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FULL RESEARCH PAPER

Identification of *Pratylenchus thornei*, the cereal and legume root-lesion nematode, based on SCAR-PCR and satellite DNA

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Abstract Two different molecular tools for the diagnosis of the cereal and legume root-lesion nematode *Pratylenchus thornei* were developed. A randomly amplified DNA (RAPD) fragment specific to *P. thornei* was identified. After sequencing the fragment, longer primers were designed that complement the terminal sequences of the RAPD fragment, and this pair of specific primers was used to amplify the sequence-characterized amplified region (SCAR). Using the developed pair of SCAR primers, the SCAR fragment specific to *P. thornei* was easily amplified with DNA extracts obtained from different life stages of the nematode. The described SCAR-PCR-based assay has the potential to be optimized for routine practical diagnostic tests. In addition, the use of a species-specific satellite DNA sequence to distinguish *P. thornei* from other *Pratylenchus* spp. is discussed.

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Introduction

Root-lesion nematodes (*Pratylenchus* spp.) are migratory, obligate endoparasites that cause severe yield losses in crops of economic significance and can be considered the second most important plant-parasitic nematodes after root-knot nematodes worldwide (Barker & Noe, 1987; Jatala & Bridge, 1990). Root-lesion nematodes penetrate, feed and migrate inside the root cortex giving rise to necrotic lesions and root cavities. Parasitism by these nematodes impairs physiological functioning of the root system which results in reduced uptake and transportation of water and nutrients within the plant (Potter & Olthof, 1993). In addition, necrotic lesions caused by *Pratylenchus* spp. may be further invaded by fungi and bacteria resulting in extensive root rots and complex diseases in a wide range of hosts (Mai, Brodie, Harrison, & Jatala, 1981; LaMondia, 2003).

Currently, the genus *Pratylenchus* includes more than 60 species (Loof, 1991) which can be differentiated only by means of minor morphological and morphometric differences. However, economic damage caused by root-lesion nematodes

to herbaceous and fruit crops throughout the world is attributable mainly to eight of the most common species, including *Pratylenchus brachyurus*, *P. coffeae*, *P. neglectus*, *P. penetrans*, *P. scribneri*, *P. thornei*, *P. vulnus* and *P. zeae* (Barker, 1998). Genetic variability among these *Pratylenchus* species has been analysed based on the comparison of the nucleotide sequences of the 26S rDNA (Al-Banna, Williamsom, & Gadner, 1997; De Luca, Fanelli, Di Vito, Reyes, & Di Giorgi, 2004). Identification of *Pratylenchus* spp. based on morphology and morphometric traits of adult females and males is time-consuming, requires ample skill and training in the observer and it is frequently inconclusive because individual specimens often vary considerably within a population (Loof, 1991). Therefore, there is a need for rapid and accurate identification procedures to facilitate quarantine inspection, implementation of integrated pest management strategies, and resistance breeding.

Pratylenchus thornei, the cereal and legume root-lesion nematode, damages many cereals and legume crops in the Mediterranean region, America, the Indian subcontinent and Australia (Castillo, Gómez-Barcina, Jiménez-Díaz, 1996; Di Vito et al., 1994; Nicol, Davies, Hancock, & Fisher, 1999; Nicol & Ortiz-Monasterio, 2004; Sharma, Smith, & McDonald, 1992; Smiley, Whittaker, Gourlie, & Easley, 2005). In addition, infection of chickpea by *P. thornei* increases the severity of root necrosis and enhances the root colonization by *Fusarium oxysporum* f.sp. *ciceris* race 5 in *Fusarium* wilt-susceptible chickpea genotypes (Castillo, Mora-Rodríguez, Navas-Cortés, & Jiménez-Díaz, 1998).

