Nematology, 2001, Vol. 3(7), 675-680

Identification of root-knot nematode species occurring in South Africa using the SCAR-PCR technique

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Received: 20 December 2000
Accepted for publication: 25 July 2001

Summary – Species of root-knot nematodes occurring in South Africa (*Meloidogyne fallax, M. chitwoodi, M. javanica, M. incognita, M. arenaria* and *M. hapla*) were identified and differentiated by the sequence characterised amplified region – polymerase chain reaction (SCAR-PCR) technique. Multiplex internal transcribed spacer (ITS)-PCR amplified a fragment in an unknown species for which no SCAR-PCR marker was available. *M. fallax*, a quarantine organism in Europe, was recorded for the first time in South Africa. The geographical distribution of *M. chitwoodi* is extended to four localities in South Africa. The SCAR-PCR technique reliably determined species composition, detecting mixed populations of *M. hapla, M. fallax* and *M. chitwoodi* on groundnut and of *M. arenaria* and *M. incognita* on *Impatiens* spp. In contrast, single species populations of *M. javanica* and *M. incognita* and the unknown species were detected in glasshouses.

Keywords – *Meloidogyne* species, *M. arenaria, M. chitwoodi, M. fallax, M. hapla, M. incognita, M. javanica*, nematode populations, ribosomal DNA.

Root-knot nematodes are the most common and destructive plant-parasitic nematode group in South Africa and adversely influence both crop quality and yield. *Meloidogyne javanica* (Treub 1885) is generally regarded as the economically most important and widespread species, followed by *M. incognita* (Kofoid & White 1919), *M. hapla* Chitwood 1949 and *M. arenaria* (Neal 1889) Chitwood, 1949. Other known South African species have a more restricted geographic distribution and include: *M. ethiopica* Whitehead 1968 in Mpumalanga and KwaZulu Natal (Fourie, 1997; Kleynhans *et al.*, 1996); *M. chitwoodi* (Golden, O'Bannon, Santo & Finley, 1980) in KwaZulu Natal and the Eastern Cape Province; *M. acronea* Coetzee, 1956 in the North West Province; *M. kikuyensis* De Grisse, 1960 in KwaZulu Natal, *M. partityla* Kleynhans, 1986 in Mpumalanga; *M. vandervegtei* Kleynhans, 1988 in KwaZulu Natal, and *M. graminicola* Golden & Birchfield, 1965 in the Northern Province (Kleynhans *et al.*, 1996).

The extensive morphological variation among and within root-knot nematode species complicates their identification (Hartman & Sasser, 1985). Accurate and reliable identification of root-knot nematodes are fundamental requirements before research programmes or proper management strategies can be implemented, particularly where quarantine organisms are concerned (Zijlstra, 2000). Recently, primer sets for sequence characterised amplified regions (SCARs) have been developed that enable sensitive and rapid identification of the species *M. incognita, M. javanica, M. arenaria* (Zijlstra *et al.*, 2000), *M. hapla, M. chitwoodi* and *M. fallax* (Zijlstra, 2000). These SCAR primers were deduced from sequences of species-specific RAPD markers. The SCAR-PCR technique is more sensitive than other existing molecular techniques and enables detection of species present in mixed populations in proportions of less than 1%.

In this study, six South African root-knot nematode isolates that were supposedly monospecific populations of *M. arenaria, M. incognita, M. javanica* and *M. hapla* were investigated by means of multiplex ITS-PCR and the SCAR-PCR techniques. The objective of this study was to determine if these populations were monospecific by identifying which species were present.

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Materials and methods

Six South African root-knot nematode populations, representing different species, were studied, namely A1, A2, I1, J1, H1 and U1 (Table 1). *Meloidogyne* spp. were initially identified by esterase and malate dehydrogenase phenotypes from single females (Esbenshade & Triantaphyllou, 1985) and by studying perineal patterns.

DNA EXTRACTION

Eggs were extracted from infested roots according to the procedure of Hussey and Barker (1973) and placed on a 20 μm-pore sieve at 20°C to allow juveniles to hatch. Second stage juveniles were collected after 3 and 7 days. These juveniles were centrifuged in a 30% sucrose solution at 2000 *g* for 2 min, washed in distilled water and pelleted in a microcentrifuge. The pellet was transferred to a mortar, frozen by liquid nitrogen and ground. DNA was extracted from the resulting fine powder as described by Zijlstra *et al.* (1997).

