Development of PCR for Specific Determination of Root-knot Nematode *Meloidogyne incognita*

Blanka TESAŘOVÁ, Miloslav ZOUHAR and Pavel RYŠÁNEK

Department of Plant Protection, Czech University of Agriculture, Prague-Suchdol, Czech Republic

Abstract

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PCR primers designed from the gene sequence for the SEC 1 oesophageal gland protein were used to specifically detect and differentiate the root-knot nematode *Meloidogyne incognita* from other species from the genus *Meloidogyne*. Amplification products were obtained from five *M. incognita* populations from different origins whereas DNA from *M. fallax*, *M. javanica*, *M. arenaria*, *M. chitwoodi* and *M. hapla* was not amplified. DNA extracted from different materials (females, root galls and spiked soil) could easily be used for *M. incognita* detection. One female gave sufficient amount of DNA for detection. Together with mitochondrial DNA this is one of the first attempts to use a gene outside of ITS regions for species specific PCR in the genus *Meloidogyne*.

Keywords: root-knot nematodes; *Meloidogyne incognita*; PCR; diagnostics; oesophageal gland protein gene

Root-knot nematodes (RKN) from the genus *Meloidogyne* are widespread in many parts of the world and cause substantial yield losses mainly in tropical and subtropical areas.

Their parasitic life style in the roots of plants results in root hyperplasia and galling which debilitates the root system (Taylor 1971).

The development of plants infested with nematodes is delayed, they may wilt and if grown in highly infested soil plants may be so badly damaged that they die before gall formation. If infestations are not high plants may appear symptomless and infestation can be overlooked. Therefore, where the presence of the nematodes is suspected symptomless plants should also be checked (Nickle 1991). The host range of *Meloidogyne* species is very wide, covering about 350 plant species from various families and thus weeds can serve as a reservoir of host plants sustaining infestation (Lišková & Sturhan 1997).

Tropical RKN have short generation cycle with a development time, depending on temperature, that can be range from between 26–56 days. Under these conditions there may be 1–5 generations per year (Decker 1969). Thus population can rise from low to very damaging levels on one crop. The nematode eggs can survive even in dry soil and it makes from RKN very dangerous parasites of plants.

Most of *Meloidogyne* species live in tropical regions only but some of them (*M. fallax*, *M. chitwoodi* and *M. hapla)* can live in field even under conditions of West and Central Europe. *Meloidogyne incognita* is one of species living in Central Europe in glasshouses only. Its presence was also proved in the Czech Republic (Zouhar *et al*. 2001).

Prevention together with fast, exact and cheap diagnostics are bases for effective plant protection against RKN. Methods using morphometric characteristics currently used for RKN detection are personnel demanding. Diagnostics on the base of DNA analysis using molecular markers may be an alternative. Its speed, accuracy and sensitivity are good prerequisites for reliable diagnosis. Relatively highly conserved sequences of internal transcribed

spacer (ITS) regions of rDNA encoding the structure of ribosomes are often used for detection purposes (Zijlstra 1997; Zijlstra *et al*. 1997; Williamson *et al*. 1997), but sequences of genes coding for other proteins can also be used if they are known. In this paper using of specific PCR with primers designed according to the sequence of such a gene is described.

MATERIALS AND METHODS

Nematodes. Populations of *M. incognita*, *M. fallax*, *M. javanica*, *M. arenaria*, *M. chitwoodi* and *M. hapla* (Table 1) were maintained on tomato plants cv. Stupické in a controlled enviroment room at 23–27°C, with 70% air humidity and 16 h day lenght. Plants were infested at 2–3 leaves stage by adding egg masses to the roots by means of a pipette. After 60 days plant material was used for DNA extraction. Nematodes were extracted from roots by a needle.

DNA extraction. Several methods of DNA extraction were tested and optimised. For the extraction of DNA from galls simple method (Zouhar *et al*. 2000) using CTAB extraction buffer (50mM Tris HCl pH 8.0, 0.7M NaCl, 10mM EDTA, 1% CTAB) was used. Galls were crushed in liquid nitrogen with pestle and mortar and after adding CTAB buffer homogenate was incubated 2 h at 60°C. A chlorophorm isoamylalcohol mixture (24:1) was used to separate DNA from proteins and other compounds. DNA was precipitated in liquid nitrogen for 20 min. After centrifugation, DNA pellet was washed in 70% ethanol, dried and resuspended in ddH_2O.

For extraction of DNA from single females or from very small number of nematodes an adapted method with proteinase K (Li *et al*. 1988) was used. Single females were crushed with glass rod in a glass minimortar with LB buffer, the mortar was rinsed with LB and the crude homogenate collected in an eppendorf which was then incubated at 37°C for one hour and at 85°C for five min. The suspension was centrifuged and supernatant precipitated with ethanol (96%) and LiCl (final conc. 1M) for one hour at –30°C. Pelleted DNA was rinsed in 70% ethanol, dried and resuspended in ddH_2O.