Polymerase-chain reaction-based methods offer new and improved possibilities for the characterization of genetic differences in *Pratylenchus* spp. to an extent that could not be approached previously with morphological observations or isozyme studies (Ibrahim, Perry, & Webb, 1995; Andrés, Pinochet, Hernández-Dorrego, & Delibes, 2000). PCR-based methods are relatively rapid, very reliable and with high discriminating potential, do not rely on the expressed products of the genome, and they are independent of environmental influence and of the stage of the nematode life cycle. Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analyses have proven useful in the identification of plant-parasitic nematodes and in determining interspecific and intraspecific variation among *Pratylenchus* species (Ouri & Mizukubo, 1999). More recently, PCR-based methods for the identification of plant-parasitic nematodes have focused on the design of specific PCR primers that amplify species-specific DNA fragments (e.g., Al-Banna, Ploeg, Williamsom, & Kaloshian, 2004; Amiri, Subbotin, & Moens, 2002; Castagnone-Sereno, Espárrago, Abad, Leroy, & Bongiovanni, 1995; Petersen & Vrain, 1996; Stanton, Hugall, & Moritz, 1997; Uehara, Mizukubo, Kushida, & Momota, 1998; Williamson, Caswell-Chen, Westerdahl, Wu, & Caryl, 1997; Zijlstra, 1997). These primers can be designed from the sequence of RAPD fragments shown to be associated with a particular nematode species, and the pair of specific primers is used to generate a sequence-characterized amplified region (SCAR) (Paran & Michelmore, 1993). In the last few years, SCAR primer sets have been developed that enable sensitive and rapid identification of several *Meloidogyne* species (Zijlstra, 2000; Ziljstra, Donkers-Venne, & Fargette, 2000; Fourie, Zijlstra, & McDonald, 2001).

A molecular diagnostic procedure alternative to PCR-based assays is the use of specific probes in hybridization assays. Satellite DNAs (satDNA) are present in the genome of almost all eukaryotic organisms and are composed of highly repetitive sequences organized as long arrays of tandemly repeated elements, present from 10^3 to 10^5 copies per haploid genome (Castagnone-Sereno, Leroy, Bongiovanni, Zijlstra, & Abad, 1999). SatDNA sequences have been characterized from a number of plant-parasitic and entomopathogenic nematodes, and have been shown to be species-specific in *Meloidogyne* (Piotte, Castagnone-Sereno, Bongiovani, Dalmasso, & Abad, 1995; Castagnone-Sereno, Leroy, & Abad, 2000), *Globodera* (Grenier, Bossis, Fouville, Renault, & Mugniéry, 2001), *Steinernema* (Grenier, Laumond, & Abad, 1995) and *Heterorhabditis* (Stack et al., 2000). Thus, satDNA-based probes offer an alternative strategy for specific identification that does not involve PCR amplification of nematode DNA.

The primary objective of this study was the identification of RAPD-PCR DNA fragments associated with *P. thornei* and the design of specific primers for the SCAR-PCR diagnosis of *P. thornei*. The species-specificity of a satDNA sequence and its diagnostic potential was also evaluated.

Materials and methods

Nematode isolates

This study comprised 28 isolates of root-lesion, root-knot, and cyst forming nematodes, as well as the stem and bulb nematode (Table 1). Those isolates were obtained from the nematode collections maintained in the Instituto de Agricultura Sostenible, Córdoba, Spain, and the Istituto per la Protezione delle Piante, Sezione di Bari, Italy. Isolates of root-lesion and of the stem and bulb nematodes were reared on carrot disks starting from a single mature female (Castillo, Trapero-Casas, & Jiménez-Díaz, 1995); root-knot nematodes were reared on tomato plants (*Lycopersicon esculentum* cv. Roma) starting from a single egg mass for each species (Hussey & Barker, 1973). The cyst forming nematodes were reared on their specific host (Table 1) starting from a single mature female cyst (Castillo & Vovlas, 2002).

DNA extraction from a mass of nematodes

Total DNA was extracted from migratory life stages of root-lesion nematodes and the stem and bulb nematode, from mature females and egg masses of root-knot nematodes, and from mature female cysts of cyst forming nematodes. Total DNA was extracted from 1 ml of nematodes in

