Nematol. medit. (2001), 29: 241-246

Department of Plant Pathology, National School of Agriculture, Meknes, Morocco
Institute of Parasitology of Russian Academy of Sciences, Moscow, Russia
Agricultural Research Centre, Crop Protection Department, Merelbeke, Belgium

AN EFFICIENT METHOD FOR IDENTIFICATION OF THE *HETERODERA SCHACHTII SENSU STRICTO* GROUP USING PCR WITH SPECIFIC PRIMERS

by

S. AMIRI, S. A. SUBBOTIN and M. MOENS

Summary. A molecular method is described for the identification of nematode species from the *Heterodera schachtii sensu stricto* group. Based on the ITS-rDNA sequence information obtained from more than 40 cyst forming nematode species, a primer SGR1 was designed, which is specific for species from the *H. schachtii sensu stricto* group. This specific primer in combination with the universal primer TW81 amplified a fragment of 850 bp from nematode DNA extracts. The primer was evaluated with 30 populations and species of the *H. schachtii sensu stricto* group and several other parasitic nematode species. Subsequent digestion of amplified PCR products by *Mva*I and *Pvu*II allowed the separation of *H. schachtii*, *H. betae* and *H. trifolii* from each other. This method of identification is highly sensitive since amplification was obtained when a single second stage juvenile or a single cyst were mixed with *Pratylenchus penetrans* or other nematode species.

Baldwin and Mundo-Ocampo (1991) listed 23 species within the Schachtii group of the genus *Heterodera*. Within the *H. schachtii sensu stricto* group, *Heterodera schachtii*, *H. betae*, *H. glycines*, *H. cajani*, *H. ciceri*, *H. trifolii*, *H. mediterranea* and *H. medicaginis* are widely distributed and some of them are major pests in certain countries (Evans and Rowe, 1998). Identification of these species is based on minor morphological and morphometrical differences and is difficult, time consuming, and requiring a lot of skill and taxonomic expertise. A solution to this problem are the molecular techniques developed during the last decade and which are becoming a popular tool for species and subspecies identification of plant-parasitic nematodes (Ferris *et al.*, 1993; Vrain and McNamara, 1994; Thiéry and Mugniéry, 1996; Szalanski *et al.*, 1997, Subbotin *et al.*, 1997; 2000). Recently, multiplex PCR techniques were developed for quick identification of several nematode species (Mulholland *et al.*, 1996; Setterquist *et al.*, 1996; Bulman and Marshall, 1997; Petersen *et al.*, 1997; Williamson *et al.*, 1997; Uehara *et al.*, 1998; Fullando *et al.*, 1999). The objective of the present study was to develop primers specific for the *H. schachtii sensu stricto* group and to combine them with universal primers in a multiplex PCR based identification method.

Materials and methods

Sequences of the ITS region of the ribosomal gene cluster of species of the *Heterodera schachtii sensu stricto* group (*H. glycines* Ichinoe, *H. medicaginis* Kirjanova, *H. schachtii*

Schmidt, *H. trifolii* Goffart) and other *Heterodera* species [*H. avenae* Wollenweber, *H. cajani* Koshy, *H. carotae* Jones, *H. ciceri* Vovlas, Greco *et* Di Vito, *H. cruciferae* Franklin, *H. fici* Kirjanova, *H. filipjevi* Stelter, *H. goettingiana* Liebscher, *H. humuli* Filipjev, *H. latipons* Franklin, *H. riparia* Subbotin *et al.*, *H. oryzicola* Rao *et* Jayaprakash, *Globodera rostochiensis* (Wollenweber) Behrens, *G. pallida* (Stone) Behrens] were obtained from Genbank (AF274384-AF274417). These sequences were aligned using ClustalW (version 1.7; Thompson *et al.*, 1994) along with other sequences of *H. schachtii* and *H. betae* Wouts, Rumpenhorst *et* Sturhan obtained by S. Amiri, S. A. Subbotin and M. Moens (unpublished). Five putative primers were selected on the base of sequence homology.

For the testing of these putative primers, eight species and 32 populations from the *H. schachtii sensu stricto* group along with several other plant parasitic nematodes were used (Table I).

The methods used for the DNA extraction and PCR amplification were similar to those described by Subbotin *et al.* (2000). Four microlitres of the DNA suspension were used as a template and added to 21 µl of the PCR mixture containing 2.5 µl of 10X Qiagen PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM of each dNTP, 0.15 µl of the putative group specific primer (Table I) and of the universal primer TW81 (0.15 nM/µl) as described by Joyce *et al.* (1994), 0.15 µl of Qiagen *Taq* DNA polymerase (5u/µl) and double distilled water added to the final volume.

