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Identification of the beet cyst nematode *Heterodera schachtii* by PCR

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Abstract

PCR-RFLPs of ITS-rDNA and PCR with species-specific primers were developed for identification of cysts and juveniles of the beet cyst nematode *Heterodera schachtii*. Restrictions of PCR product by *Mva*I or *Scr*FI distinguish *H. schachtii*, *H. betae*, *H. trifolii* and *H. medicaginis*. RFLP profiles with eight restriction enzymes for these four nematode species are presented. Based on Internal Transcribed Spacer sequences of populations from several Schachtii group species, a specific primer for *H. schachtii* was designed, permitting amplification of the target sequence from juveniles and cysts of the beet cyst nematode. A duplex PCR protocol tested with a wide range of nematode samples is described.

Introduction

The beet cyst nematode, *Heterodera schachtii*, is a major pest in sugar beet (*Beta vulgaris*) production. *Heterodera schachtii* attacks over 200 plant species within 95 genera from 23 different plant families; most hosts are found in both the Chenopodiaceae and Cruciferae (Steele, 1965). The species is widespread in most European countries, the USA, Canada, the Middle East, Africa, Australia and South America (Baldwin and Mundo-Ocampo, 1991; Evans and Rowe, 1998). The nematode causes serious yield reductions and decreases sugar content of sugar beet wherever the crop is grown. In European countries the annual yield losses were estimated at ca. 90 million Euro (Müller, 1999).

Heterodera schachtii belongs to the *H. schachtii sensu stricto* group, which also contains *H. betae*, *H. ciceri*, *H. daverti*, *H. galeopsidis*, *H. glycines*, *H. lespedezae*, *H. medicaginis*, *H. rosii* and *H. trifolii* (Subbotin et al., 2000). Minor morphological and morphometrical differences can only distinguish all of these species from each other. In this group only *H. schachtii*, *H. betae* and *H. trifolii* are of economic importance for West-European agriculture. The yellow beet cyst nematode, *H. betae*, previously named as *H. trifolii* forma specialis *betae* or beet race of *H. trifolii*, was recently described by Wouts et al. (2001). It infects sugar beets, peas and beans and is recorded from the Netherlands (Maas and Heijbroek, 1981), Sweden (Andersson, 1984), Switzerland (Vallotton, 1985), Germany (Schlang, 1990), France (Bossis et al., 1997) and Italy (Ambrogioni et al., 1999). *Heterodera trifolii* is more wide spread and can be of importance in clover.

It has been demonstrated that for routine identification of plant-parasitic nematodes, DNA based diagnostics are quicker than the traditional strategy using morphology and morphometrics (Vrain and McNamara, 1994; Powers et al., 1997). Restriction fragment length polymorphism analysis (RFLPs) of the Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) can be used to differentiate *H. schachtii* from closely related species of the *H. schachtii* group. Digestion of the PCR-amplified product by the restriction enzymes *Fok*I and *Mva*I produce unique

RFLP profiles for several populations of this species (Szalanski et al., 1997; Subbotin et al., 2000).

PCR with a species-specific primer can also be used successfully for species differentiation and constitutes a major step forward in developing DNA diagnostics. This approach allows to detect one or several nematode species by using a single PCR test and decreases the diagnostic time and costs (Mulholland et al., 1996; Setterquist et al., 1996; Bulman and Marshall, 1997; Petersen et al., 1997; Williamson et al., 1997; Uehara et al., 1998; Zijlstra et al., 1995; Subbotin et al., 2001a).

The objective of this work was to improve the identification of beet cyst nematode species with molecular methods. Therefore, we verified the applicability of the PCR-RFLP diagnostic technique to a large collection of *H. schachtii* populations and tried to obtain RFLP diagnostic profiles for *H. schachtii, H. betae*, *H. trifolii* and *H. medicaginis*. We also report on the development of a rapid and precise method for the diagnosis of juveniles and cysts of *H. schachtii* using a duplex PCR with a species-specific primer.