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

Nematode species	Code	Host	Country location
Pratylenchus thornei	1.1[a,b]	Chickpea	Cañete de las Torres, Córdoba (Spain)
Pratylenchus thornei	1.2[a,b]	Chickpea	Jerez, Cádiz (Spain)
Pratylenchus thornei	1.3[a,b]	Chickpea	Santaella, Córdoba (Spain)
Pratylenchus thornei	1.4[a,b]	Chickpea	Marchena, Sevilla (Spain)
Pratylenchus thornei	1.5[a,b]	Chickpea	Tel-Hadya, Aleppo (Syria)
Pratylenchus neglectus	2.1[b]	Wheat	Cerignola, Foggia (Italy)
Pratylenchus neglectus	2.2[b]	Olive tree	Castro del Río, Córdoba (Spain)
Pratylenchus neglectus	2.3[b]	Olive tree	Úbeda, Jaén (Spain)
Pratylenchus fallax	3.1[a,b]	Olive tree	La Luisiana, Sevilla (Spain)
Pratylenchus vulnus	4.2[a,b]	Olive tree	Villaverde del Río, Sevilla (Spain)
Pratylenchus penetrans	5.1[a,b]	Olive tree	Pedrera, Sevilla (Spain)
Pratylenchus penetrans	5.2[b]	Apple tree	Canada
Pratylenchus penetrans	5.3[b]	Apple tree	Rhenes (France)
Pratylenchus penetrans	5.4[b]	Apple tree	Wisconsin (USA)
Pratylenchus penetrans	5.5[b]	Chickpea	Gravina, Bari (Italy)
Zygotylenchus guevarai	7.1[a,b]	Olive tree	Baeza, Jaén (Spain)
Radopholus similis	8.1[a,b]	*Anthurium* sp.	Madeira (Portugal)
Ditylenchus dipsaci	9.1[a,b]	Faba bean	Bari (Italy)
Meloidogyne artiellia	S[a,b]	Chickpea	Tel-Hadya, Aleppo (Syria)
Meloidogyne artiellia	I[a,b]	Chickpea	Monopoli, Bari (Italy)
Meloidogyne arenaria	Ma[a,b]	Olive	Villaverde del Río, Sevilla (Spain)
Meloidogyne incognita	Mi[a,b]	Olive	Alcolea, Córdoba (Spain)
Meloidogyne javanica	Mj[a,b]	Olive	Córdoba (Spain)
Heterodera mediterranea	HmA[a,b]	Wild Olive	Zahara de los Atunes, Cádiz (Spain)
Heterodera mediterranea	HmI[a,b]	Lentisc	Torre Canne, Brindisi (Italy)
Heterodera mediterranea	HmU[a,b]	Olive	Utrera, Sevilla (Spain)
Heterodera ciceri	Hc[b]	Chickpea	Tel-Hadya, Aleppo (Syria)
Globodera rostochiensis	Gr[b]	Potato	Polignano, Bari (Italy)

[a] Nematode isolates used for RAPD-PCR analyses

[b] Nematode isolates used for SCAR-PCR analyses

solution by crushing them into an eppendorf tube using a pestle. The solution with the crushed nematodes was then incubated at 37°C for 3 h in 200 µl of extraction buffer (10 mM Tris–HCl: pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, and 200 µg ml^{-1} proteinase K) as described by Uehara et al. (1998). The lysate was mixed with 1 volume of phenol/chloroform/isoamylalcohol (25:24:1) and centrifuged (30 min, 14000 rpm, 4°C). The supernatant was digested with RNAase (20 µg ml^{-1}) and extracted with chloroform:isoamylalcohol (24:1). After a new centrifugation, DNA in the upper phase was precipitated with 2 volumes of 95% ethanol (4°C), washed twice with 70% ethanol, and resuspended in 25 µl ultrapure water.

DNA extraction from a single female nematode

Single female nematodes (five repetitions) belonging to isolate 1.1 of *Pratylenchus thornei* species were used. The nematodes were maintained on chickpea plants under appropriate conditions in a growth chamber. The female nematodes were hand-picked from the root tissues under a stereomicroscope and stored at –80°C until used. Total DNA was purified from individual females as follows: each female was placed in 15 µl of lysis buffer (50 mM KCl, 10 mM Tris–HCL pH 8, 2.5 mM $MgCl_2$, 60 µg ml^{-1} Proteinase K, 0.45% NP40, 0.45% Tween 20 and 0.01% gelatine) and successively incubated at 65°C for 1 h and at 94°C for 10 min. Then the lysates were placed on ice until their use in PCR analyses.

RAPD analysis

RAPD-PCR amplification reactions (25 µl) consisted of 2.5 µl of 10x reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0 [25°C], 1% v/v Triton X-100), 3 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of primer, 0.75 U of Eco*Taq* DNA Polymerase (Ecogen, S.R.L., Barcelona, Spain), and 50–100 ng of genomic DNA. RAPD reactions were carried out using 12 different 10-mer random primers of the OPE primer set (OPE02–OPE13) from Operon (Operon Technology, Alameda, CA, USA). Reactions were performed in 2400 and 9600 thermocyclers (Perkin-Elmer, Norwalk, CT), or in a PTC 100 thermocycler (MJ Research, Watertown, MA). Reaction conditions were: 4 min of denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min of annealing at 36°C and 3 min of extension at 72°C. The final cycle consisted of 1 min of annealing followed by 6 min at 72°C to produce fully double-stranded DNA fragments. The temperature between annealing and extension increased at 0.6°C s^{-1}. Amplification products were separated by electrophoresis on 1% agarose gels at 1.5 V cm^{-1}, stained with ethidium bromide, and visualised under UV light. The 0.1-kb DNA ladder XIV size marker was used for electrophoresis (Boehringer-Manheim, Barcelona, Spain). All reactions were repeated at least twice and always included negative (no template DNA) controls.