The use of DNA in a crude homogenate was also optimised, especially for use for quick diagnostics. One small gall was homogenised with a pellet pestle with 50 µl of reaction buffer for Taq DNA polymerase (Promega) in a microtube. Homogenate was 5 time frozen in liquid nitrogen and thawed at 60°C and then incubated at 60°C for one hour. After centrifugation supernatant was removed to a sterile eppendorf tube and used for PCR.

DNA from soil was extracted by slightly modified method of Yeates *et al*. (1998). Soil spiked with root galls or females (1 g of soil with 100 mg of gall tissue or 10 females, respectively) was homogenized with 2 ml of TNE with pestle and mortar and incubated at 65°C for one hour. After centrifugation, supernatant was removed into sterile tube, 1 ml of TNE was added to the pellet and incubation at 65°C and centrifugation were repeated. Supernatants were pooled and 0.5 volume of 30% PEG in 1.6M NaCl was added. After 2 h of incubation at room temperature the mixture was centrifuged and pellet was resuspended in 200 µl of TE. 7.5M solution of potassium acetate was added to achieve final concentration 0.5M and mixture was chilled on wet ice for 5 min. After centrifugation the supernatant was removed and mixed with an equal volume of phenol-chloroform mixture (1:1), the mixture was vortexed and centrifuged. The water phase was removed and a 2.5 volume of ethanol with 10% of 8M LiCl was added. Further steps were the same as mentioned in above protocols. Extracted DNA was quantified by spectrophotometer.

Table 1. Populations of *Meloidogyne* species used in this study and their origin

Species	Source
M. incognita	Czech University of Agriculture, Czech Republic
M. incognita	University of Evora, Portugal
M. incognita	Egypt
M. incognita	Egypt
M. incognita	SCRI Dundee, Scotland
M. fallax	PRI Wageningen, the Netherlands
M. javanica	PRI Wageningen, the Netherlands
M. arenaria	PRI Wageningen, the Netherlands
M. chitwoodi	PRI Wageningen, the Netherlands
M. hapla	Semice, Czech Republic
M. hapla	Mělník, Czech Republic
M. hapla	PI Košice, Slovak Republic

PCR. Primers were designed from published sequence of *Meloidogyne incognita* DNA which codes for putative oesophaegal gland protein SEC-1 in locus MIU09180 (Ray *et al*. 1994) using program Primer Input 5. The positions and sequences of primers are given in Scheme 1. The predicted lenght of product is 502 bp.

Several DNA polymerases were tested: DynaZyme (Finnzymes), Taq (Promega), Tfl (Promega), and Red Taq (Sigma). Generally each 25 µl reaction mixture contained 1.5mM of $MgCl_2$, 0.2mM of each dNTP, 12.5pmol of both primers, 1.5–2.0 units of DNA polymerase and 100–500 ng of extracted DNA. PCR was performed in a PTC 200 thermocycler (MJ Research) programmed to give 94°C for 4 min, then 40 cycles (50 cycles for DNA from soil) 94°C for 1 min, 60°C for 1 min, 72°C for 90 s, and final elongation phase at 72°C for 5 min. A UV transilluminator was used to visualise the products after electrophoresis in 1% agarose gel with ethidium bromide.

RESULTS

The primers amplified the same size of product from the DNA of all five populations of *M. incognita* irrespective of their origin. Amplification products from DNA from all the other species tested were never obtained (Figure 1). Optimal annealing temperature for the primers was 60°C. In addition to producing an amplification product from *M. incognita* DNA from all geographic locations all sources of the DNA (isolated females, galls and soil) also produced the appropriate products. The amount of DNA extracted from one female was quite sufficient to give an easily visible product. The simplified PCR protocol with DNA from a crude homogenate was also successful. When attempting to extract DNA from soil it was found that at least ten females needed to be added to 1 g of soil for successful detection (Figure 2). All DNA polymerases used (DynaZyme, Taq, Red Taq, Tfl) gave the same results (data not shown). In all cases

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 721 CACTCCCTCAAAGGCTTTAAATAGCAAAGGGCAAGTAAGGATGCTCTGACTATTGTGAGA
                                  >>>>>>>>>>>>>>>>>>>>
 781 CCATTTTGATTATAAGTCGTCTTAAATTTGTTCGGTAACTAGATTGGATAACTCACAAGT

