European Journal of Plant Pathology **110:** 855–860, 2004.
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Short communication

A PCR test to detect the cereal root-knot nematode *Meloidogyne naasi*

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Accepted 1 March 2004

Key words: diagnostics, ITS, ribosomal DNA

Abstract

The cereal root-knot nematode *Meloidogyne naasi* can cause serious cereal crop losses. The nematode is also found in agricultural fields where non-host crops are grown. Control of *M. naasi* can be based on preventing its spread, host resistance and crop management as well as on the design of crop rotation systems. Detection methods are required for these purposes and can also be helpful for inspection services and experimental research. This study describes the development of a simple PCR test that enables the detection of *M. naasi*. Alignment of sequences of rDNA-ITS fragments of *M. naasi* and five other *Meloidogyne* species was used to design the *M. naasi* specific forward primer N-ITS. Together with the reverse primer R195 *M. naasi* specific amplification was achieved.

Root-knot nematodes are important pest organisms causing serious crop losses worldwide. More than 90 species have been described (Karssen, 2000). Root-knot nematode species that can be found in the Netherlands are *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. naasi* and the recently described *M. minor* (Karssen et al., in press). They can be present either alone or as mixtures. The former two have acquired importance as quarantine pests while *M. hapla* has become increasingly important. All three species cause considerable damage on most agricultural crops. The cereal root-knot nematode *M. naasi* is also found frequently in agricultural areas, but its relevance is limited to grasses and cereals (Franklin, 1973; Jepson, 1987). The fact that *M. naasi* can seriously reduce the yield of wheat and barley crops, resulting in losses of up to 70% (Gooris and d'Herde, 1977; Cook and Guile, 1986) demonstrates that *M. naasi* is also an economically important pest. Incidentally, *M. naasi* has been reported from a few dicotyledonous plants such as sugar beet and weeds (Karssen and van Hoenselaar, 1998). *Meladogyne naasi* has also be found in soils where potatoes are grown.

Species identification based on differences in morphological characters (Hartman and Sasser, 1985) requires a lot of skill and is often inconclusive when only individual nematodes are available. Isozyme analysis is another way to identify species of the genus *Meloidogyne* (Esbenshade and Triantaphyllou, 1985). However, for reliable results, isozyme analysis can only be done with mature females. With respect to routine diagnostic assays, PCR based tests offer an attractive alternative, since they are DNA based techniques and they do not rely on the expressed products of the genome and are independent of environmental or developmental stages. In addition they are reliable, sensitive, relatively fast, easy to perform and do not require nematological expertise.

For the PCR based detection of the species *M. chitwoodi*, *M. fallax* and *M. hapla* several methods have been described (Petersen and Vrain, 1997; Zijlstra et al., 1995; 1997; Petersen et al., 1997; Zijlstra, 1997; 2000; Wishart et al., 2002). Reliable PCR based detection of *M. naasi* however had not been accomplished before.

This paper describes the development of a PCR test enabling the detection of *M. naasi* using

primers derived from the ITS sequence of *M. naasi*. In order to design *M. naasi* specific PCR primers, previously determined sequences of the ITS regions of *M. naasi*, *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. minor* and *M. incognita* (Zijlstra, 1997; Karssen et al., submitted) were compared. The alignment of these rDNA sequences was performed using the computer program DNA-STAR Megalign (DNASTAR Inc., Madison, WI) and is shown in Figure 1. For this study a forward primer specific for *M. naasi* was designed. Its position on the sequences is shown in Figure 1: N-ITS: 5′-CTCTTTATGGAGAATAATCGT-3′. As a reverse primer (R195) was used: 5′-CCTCCGC TTACTGATATG-3. The other primers shown in Figure 1 have been described previously (Zijlstra, 1997).