Cloning and sequencing of RAPD fragments, and primer design for SCARs

Selected RAPD fragments were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany), and cloned into the PCR 2.1-TOPO vector of the TOPO-TA Cloning kit (Invitrogen, Groningen, The Netherlands), according to the manufacturer's instructions. Recombinant plasmids were screened for insert size, and plasmid DNA from the correct insert-containing clones was extracted using the Qiagen Plasmid Minikit (Qiagen, Hilden, Germany). Sequencing of the inserts was performed in both directions using the Big Dye Terminator Sequencing kit (Perkin Elmer) and the Applied Biosystems apparatus (model ABI 310). Specific PCR primers were designed from the sequences of two clones (clones PthB and PthC) using the Primer Select 3.11 programme of the 'DNA Star' software (Madison, WI, EEUU). The specific PCR oligonucleotide primers (Table 2) were synthesized by Genset (Paris, France).

Southern blot

RAPD fragments amplified with primer OPE13 were resolved on 1% agarose gels and transferred onto Nylon Membranes (Roche Diagnostics, Manheim, Germany). Insert PthC was labelled

Table 2 Nucleotide sequence of primer sets designed for SCAR-PCR analyses

Name	Primers sequences	Size of SCAR (bp)	Annealing temperature (°C)
Pth*f*	TTC GGA AGA CAA TAA ATC	1078	47
Pth*r*	TCC AAA ATG AAA TAA TAA A		
Pthsat *f*1	CCC GAT TCG GAT TGA ATG CG	Ladder-like pattern	58
Pthsat *f*2	AGC CGT CTG CCA ATG TTT AAT AAG CAA TAA		
Pthsat *r*1	CCC GAT TCG GAA AGG GAC GA		

Primer pair Pth *f*/Pth *r* was derived from the external sequence of clone C. Primer pairs Pthsat *f* 1/Pthsat *r*1 and Pthsat *f* 2/Pthsat *r*1 were derived from the external sequence of the B clone, and the first includes the RAPD primer sequence (underlined nucleotides)

using DIG-11-dUTP [digoxigenin-3-O-methylcarbonil-amino-caproyl-5-(3-aminoallyl)-uridine-5́-triphosphate] (Boehringer-Mannheim, Barcelona, Spain) and hybridized back to the original OPE 13-RAPD southern blot. Prehybridization and hybridization treatments were performed under high stringency conditions, as described by Sambrook, Fritsch, & Maniatis (1989).

Specific PCR reactions

Oligonucleotide primers designed from sequences of inserts PthB and PthC (Table 2) were used in specific PCR reactions. Amplification reactions for SCARs were performed in 25 µl reaction volumes containing 2.5 µl of 10× reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.6 U of Eco*Taq* DNA Polymerase (Ecogen), 0.3 µM of each primer, and 50–100 ng of genomic DNA. For SCAR amplifications, reaction conditions were: denaturation at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at the corresponding hybridization temperature (see Table 2), and 1 min at 72°C. The final cycle consisted of 1 min of annealing followed by 6 min at 72°C to produce fully double-stranded DNA fragments. Oligonucleotide primer set designed from sequence of insert PthC (Table 2) was also used in specific PCR reactions using 5 µl of DNA extracted from a single nematode of isolate 1.1 as a template in amplification reactions (25 µl) as above. 50 ng of DNA extracted from a mass of nematodes of the same isolate 1.1 was used as a template in a positive control reaction, while a mixture without template DNA was used as the negative control. The reaction conditions for the specific amplification were: denaturation at 94°C for 2 min, followed by 40 cycles of 1 min at 94°C, 1 min at 42°C and 1 min at 72°C. The final cycle consisted of 1 min of annealing followed by 5 min at 72°C to produce fully double-stranded DNA fragments.

Dot blot

A selected fragment (32 nt: ACCGGGTTCC C**TAAAGAAT**CGTCCCTTTCCGA) of the sat-DNA sequence contained in insert PthB (see Results) was synthesized and labelled with DIG-11-dUTP [digoxigenin-3-O-methylcarbonil-amino-caproyl-5-(3-aminoallyl)-uridine-5́-triphosphate] by Genset (Paris, France), and used as a probe in dot-blot experiments using 50 ng of total DNA of all isolates listed in Table 1 except *P. thornei* isolates 1.4 and 1.5. To prepare the dot blot, the DNA was first diluted to 5 ng μl^{-1}, then denatured with a volume of 0.2 M NaOH and incubated at room temperature for 10 min. This DNA solution (10 µl) was dotted onto Nylon Membranes (Roche Diagnostics, Manheim, Germany) and neutralised with 4 M ammonium acetate. Pre-hybridization and hybridization treatments were done under high stringency conditions, as described by Sambrook et al. (1989).