MULTIPLEX ITS AMPLIFICATION

DNA fragments containing ITS-regions were amplified by PCR using the ITS-specific primers as described by Zijlstra (1997) with the only difference that reactions were performed in volumes of 25 μl. Based on sequence information of ITS-regions of *M. chitwoodi*, *M. fallax*, *M. hapla* and *M. incognita* species, specific 'forward' PCR-primers had been designed. Three forward primers in combination with one common reverse primer were used in a single PCR reaction. Root-knot nematode species in samples tested can thus be identified in a single step by the size of their amplified fragments. A control reaction without template DNA was included in each PCR experiment. Following DNA amplification, 12 μl of the products were analysed on a 1% agarose gel.

SCAR-AMPLIFICATION

Amplification reactions with SCAR primers were performed as described in Zijlstra (2000) and Zijlstra *et al.* (2000). Following DNA amplification, 12 μl of the products were analysed on a 1% agarose gel. The SCAR-PCR based identification technique was developed by selecting first the length variant species-specific RAPD fragments for *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. incognita*, *M. javanica* and *M. arenaria*. After sequencing the fragments, longer primers were designed to complement the

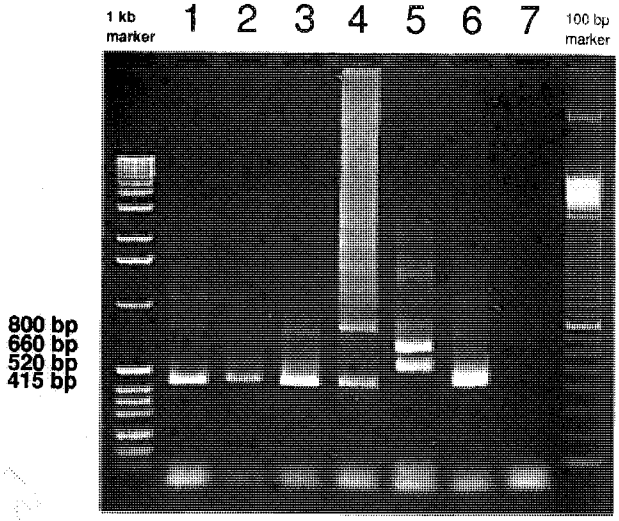

Fig. 1. *Amplification products of PCR reactions using forward primers H-18S, CF-ITS and I-ITS and reverse primer HCFI-28S and 1 to 10 ng of template DNA of* Meloidogyne *isolate: A1, lane 1; A2, lane 2; I1, lane 3; J1, lane 4; H1, lane 5; U1, lane 6; no template DNA control, lane 7.*

Table 1. *Regional and host origins and morphological and isozyme identifications of six root-knot nematode (*Meloidogyne *spp.) populations from South Africa and their identification multiplex ITS-PCR and SCAR-PCR.*

Code	Origin	Host	Morphology and isozymes	ITS-PCR	SCAR-PCR
A1	Durban	*Impatiens* spp.	*M. arenaria*	Tropical species	*M. arenaria* and *M. incognita*
A2	Stellenbosch	Tomato	*M. arenaria*	Tropical species	*M. arenaria*
I1	Vaalharts	Soybean	*M. incognita*	Tropical species	*M. incognita*
J1	Middelburg	Soybean	*M. javanica*	Tropical species	*M. javanica*
H1	Vaalharts	Groundnut	*M. hapla*	*M. hapla* and *M. chitwoodi* and/or *M. fallax*	*M. hapla*, *M. chitwoodi* and *M. fallax*
U1	Stellenbosch	Tomato	Unidentified	Tropical species	Unidentified

terminal sequences of the polymorphic DNA fragments. The resulting pairs of primers were used to generate the sequence-characterised amplified regions (SCARs). Using the developed pairs of SCAR primers, SCAR fragments of *M. chitwoodi* (800 bp), *M. fallax* (515 bp), *M. hapla* (610 bp), *M. incognita* (1200 bp), *M. javanica* (670 bp) or *M. arenaria* (420 bp) are easily amplified from DNA extracts from nematodes.

Results

The 415 bp fragment, representing the group of tropical root-knot nematode species such as *M. arenaria*, *M. incognita* and *M. javanica* (Zijlstra, 1997), is seen in lanes 1, 2, 3 and 4 representing isolates A1, A2, I1 and J1, respectively (Fig. 1). In lane 4, there is an additional fragment of about 800 bp, while lane 5 (isolate H1) shows a fragment of 660 bp, representing *M. hapla*, and a fragment of about 520 bp, representing *M. chitwoodi* (525 bp) and/or *M. fallax* (517 bp – Zijlstra, 1997) (Fig. 1). Lane 6 (isolate U1) also showed a 415 bp fragment for this unidentified *Meloidogyne* species (Fig. 1).