DNA was isolated from juveniles using the High Pure PCR template preparation kit (Roche). DNA was extracted from single juveniles as described by Harris et al. (1990). Nematode isolates used are listed in Table 1. Second stage juveniles of *M. chitwoodi* isolate Co, *M. fallax* isolate Fa, *M. hapla* isolate Hk, *M. incognita* isolate Ia, *M. minor* isolate Ma and *M. naasi* isolate Ne were used for the construction of nematode mixtures as described in Zijlstra et al. (1997). The selected primers for *M. naasi* amplification were tested in a *M. naasi* PCR assay. The PCR test was tested on all isolates listed in Table 1 for specificity to *M. naasi* and conditions were optimised. The *M. naasi* PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.6 μM of each of the primers N-ITS and R195, 1 unit of Taq DNA polymerase (Roche), 3 ng of DNA or the crude DNA extract from a single juvenile, and deionized water to a volume of 25 μl. PCR-amplification conditions were as follows: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, repeated for 35 cycles. To amplify from single juveniles, the same conditions were used, except that the number of cycles was increased from 35 to 45 and the reaction volume was 50 μl. Figure 2 shows some of the results. Figures 2 and 3 shows that when *M. naasi* DNA was present in the template DNA, a 433 bp fragment was amplified as was expected from Figure 1. No amplification could be observed when DNA of *M. incognita*, *M. javanica*, *M. arenaria*, *M. chitwoodi*, *M. fallax*, *M. minor* or *M. hapla* was used as template DNA (Figures 2 and 3). *M. naasi* was also detected in mixtures of *M. naasi* with *M. incognita*, *M. chitwoodi*, *M. fallax*, *M. minor* or *M. hapla* (Figure 3). When template consisted of DNA from *Pratylenchus penetrans*, no amplified products were observed (data not shown). These data show that the primer set N1-ITS/R195 provides a good tool to identify the species *M. naasi*.

Previously a multiplex PCR that simultaneously identified *M. hapla*, *M. chitwoodi*, *M. fallax* and *M. incognita* in mixtures, using the primers H-18S, CF-ITS, I-ITS and R195 (Figure 1) in one reaction was developed (Zijlstra, 1997). Attempts were made to develop a multiplex PCR system combining the primers H-18S, CF-ITS, N-ITS and R195 shown in Figure 1. Only amplification of the 433 bp *M. naasi* fragment in a multiplex PCR occurred when the template offered contained *M. naasi* DNA only. When DNA of *M. hapla*, *M. chitwoodi* or *M. fallax* was present, the amplification of the 433 bp fragment failed, whereas the expected products for the other species were produced (data not shown). Changes in annealing temperature, concentrations of primers or MgCl2 did not improve the situation. Probably there is a preferential annealing of the other two forward primers H-18S and CF-ITS that hampers the annealing of N1-ITS in the multiplex system. Nevertheless the simplex PCR test presented here proved to be a reliable tool for the detection of *M. naasi*. The 433 bp amplicon was easily amplified from DNA extracts of isolates of *M. naasi* and of mixtures of *Meloidogyne* species that can be found in agricultural areas. The method was sensitive enough to amplify the 433 bp fragment from the crude extract of a single juvenile.

This test offers an attractive alternative to replace identification methods based on morphological characters or isozyme patterns. The latter two methods are time consuming, they require specific developmental stages and are not always conclusive. However they remain valuable complementary methods, especially for research purposes and for the discovery of new species or type variants.

This PCR test was developed to detect *M. naasi* in agricultural samples. Therefore its specificity to *M. naasi* was examined in the presence of relevant nematodes. Although it is expected that the test also allows detection of *M. naasi* in samples from a