Results

RAPD-PCR analysis

The 12 OPE primers tested generated banding patterns from DNA of all the nematode isolates in this study, and those patterns were reproducible

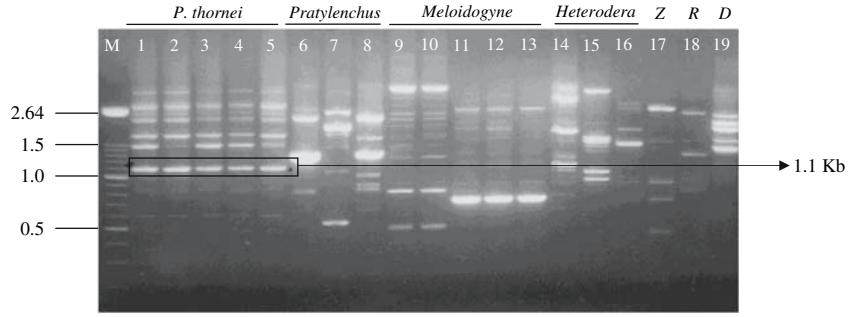

Fig. 1 RAPDs generated by primer OPE-13 using total DNA from isolates of different nematode species. Numbers on the left side are the sizes of the 0.1 kb DNA ladder XIV (Boehringer-Manheim) (Lane M). Number on the right side is the size of the *P. thornei* associated fragment (boxed bands). Lanes correspond to the following nematode isolates: lanes 1–5: *P. thornei* isolates (1.1, 1.2, 1.3, 1.4 and 1.5, respectively); lane 6: *Pratylenchus fallax*; lane 7: *Pratylenchus vulnus*; lane 8: *Pratylenchus penetrans* (5.1); lanes 9–10 *Meloidogyne artiellia* ; lane 11 Meloidogyne arenaria; lane 12 Meloidogyne incognita; lane 13 Meloidogyne javanica; lanes 14 to 16 Heterodera mediterranea isolates (HmA, HmI, HmU, respectively); lane 17 Zygotylenchus guevarai; lane 18 Radopholus similis; lane 19 Ditylenchus dipsaci. Codes refer to Table 1

in repeated amplifications. No band was amplified in any of the control (without DNA) reactions. Primer OPE-13 produced a major amplification product 1.1 kb in size that was present in total DNA of all the *P. thornei* isolates. However, this RAPD band was not amplified using total DNA from other *Pratylenchus* spp., *Meloidogyne* spp., *Z. guevarai*, *R. similis*, *Heterodera* spp., and *D. dipsaci* (Fig. 1). Thus, this *P. thornei*-associated DNA fragment was selected for further studies.

Identification and characterization of the *P. thornei*-associated RAPD fragment

The 1.1 kb *P. thornei*-associated fragment was purified and cloned, and four correct insert size-containing clones (clones PthA–PthD) were selected. The insert sizes of the four clones were: PthA, 1097 bp; PthB, 926 bp; PthC, 1115 bp; and PthD, 1087 bp. Individual hybridization of each insert with the initial RAPD pattern generated with primer OPE 13 confirmed the co-presence of the four different inserts in the same RAPD fragment in the gel. Labelled inserts PthB and PthC never hybridized with any fragment of the RAPD patterns amplified by primer OPE 13 using DNA of nematode isolates other than *P. thornei.* On the contrary, inserts PthA and PthD gave a weak hybridization signal at the same position in the gel from all the nematode isolates studied, although no band was visible after RAPD amplification (Fig. 1).

Development of *P. thornei*-specific SCAR primers and SCAR-PCR

An oligonucleotide primer pair was designed from sequences of insert PthC (Table 2) that was further used in specific-PCR assays. Primer set Pth*f*/Pth*r* amplified a 1078 bp fragment when DNA from *P. thornei* isolates 1.1, 1.2, 1.3, 1.4 and 1.5 were used as templates (Fig. 2). No amplification occurred using that primer pair with total DNA from other *Pratylenchus* spp., *Meloidogyne* spp., *Z. guevarai*, *R. similis*, *Heterodera* spp., and *D. dipsaci* (Data not shown).