Materials and methods

Nematode populations

Fifty-eight populations of different species of cyst-forming nematodes were used in this study (Table 1). All *H. schachtii* populations, as well as two populations of *H. betae* (Münster, Germany; Berkane, Morocco), and one population of the clover cyst nematode *H. trifolii* (New Zealand), *Globodera rostochiensis*, *G. pallida* and *Pratylenchus penetrans* were identified by their morphometrics and morphological characters. The remaining cyst-forming nematode species were previously identified based on their morphometrics and morphological characters and rDNA-RFLPs (Subbotin et al., 2000).

Populations were maintained in pots kept in glasshouses or directly extracted from field soil samples. Cysts were extracted from the soil (Seinhorst, 1964) and kept in Eppendorf tubes at room temperature during several weeks or months before use.

Sample preparation for molecular studies

Either several cysts, one cyst, or single juveniles alone or in a mixture with other nematode species were transferred into an Eppendorf tube containing 8 µl distilled water and 10 µl nematode lysis buffer (500 mM KCl, 100 mM Tris–HCl pH 8.0, 15 mM $MgCl_2$, 1.0 mM DTT, 4.5% Tween 20) and crushed with an microhomogenisor Vibro Mixer (Zurich, Switzerland) for 2.5–3 min. Two microlitre proteinase K (600 µg ml^{-1}) (Promega Benelux, Leiden, The Netherlands) were added and the tubes were incubated at 65 °C (1 h) and 95 °C (10 min) consecutively and finally centrifuged (1 min; 16 000*g*). The DNA suspension was stored at −20 °C and used for further study.

PCR-RFLP

Ten microlitres of the DNA suspension were added to the PCR reaction mixture containing: 10 µl 10X Qiagen PCR buffer, 20 µl 5X Q-solution, 200 µM of each dNTP (*Taq* PCR Core Kit, Qiagen, Germany), 1.5 µM of each primer (synthesised by Life Technologies, Merelbeke, Belgium), 0.8 U *Taq* Polymerase (5 U/µl) (*Taq* PCR Core Kit, Qiagen, Germany) and double distilled water to a final volume of 100 µl. Primers TW81 and AB28 (Table 2, Figure 1) as described by Joyce et al. (1994) were used. The DNA-amplification profile carried out in a GeneE (New Brunswick Scientific, Wezembeek-Oppem, Belgium) DNA thermal cycler consisted of 4 min at 94 °C; 35 cycles of 1 min at 94 °C,

Table 1. Species and populations of cyst nematodes tested in this study

Species	Population origin	RFLPs	PCR with specific primer
Heterodera schachtii	Molembaix, Belgium	+	+
	Hermé, Belgium	+*	+
	Gingelon, Belgium	+	+
	Hérines, Warcoing, Belgium	+	+
	Quiévrain, Belgium	+	+
	Ohain, Belgium	+	+
	Meerdonk, Belgium	−	+
	Boire Glom, Belgium	−	+

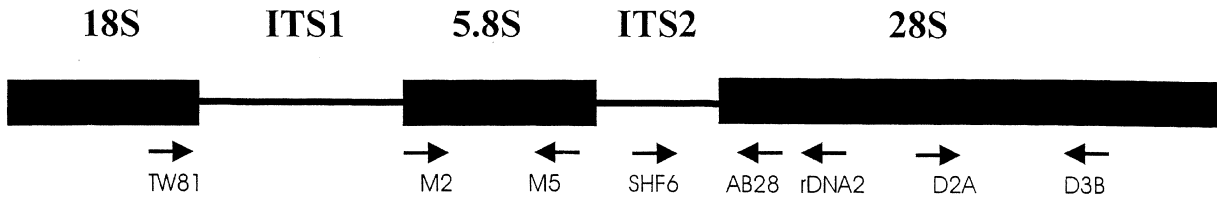
Table 1. (*Continued*)

