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Short communication

Identification of the tropical root-knot nematode species *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using a multiplex PCR assay

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Root-knot nematodes (*Meloidogyne* spp.) have gained importance due to their widespread distribution, their host range and damage potential (Hussey & Janssen, 2002). A recent survey on root-knot nematodes in Europe by Wesemael *et al.* (2011) showed that out of the 90 species described so far, 23 have been found in Europe. Furthermore, this survey showed that three species, namely *Meloidogyne incognita*, *M. arenaria* and *M. javanica*, are the most prevalent in southern European countries and in protected cultivation systems in the northern parts of Europe. These species can cause significant economic losses due to yield and quality reduction on high value crops. Root-knot nematode species are normally identified using morphological features and morphometrics on second-stage juveniles (J2), males, on the perineal patterns of mature females or isozyme phenotyping of females (Hunt & Handoo, 2009). As the J2 stage is readily available from soil, and identifying this stage is most useful for making appropriate management decisions, several molecular methods, such as the use of restriction fragment length polymorphisms (RFLP), satellite DNA probes, sequence characterised amplified regions (SCAR) and real-time PCR assays, have become the preferred method for identification (Blok & Powers, 2009). However, few of these methods enable the simultaneous identification of several nematode species within one sample.

Until recently, our laboratory routinely used the molecular diagnostic key by Adam *et al.* (2007) to identify *Meloidogyne* spp. specimens obtained from samples such as roots or soil. However, using the MiF/MiR SCAR primers (Meng *et al.*, 2004), PCR amplification failed with several *M. incognita* populations. Alternatively, the SCAR primers Inc-K14-F/R (Randig *et al.*, 2002) proved more reliable and highly specific. However, following the key as described by Adam *et al.* (2007), every identification of a suspected tropical nematode species can require up to three PCR reactions and, in case of failure of a PCR amplification, supplementary RAPD-PCR amplifications are needed for species identification. This can be both time and material consuming.

The objectives of this study were therefore to develop a multiplex PCR protocol in part based on previously published highly specific and reliable SCAR-based PCR assays for *M. arenaria* and *M. javanica*, combined with a new specific assay for *M. incognita*. This multiplex assay should enable fast and reliable molecular diagnostics producing species-specific amplicons that can be easily separated by gel electrophoresis.

All root-knot nematode populations used in this study (Table 1) were reared on tomato (cv. Moneymaker) in a glasshouse. The identification was done according to Adam *et al.* (2007) using species-specific PCR assays where available. For additional species, correct identification was confirmed following a barcoding protocol according to Holterman *et al.* (2011).

Two previously published lysis buffer protocols were utilised to extract DNA from single J2 (Holterman *et al.*, 2006, 2012). The DNA was stored at −20°C until further

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Table 1. Isolates and sources of *Meloidogyne* spp. populations used in this study.

Species	Number of isolates	Source	Origin
M. incognita	3	Tomato	Reichenau, DE[1)]
M. incognita	2	*Anubias barteri*	Taiwan
M. incognita	1	Tomato	E6478, reference culture, G. Karssen, PD[2)], NL
M. incognita	2	Tomato	Ticino, CH[1)]
M. incognita	1	Tomato	California, USA
M. incognita	1	*Passiflora* spp.	Aargau, CH[1)]
M. incognita	3	Tomato	Gordola, Ticino, CH[1)]
M. arenaria	1	Tomato	Ticino, CH
M. arenaria	2	Tomato	Zurich, CH
M. arenaria	1	Tomato	C6625, reference culture, G. Karssen, PD, NL
M. arenaria	1	Tomato	California, USA
M. javanica	2	Tomato	E1387, reference culture, G. Karssen, PD, NL
M. javanica	3	Tomato	California, USA
M. javanica	3	Tomato	E9654, reference culture, G. Karssen, PD, NL
M. hapla	3	Rose	China
M. hapla	3	Rose	Aargau, CH
M. hapla	3	Tomato	Wallis, CH
M. hapla	1	Egg plant	Lucerne, CH
M. hapla	1	Tomato	E6345, reference culture, G. Karssen, PD, NL
M. hapla	1	Carrot	Bern, CH
M. enterolobii	3	Tomato	Wauwil, CH
M. enterolobii	1	Cucumber	Daettwil, CH
M. chitwoodi	1	Potato	Julius-Kühn Institute, Münster, DE
M. fallax	4	Tomato	Wallis, CH
M. ethiopica	1	Tomato	Agricultural Institute, Ljubljana, SLO

[1)] Used for sequencing the 399 bp amplicon.
[2)] Plant Protection Service, Wageningen, The Netherlands.

use. To develop *M. incognita*-specific primers, the 399 bp amplicon produced using the Inc-K14-F/R SCAR primers (Randig *et al.*, 2002) was sequenced. PCR reactions were performed in triplicate with DNA from four *M. incognita* populations (Table 1) on a Genius Thermocycler (Techne) using Qiagen HotStar Taq Mastermix (Qiagen) in a 20 μl reaction volume composed of 10 μl Mastermix, 0.5 μl crude DNA extract, 0.3 μM of each primer and brought to volume with MilliQ water. Amplification was performed by an initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 40 s, 64°C for 1 min and 72°C for 2 min, and with a final extension cycle of 72°C for 7 min. PCR products were cleaned using NucleoFast 96 PCR plates (Macherey-Nagel) and *ca* 25 ng DNA was then used in 8 μl sequencing reactions with 2 μl of BigDye Terminator reaction mix (Applied Biosystems) and 0.5 μM of one of the primers Inc-14K-F and Inc-14K-R. All sequencing reactions were performed on both strands on an Applied Biosystems 3130xl system (Applied Biosystems). The consensus sequence was used to design primers with the program FastPCR (Vers. 6.0.188; Kalendar *et al.*, 2009). The primers Mi2F4 (5′-ATG AAG CTA AGA CTT TGG GCT-3′) and Mi1R1 (5′-TCC CGC TAC ACC CTC AAC TTC-3′), yielding an amplicon size of 300 bp, were selected for further specificity testing.