Development of primers that amplify a *P. thornei*-specific satDNA sequence

Two different oligonucleotide primer pairs (Pthsat*f*1/Pthsat*r*1 and Pthsat*f*2/Pthsat*r*1) were designed from sequence of insert PthB (Table 2) and both amplified a ladder-like pattern with DNA of *P. thornei* isolates as the template (Fig. 3). That indicates the presence of a tandemly repeated sequence, characteristic of a satDNA. Sequencing showed that this satDNA

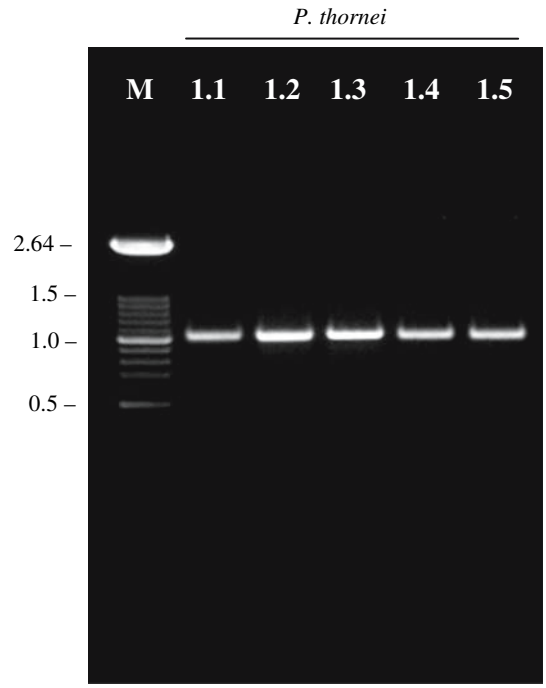

Fig. 2 Amplification product obtained after specific PCR with DNA of five different *P. thornei* isolates (1.1–1.5) using primers Pthf/Pthr. Numbers on the left side are the sizes of the 0.1-kb DNA ladder XIV (Boehringer-Manheim) (Lane M)

consisted of 17 consecutive repetitions (31–32 bp long; [**TRAAGAAT**YGTCCYTYTYCKAACY GRWTYYC(C)]) located between base 393 and base 907 of the PthB sequence. Each repetition contained a core region composed of 8 nucleotides (TRAAGAAT). The flanking regions, located at the ends 5′ (5 nt) and 3′ (9 nt) of the core region, varied in 1 or 2 nts between repetitions (Fig. 4).

Dot-blot experiment

Purified total DNA of each of nematode isolates listed in Table 1 (except *P. thornei* isolates 1.4 and 1.5) was dot-blotted onto a nylon membrane and hybridized with the DIG labelled sat DNA sequence, as described in Materials and methods. Hybridisation occurred with all the *P. thornei* isolates (1.1, 1.2 and 1.3) but not with any other nematode isolates in this study (Fig. 5)

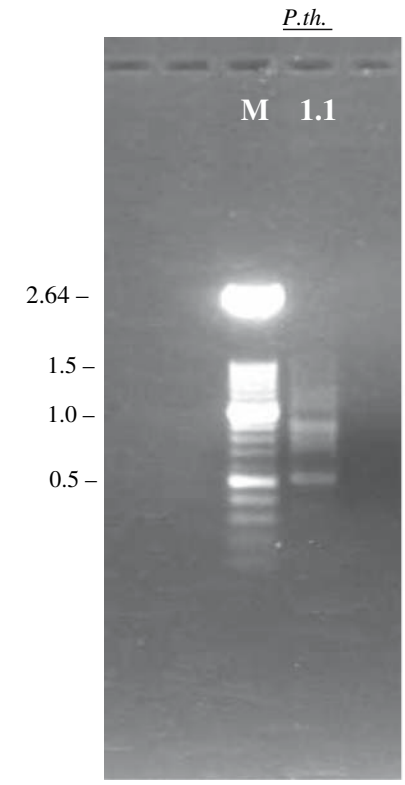

Fig. 3 Typical amplification of a satDNA sequence using the primer sets Pthsat*f*1/Pthsat*r* or Pthsat*f*2/Pthsat*r*1 and DNA of *P. thornei* isolates (in this case isolate 1.1). DNA products were separated on a 1% agarose gel and stained with ethidium bromide. Numbers on the left side are the sizes of the 0.1-kb DNA ladder XIV (Boehringer-Manheim) (Lane M)

Specific PCR reactions with DNA of a single nematode

Five micro liter of the total DNA extracted from a single female of *P. thornei* isolate 1.1 were enough to amplify the species-specific SCAR fragment when used as template with primer set Pthf/Pthr (Fig. 6)

Discussion

Accurate identification of plant-parasitic nematode species is a critical component of many areas of plant pathology, especially for quarantine inspection and management strategies. The use of molecular genetic techniques, particularly