For the multiplex PCR, two universal primers D3A and D3B (0.15 nM/µl) as described by Al-Banna *et al.* (1997) were added to the PCR mixture (Table II). The combination of the group specific primer and the universal primer TW81 amplifies a fragment beginning at the end of the 18S gene up to a few bases inside ITS2 region of the ribosomal cluster (Fig. 1). The D3A and D3B primers amplify the D3 expansion region of the large subunit of the ribosomal gene 28S. Amplifi-

TABLE I - *Nematode populations used in this study.*

Species	Population origin
Heterodera schachtii	Molembaix, Belgium
H. schachtii	Hermé, Belgium
H. schachtii	Göttingen, Germany
H. schachtii	Schladen, Germany
H. schachtii	Aisne, France
H. schachtii	Nord, France
H. schachtii	Marne, Frnce
H. schachtii	Ouled Mbarek, Tadla, Morocco
H. schachtii	Madagh, Berkane, Morocco
H. schachtii	Rutten, Netherland
H. schachtii	Borsel, Netherland
H. schachtii	Teckomatorp, Sweden
H. schachtii	Slottaquirden, Sweden
H. schachtii	BBA, Germany
H. schachtii	Kerma, city Kerman, Iran
H. schachtii	Bologna, Italy
H. betae	Gelber, Germany
H. betae	Berkane, Moulyia, Morocco
H. trifolii	Moscow region, Russia
H. trifolii	Merelbeke, Belgium
H. glycines	Arkansas, USA
H. glycines	Pinganlin, Gongzhaling, Jinkin provine China
H. glycines	Klanha, Gongzhaling, Jinkin provine China
H. glycines	Heze, Shanong province, China
H. glycines	Zhangjiakou, Hebei province, China
H. glycines	Brazil
H. riparia	Moscow region, Russia
H. humuli	Chuvashija, Russia
H. medicaginis	Stavropol region, Russia
H. cajani	India
H. ciceri	Syria
H. sonchophyla	Estonia
Heterodera sp. 1	Knokke, Belgium (*Atriplex litoralis*)
Heterodera. sp. 2	Germany (*Rumex* sp.)
H. avenae	Gharb, Morocco
H. avenae	Moorslede, Belgium
Globodera rostochiensis	Poperinge, Belgium
G. pallida	Koekelare, Belgium
Radopholus similis	Villa Clara, Cuba
Pratylenchus penetrans	Lokeren, Belgium

TABLE II - *Sequences of primers used in multiplex PCR for detecting* Heterodera schachtii *group species.*

Primer	Sequence
TW81	5'-GTTTCCGTAGGTGAACCTGC-3'
D3A	5'-GACCCGTCTTGAAACACGGA-3'
D3B	5'-TCGGAAGGAACCAGCTACTA-3'
SGR1	5'-GTACATGATCCCACGAAGC-3'

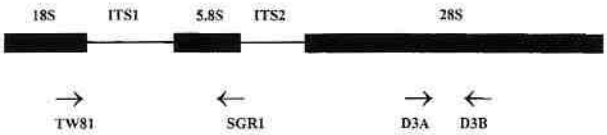

Fig. 1 - Position of the primer used in the multiplex PCR.

cation was performed in a MJ Research PTC-200 Peltier Thermal Cycler (MJ Research, Inc., MA, USA). The PCR programme consisted of four minutes at 94 °C followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 1.5 minutes at 72 °C. An additional ten minutes extension at 72 °C completed the programme. 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 0.8% agarose gel (100 V, 30-45 min), stained with ethidium bromide, visualised and photographed under UV light. Multiplex PCR was repeated several times with the same sample to verify the results.

In a further step, 2-5 microlitres of each PCR-product obtained with the combination of TW81 and the group specific primer SGR1 were digested with *Mva*I, *Puv*II or *Rsa*I in the buffer stipulated by the manufacturer. The digested DNA was loaded on a 1.5% agarose gel (100V for 1.5 h), stained with ethidium bromide, visualised and photographed under UV light.

To examine the sensitivity of the method attempts were made to identify species of the *H. schachtii sensu stricto* group in different combinations with other nematode species. Therefore, combinations were made of a single cyst of *H. schachtii sensu stricto* species with (1) a single cyst of a species not belonging to this group, or with (2) 12 individuals of *Pratylenchus penetrans* (Cobb) Filipjev *et* Shuurmans Stekhoven, *Meloidogyne arenaria* (Neal) Chitw. (J_2), *Radopholus similis* (Cobb) Thorne or dorylaimids. Finally, combinations of a single second stage juvenile of *H. schachtii* (Morocco) with different numbers of *P. penetrans* individuals were examined. The experiments were repeated four times.