 841 GCCAAAAATTTCTTTTCTTTACAGTTAGTTCTTAATTTTTGTCCATTTAAACTTACAATT

 901 AAGATGGATGATGAAAATAAGAAACTGCGTTCTAAAAACGATCAGTTCTCTTCGCAATTG

 961 GAAAAACAGCTTAAGGATTTGGAACATTTGCGCGCTGAGAATGATCAATTAAAACGAGAA

1021 AAGAATGAACAAGACGAACGTCATGTCATCTTTGAACAAACTGTAAAGAACAAGCTTATT

1081 GAGACACATCAAGTTTGTTTTCTTTTTTTTTGGACTTAATCGTTTTTAGTTGGGCGATGA

1141 AAACAAAAAGTTACGTTTGAAATGTGAGCAATTTTGTGAGCAATTGGAGAAGAAGAAAAC

1201 AGAGATTGCTAAGTTTTGCGAAGATCGCGACGTGGCTATGAAAGAGGTGCTTAAACTTCG
                                  <<<<<<<<<<<<<<<<<<<<
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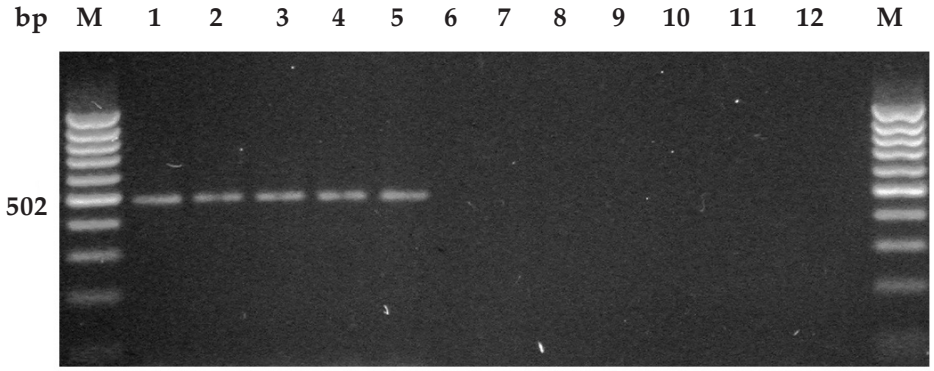
Scheme 1. Amplified part of sequence of the gene for putative oesophaegal gland protein SEC 1 of *M. incognita* and position of primers



M: molecular marker
Lane 1: DNA from *M. incognita* (Czech Republic)
Lane 2: DNA from *M. incognita* (Portugal)
Lane 3: DNA from *M. incognita* (Egypt, isolate 1)
Lane 4: DNA from *M. incognita* (Egypt, isolate 2)
Lane 5: DNA from *M. incognita* (SCRI Dundee)
Lane 6: DNA from *M. arenaria* (PRI Wageningen)
Lane 7: DNA from *M. javanica* (PRI Wageningen)
Lane 8: DNA from *M. fallax* (PRI Wageningen)
Lane 9: DNA from *M. chitwoodi* (PRI Wageningen)
Lane 10: DNA from *M. hapla* (Slovakia)
Lane 11: DNA from *M. hapla* (Czech Republic, isolate 1)
Lane 12: DNA from *M. hapla* (Czech Republic, isolate 2)
M: molecular marker

Figure 1. Results of PCR using DNA extracted from females of *M. incognita* and other species from the genus *Meloidogyne*

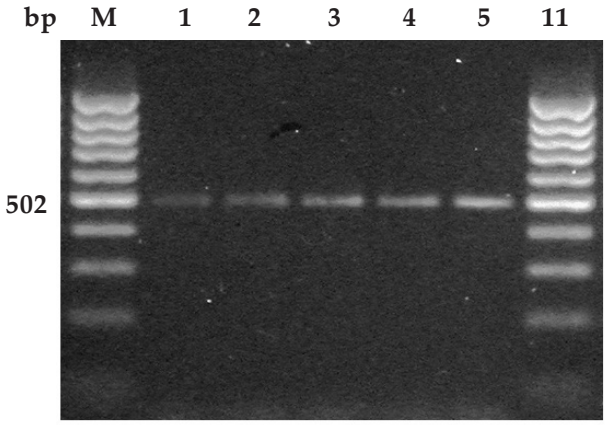


Figure 2. Results of PCR using DNA extracted from various materials containing *M. incognita*

M: molecular marker
Lane 1: DNA extracted from spiked soil (ten females/g)
Lane 2: DNA extracted from spiked soil (100 mg of root gall tissue/g)
Lane 3: DNA in crude gall homogenate
Lane 4: DNA extracted from one isolated female
Lane 5: DNA extracted from root galls
M: molecular marker

products were obtained only if sufficient number of cycles was done (40 cycles for DNA from females and galls, 50 cycles for DNA from soil).