Species	Population origin	RFLPs	PCR with specific primer
	Deftinge, Belgium	−	+
	Wez, Belgium	−	+
	Attre, Belgium	−	+
	Limon, Belgium	−	+
	Bourgois, Belgium	−	+
	Göttingen, Germany	+	+
	Schladen, Germany	+	+
	Kitsingen, Germany	+	+
	Aisne, France	+	+
	Nord, France	+	+
	Marne, France	+	+
	Finistère, France	+	+
	Rondebult, South Africa	+	+
	Ouled Mbarek, Morocco	+*	+
	Bouareg, Morocco	+	+
	Madagh, Morocco	−	+
	Mout aruif, Morocco	−	+
	Rutten, the Netherlands	+	+
	Borsel, the Netherlands	−	+
	Achthuizen, the Netherlands	+	+
	Stellendam, the Netherlands	+	+
	Teckomatorp, Sweden	+	+
	Slottaquirden, Sweden	+	+
	Fide, Sweden	+	+
	Kastlösa, Sweden	+	+
	Münster, BBA, Germany	+	+
	Kerma, Iran	+	+
	Bologna, Italy	+	+
H. betae	Münster, BBA, Germany	+*	+
	Berkane, Morocco	+	+
H. trifolii	Moscow region, Russia	−	+
	New Zealand	+*	+
H. glycines	Arkansas, USA	−	+
	Pinganlin, China	−	+
	Klanha, China	−	+
	Heze, China	−	+
	Zhangjiakou, China	−	+
	Brazil	−	+
H. medicaginis	Stavropol region, Russia	+*	+
H. cajani	India	−	+
H. ciceri	Syria	−	+
H. sonchophila	Estonia	−	+
Heterodera sp. 1	*Artiplex* sp., Belgium	−	+
Heterodera sp. 2	*Rumex* sp., Germany	−	+
H. avenae	Moorslede, Belgium	−	+
	Gharb, Morocco	−	+
H. riparia	Moscow region, Russia	−	+
H. humili	Chuvashija, Russia	−	+
Globodera rostochiensis	Poperinge, Belgium	−	+
G. pallida	Koekelare, Belgium	−	+
Pratylenchus penetrans	Lokeren, Belgium	−	+

+: PCR product digested with 3 enzymes (*Mva*I, *Pvu*II and *Rsa*I).
+*: PCR production digested with 8 enzymes (*Alu*I, *Ava*I, *Bse*NI, *Cfo*I, *Eco*72I, *Mva*I, *Rsa*I and *Scr*FI).

Table 2. Primer sequences

Primer	Sequence 5′–3′	Reference
TW81	GTTTCCGTAGGTGAACCTGC	Joyce et al. (1994)
AB28	ATATGCTTAAGTTCAGCGGGT	Joyce et al. (1994)
5.8SM2	CTTATCGGTGGATCACTCGG	Zheng et al. (2000)
5.8SM5	GGCGCAATGTGCATTCGA	Zheng et al. (2000)
SHF6	GTTCTTACGTTACTTCCA	Present study
rDNA2	TTTCACTCGCCGTTACTAAGG	Vrain et al. (1992)
D2A	ACAAGTACCGTGAGGGAAAGTTG	De Ley et al. (1999)
D3B	TCGGAAGGAACCAGCTACTA	De Ley et al. (1999)

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H.schachtii (10-Gem)  :CTACTTGTACGTGTTCTTACGTTACTTCC--AGCTCGGCTGTGGGGTTTTGGTGTGCT
H.schachtii (12-1-Bel):T.......................G.TC.........................
H.schachtii (1-2-Mor) :T.......................G.TC.........................
H.betae     (3-4-Mor) :T.................T.....G.TC.........................
H.trifolii  (1-Mos)   :T.................T.....G.TC.........................
H.glycines            :T.......................G.TC.........................
H.medicaginis         :T.......................G.TC.........................
H.ciceri    (15-Syr)  :T................G......G.TC.........................
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Figure 1. Position of primers used in PCR and alignment of a fragment of rDNA sequences of *Heterodera schachtii* and closely related species. The bold and underlined characters indicate the sequence of the specific primer SHF6.

1.5 min at 55 °C and 2 min at 72 °C; followed by a final elongation step of 10 min at 72 °C. After DNA amplification, 5 µl product was run on a 1% agarose gel (100 V, 45 min). The remainder was stored at −20 °C.

For twenty-five *H. schachtii* populations and two *H. betae* populations (Table 1), 5 µl PCR product was digested with *Mva*I, *Rsa*I and *Pvu*II. Additionally, for one population of *H. schachtii*, *H. betae, H. trifolii* and *H. medicaginis* (Table 1), the PCR product was also digested with each of following enzymes: *Alu*I, *Ava*I, *Cfo*I (Promega, Leiden, The Netherlands) *BseN*I, *Eco*72I, *Mva*I, *Rsa*I (MBI Fermentas, St. Leon-Rot, Germany) and *ScrF*I (Eurogenetec, Seraing, Belgium) according to the manufacturer's instructions. The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis (100 V, 2.5 h), stained with ethidium bromide, visualised and photographed under UV-light. Procedures for obtaining PCR-amplified products and endonuclease digestion of these products were repeated at least twice to verify the results.

