Development and Validation of LNA-Based Quantitative Real-Time PCR Assays for Detection and Identification of the Root-Knot Nematode *Meloidogyne enterolobii* in Complex DNA Backgrounds

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ABSTRACT

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Meloidogyne enterolobii is a quarantine root-knot nematode posing a major threat to agricultural production systems worldwide. It attacks many host plants, including important agricultural crops, ornamentals, and trees. *M. enterolobii* is a highly virulent and pathogenic root-knot nematode species, able to reproduce on plants resistant to other *Meloidogyne* spp. Significant crop damage has been reported in Asia, South America, Africa, the United States, France, and greenhouses in Switzerland. To identify potential introduction pathways and ensure appropriate phytosanitary measures and management strategies, accurate detection and identification tools are needed. Therefore, two real-time

quantitative polymerase chain reaction (PCR) assays based on the second intergenic spacer region of the ribosomal DNA cistron and the cytochrome oxidase c subunit I (COI) gene using locked nucleic acid probes were developed and validated for fast and reliable detection and identification of *M. enterolobii*. Analytical specificity was confirmed with 16 *M. enterolobii* populations, 16 populations of eight closely related *Meloidogyne* spp., and four species from other nematode genera. Optimizing and testing the assays on two real-time PCR platforms revealed an analytical sensitivity of one juvenile in a background of 1,000 nematodes and the intended limit of detection of one juvenile per 100 ml of soil. Both assays performed equally well, with the COI-based assay showing a slightly better performance concerning detection of *M. enterolobii* target DNA in complex DNA backgrounds.

Additional keyword: quantification.

Root-knot nematodes (*Meloidogyne* spp.) pose a significant risk to agricultural production systems worldwide. *Meloidogyne enterolobii* (syn. *M. mayaguensis*) (Karssen et al. 2012) is a polyphagous species, with a wide host plant range, including ornamentals and important agricultural crops (Anonymous 2014). *M. enterolobii* is considered to be a highly virulent and pathogenic root-knot nematode species, able to develop and reproduce on host plants carrying resistance to the mayor tropical root-knot nematodes (Brito et al. 2007; Kiewnick et al. 2009) as well as resistant cucumber root stock (Kiewnick et al. 2011). Recently, *M. enterolobii* was found damaging cotton and soybean in the United States (Ye et al. 2013) and causing severe damage and loss of whole production areas for watermelon in Mexico (Ramirez-Suarez et al. 2014). More findings of significant damage in crops such as potato and carrot indicate the growing importance of this species (Onkendi and Moleleki 2013; Wang et al. 2014). The ability of *M. enterolobii* to reproduce on plants with known sources of resistance to root-knot nematodes makes the management of this species difficult, particularly in organic farming systems, where chemical control is not an option.

Based on several interceptions of ornamental plants infested with *M. enterolobii* by the Dutch and German authorities, this species was added to the European Plant Protection Organization A2 list in 2010 and, therefore, is recommended for regulation as a quarantine species (Castagnone-Sereno 2012). Based on the widespread distribution and potential to establish in the Mediterranean region and subtropical regions, several countries now have designated *M. enterolobii* as a quarantine pest (Elling 2013; Kiewnick et al. 2014).

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The recent reports on the spread of *M. enterolobii* demonstrate that there are pathways for introduction which have to be identified. To ensure that appropriate phytosanitary measures and management strategies are at hand to protect agricultural production systems worldwide against quarantine nematodes, reliable detection and identification tools are needed.

Currently, root-knot nematode identification is mainly based on morphological characteristics or isozyme analysis, which are time-consuming and require mature females and expert knowledge (Anonymous 2011). A number of molecular assays have been developed (Castagnone-Sereno 2012) for *M. enterolobii* species identification but these are not single-tube assays and are lacking sensitivity in detecting low amounts of target DNA in complex DNA backgrounds. Recently, DNA barcoding was evaluated as a diagnostic tool to identify *M. enterolobii* and closely related species (Kiewnick et al. 2014). It was demonstrated that the cytochrome oxidase I (COI) region was well suited for species delimitation and offered potential to develop assays for detection of *M. enterolobii* in complex DNA backgrounds (Kiewnick et al. 2014).

