Wilmington, DE, USA). This solution was used for a dilution process. Resulting solutions were used in such a way that DNA amounts of 1 ng, 0.5 ng, 0.1 ng, 50 pg, 10 pg, 5 pg and 1 pg per reaction were assayed.

Results

Based on rDNA sequence differences between *M. minor* and the other mentioned *Meloidogyne* species listed in Table 1, the potentially best discriminating primers/probe combination was selected: the primers Mminor_f299 (5′-CCGTGACTGAATATGA GGTGA-3′) and Mminor_r362 (5′-GAGGCTCAT TAAGTCTTACGATTAT-3′) were chosen together with MGB Taqman probe P_Mm_MGB321 (5′-FAM-ATGTTAGGATTATCG-MGBNFQ-3′) where the probe and the combination of the forward and reverse primer are specific for *M. minor* (Fig. 1).

The analytical specificity of the real-time PCR assay was determined experimentally using 2 μl (1 ng/μl) genomic DNA (method 1) from the isolates listed in Table 1. FAM fluorescence could only be measured when the assay contained DNA of *M. minor*. No FAM signals were obtained when DNA isolated from six other *Meloidogyne* species was offered as template DNA (Fig. 2). The generic PCR on the DNA showed that the quality of the DNA was sufficient for amplification. The real-time PCR resulted in an *M. minor* amplicon of 64 bp.

The detection limit of the assay was demonstrated with a DNA dilution series of *M. minor* isolate D4826. The primers/probe combination displayed a linear range (Fig. 3). The range for detection of *M. minor* expands beyond the range of 1 ng (Ct = 23.7) to 5 pg (Ct = 34.7), and the correlation between the Ct value and known quantities of DNA was high (r^2 = 0.9872) in this range. The *M. minor* specific real-time PCR assay was also assayed on DNA extracted from a single juvenile of the different *M. minor* populations D4826, WVR.P5 and H.8A listed in Table 1. The real-time

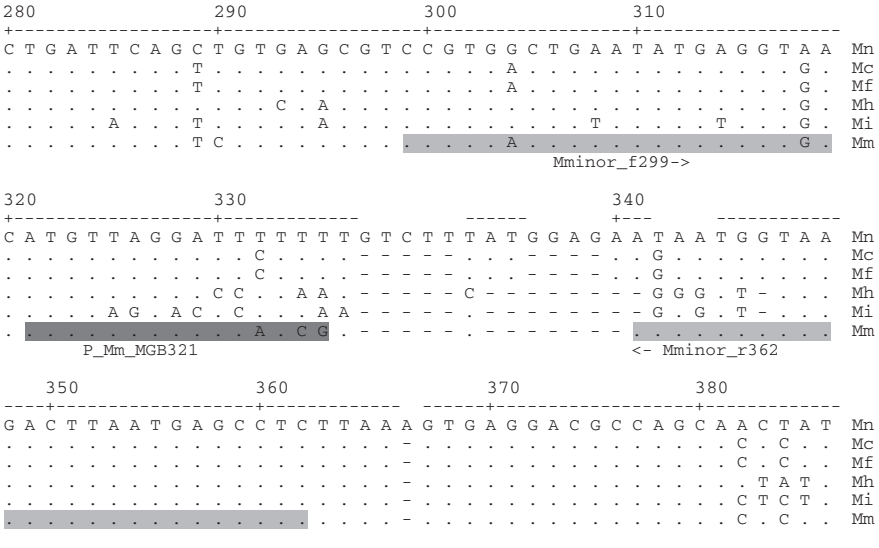

Fig. 1 Alignment of fragments of rDNA-ITS sequences of *Meloidogyne naasi* (Mn), *M. chitwoodi* (Mc), *M. fallax* (Mf), *M. hapla* (Mh), *M. incognita* (Mi) and *M. minor* (Mm). The locations of the primers Mminor_f299 and Mminor_r362 and probe P_Mm_MGB321 are highlighted in grey tones

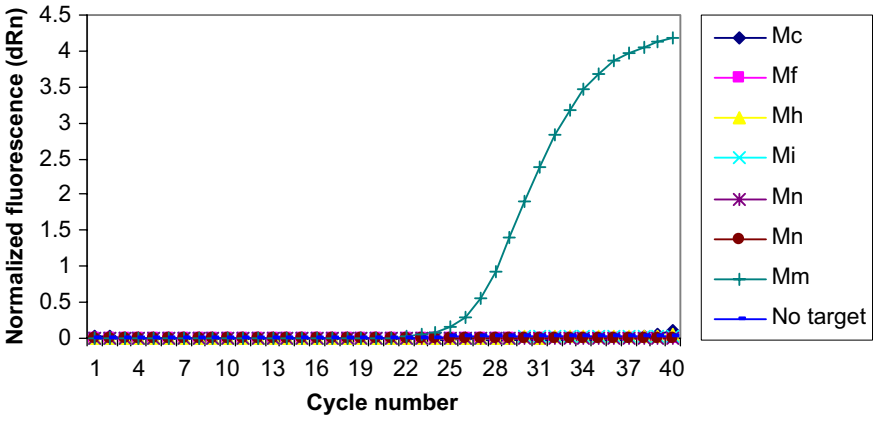

Fig. 2 Specificity of the real-time PCR for *Meloidogyne minor*. Typical results of amplification plots showing the assaying of 2 ng amounts of genomic DNA of isolates of *Meloidogyne* species measuring FAM signal. Mn, *M. naasi*; Mc, *M. chitwoodi*; Mf, *M. fallax*; Mh, *M. hapla*; Mi, *M. incognita*; Mm, *M. minor*