PCR with the *M. hapla* specific SCAR primer set Fh/Rh resulted in the *M. hapla* 610 bp SCAR fragment (Zijlstra, 2000) being amplified when template DNA from isolate H1 was used (Fig. 2, lane 5). No amplification could be observed when DNA from the other isolates was used as template (Fig. 2, lanes 1, 2, 3, 4, 6; Table 1).

PCR with the *M. chitwoodi* specific SCAR primer set Fc/Rc resulted in the *M. chitwoodi* 800 bp SCAR fragment (Zijlstra, 2000) being amplified when template DNA from isolate H1 was used (Fig. 3, lane 1; Table 1).

PCR with the *M. fallax* specific SCAR primer set Ff/Rf resulted in the *M. fallax* 515 bp SCAR fragment (Zijlstra,

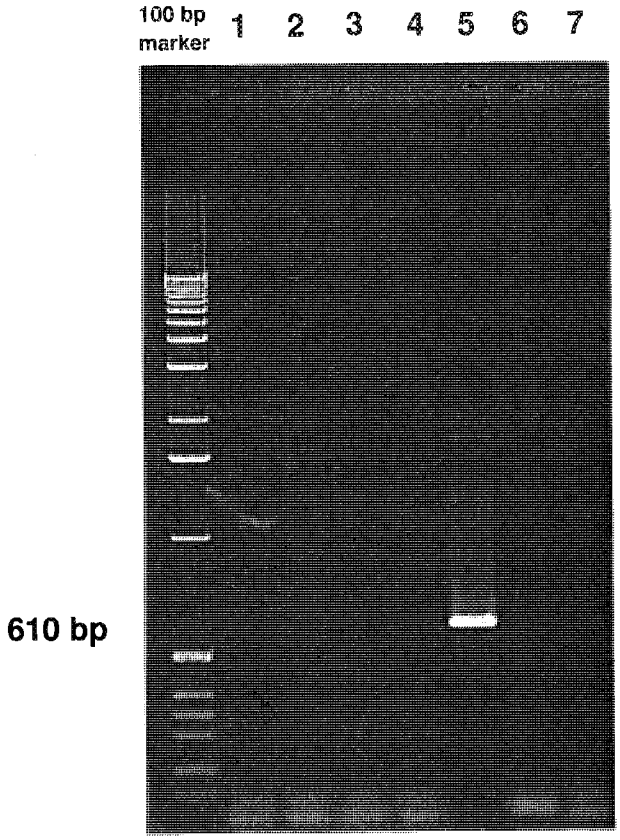

Fig. 2. *Amplification product of PCR reactions using primers Fh and Rh and 1 to 10 ng of template DNA of* Meloidogyne *isolate: A1, lane 1; A2, lane 2; I1, lane 3; J1, lane 4; H1, lane 5; U1, lane 6; no template DNA control, lane 7.*

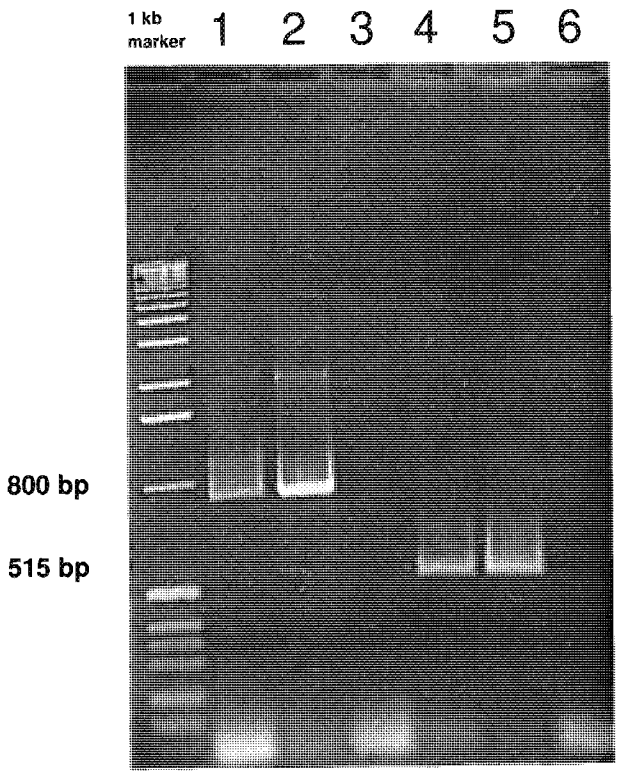

Fig. 3. *Amplification products of PCR reactions using primers Fc and Rc (lanes 1 to 3) or Ff and Rf (lanes 4 to 6) and 1 to 10 ng of template DNA of* Meloidogyne *isolates: H1, lanes 1 and 4;* M. chitwoodi *reference population, lane 2;* M. fallax *reference population, lane 5; no template DNA control, lanes 3 and 6.*