Real-time polymerase chain reaction (PCR) assays are well suited for detection and identification of plant-parasitic nematodes, including *Meloidogyne* spp. (Berry et al. 2008; De Weerd et al. 2011; Zijlstra and Van Hoof 2006). However, most of these assays are based on the noncoding internal transcribed spacer (ITS) region. Only recently, it was demonstrated that barcoding regions such as the small subunit of the ribosome (SSU) are well suited for the development of real-time PCR assays (Rybarczyk-Mydlowska et al. 2012). These assays allowed for detection and correct identification of target nematode DNA in complex DNA backgrounds obtained from nematode suspensions containing thousands of nematode specimens, including closely related species (Rybarczyk-Mydlowska et al. 2012).

The objective of our study was to develop a fast and reliable quantitative PCR assay that distinguishes *M. enterolobii* from

closely related species. Furthermore, the assay should be sensitive enough to detect one second-stage juvenile (J_2) in nematode suspensions obtained from field soil or root samples. This specific detection should be robust and independent from the real-time PCR platform and chemistry used. Therefore, two different real-time PCR assays based on a noncoding second intergenic spacer (IGS2) region and on one coding COI region were developed. The IGS2 region was selected because several PCR assays are based on this variable noncoding region on the ribosomal DNA (rDNA) (Castagnone-Sereno 2012), although a high intraindividual variation was shown for *M. enterolobii* (Holterman et al. 2012). In contrast, the COI gene represents mtDNA showing an intrinsically lower intraspecific variation with a good discriminatory power between closely related *Meloidogyne* spp. (Kiewnick et al. 2014).

To further enhance specificity of the assays, locked nucleic acid (LNA) probes were used. Advantages of using LNA probes are that they have nonfluorescent dark quenchers at the 3' end and, therefore, avoid background fluorescence. They are also much shorter than normal TaqMan and minor groove binding probes and have a length of only 8 to 9 nucleotides. LNA probes are locked nucleic acids due to a methyl-bridge between the O-Atom on the second C-Atom and the fourth C-Atom on the ribose ring, which are DNA nucleotide analogs with higher affinity to target DNA (Josefsen et al. 2009). Thus, they

provide a higher specificity and detection rate of short target DNA sequences to distinguish between species.

The overall goal of this study was to determine the suitability of either a coding (COI) or noncoding (IGS2) region-based assay to serve as a highly specific and sensitive real-time PCR assay for detection and identification of *M. enterolobii* in complex DNA backgrounds.

MATERIALS AND METHODS

Primer and probe design. Primers and LNA probes for detection of *M. enterolobii* were developed using the online Universal ProbeLibrary Assay Design Center (www.universalprobelibrary.com; Roche Applied Science, Mannheim, Germany). Sequences of the IGS2 and the COI region obtained from six *M. enterolobii* populations (Holterman et al. 2012; Kiewnick et al. 2014; Table 1) were used to detect sequence regions, specific for *M. enterolobii* and suitable for the design of primers flanking the probe binding sites. The software Primer3 selected the primers Ment50F/R and Ment17F/R for use with the LNA probes 50 and 17, respectively (Table 2). The primers Ment50F/R amplify a fragment of 63 bp, whereas the primers Ment17F/R amplify a fragment of 66 bp. The two probes are very specific LNA probes that contain FAM as reporter dye at the 5' end and a nonfluorescent dark quencher at the 3' end (Josefsen et al. 2009).