Cloning and sequencing

PCR products of three populations of *H. schachtii* (Ohain, Belgium; Münster, Germany; Ouled Mbarek, Morocco) and of one population of *H. betae* (Berkane, Morocco) were excised from 1% TBE-buffered agarose gels using the QIAquick Gel Extraction Kit (Qiagen), cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega, Leiden, the Netherlands). Several clones of each species were isolated using blue/white selection and submitted to PCR with vector primers. The PCR product from each clone was digested by *Mva*I, *Rsa*I or *Pvu*II to identify the ITS haplotype. From each population three clones were selected and sequenced in both directions using two vector primers, one internal forward primer 5.8SM2 and one internal reverse primer 5.8SM5 or TW81 (Table 2) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems Benelux, The Netherlands) according to the manufacturer's instructions. The program

Table 3. Sizes of restriction fragments (bp) of rDNA-ITS region of *Heterodera schachtii, H. betae, H. trifolii,* and *H. medicaginis* based on RFLPs and sequence data

Enzymes	*Heterodera schachtii*	*Heterodera betae*	*Heterodera trifolii*	*Heterodera medicaginis*
*Alu*I	*15, 44**, 166, 180, 280, 339	*15, 44*, 166, 180, 282, 339	*15, 44*, 166, 180, 281, 339	*15, 44*, 166, 181, 282, 341
*Ava*I	112, 362, 550	112, 364, 550	112, 363, 550,	113, 364, 552
*Bse*NI	62, 408, 554	*22, 36*, 62, 408, *498*, 520	*22*, 62, 408, 533	410, 619
*Cfo*I	*46*, 105, 146, 309, 418	*46*, 105, 146, 309, 420	*46*, 105, 146, 309, 419	*46*, 106, 146, 311, 420
*Eco*72I	*236, 287,* 501, 523	503, 523	502, 523	504, 525
*Mva*I	73, 137, 193, 620, 758, 814, 951	73, *137*, 196, *620*, 757, *953*	73, 196, 756	74, 196, *331, 428,* 759
*Rsa*I	*6*, 205, *376, 437,* 813	*6*, 205, 217, 598, 815	*6*, 205, 217, 597	*6*, 206, 817
*Scr*FI	73, 137, 196, *289*, 331, 426, 525	73, 196, 757, 953	73, 196, 756	74, 196, 331, 428

**Italics*: poorly visualised restriction bands on 1.5% agarose gel.

used for all sequencing reactions was: 30 s at 94 °C, 30 s at 50 °C and 3 min 30 s at 60 °C, repeated for 25 cycles. The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems Inc., Gaithersburg, Maryland, USA). Sequences were run on a 377 DNA Sequencer (PE Applied Biosystems, Warrington, UK). The ITS sequences of *H. schachtii* are deposited in the GenBank database.

Sequence analysis

DNA sequences were edited with Chromas 1.45 (©1996–1998 Conor McCarthy) and aligned using ClustalX 1.7 (Thompson et al., 1994). Based on the obtained ITS-RFLPs and sequence data along with several sequences of ITS clones of *H. trifolii* and *H. schachtii* (Subbotin et al., 2001, unpublished data), and using WebCutter 2.0 (http://www.firstmarket.com/cutter/cut2.html) we calculated the length of the restriction fragments obtained for *H. schachtii*, *H. betae*, *H. trifolii* and *H. medicaginis* after ITS digestion with eight enzymes (Table 3).

PCR with species-specific primers

Several published sequences of *H. schachtii* group species (Zheng et al., 2000; Subbotin et al., 2001b) were used in the analysis and selection of putative species-specific primers (Figure 1). Several putative specific primers for *H. schachtii* were manually selected based on sequence differences.