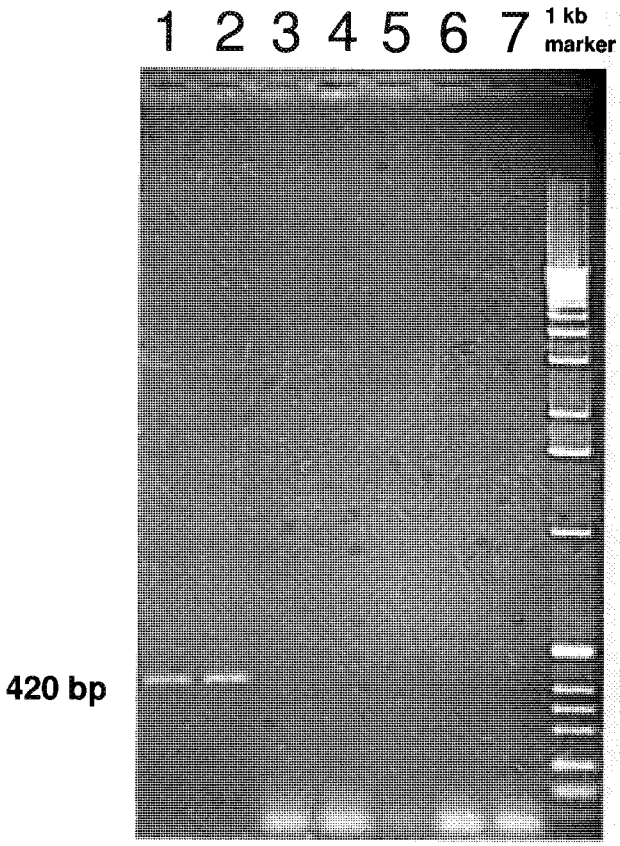

Fig. 4. *Amplification product of PCR reactions using primers Fa and Ra and 1 to 10 ng of template DNA of* Meloidogyne *isolate: A1, lane 1; A2, lane 2; I1, lane 3; J1, lane 4; H1, lane 5; U1, lane 6; no template DNA control, lane 7.*

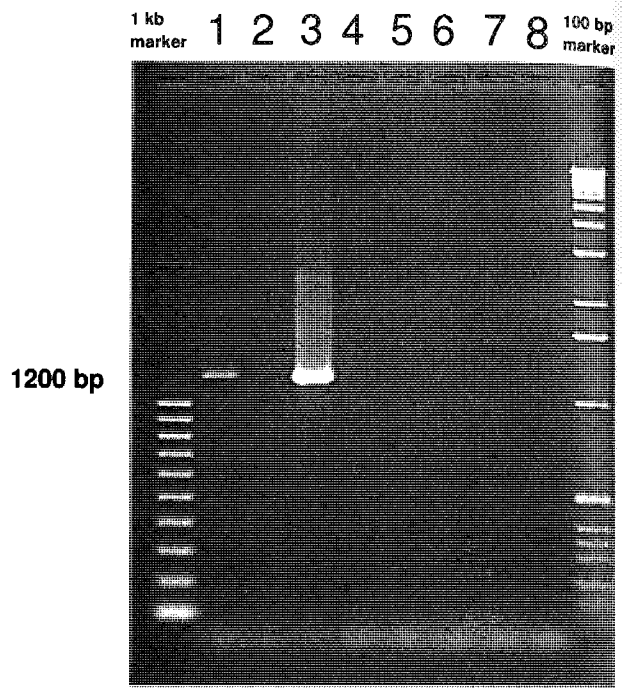

Fig. 5. *Amplification product of PCR reactions using primers Fi and Ri and 1 to 10 ng of template DNA of* Meloidogyne *isolate: A1, lane 1; A2, lane 2; I1, lane 3; J1, lane 4; H1, lane 5; U1, lane 6; no template DNA control, lanes 7 and 8.*

2000) being amplified when template DNA from isolate H1 was used (Fig. 3, lane 4; Table 1).

PCR with the *M. arenaria* specific SCAR primer set Fa/Ra resulted in the *M. arenaria* 420 bp SCAR fragment Zijlstra *et al.* (2000) being amplified when template DNA from isolates A1 and A2 were used (Fig. 4, lanes 1, 2). No amplification could be observed when DNA from the other isolates was used as template (Fig. 4, lanes 3, 4, 5, 6; Table 1).