After optimizing primer and probe concentrations according to the manufacturer's recommendations, the real-time PCR was performed in reaction volumes of 20 μ l containing 4 μ l of template DNA, 900 nM each primer, 200 nM probe, 10 μ l of TaqMan Environmental PCR Master Mix (Applied Biosystems, Zug, Switzerland) for the use in the Applied Biosystems 7500 FAST Real-Time PCR instrument (Applied Biosystems), or 10 μ l of Roche LC480 Probes Master if using the Roche LC480 Light cycler instrument (Roche, Basel, Switzerland). On the ABI 7500 FAST instrument, the baseline was set automatically and the fluorescence threshold manually at a predetermined value of 0.05 using the COI probe 17 and 0.2 using the IGS2 probe 50. These values were based on in-house validation data obtained during experiments for adjusting primer and probe concentrations for optimum performance of the assays (S. Kiewnick, unpublished data). Cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C using the ABI 7500 FAST instrument. On the Roche Light Cycler platform (LC480), cycling conditions were 5 min at 95°C; followed by 45 cycles of 10 s at 95°C, 1 min at 60°C, and 3 s at 72°C; and a final cooling step at 40°C for 10 s. Unless otherwise stated, all PCR were performed in duplicate.

DNA extraction. All *Meloidogyne* populations representing different geographical regions used in this study (Table 1) belonged to reference collections, and species was confirmed using species-specific PCR assays (Kiewnick et al. 2013; Tigano et al. 2010) or following a DNA-barcoding protocol (Kiewnick et al. 2014). DNA was extracted from single or multiple *Meloidogyne* J_2 in 50 or 200 μ l of lysis buffer according to Holterman et al. (2006). Lysis took place in a Thermomixer (Eppendorf, Hamburg, Germany) at 65°C and 750 rpm for 2 h, followed by 5 min of incubation at 99°C (Holterman et al. 2006). Lysates were immediately used for real-time PCR or stored at -20°C until further use. Unless otherwise stated, all DNA samples were diluted 1:4 using Milli-Q-water and 4 μ l was used as template for real-time PCR assays.

Specificity testing. Specificity of real-time PCR assays was assessed with DNA from 36 individual nematode populations, including 16 *M. enterolobii* populations, 16 closely related other *Meloidogyne* spp., and 4 species from different genera (Table 1).

Sensitivity and quantitative detection. In order to produce standard curves that relate cycle threshold (C_t) values to number of target nematodes, *M. enterolobii* DNA standards were obtained by extracting DNA from 60 J_2 in 200 μ l of lysis buffer. Three individual DNA extractions were performed, pooled, and diluted to reach a final

TABLE 1. Species and populations used to validate the specificity of the real-time polymerase chain reaction (PCR) assays for the detection and identification of *Meloidogyne enterolobii*

Nematode species	Population or isolate	Source ^a
<i>Meloidogyne enterolobii</i>	M. ma.-I	Switzerland
	M. ma.-II	Switzerland
	N01-514-18B	United States
	Y06-111-18B	United States (Brazil)
	N2-283-14B.7	United States
	N2-283-14B.12	United States
	N2-283-14B.1	United States
	N2-283-14B.2	United States
	N2-283-14B.6	United States
	N3-18B.8	United States
	N3-18B.11	United States
	N1-514-3B.8	United States
	N1-514-3B.10	United States
	E1470	Netherlands/PD (Type China)
	E3504	Netherlands/PD
	10.108	France (Senegal)
<i>M. incognita</i>	Passiflora	Switzerland
	Reichenau II	Germany
	E4678	Netherlands/PD
	Gordola	Ticino, Switzerland
	Population-11	Germany/JKI
<i>M. hapla</i>	Population-Bamberg	Germany/JKI
	E6345	Netherlands/PD
<i>M. fallax</i>	Seeland-32	Switzerland
	VS-6-4-15	Switzerland
<i>M. arenaria</i>	HSW	Switzerland
	C6625	Netherlands/PD
<i>M. javanica</i>	E9654	Netherlands/PD
	E1387	Netherlands/PD
<i>M. ethiopica</i>	SL	Slovenia
<i>M. chitwoodi</i>	JKI Muenster	Germany/JKI
<i>M. graminicola</i>	Bonn University	Germany/BU
<i>Bursaphelenchus xylophilus</i>	8A	Portugal
<i>B. mucronatus</i>	PtO	Portugal
<i>Nacobus aberrans</i>	...	France
<i>Globodera rostochiensis</i>	...	Switzerland