Two microlitres of the template DNA suspension (see sample preparation) were added to a PCR mixture containing 2.5 µl 10X Qiagen PCR buffer, 5 µl 5X Q-solution, 0.5 µl 10 mM dNTP mix, 1.5 U *Taq* Polymerase (5 U/µl), 12 µl double distilled water, and one or two sets of primers (Table 2). The first set contained 1.5 µM of the putative species-specific primer and 1.5 µM of the universal primer. The second set contained 1.5 µM of each of the universal primers D2A and D3B (De Ley et al., 1999) (Table 2, Figure 1). Amplification was performed in a MJ Research PTC-200 Peltier Thermal Cycler (MJ Research, Inc. MA, USA). The PCR programme consisted of 4 min at 94 °C, 10 cycles of 30 s at 94 °C, 40 s at 45 °C and 1 min at 72 °C; 20 cycles of 30 s at 94 °C, 40 s at 55 °C and 1 min at 72 °C, and 10 min at 72 °C. A negative control containing the PCR mixture without DNA template was also run. Five microlitres of each amplified sample were analysed by electrophoresis in a 1% agarose gel (100 V, 30–45 min), stained with ethidium bromide, visualised and photographed under UV-light. Duplex PCR were repeated several times with the same sample to verify the results.

To determine the sensitivity of the PCR with different combinations of specific and universal primers at different amounts of target DNA, the total DNA was extracted from a single cyst. The DNA concentration was measured with a Pharmacia GeneQuant RNA/DNA Calculator and serially diluted in sterile water. Two microlitre of DNA suspension (36, 18, 9, 4.5, 2.3, 1.2 or 0.6 ng) was added to a tube containing 20 µl PCR mixture with the primer combination. Concentrations of the species-specific PCR products were estimated on agarose gel with Lambda DNA/HindIII marker (Promega) using Kodak Digital Science 1D imaging system. Each dilution/primer combination was examined in three different PCR reactions.

Results

PCR amplification and RFLP analysis

PCR with primers TW81 and AB28 yielded a single fragment of approximately 1025 bp for all studied

cyst forming nematode populations. No PCR products were obtained in the negative control lacking DNA template. *Mva*I (Figure 2A), *Rsa*I (Figure 2B) or *Pvu*II (data not shown) digestion of the PCR product generated RFLP patterns similar for all *H. schachtii* populations. Intraspecific differences were observed between the two *H. betae* populations after *Rsa*I digestion (Figure 2B). For all populations of *H. schachtii* and *H. betae*, the sum of the restricted fragment lengths generated by *Mva*I, *Rsa*I and *Scr*FI was higher than the length of the unrestricted amplified product suggesting a heterogeneity of ITS regions was present in the genome of both species.

Digestion of the PCR product obtained from *H. schachtii* (Figure 3A), *H. betae* (Figure 3B), *H. trifolii* (Figure 3C) and *H. medicaginis* (Figure 3D) yielded some distinct RFLPs. *Mva*I, *Rsa*I and *Scr*FI clearly separated the populations of the four studied species from each other (Table 3). The *Bse*NI pattern distinguished *H. schachtii* and *H. medicaginis* from other species.

PCR with species-specific primer

For the development of the direct PCR identification of *H. schachtii* we used two sets of primers. The first set included the putative specific primer and a universal primer. Based on the analysis of sequence differences between *H. schachtii*, *H. betae*, *H. trifolii*, *H. ciceri*, *H. glycines* and *H. medicaginis* (Figure 1) we identified manually several putative primers. Fourteen unique nucleotide mutations were detected. These are present in ITS sequences of more than one haplotype of *H. schachtii* and absent in sequences of related species. Tests with twelve selected putative primers (data not shown) yielded reliable results with only one species-specific primer SHF6 (Table 2), which was used in further tests in combination with AB28 or rDNA2 (Figure 1). The second set of primers contained the universal primers D2A and D3B. These primers amplify the D2–D3 expansion region of the 28S gene and indicate the presence of template nematode DNA in the sample, and as a consequence prove the success of the PCR reaction. Results of duplex PCR with these two sets of primers are presented in Figure 4. The SHF6 and AB28 primer or rDNA2 primer combination amplified a fragment of ca. 200 bp or ca. 255 bp, respectively. The fragment amplified by the universal primers D2A and D3B is about 800-bp long.