Results and discussion

For three of the five putative group specific primers, the PCR repeatedly yielded more than one band; the PCR with a fourth primer failed to amplify a DNA fragment for some samples. As a consequence these four primers were discarded. Only primer SGR1 (Table II) yielded a single fragment of 850 bp for all samples and was used for further tests of the large list of *H. schachtii* group populations (Table I). All species belonging to this group yielded a similar DNA fragment (Fig. 2). Fig. 3 shows a fragment of the sequence alignment of cyst nematode species indicating the sequence of the SGR1 primer.

For the development of the multiplex PCR, the two universal primers D3A and D3B were added to the primers SGR1 and TW81. The combination D3A and D3B amplified the D3 ex-

Fig. 2 - Testing the combination of primer TW81 and the group specific primer SGR1 on species and populations belonging to the *Heterodera schachtii sensu stricto* group. L: 100 bp DNA ladder 1-13: *H. schachtii*; 14-15: *H. glycines*; 16-17: *H. betae*; 18: *H. trifolii*.

```
H.glycines      : GTGC-G--T--TGCT---T--CGTGGGATCATG--TAC T----T--GT----ACGTGTTC-TTAC
H.medicaginis   : ....-.--.--....---.--.....--....----.--.----.....-....
H.trifolii      : ....-.--.--....---.--.....--....----.--.----.....-....
H.schachtii     : ....-.--.--....---.--.....--....----.--.----.....-....
H.cajani        : -...-.--.GT....C--.--...AT.....-C.TT.----.--.A----G.....-A...
H.cruciferae    : A...-.--.GT.TAAC--.--...T.....-CGGAC---AC--.------.....-..T.
H.carotae       : A...-.--.GT.TAAC--.--...T.....-CGGAC---AC--.------.....-..T.
H.goettingiana  : A...-.--.GT.ATAC--.--...A.....-CGGAC---AC--.------.....-..T.
H.avenae-France: .....C--.GT.ATGT--.--G...A.....TCGG.A----.--.-----.....-..G.
H.filipjevi     : .....C--.GT.ATGT--.--G...A.....TC.G.A----G--.-----.....-..G.
H.latipons      : ....-.--.GT.ATGT--.--G...A.....TC.G..----.--.-----.....-..G.
H.riparia       : ....-.--.GT..A.C--.--.....TC.GT.----.--.-----.....-..G.
H.humuli        : ....-.--.GT..A.C--.--.....TC.GT.----.--.-----.....-..G.
H.fici          : ....-.--.GT..T.C--.--.....TC.GT.----.--.-----.....-..G.
H.oryzicola     : .....A--.GT.-TGCAA.GA.....G..TG.GA.--GA.----A-AC...C....-C--.
G.rostochiensis: ....-.--.GT.TT--TT.G-.-.AA.....C.----..---TC--.G-----.....-..G.
G.pallida       : ....-.--.GT.TTA-TT.G-.-.AA.....C.----..---TC--.G-----.....-..G.
C.estonica      : ....-.--.GT.TTG-AC.G-.-.AA.....C.----.----CC--.GT----.....-..G.
```

Fig. 3 - Alignment of a fragment of rDNA sequences *H. schachtii* group species and some other plant parasitic nematode species. The bold characters indicate the sequence of primer SGR1.

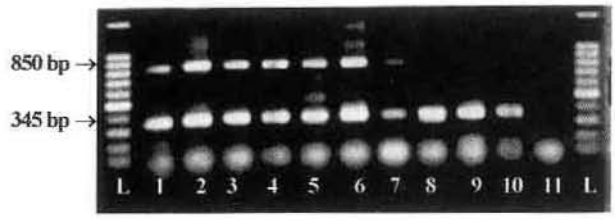

Fig. 4 - Multiplex PCR with the group specific primer of the *H. schachtii* group; L: 100 bp DNA ladder 1-3: *H. schachtii*; 4: *H. betae*; 5: *H. trifolii*; 6: *H. glycines*; 7: *H. ciceri*; 8: *H. humuli*; 9: *H. avenae*; 10: *Globodera pallida* and 11: negative control.

pansion region of the 28S gene (ca. 345 bp; Al-Banna *et al.*, 1997) and indicated the presence of template nematode DNA in the sample. Multiplex PCR with these two sets of primers was performed on single cyst samples of all *H. schachtii* group species being studied and on eight populations of other cyst forming and non-cyst forming nematode species. Samples from the *H. schachtii* group always yielded two distinct fragments (345 bp and 850 bp); all other samples showed a single fragment (345 bp) (Fig. 4).