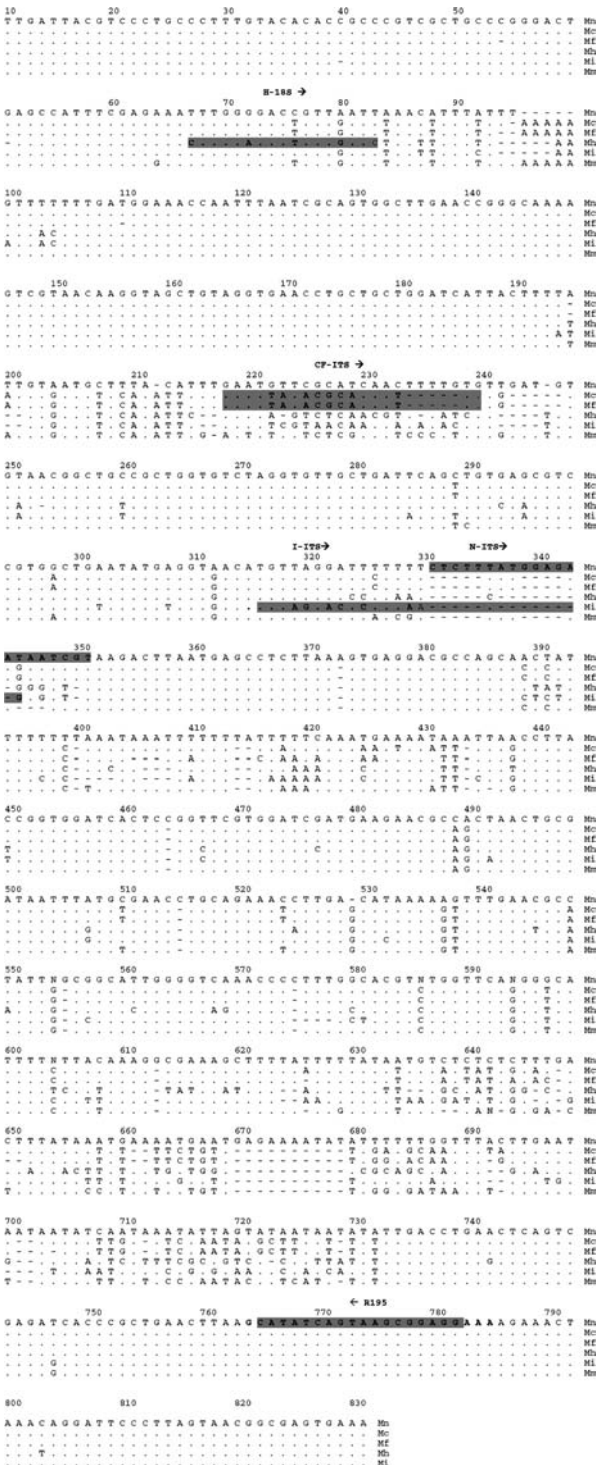

Figure 1. Alignment of fragments of rDNA ITS sequences of *M. naasi* (Mn), *M. chitwoodi* (Mc), *M. fallax* (Mf), *M. hapla* (Mh), *M. incognita* (Mi) and *M. minor* (Mm).The locations of primers H-18S, CF-ITS, I-ITS, N-ITS and R195 have been marked with arrows.

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

Code	Location	Isolate	Source
M. incognita			
Ia	The Netherlands		PRI[1]
Ib	The Netherlands	Inc568-93	PD[2]
Io	Senegal	Line 16	IRD[3]
Ip	Burkina Fasso	Line 17	IRD
Ir	Chad	Line 18	IRD
M. javanica			
Ja	Unknown		PRI
Jb		C3059	PD
Jh	Burkina Fasso	Line 22	IRD
Ji	Burkina Fasso	Line 23	IRD
Jj	Spain	Line 24	IRD
M. arenaria			
Ab	Ivory Coast		IRD
Ag	French West Indies	Line 31	IRD
Ah	The Netherlands	Xa	PD
M. chitwoodi			
Co	The Netherlands	Horst	AGV[4]
Cbd	Washington, USA	WAMC16, race 2	WSU[5]
Cbh	California, USA	CAMC2, race 3	WSU
M. fallax			
Fa	The Netherlands	CHB	AGV
M. hapla			
Hh	The Netherlands		AGV
Hk	The Netherlands	Sl.92	AGV
Han	South Corea	C2346	PD
Hbq	Hungary	C6611	PD
M. minor			
Ma	The Netherlands		PD
M. naasi			
Nc	The Netherlands	C6190	PD
Nd	The Netherlands	D5133	PD
Ne	The Netherlands		PD
Pratylenchus penetrans			
Pp			PD

[1] Plant Research International B.V., Wageningen, the Netherlands.
[2] Plant Protection Service, Wageningen, the Netherlands.
[3] IRD, Montpellier, France.
[4] Research Unit for Arable Farming, Field Production of Vegetables and Multifunctional Agriculture, Lelystad, the Netherlands.
[5] Washington State University, Prosser, USA.

different habitat, it is advised to verify its specificity under such circumstances. For instance, in dunes, *M. naasi* co-occurs with the grass root-knot nematodes *M. duytsi* and *M. maritima* (Karssen, 2000). To detect *M. naasi* in samples of coastal areas, one should be aware that these two species have not been included in the evaluation of the specificity to *M. naasi*.