PCR with the *M. incognita* specific SCAR primer set Fi/Ri resulted in the *M. incognita* 1200 bp SCAR fragment (Zijlstra *et al.*, 2000) being amplified when template DNA from isolates A1 and I1 were used (Fig. 5, lanes 1, 3). No amplification could be observed when DNA from the other isolates was used as template (Fig. 5, lanes 2, 4, 5, 6; Table 1).

PCR with the *M. javanica* specific SCAR primer set Fj/Rj resulted in the *M. javanica* 670 bp SCAR fragment (Zijlstra *et al.*, 2000) being amplified when template DNA from isolate J1 was used (Fig. 6, lane 4). No amplification could be observed when DNA from the other isolates was used as template (Fig. 6, lanes 1, 2, 3, 5, 6; Table 1).

Discussion

Multiplex ITS-PCR indicates that isolate H1 contained not only *M. hapla*, but also *M. chitwoodi* and/or *M. fallax*. This sample was therefore also tested with *M. chitwoodi* and *M. fallax* SCAR-primers. These reactions confirmed the presence of *M. chitwoodi*, but also of *M. fallax*. The *M. hapla* SCAR-reaction confirmed the presence of *M. hapla* in isolate H1 but not in the others. The presence of tropical species in other samples was evident from multiplex ITS-PCR and SCAR reactions confirmed them as *M. arenaria, M. incognita* and *M. javanica*. Although multiplex ITS-PCR indicated that isolate U1 belonged to the tropical group, no fragment was amplified with the other six available SCAR-PCR primers. Isolate

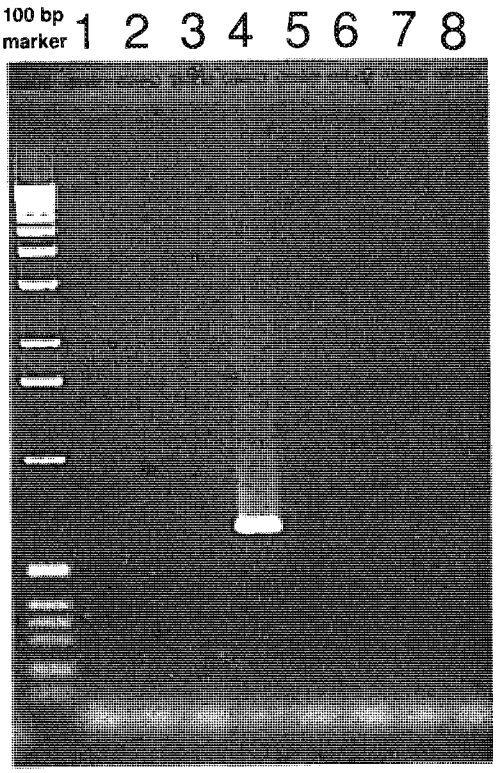

Fig. 6. *Amplification product of PCR reactions using primers Fj and Rj and 1 to 10 ng of template DNA of* Meloidogyne *isolate: A1, lane 1; A2, lane 2; I1, lane 3; J1, lane 4; H1, lane 5; U1, lane 6; no template DNA controls, lanes 7 and 8.*

U1 thus remains unidentified, as no SCAR-PCR markers are currently available for molecular identification of this species. Molecular techniques indicated that *M. javanica*, *M. incognita* and the unidentified species occurred in monoculture greenhouse populations, while *M. hapla*, *M. chitwoodi* and *M. fallax* were present as a mixed population on groundnut from the Vaalharts area (Northern Cape). *M. arenaria* and *M. incognita* also occurred as a mixed population on *Impatiens* spp. flower roots from Durban (KwaZulu Natal).

The presence of *M. fallax* in South African soils has not previously been reported. SCAR-PCR on the *M. hapla* Vaalharts population resulted in amplification of a product of identical size as to the *M. fallax* reference population. Therefore, this species should be considered as *M. fallax*. At present, the host range for this species in South Africa is unknown.

The presence of *M. chitwoodi* on groundnut at Vaalharts is surprising. Although groundnut cv. Florrunner is a non-host to this species (O'Bannon & Nyczepir, 1982), the South African groundnut cv. Sellie supported *M. chitwoodi* from this site. The geographical distribution of this species has until recently been restricted to only three localities in South Africa, namely Boston and Mooi River in KwaZulu Natal and Montgomery in the Eastern Cape (Kleynhans, 1991).

The SCAR-PCR method used in this study proved not only to be a reliable tool for routine diagnostic identification of root-knot nematode populations, but also enabled differentiation between individuals of *M. chitwoodi*, *M. fallax*, *M. hapla*, *M. incognita*, *M. javanica* and *M. arenaria* occurring in mixed populations.

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