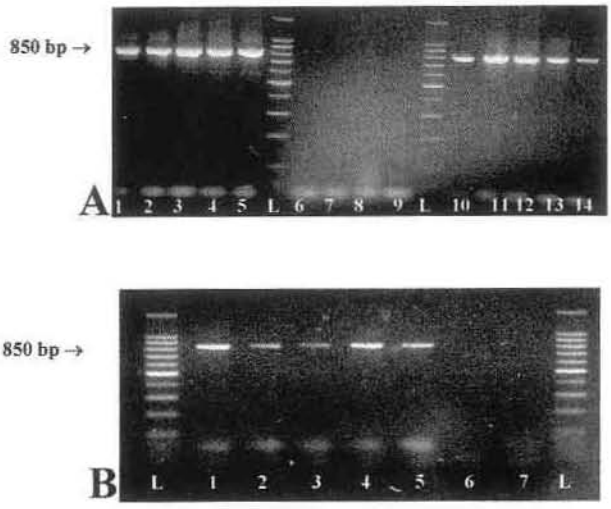

Fig. 5 - Test of the sensitivity of the group specific primer SGR1: L: 100 bp DNA ladder A: 1: single cyst of *H. schachtii*; 2-5: single cyst of *H. schachtii* mixed with 12 individuals of *Radopholus similis, Meloidogyne arenaria*, dorylaimids and *Pratylenchus penetrans*, respectively; 6-9: 12 *R. similis, M. arenaria*, dorylaimids and *P. penetrans* without *H. schachtii*; 10-14: single cyst of *H. schachtii*. B: 1: single juvenile of *H. schachtii*; 2-5: single juvenile in mixture with increasing numbers of *P. penetrans* 1:1, 1:10, 1:100 and 1:200, respectively; 6 and 7: 100 and 200 *P. penetrans* without *H. schachtii*.

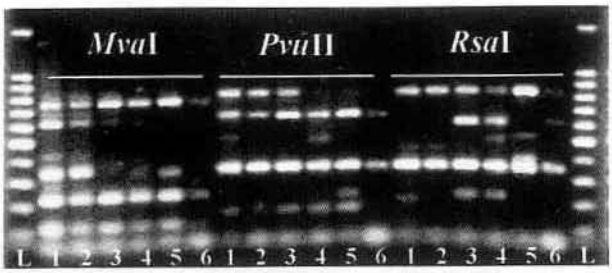

Fig. 6 - ITS-RFLPs of species belonging of the *H. schachtii* group from the PCR product obtained with the group specific primer after restriction with *Mva*I, *Pvu*II or *Rsa*I. L: 100 bp DNA ladder 1-2: *H. schachtii*, 3: *H. betae*, 4: *H. trifolii*, 5: *H. glycines* and 6: *H. ciceri*.

The primer combination was able to detect single cysts and even single second stage juveniles of the *H. schachtii* group in mixtures with other nematodes. Only samples containing one of these stages yielded the group specific band (850 bp) after amplification with the SGR1-TW81 primer combination (Fig. 5).

Subsequently, the PCR product obtained with the combination of SGR1 and TW81 was digested with *Mva*I, *Pvu*II or *Rsa*I. *Mva*I clearly separated *H. schachtii* from the closely related and morphologically poorly distinguished species from the *H. schachtii* group. *Pvu*II separated *H. betae* from *H. trifolii*. *Rsa*I did not distinguish the *H. schachtii* group species (Fig. 6).

The development of the specific group primers is based essentially on sequence information, which is needed from many populations belonging to the same group or to other groups. This information can allow the separation of populations with small or large differences in sequences. This technique is particularly interesting as a first step in the detection of species belonging to the *H. schachtii* group with a simple PCR. In a second step, the species can be identified using RFLP patterns obtained with appropriate enzymes. Our group specific primer SGR1 separates all tested species belonging to the *H. schachtii sensu stricto* group which were at our disposal. This rapid and reliable method of identification of the *H. schachtii* group species based on multiplex PCR allows a precise detection of juveniles and cysts either alone or in a mixture with other nematode species.