DISCUSSION

The PCR primers studied proved to be a powerful tool for nematode determination.

They were found to be very specific and sensitive (one female or larva gives sufficient amount of DNA for analysis).

The only crutial prerequisite was having a sufficient number of PCR cycles. This could be due to relatively low amount of target DNA in each sample. All the source material investigated containing the target DNA (isolated females, roots, soil) and very different methods of DNA extraction, including the simplified protocol, could be used for detection.

This is in contrast to species determination using morphometric characteristics where more individuals are usualy required (Nickle 1991) and direct detection of nematodes in soil is impossible. So, our PCR method adds to the group of molecular methods of *Meloidogyne* species diagnostics (Williamson *et al*. 1997; Zijlstra 1997; Zijlstra *et al*. 1997). However, together with mitochondrial DNA (Dutova *et al*. 2002), this is one of the first cases

when a known gene sequence outside of rDNA region has been used for primer design. The difference is in the fact that using ribosomal DNA species-dependent minor modifications in ITS regions can be utilised and several species can be detected and differentiated by the same primer pair which amplifies different product lenghts from different species enabling differentiation of several species in one reaction. The problem may be if the product lenght differs only slighty between two species as in the case of *M. chitwoodi* and *M. fallax* (Zijlstra 1997). Moreover, product 436 bp long, which corresponds to *M. incognita* is of the same length also for *M. arenaria* and *M. javanica,* thus hampering unambiguous determination of *M. incognita*. Zijlstra *et al*. (2000) tried to solve the problem using SCAR based PCR to find genetic markers for differentiation of these species. However, the positions of target sequences annealing to these primers are not exactly known and obtaining of reliable results may be problematic. The method of DNA extraction and quality of extracted DNA may be crutial in this case (Zouhar, unpublished data). Sometimes, problems may also arise if there are very similar sequences in the ITS region and species specific primers are to be used. This is the case with *Globodera pallida* and *G. rostochiensis* where differentiation requires very precise adjustment of annealing temperature of both primers to prevent their nonspecific binding (Zouhar *et al*. 2000). The advantage of using ITS sequences for detection is in their relatively high abundance as there are multiple copies in the genome. Thus the higher amount of template DNA can mean that fewer PCR cycles are required to obtain detecable amplification product. The advantage of our system for *M. incognita* detection is that it is species specific without the problems of nonspecific primer binding because genes for oesophageal gland protein are probably more different among various species then relatively highly conserved ITS regions. Unfortunately, there is not too much information about sequences of genes for SEC proteins of other *Meloidogyne* species avaiable with the exception of a small part of this gene of *M. hapla* (McCarter 1999). On the other hand, our method could also be so specific due to the fact that one primer is in the coding region and the another is in non-coding region of the gene. Thus, even if SEC proteins were very similar or identical, the non-coding sequences may be more different among various species to enable their differentiation. Anyway, our results with isolates of *M. incognita* from geographically very distant regions showed reliability, specifity and sensitivity of determination. So, we conclude that our PCR method is a very useful molecular tool for routine identification of *M. incognita* either alone or for confirmation of results obtained by other methods.

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Souhrn

Tesařová B., Zouhar M., Ryšánek P. (2003): **Vývoj metody PCR pro specifickou detekci hálkotvorného háďátka** *Meloidogyne incognita*. Plant Protect. Sci., **39**: 23–28.

Hálkotvorné háďátko *Meloidogyne incognita* bylo specificky detekováno a odlišeno od ostatních druhů rodu *Meloidogyne* metodou PCR s použitím primerů navržených na základě sekvence DNA genu pro SEC 1 protein jícnové žlázy. Pět populací *M. incognita* různého původu bylo spolehlivě detekováno, zatímco DNA z *M. fallax, M. javanica, M. arenaria, M. chitwoodi* a *M. hapla* nebyla amplifikována. DNA extrahovaná z různých materiálů (samičky, kořenové hálky, zemina) byla s úspěchem použita k detekci *M. incognita*. K detekci postačuje množství DNA extrahované z jediné samičky. Spolu s již popsaným využitím mitochondriální DNA je toto jeden z prvních pokusů o použití sekvence genu mimo oblast rDNA pro druhově specifickou PCR u rodu *Meloidogyne.*

Klíčová slova: hálkotvorná háďátka*; Meloidogyne incognita*; PCR; diagnostika; gen pro protein jícnové žlázy

Corresponding author:

Ing. Miloslav Zouhar, Ph.D, Česká zemědělská univerzita v Praze, Agronomická fakulta, katedra ochrany rostlin, 165 21 Praha 6-Suchdol, Česká republika
tel.: + 420 224 382 595, fax: + 420 234 381 801, e-mail: zouhar@af.czu.cz