These two sets of primers were further tested in a PCR with template DNA from single second stage juveniles, single cysts or multiple cysts from 36 populations of *H. schachtii*. All samples containing *H. schachtii* always yielded two distinct fragments; all samples without *H. schachtii* showed only one fragment of 800 bp (Figure 4). No distinct additional amplification products were observed.

The DNA extraction method described above yielded approximately 72 ng/µl DNA from a single cyst. The results of the sensitivity test of the PCR using different combinations of specific and universal primers are shown in Figure 5. Specific amplified PCR products were obtained in all samples containing target DNA, even when 0.6 ng DNA or ca. 1/1000 cyst was used in the PCR. The primer combination SHF6 with AB28 yielded more amplification

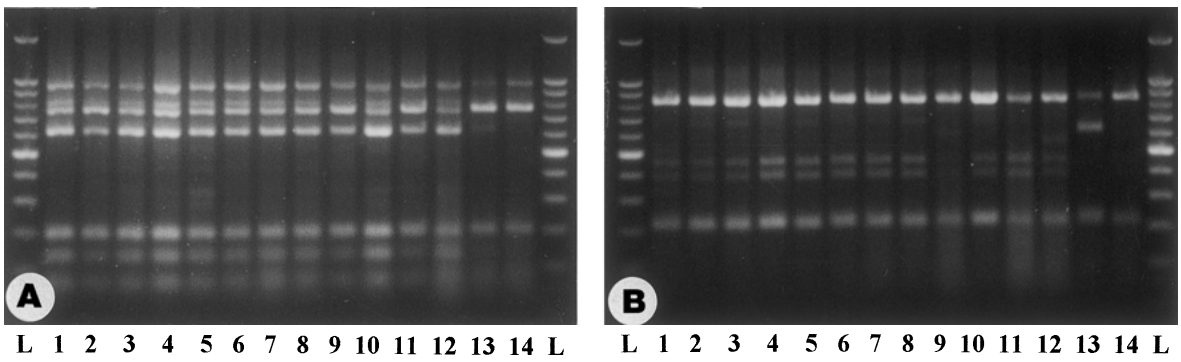

Figure 2. Restriction fragments of amplified ITS regions of *Heterodera schachtii* and *H. betae*. A: *Mva*I; B: *Rsa*I. L: 100 bp DNA ladder (Promega), 1–3: *H. schachtii* (Belgium), 4 and 5: *H. schachtii* (Germany); 6 and 7: *H. schachtii* (France); 8: *H. schachtii* (Iran); 9 and 10: *H. schachtii* (Morocco); 11: *H. schachtii* (the Netherlands); 12: *H. schachtii* (Sweden); 13: *H. betae* (Germany) and 14: *H. betae* (Morocco).