^a JKI = Julius-Kuehn Institute, Muenster, Germany; BU = Bonn University, Germany; PD = Plant Diagnostics, Wageningen, The Netherlands; SL = Plant Diagnostic lab Slovenia; United States = Florida Department of Agriculture.

concentration of 0.3 J₂ per reaction. Standards were further diluted to adjust the concentrations to 0.3, 0.06, 0.03, 0.003, to 0.0003 *M. enterolobii* J₂ per 20-μl reaction. Based on this dilution series, standard curves of *M. enterolobii* were prepared by defining the slope (*k*) and the *y* intercept of the regression line describing the linear relationship between log number of J₂ per reaction and the corresponding C_t values. In addition, the slope of the linear regression line between logarithmic values of J₂ counts (x-axis) and C_t values (y-axis) was used to calculate the amplification efficiency, $E = (10^{(-1/k)}) - 1$, where a value of 1.0 corresponds to 100% amplification efficiency (Pfaffl 2001).

Selectivity. Selectivity as defined by the standard PM7/98 (Anonymous 2010) is not relevant for nematode identification because they are previously isolated from the matrix (soil or roots). However, if a test is used for detection, insensitivity to variation in matrix (in this case nontarget background nematodes) should be determined. To rule out cross-interference (real-time PCR inhibition) by nontarget DNA, the standard curves described above were prepared with the addition of nontarget background nematode DNA. To each DNA dilution of the standard curve (0.3 to 0.0003/reaction), 1 μl of template DNA obtained from 1,000 nematodes extracted in 200 μl of lysis buffer was added. Standard curves with the presence of background DNA were generated as described above and compared with control standard curves in the absence of background DNA.

In order to determine whether the presence of nontarget background DNA affects the detection and quantification of *M. enterolobii*, slopes of the linear regression describing the standard curves were compared according to Zar (1999).

Detection and quantification of *Meloidogyne enterolobii* For experiments using *Meloidogyne*-free nematode suspensions as nontarget backgrounds, field soil samples from an organic farm near Wauwil, Switzerland were extracted using the centrifugation flotation method (Van den Berg et al. 2014). Suspensions contained plant-parasitic and saprophagous nematodes and were adjusted to approximately 1,000 nematodes/ml. Afterward, 1 ml of nematode suspension was transferred to a 1.5-ml Eppendorf tube. Following centrifugation for 10 min at 8,000 × *g*, the supernatant was carefully removed until a final volume of 100 μl was reached. After adding an

equal volume of lysis buffer, lysis was done as described above and DNA was stored until further use at –20°C.

In order to produce standards to relate C_t values to the number of target nematodes in a nontarget background, 1, 2, 5, or 10 handpicked *M. enterolobii* J₂ were added to each nematode sample. DNA was extracted as described above. As a control, 1, 2, 5, and 10 handpicked J₂ were transferred to Eppendorf tubes containing 100 μl of water only and DNA extraction was performed as described above. Standard curves with and without background nontarget nematodes were prepared in triplicate and the experiment was conducted twice. DNA samples were diluted 1:4 using Milli-Q-water and 4 μl was used as template for real-time PCR assays.

Spiked Bonsai soil and root samples. In order to confirm the detection limits of the real-time PCR assays in the presence nontarget background nematodes, including other *Meloidogyne* spp. such as *M. incognita* and *M. javanica*, substrate and root samples from Bonsai plants (*Ficus microcarpa*), collected during a routine import control were utilized. Nematode suspensions were obtained after incubation of triplicate root and substrate samples (50 g each) from three independent import samples for 3 days in a mist chamber at 23°C. Each suspension (approximately 1,000 nematodes) was then checked microscopically for the presence of *Meloidogyne* spp. individuals and other plant-parasitic and saprophagous nematodes. Afterward, samples were divided, one-half (approximately 500 nematodes) was spiked with one handpicked *M. enterolobii* J₂, and DNA extraction was performed as described above. DNA samples were diluted 1:4 using Milli-Q-water and 4 μl was used as template for real-time PCR assays. The positive internal control contained one *M. enterolobii* juvenile in water only and the experiment was conducted twice.