CCCGATTCGGATTGAATGCGCAAATTGGCTTTAAAGCCTAGCCGTCTGCCAATGTTTAATAAGCAATAAAT
TTCGGTTCGTTTTCACAATCTAAAAAAGAAAAATTGTTAATACACAAATTTTAAGCGCAGGGATGGGAGTG
AGTGTAGTACGTAGAAGCAAGTTTTAAGTTTTAACAAGGACACATGAGACATGACACATGACTAATGTTTG
AATTTAAGGTTACMTATGCCGACCCGACCAGCTTCAGATCAGAGGATTAATTTAAACGAGCGTGTGGTGCC
GCTTCGCGGCACCTTCACGCTCTTTCGTAGTTACCGAATATCGATTCTCTAAGATTTCTCCGTTTGATACA
ATTCCCTAAAAAATCATTCATTTCCGAACCGATTCCA**TAAAGAAT**CGTCCTTTTCCGAACCGGATTTCC**TG**
AAGAATTGTCCCTCTTCTAACCGATTCTC**TAAAGAAT**CGCCCCTTTCCAAACCGGATTCCC**TACAGAAT**TG
TCCCTCTTCTAACCGATTCTC**TAAAGAAT**TGTCCCTCTTCTAACCGATTCCC**TAAAGAAT**CGTCCCTTTCC
GAACTGGATTCCC**TAAAGAAT**CGTCCCTTTCCGAACCGGGTTCCC**TAAAGAAT**CGTCCCTTTCCGAACCGG
GTTCCC**TAAAGAAT**CGTCCCTTTCCGAACCGGGTTCCC**TAAAGAAT**CGTCCCTTTCCGAACCGGATTCCC**T**
GAAGAATCGTCCCTTTCCGAACCGGGTTCCC**TAAAGAAT**CGTCCCTTTCCGAACCGGGTTCCC**TAAAGAAT**
CGCCCCTTTCCAAACCGGATTCCC**TACAGAAT**TGTCCCTCTTCTAACCGATTCCC**TAAAGAAT**TGTCCCTC
TTCTAACCGATTCCC**TAAAGAAT**CGTCCCTTTCCGAACTGGATTCCC**TAAAGAAT**CGTCCCTTTCCGAATC
GGG

Fig. 4 Top: Nucleotide sequence corresponding to the monomer of *P. thornei*-sat DNA (core region is in bold). Bottom: sequential monomers aligned for nucleotide base-pair examination

those based on PCR and SCAR primers, is one of the best choices for diagnostic purposes and it has been used for the specific identification of several nematode species (Da Conceição, Dos Santos, Abrantes, & Santos, 2003; Zijlstra, 2000). The goal of this study was to design specific PCR primers that would allow differentiation of *P. thornei* from other *Pratylenchus* spp. and other nematode genera. Our results demonstrate that SCAR markers can be obtained from the sequencing of *P. thornei*-specific reliable RAPD markers. To find species-specific RAPD-fragments, RAPD-PCRs were performed with DNA from a variety of nematode populations (Table 1) and the selected fragments were cloned and sequenced. The SCAR primers developed from these sequences proved useful in discriminating *P. thornei* from other *Pratylenchus* spp. and root-lesion nematodes, as well as root-knot and cyst forming nematodes that may simultaneously co-infest soil or roots of susceptible crops of the former species. The PCR-assays using one pair of species-specific SCAR primers yielded large amounts of the desired amplification product, regardless of the source of template DNA. The SCAR fragment of *P. thornei* was easily amplified from DNA extracts of a mass of nematodes. Furthermore, the procedure was sensitive enough to amplify the SCAR fragment from the crude total DNA extract of a single nematode.

Repetitive sequences known as satDNA have been characterized in a number of nematodes of agronomic interest, and because of their specificity and reiteration in the genome, they have provided very powerful tools to discriminate between closely related species for which specific