Initially, a gradient PCR was performed combining primers Far/Rar and Fjav/Rjav (Zijlstra *et al.*, 2000) with primers Mi2F4 and Mi1R1. Twenty μl PCR reactions were performed as described above with pooled crude DNA (0.5 μl each) from *M. incognita*, *M. javanica* and *M. arenaria* J2 and 0.5 μM of each primer on a Mastercycler gradient (Eppendorf). Amplification was performed by an initial denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 40 s, 60 $\pm$ 5°C for 1 min and 72°C for 2 min, and with a final extension cycle of 72°C for 7 min. Successful amplification was checked on a 1.2% agarose gel with TBE buffer (pH 9) after staining with

ethidium bromide. The final validation of the diagnostic sensitivity and specificity (Anon., 2010) was done with *Meloidogyne* populations listed in Table 1. The PCR was performed with 1 μl crude template DNA. Twenty μl PCR reactions were performed as described above with 0.5 μM each of primers Far/Rar and Mi2F4/Mi1R1, 0.2 μM each of primers Fjav/Rjav and an annealing temperature of 59°C. Successful amplification, as well as the absence of unspecific amplification products was checked as described above.

Gradient multiplex PCR with a mixture of DNA from *M. incognita*, *M. arenaria* and *M. javanica* produced amplification products of 670, 420 and 300 bp, respectively. No non-specific amplification products were observed. The annealing temperature of 59°C was chosen for further testing as it produced similar intensities of bands (Fig. 1). However, as the intensity of bands for *M. arenaria* (420 bp) and *M. incognita* (300 bp) was less compared to *M. javanica* (670 bp), primer concentration of Fjav/Rjav was decreased to 0.2 μM. Furthermore, band intensity varied with different loadings of DNA/reaction, although correct identification of species was always possible.

Testing the final multiplex PCR protocol with 14 *Meloidogyne* populations (Table 1), demonstrated a diagnostic sensitivity of 100% (Anon., 2010). The DNA extraction protocol used had no effect on the PCR results. The specificity of the multiplex assay was also 100% (Anon., 2010) when tested with 11 populations of *M. hapla*, *M. enterolobii*, *M. fallax*, *M. chitwoodi* and *M. ethiopica* (Table 1).

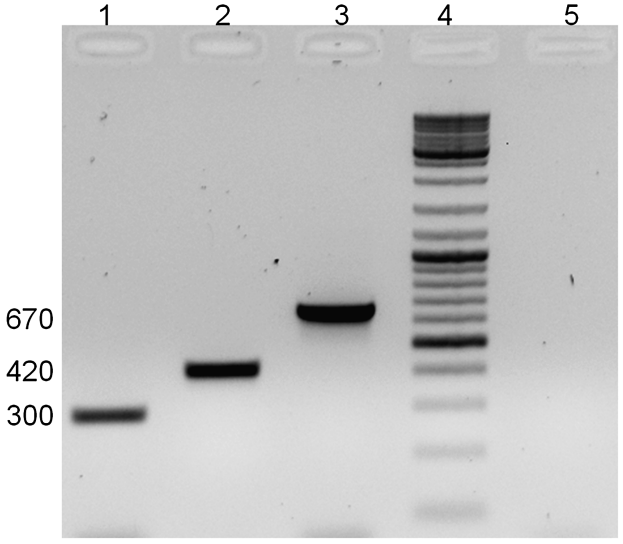

Fig. 1. Example of differential amplification of *Meloidogyne incognita*, *M. arenaria* and *M. javanica* in multiplex PCR assay using primers Mi2F4/Mi2R1, Far/Rar and Fjav/Rjav. Lane 1 = *M. incognita* (Gordola, CH); lane 2 = *M. arenaria* (C6625, NL); lane 3 = *M. javanica* (E-9654, NL); lane 4 = Molecular marker; lane 5 = Water control.

Rapid diagnostics of *Meloidogyne* species obtained at the J2, female or male stage is critical for making management decisions or in support of plant health inspection services. The multiplex PCR protocol described is an improvement in terms of reduction in time and material needed for diagnostics of the three most important species for Europe (Wesemael *et al.*, 2011). Combined with an efficient, easy to use DNA extraction protocol, identification can be done within 1 day. This multiplex PCR protocol was successfully tested in our laboratory with more than 200 samples. The specificity of the multiplex PCR protocol confirmed previous studies (Zijlstra *et al.*, 2000; Randig *et al.*, 2002; Adam *et al.*, 2007). The slightly lower intensity of the amplification product obtained with *M. incognita* primers Mi2F4/Mi1R1 might be due to a lower copy number of the targeted region (Adam *et al.*, 2007). However, decreasing the Fjav/Rjav primer concentration compensated for this effect. Together with the primers 194/195 (Adam *et al.*, 2007), seven species, *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, *M. chitwoodi*, *M. fallax* and *M. enterolobii*, can be identified with two PCR reactions. This multiplex PCR method has the potential for use in routine molecular diagnostics as it allows for rapid identification of three tropical *Meloidogyne* species with a wide host range found in similar geographical regions in support of integrated nematode management strategies.

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