RESULTS

Analytical specificity of real-time PCR assays. Depending on the real-time PCR chemistry and platform used, DNA from 0.3 J₂ per reaction resulted in C_t values of 24.8 to 25.08 for IGS probe 50 and C_t values of 25.1 to 26.5 for COI probe 17 (data not presented). Using either real-time PCR assay, increase in fluorescence was only

TABLE 2. Primer combinations and locked nucleic acid (LNA) probes used in assays for detection and identification of *Meloidogyne enterolobii*

Target, gene region	Primer, probe	Sequence (5'–3')	Amplicon length (nt)	T _m (°C)
<i>M. enterolobii</i> (IGS2)	Ment50F	CCT AAT TTT TTC TCC AGC TGC AAT	63	60
	Ment50R	TCA ATG GCA ATT CCT GAC AAT		60
	LNA probe 50 ^a	GCT CCA GA ^b		-
<i>M. enterolobii</i> (COI)	Ment17F	TGT GGT GGC TCA TTT TCA TTA	66	59
	Ment17R	AAA AAC CCT AAA AAT ACC CCA AA		59
	LNA probe 17 ^c	AGG AGC TG ^b		-

^a Roche catalog number 04688112001.

^b Probe sequence as provided by the universal Profinder software.

^c Roche catalog number 04686900001.

TABLE 3. Performance characteristics of real-time polymerase chain reaction (PCR) assays base on cytochrome oxidase I (probe 17) and intergenic spacer (probe 50) on two platforms (ABI and Roche) using standard curves based on serial dilutions of DNA from *Meloidogyne enterolobii* second-stage juveniles (J₂)

Real-time PCR assay	Number of experiments	Real-time platform	Dynamic range (J ₂ per reaction) ^a		Linear regression ^b			LOD ^c		
			Low limit	High limit	Slope (<i>k</i>)	R ²	<i>E</i> (%)	J ₂ per reaction	Average C _t	C _t SD
LNA ^d Probe 17	7	ABI 7500 Fast	0.003	0.3	-3.16	0.982	107.4	0.0003	35.2	1.06
	6	Roche LC480	0.003	0.3	-3.41	0.985	96.5	0.0003	36.0	1.21
LNA Probe 50	8	ABI 7500 Fast	0.003	0.3	-3.50	0.994	93.4	0.0003	35.2	0.73
	5	Roche LC480	0.003	0.3	-3.42	0.992	96.2	0.0003	35.6	0.10

^a Range of concentrations for which cycle threshold (C_t) values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of C_t values × log J₂ per reaction).

^b Linear regression of all positive samples in a plot of C_t values against logarithmic number of *M. enterolobii* J₂; *k* = slope of the linear regression line, R² = average square regression coefficient, and *E* = efficiency of amplification.

^c LOD = limit of detection, for the purpose of this study defined as concentration at which at least two of the triplicate reactions were positive (i.e., detecting fewer than 0.0003 J₂ per reaction).

^d LNA = locked nucleic acid.

detected in samples containing *M. enterolobii* DNA, demonstrating an analytical specificity of 100% (Table 1).

Sensitivity and detection range of real-time PCR assays. To determine the sensitivity of the real-time PCR assays, serial dilutions of *M. enterolobii* DNA were tested on two real-time PCR platforms (Table 3). When C_t values were plotted against the log number of J_2 per reaction, a significant linear relationship was found for the COI probe 17, with slopes of -3.16 and -3.41 , R^2 values of 0.982 and 0.985 , and efficiencies of amplification of 107.4 and 96.5% for the ABI and Roche platform, respectively. Similarly, linear relationships were found for IGS probe 50 with slopes of -3.50 and -3.42 , R^2 values of 0.994 and 0.992 , and efficiencies of amplification of 93.4 and 96.2% for the ABI and Roche system, respectively. For both assays, a high linearity was observed over the range of 0.3 to $0.003 J_2$ per reaction (Table 3). The C_t value increased with the degree of DNA dilution, with the lowest