Acknowledgements. This study was partly granted by the Royal Belgian Institute for Sugar Beet Improvement and by the Belgian Federal Ministry of Agriculture (subsidised research). S. Amiri received a grant from the Belgian Technical Co-operation. S. A. Subbotin is grateful for his NATO Research Fellowship. The authors thank Drs S. Anderson (Sweden), G. Caubel (France), E. Krall (Estonia), O. Hermann (Belgium), W. Heijbroek (the Netherlands), D. Peng (China), R. Robbins (USA), J. Rowe (UK), D. Sturhan (Germany), R. Tacconi (Italy), R.C.V. Tenente (Brazil), N. Vovlas (Italy), L. Waeyenberge (Belgium) for providing nematode material.

Literature cited

Al-Banna L., Williamson V. and Gardner S. L., 1997. Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Molecular Phylogenetics and Evolution, 7*: 92-102.

Baldwin J. G. and Mundo-Ocampo M., 1991. Heteroderinae, cyst- and non- cyst -forming nematodes. Pp. 275-362. *In*: (Nickle, W. R. ed). A manual of agricultural nematology. New York, NY, USA Marcel Dekker Inc.

Bulman S. R. and Marshall J. W., 1997. Differentiation of Australian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science, 25*: 123-129.

Evans K. and Rowe J. A., 1998. Distribution and economic importance. Pp. 1-30. *In*: (Sharma, S. B. ed) The cyst nematodes. London, UK, Kluwer Academic, Publishers.

Ferris V. R., Ferris J. M. and Faghihi J., 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology, 16*: 177-184.

Fulland A., Barrena E., Viribay M., Barrena I., Salzar A. and Ritter E., 1999. Identification of potato cyst nematode species *Globodera rostochiensis* and *G. pallida* by PCR using specific primer combinations. *Nematology, 1*: 157-163.

Joyce S. A., Reid A., Driver F. and Curran J., 1994. Application of polymerase chain reaction (PCR) methods to identification of entomopathogenic nematodes. Pp. 178-187. *In*: "COST 812 Biotechnology: Genetics of en-

tomopathogenic nematode-bacterium complexes". (A. M. Burnell, R.-U. Ehlers, and J. P. Masson, eds)., Proceedings of Symposium and Workshop, St. Patrick's College, Maynooth, Co. Kildare, Ireland. Luxembourg, European Commission, DG XII.

MULHOLLAND V., CARDE L., O'DONNEL K. L., FLEMING C. C. and POWERS T. O., 1996. Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. Pp. 247-252. *In*: (Marshall, G. ed) Proceeding of diagnostics in Crop Production Symposium. British Crop production Council, Fernham, UK.

PETERSEN D. J., ZIJLSTRA C., WISHART J., BLOK V. and VRAIN T. C., 1997. Specific probes efficiently distinguish root-knot nematode species using signature sequence in the ribosomal intergenetic spacer. *Fundamental and Applied Nematology, 20*: 619-626.

SETTERQUIST R. A., SMITH G. K., JONES R. and FOX G. E., 1996. Diagnostic probes targeting the major sperm protein gene that may be useful in the molecular identification of nematodes. *Journal of Nematology, 28*: 414-421.

SUBBOTIN S. A., STURHAN D., WAEYENBERGE L. and MOENS M., 1997. *Heterodera riparia* sp. n. (Tylenchida:Heteroderidae) from common nettle, *Urtica dioica* L., and rDNA-RFLP separation of species from the humuli group. *Russian Journal of Nematology, 5*: 143-157.

SUBBOTIN S. A., WAEYENBERGE L. and MOENS M., 2000. Identification of cyst forming nematode of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLPs. *Nematology, 2*: 153-164.

SZALANSKI A., SUI D. D., HARRIS T. S. and POWERS. T. O., 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology, 29*: 255-267.

THIÉRY M. and MUGNIÉRY D., 1996. Intraspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous. *Fundamental and Applied Nematology, 19*: 471-479.

THOMPSON J. D., HIGGINS D. G. and GIBSON T. J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*: 4673-4680.

UEHARA T., MIZUKUBO A. and MOMOTA Y., 1998. Identification of *Pratylenchus coffeae* and *P. loosi* using specific primers for PCR amplification of ribosomal DNA. *Nematologica, 44*: 357-368.

VRAIN T. C. and MCNAMARA D. G., 1994. Potential for identification of quarantine nematode by PCR. *EPPO Bulletin, 24*: 453-458.

WILLIAMSON V. M., CASWELL-CHEN E. P., WESTERDAHL B. B., WU F. F. and CARYL G., 1997. A PCR assay to identify and distinguish single juveniles of *Meloidogyne hapla* and M. *chitwoodi*. *Journal of Nematology, 29*: 9-15.

Accepted for publication on 13 September 2001.