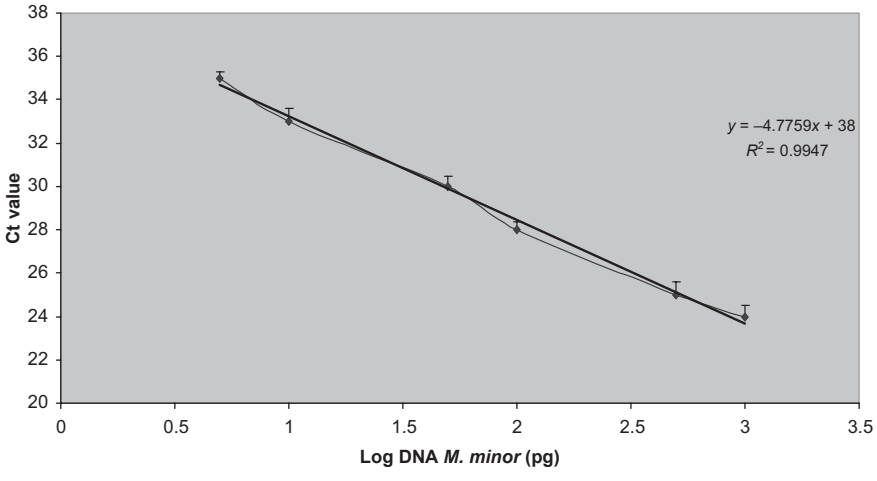

Fig. 3 Standard curve demonstrating the quantification of genomic DNA of *Meloidogyne minor*. Ct values shown are mean values for 3-fold reactions; error bars represent standard deviations. Ct values are plotted against DNA amounts of *M. minor*. Linear regression analysis: k = slope of linear regression between logarithmic values of DNA quantity and Ct values (−4.7759); R^2 = average squared regression coefficient (0.9947); E = efficiency of amplification (1.62). $E = 10^{-1/k}$

PCR assay resulted in a detectable FAM signal for each DNA extraction method for single juveniles used, demonstrating its robustness: average Ct = 28.8, SD = 0.7. TaqMan assays using DNA from three DNA extraction replications always showed similar Ct values (for method 2 average Ct = 29.1, SD = 0.9; for method 4 average Ct = 28.7, SD = 0.7; for method 3, no DNA extraction replicates have been tested), proving the repeatability of the assay. In addition, juveniles from population H.8A reared from as a single egg-mass culture were assayed in two different labs (PD and ILVO) and although different DNA extraction methods were used (1 and 2, respectively) the same positive identification of *M. minor* was obtained, demonstrating the robustness of the assay (data not presented).

Discussion

The real-time PCR assay as described here is a rapid, specific assay and easy to perform for the identification of *M. minor*. Robustness, repeatability and reproducibility of the assay were shown. The assay is sensitive enough to be successfully used on impure DNA extracted from single juveniles. Therefore, the detection limit is sufficiently low for the scope of the assay to identify a single nematode isolated from soil or plant samples. To prevent the scoring of false negatives, a generic PCR on the DNA to be analyzed is needed to show that the quality of the DNA is sufficient for amplification.

Interestingly, comparable TaqMan assays for *Meloidogyne* species *M. chitwoodi* and *M. fallax* are more sensitive (Zijlstra and van Hoof 2006). These easily detected 100 fg amounts of DNA with a Ct value of approximately 35, whereas in the *M. minor* TaqMan assay presented here a Ct value of 35 represented the amount of 5 pg. An explanation for this could be that the amplification efficiency E (Ramakers et al. 2003) of the *M. minor* TaqMan assay is not very high ($E = 10^{(-1/-47\ 759)} = 1.62$) in comparison with the TaqMan assays for *M. chitwoodi* ($E = 10^{(-1/-3566)} = 1.90$) and *M. fallax* ($E = 10^{(-1/-3600)} = 1.89$) (Zijlstra and van Hoof 2006). However, the TaqMan assay described by Zijlstra and van Hoof (2006) is a 3-steps protocol with cycling temperatures of 95, 54 and 72°C, whereas the *M. minor* TaqMan assay is a 2-step protocol with cycling temperatures of 95 and 58°C. Perhaps a 3-step protocol could enhance the efficiency of the *M. minor* TaqMan assay.

The designed real-time PCR assay offers a valuable alternative for identification methods based on morphological characters, isozyme patterns or host range. The latter methods require specific developmental stages and are not always conclusive. However, they remain valuable methods for identification and research purposes. The described real-time PCR assay is a convenient, reliable and fast assay for the identification of *M. minor*. It can successfully be applied for the identification of isolated single juveniles extracted from soil or plant samples. Moreover, the technique enables quantification which can be very useful for research studies such as population dynamics, but additional research would be required to explore this.

Acknowledgements

This research was supported by FAVV, Nationaal Referentielaboratorium voor Plantenziekten. We thank Richard van Hoof for his helpful comments on this manuscript.

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