Fig. 1. Standard curves of *Meloidogyne enterolobii* DNA from nematode suspensions containing 1,000 nematodes spiked with 1, 2, 5, and 10 hand-picked second-stage juveniles (J_2) in real-time polymerase chain reaction assays. Threshold cycle (C_t) values are plotted against the log number of J_2 in 200 μ l of lysis buffer. Data points are means ($\pm$ standard error) from two experiments. Intergenic spacer (IGS) ABI: $y = -3.260x + 30.23$, $R^2 = 0.978$; IGS Roche: $y = -3.309x + 30.75$, $R^2 = 0.983$; cytochrome oxidase I (COI) ABI: $y = -3.772x + 31.97$, $R^2 = 0.945$; COI Roche: $y = -3.496x + 31.12$, $R^2 = 0.998$.

concentration of $0.0003 J_2$ resulting in the highest C_t values of 35.2 to 36.0 for COI probe 17 and 35.2 to 35.6 for IGS2 probe 50, depending on the platform used.

Selectivity and detection of *M. enterolobii* in complex nematode DNA backgrounds. To test the insensitivity of the real-time PCR assays to matrix variation (in this case, complex nematode DNA backgrounds), template DNA extracted from 1,000 soil nematodes was added to *M. enterolobii* standard curve template DNA samples. As expected, the addition of large amounts of complex DNA background slightly reduced the sensitivity of the two assays (S. Kiewnick, unpublished data). However, statistical analysis of the slopes of the linear regressions according to Zar (1999) did not reveal significant differences. The reductions of sensitivity resulting in slightly increased C_t values were independent from the platform or primer probe combination tested (S. Kiewnick, unpublished data).

Standard curves of *M. enterolobii* DNA from nematode suspensions containing 1,000 nematodes spiked with 1, 2, 5, and 10 handpicked J_2 were generated by plotting the C_t values against the log number of *M. enterolobii* J_2 in 200 μ l of lysis buffer. For all combinations of primers and probe and platform used, highly significant linear relationships with R^2 values of 0.945 or higher were found (Fig. 1). Single nematodes generated C_t values of 31.06 and 31.61 for COI probe 17 and values of 30.07 and 30.58 for IGS probe 50 on the ABI and Roche platform, respectively (Fig. 1). Slopes of the linear regressions ranged between -3.26 and -3.77 .

Spiked Bonsai soil and root samples. To further validate the specificity of the developed assays, single specimens were added to suspensions containing nontarget background nematodes, including other *Meloidogyne* spp., obtained from Bonsai plants substrate and root samples. In the *M. enterolobii*-spiked samples, the target DNA could be detected with C_t values averaging 29.83 and 29.77 for probe 50 on the ABI and Roche platform, respectively. Similarly, spiked samples gave average C_t values of 31.05 (ABI) and 31.19 (Roche) for probe 17. These C_t values are in the expected range and represent the signal for a single J_2 in a complex nontarget DNA background. For nonspiked samples, average C_t values were 38.03 and 36.04 for IGS probe 50 and 39.83 and 38.02 for COI probe 17 and, thus, confirmed the absence of *M. enterolobii* J_2 in these samples (Table 4).

DISCUSSION

The described quantitative real-time PCR assays allowed a rapid and specific detection and identification of *M. enterolobii*. Both

TABLE 4. Detection of *Meloidogyne enterolobii* in nonspiked and spiked (one J_2) nematode suspensions obtained from Bonsai soil and root samples by two real-time polymerase chain reaction assays (cytochrome oxidase I probe 17 and intergenic spacer probe 50) on two platforms (ABI and Roche)