When the PCR test is optimised into a fast practical test for detection of *M. naasi* in agricultural samples of soil or plant material it should be very helpful in preventing the spread of this

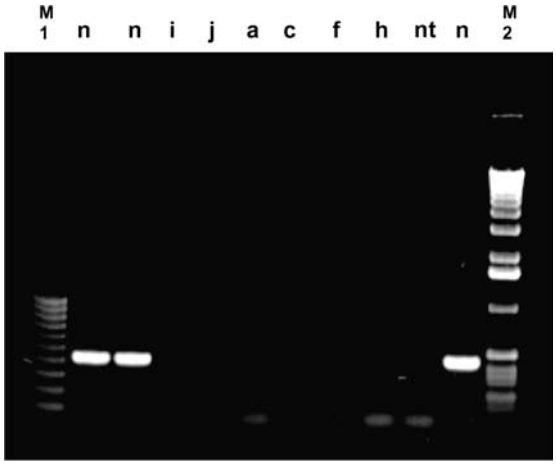

Figure 2. Typical amplification products of PCR reactions using the primers N-ITS and R195 using 3 ng template DNA of *M. naasi* (n), *M. incognita* (i), *M. javanica* (j), *M. arenaria* (a), *M. chitwoodi* (c), *M. fallax* (f) and *M. hapla* (h). M1: 100 bp size marker; nt: no template control; M2: 1 kb size marker.

damaging nematode and thereby reducing economically important crop losses.

Acknowledgements

G. Karssen, L. Molendijk, H. Mojtahedi and A. McDonald are greatly acknowledged for providing nematode isolates. We thank M. Fargette for providing DNA samples of isolates Io, Ip, Ir, Jh, Ji, Jj, Ab and Ag. The maintenance, propagation and harvest of the nematodes by L. Poleij and the experimental assistance by D. van der Wal are very much appreciated.

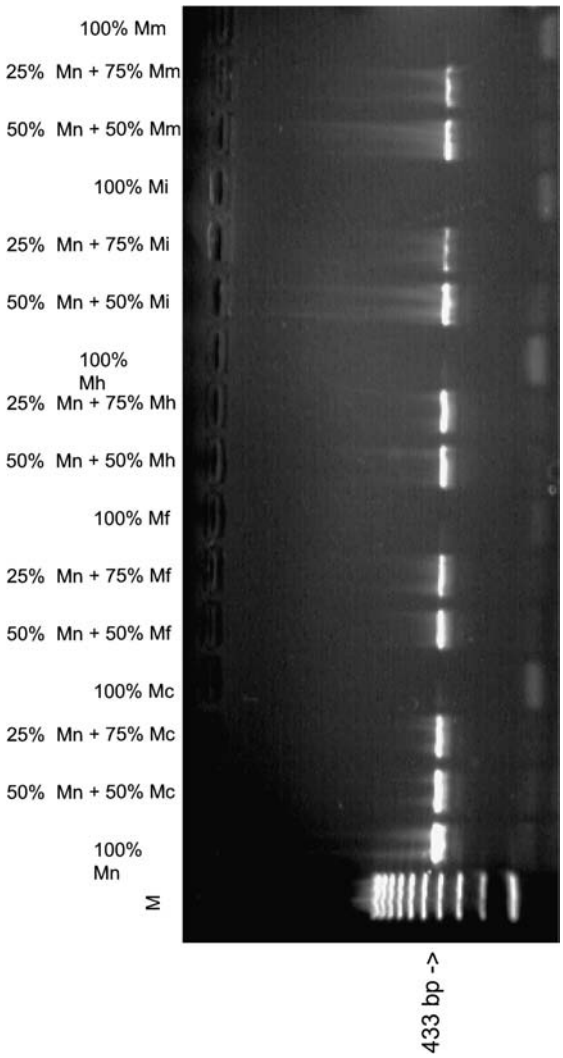

Figure 3. Typical amplification products of PCR reactions using the primers N-ITS and R195 using 3 ng template DNA from nematode samples consisting of *M. naasi* (Mn) and/or *M. chitwoodi* (Mc), *M. fallax* (Mf), *M. hapla* (Mh), *M. incognita* (Mi) and/or *M. minor* (Mm). M: 100 bp size marker.

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