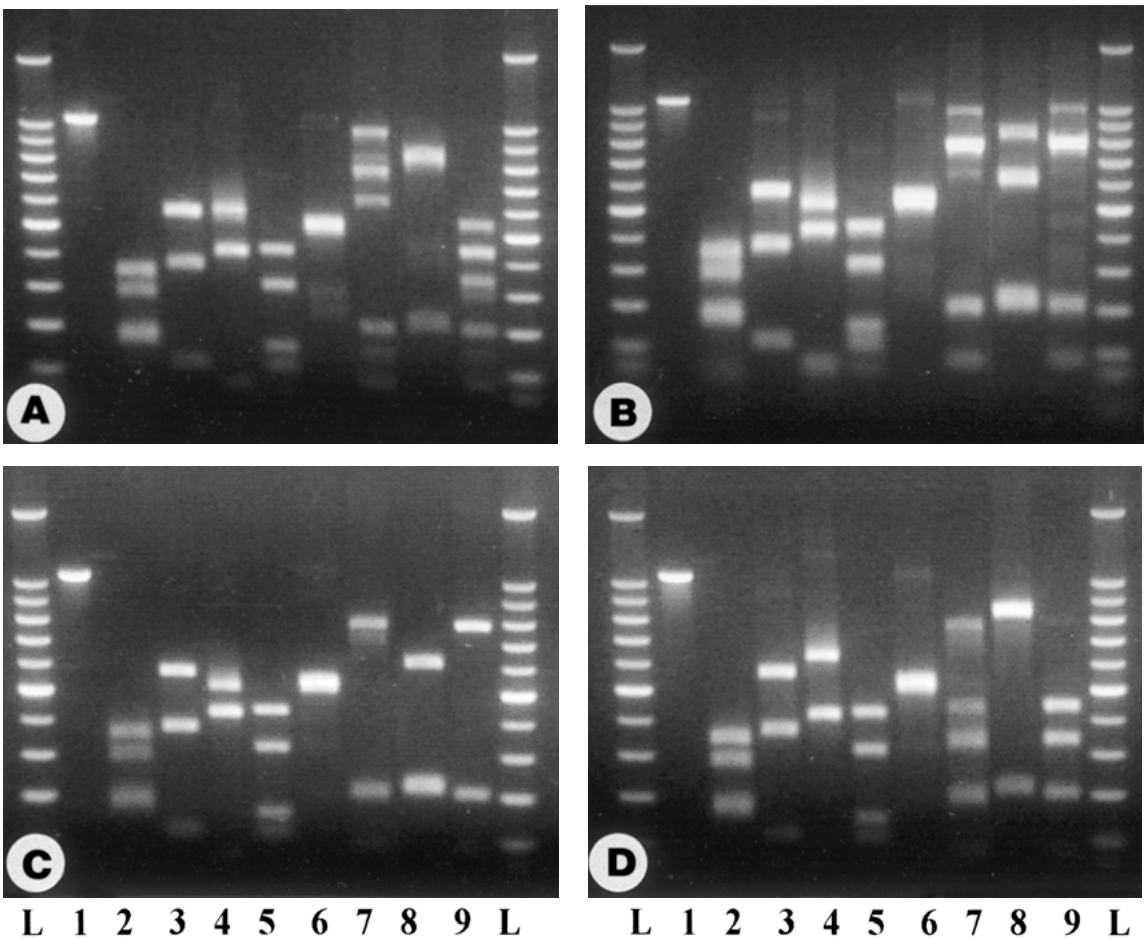

Figure 3. ITS-RFLPs of cyst forming nematode species after endonuclease restriction. A: *Heterodera schachtii* (Belgium); B: *H. betae* (Germany); C: *H. trifolii* (New Zealand); D: *H. medicaginis* (Russia). L: 100 bp DNA ladder (Promega), 1: unrestricted PCR product, 2: *Alu*I; 3: *Ava*I; 4: *Bse*NI; 5: *Cfo*I; 6: *Eco*72I; 7: *Mva*I; 8: *Rsa*I; 9: *Scr*FI.

products, than that with rDNA2 in corresponding variants.

Discussion

The present investigation clearly demonstrated that rDNA-RFLP can distinguish *H. schachtii* from morphologically similar species and confirms previous results (Szalanski et al., 1997; Subbotin et al., 2000; Wouts et al., 2001). Our studies, however, provide additional information on the *H. schachtii* diagnostics and the variability of the ITS sequences. It does not reveal intraspecific variation in the ITS with respect to *Mva*I restriction sites and supports the diagnostic usefulness of this enzyme.

Differences in RFLP profiles between *H. schachtii* and *H. betae* were often marked by the presence of additional bands of different intensity, indicating heterogeneity and the presence of several ITS haplotypes within the genome of individuals of both species. ITS heterogeneity was previously reported for several species from the Schachtii group (Szalanski et al., 1997; Subbotin et al., 2000). Such diversity of paralogous ITS-rDNA can complicate species diagnostics based on RFLPs because under standard PCR conditions, the low-stabile and high-stabile paralogues have different rates of amplification (Buckler et al., 1997; Fenton et al., 1998), and finally produce various RFLP profiles. Our study also showed that *Mva*I and *Scr*FI produced rather complex RFLP profiles containing a fragment with different intensities but