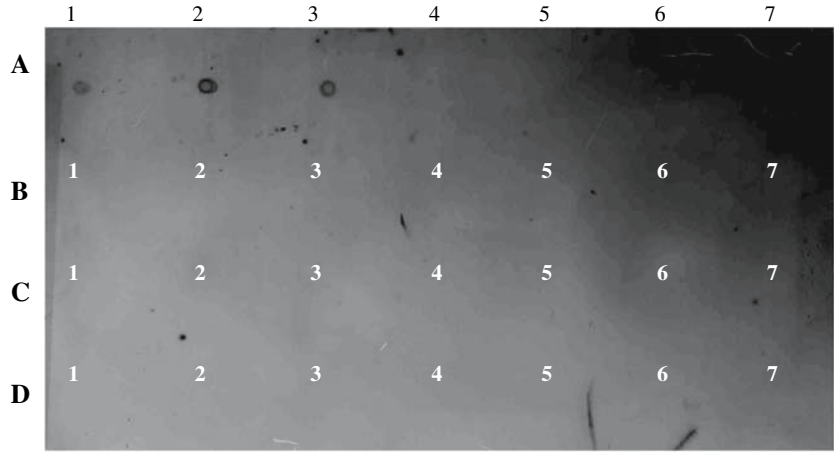

Fig. 5 Dot-blot experiment. A dot-blot of the isolates listed in Table 1 (except *P. thornei* isolates 1.4 and 1.5) was successfully hybridized with a DIG-11-dUTP labelled satDNA probe from *P. thornei* isolate 1.1. Points correspond to the following nematode isolates: A1 = 1.1, A2 = 1.2, A3 = 1.3, A4 = 2.1, A5 = 2.2, A6 = 2.3, A7 = 3.1, B1 = 4.2, B2 = 5.1, B3 = 5.2, B4 = 5.3, B5 = 5.4, B6 = 5.5, B7 = 7.1, C1 = 8.1, C2 = 9.1, C3 = S, C4 = I, C5 = Ma, C6 = Mi, C7 = Mj, D1 = HmA, D2 = HmI, D3 = HmU, D4 = Hc, and D5 = Gr. Codes refer to Table 1

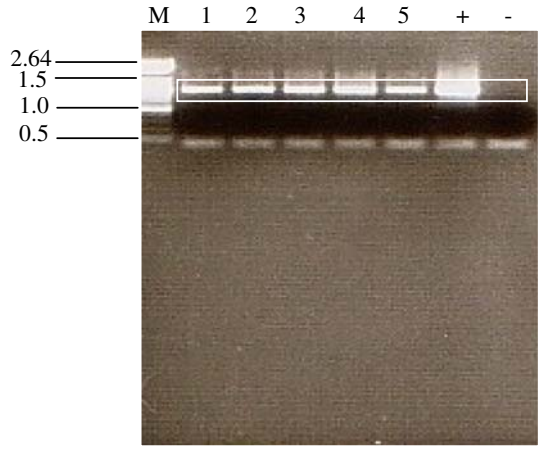

Fig. 6 Specific SCAR-PCR (boxed bands) generated by oligonucleotide primer set Pth*f*/Pth*r* using total DNA from five single females belonging to isolate 1.1 of *P. thornei*. Numbers on the left side are the sizes of the 0.1 kb DNA ladder XIV (Boehringer-Manheim) (Lane M). Lanes 1–5 correspond to the DNA of five single nematodes. Lane + correspond to the positive control (SCAR-PCR amplification using DNA extracted from a mass of nematodes of isolate 1.1 of *P. thornei*). Lane—correspond to the negative control (without DNA)

markers are needed (Grenier, Castagnone-Sereno, & Abad, 1997). Here, we present evidence that such sequences may be of interest for the development of a routine diagnostic procedure for the identification of *P. thornei*. From a practical point of view, the dot blot procedure described here presents technical features that should be of interest for such routine protocol. First, after hybridization against the blotted target, the hybridization solution still contains large amounts of DIG-labelled probe that has not annealed, which is stable for at least 1 year when stored at –20°C and can be reused in hybridization experiments (Castagnone-Sereno et al., 1999). Second, because of the repetitive nature of satDNA, the probe could identify the crude extract obtained from a single nematode, without the need for any time-consuming DNA purification step.

In conclusion, results of this study provide two different DNA-based methods to identify *P. thornei*. Although several DNA-based methods have been used to identify *Pratylenchus* species or populations (Al-Banna et al., 2004; Duncan et al., 1999; Ouri & Mizukubo, 1999; Pinochet, Cenis, Fernández, Doucet, & Marull, 1994; Uehara et al., 1998; Waeyenberge, Ryss, Moens, Pinochet, & Vrain, 2000;), this is the first report of SCAR-based identification of a *Pratylenchus* species. The SCAR-PCR method described here has potential application to routine diagnostic purposes using DNA extracts from different life cycle stages, soil samples or infected plant

material. Additionally, the satDNA sequence identified can be used as a sensitive and reliable probe to separate *P. thornei* from other closely related species. However, validation of these methods with additional *P. thornei* populations from other geographic origins, and with other closely related species, particularly *P. mediterraneus* populations (De Luca et al., 2004), is required.

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