Sample	Sample ID	<i>Meloidogyne</i> spp. (n) ^b	C_t values ^a			
			Probe 50		Probe 17	
			ABI	Roche	ABI	Roche
Nonspiked Soil	372	<10	38.23	35.99	40.00	37.20
	411	>40	38.26	36.09	40.00	38.20
	373	>40	37.83	36.84	39.96	39.72
	372	<20	38.36	36.81	39.78	37.99
Roots	411	>40	38.89	35.60	40.00	38.36
	373	<20	36.63	34.92	39.22	36.66
	Average		38.03	36.04	39.83	38.02
Spiked ^c Soil	372	<10	29.00	28.98	30.83	30.65
	411	>40	30.04	29.86	31.25	31.22
	373	>40	29.84	29.73	31.08	31.04
	372	<20	30.00	29.99	30.90	31.27
Roots	411	>40	29.86	29.94	30.92	31.49
	373	<20	30.22	30.11	31.33	31.49
	Average	...	29.83	29.77	31.05	31.19
Control: <i>M. enterolobii</i> J_2 in H_2O		...	29.08	29.20	30.82	30.57

^a C_t = cycle threshold.
^b Range of *M. incognita* juveniles per sample; average range of three replicate extractions. In sample number 411, both *M. incognita* and *M. javanica* were found.
^c Spiked samples = one handpicked *M. enterolobii* J_2 was added before DNA extraction.

assays showed a high specificity and an analytical sensitivity of one J_2 in a background of 1,000 nematodes. This represents a detection limit of one J_2 in a sample obtained from 100 ml of soil containing up to 1,000 individuals from other nematode species. The assays were highly repeatable and reproducible, independent of the chemistry and real-time PCR platform used. Both assays performed well, with the COI-based assay showing a slightly better specificity when tested on samples obtained from roots and substrate of Bonsai plants (Table 4).

Real-time PCR and, in particular, quantitative assays have gained a lot of popularity in recent years (Rybarczyk-Mydlowska et al. 2012). The majority of these assays were based on noncoding ITS regions rather than coding genes or gene regions. However, a recently developed SYBR Green I assay based on the SSU rDNA, a relatively conserved coding region with low intraspecific variation, performed very well in the detection and identification of four *Aphelenchoides* spp. (Rybarczyk-Mydlowska et al. 2012).

Designing the presented assays using the universal probe library resulted in a high specificity, even the assay based on the noncoding IGS region. The utilized IGS region was shown to be unsuitable for DNA barcoding or high-resolution melting curve analysis due to high intraindividual variation (Holterman et al. 2012; Kiewnick et al. 2014). Conversely to the IGS region, the COI gene as barcoding region contains good phylogenetic information and an extremely low within-species variation (Kiewnick et al. 2014). Consequently, the COI-based assay using probe 17 is better suited, as was demonstrated with the analysis of samples from Bonsai roots and substrate. In this experiment, some of the unspiked samples showed a slight cross reaction when the IGS probe-50-based assay was used (Table 4).

DNA extraction is a critical step for successful detection and identification of *M. enterolobii*. The chosen lysis buffer protocol proved to be very efficient in a number of studies (Holterman et al. 2006, 2012; Kiewnick et al. 2014), including studies on quantitative DNA-based monitoring of nematode assemblages (Vervoort et al. 2012). Because the lysis buffer protocol did not include any purification or PCR inhibitor removal step, the developed LNA probe-based assays proved very robust for detection of target DNA in complex DNA backgrounds.

The detection limit of one J_2 in a background of 1,000 other nematodes is comparable with or lower than other real-time PCR assays developed for *Meloidogyne* spp. (Agudelo et al. 2011; De Weerd et al. 2011; Zijlstra and Van Hoof 2006). Based on the calculation by Zijlstra and Van Hoof (2006), as little as 5% of the genomic DNA of one *M. enterolobii* J_2 is sufficient for detection and identification.

The use of gene regions for the purpose of species detection requires a thorough investigation of the intraspecific variation (Rybarczyk-Mydlowska et al. 2012). In this light, we selected the COI-based assay (probe 17) for further use as a routine diagnostic and detection tool. The COI barcoding region provided sufficient informative nucleotide positions to develop this LNA probe-based assay and demonstrated a higher specificity when import control samples were used as source for complex DNA backgrounds. The developed assay can easily be implemented on standard real-time PCR platforms available at research and agricultural inspection service laboratories to speed up routine diagnostics. To our knowledge, this is the first use of an LNA probe-based quantitative real-time PCR assay for detection and identification of *M. enterolobii*.

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