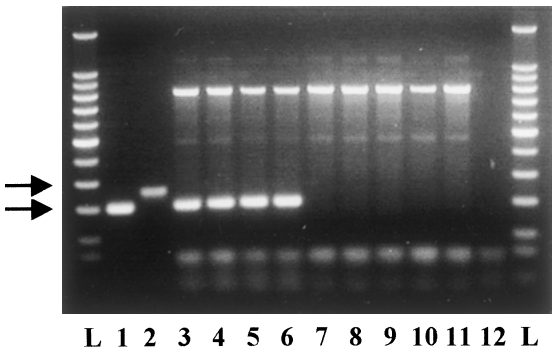

Figure 4. PCR on single cysts with species-specific primer SHF6: L: 100 bp DNA ladder (Promega), 1: Primer combination SHF6-TW81 and 2: Primer combination SHF6-rDNA2 with same amount of template DNA. 3–11: Duplex PCR. 3–6: *Heterodera schachtii*; 7: *H. betae*; 8: *H. trifolii*; 9: *H. glycines*; 10: *H. ciceri*; 11: *H. humuli*; 12: negative control without DNA. Arrows indicate specific bands.

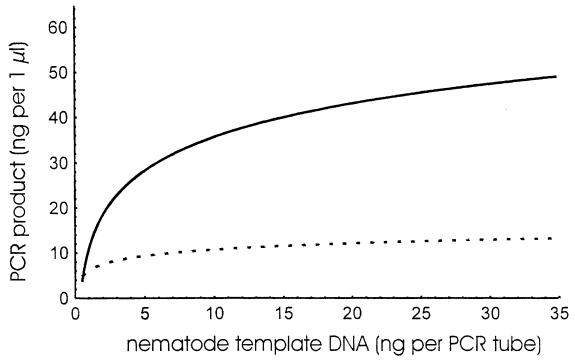

Figure 5. Efficiency of PCR with combinations of the *H. schachtii* specific-primer and universal primers AB28 (continuous line) or rDNA2 (dotted line). Curves are produced by from data obtained in three replicates.

constant and not subject to PCR conditions. We therefore conclude that the RFLP profiles generated by these enzymes are useful for separating *H. schachtii*, *H. betae*, *H. trifolii* and *H. medicaginis*.

Although Wouts et al. (2001) used another set of primers to amplify the ITS region, our results support their data and show that *H. betae* can easily be distinguished from *H. schachtii* by *Mva*I. The same authors did not detect any intraspecific variation in the ITS regions of four German and Dutch populations of *H. betae*. Our study, however, using populations of a broader geographical origin, allowed concluding for that on the base of restriction patterns obtained by *Rsa*I. The *Rsa*I pattern obtained for the *H. trifolii* population from New Zealand was different from that of previously studied European populations (Subbotin et al., 2000). As a consequence *Rsa*I cannot be considered as an appropriate enzyme for species differentiation of the Schachtii group.

PCR-RFLPs are especially suited to identify cysts or juveniles of monospecific populations. However, this strategy does not allow mixed species populations to be identified. To overcome this limitation in the *H. schachtii* detection, PCR with a species-specific primer was developed. Unlike standard PCR-based approaches, this technique is based upon allele-specific amplification, and uses a combination of primers in each PCR reaction. The first combination contains a species-specific primer, which is designed to match at a species-specific allele. The second contains two universal primers, which detect DNA of all nematode species and so indicate if the PCR has worked well. We restricted ourselves to the design of a primer, which, amplifies in combination with a universal primer only one haplotype of *H. schachtii*. A similar approach was used for the construction of a specific primer for *H. glycines* (Subbotin et al., 2001a). Our method detected correctly all *H. schachtii* populations that were studied, and allow concluding that the haplotype we selected is present in all populations. No positive reaction was observed with any of other cyst forming nematodes species we examined.

The method is of important relevance to regulatory services where information on species identity is limited. As the method is based on the detection of a species-specific DNA nucleotide, it can be used for the identification of all developmental stages of *H. schachtii* at low population density and at any time of the season. It allows identification in 5 h including DNA extraction. Detection in mixtures can be improved by optimising the conditions of both the DNA extraction and the PCR. Further tests with more species and populations of the *H. schachtii* group and with wider geographical origins will prove the general applicability of our results and confirm the robustness of the method.

The presence of *H. betae* in Morocco is reported here for the first time. The population was isolated from a heavy soil in Berkane in the Moulyia irrigated area. In this region, from where also the first record of *H. schachtii* in Morocco was reported (Anon., 1983), sugar beet is rotated every second year with cereals. Accurate and fast identification of the yellow beet cyst nematode in other beet growing areas is very important for planning of plant protection measures.

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