Nematology, 2001, Vol. 3(4), 365-371

A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR

Sergei A. SUBBOTIN [1,*], Deliang PENG [2] and Maurice MOENS [3]

[1] *Institute of Parasitology of Russian Academy of Sciences, Leninskii prospect 33, Moscow, 117071, Russia*
[2] *Institute of Plant Protection of Chinese Academy of Agricultural Sciences, Beijing 100094, China*
[3] *Agricultural Research Centre, Crop Protection Department, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium*

Received: 1 July 2000; revised: 1 February 2001
Accepted for publication: 6 February 2001

Summary – A method for rapid identification of juveniles and cysts of the soybean cyst nematode based on PCR with species specific primers is described. The PCR assay was tested on 53 populations originating from China, Russia, USA and Brazil. A single cyst or second stage juvenile of *Heterodera glycines* alone or in a mixture with other soil inhabiting nematodes was detectable.

Keywords – cysts, second stage juveniles, species specific primers.

The soybean cyst nematode *Heterodera glycines* Ichinohe, 1952 is a major pest on soybeans in China, the USA and the Russian Far East. The species is also reported from Argentina, Brazil, Canada, Colombia, Egypt, Indonesia, Japan and Korea (Noel, 1985; Eroshenko *et al.*, 1990; Baldwin & Mundo-Ocampo, 1991; Liu *et al.*, 1997; Evans & Rowe, 1998). *H. glycines* belongs to the *schachtii* group which includes species only differing in minor morphological and morphometrical characters (Graney & Miller, 1982; Sikora & Maas, 1985). Identification of these species requires considerable skill and is time consuming and difficult, even for taxonomists. In practice, cyst forming nematodes are assumed to be *H. glycines* if found infesting a field with a soybean history (Riggs & Niblack, 1993).

DNA-based diagnostics provide an attractive solution to problems associated with identification. Molecular probes developed for detection of *H. glycines* (Besal *et al.*, 1988) are not yet widely available (Riggs & Niblack, 1993). Moreover, this approach has several limitations for routine diagnostics because it requires both a large amount of DNA and some technical skill. The development of the polymerase chain reaction (PCR) technology has opened new opportunities in nematode diagnostics. Restriction fragment length polymorphism (RFLP) analyses of the internal transcribed spacers (ITS) of the ribosomal DNA (rDNA) became a popular tool for identification of cyst forming nematode species (Thiéry & Mugniéry, 1996; Bekal *et al.*, 1997; Orui, 1997; Subbotin *et al.*, 1997, 1999, 2000; Szalanski *et al.*, 1997). It has been shown that digestion of the PCR product obtained after amplification of the ITS1-5.8S-ITS2 region with different combinations of restriction enzymes allows clear separation of most cyst forming nematode species from each other. *H. glycines* can be distinguished from other species (*H. ciceri, H. medicaginis, H. schachtii* and *H. trifolii*) of the *schachtii sensu stricto* group by the restriction enzyme *Ava*I (Subbotin *et al.*, 2000; Zheng *et al.*, 2000).

PCR with specific primer combinations or multiplex PCR constitutes a major development in DNA diagnostics and allow the detection of one or several species in a mixture by a single PCR test, decreasing diagnostic time and costs. This technology has found wide application in medicine for diagnosis of infective and genetic diseases, and in plant pathology for the diagnosis of fungal (Bridge *et al.*, 1998) and bacterial pathogens (Louws *et al.*, 1999). In nematology, this diagnostic tool has been developed for identification of *Globodera pallida* and *G. rostochiensis* (Mulholland *et al.* 1996; Bulman & Marshall, 1997; Fullaondo *et al.*, 1999), *Pratylenchus penetrans* and *P. scribneri* (Setterquist *et al.*, 1996), *Meloidogyne chitwoodi* and *M. fallax* (Petersen *et al.*, 1997), *M. chitwoodi* and

* Corresponding author, e-mail: s.subbotin@clo.fgov.be
Present address: Agricultural Research Centre, Crop Protection Department, Burg. van Gansborghelaan 96, 9820 Merelbeke, Belgium.

M. hapla (Williamson *et al.*, 1997) and *P. coffeae* and *P. loosi* (Uehara *et al.*, 1998).

In this article, based on the results of previous sequence studies (Zheng *et al.*, 2000), we describe a rapid and reliable method for the diagnosis of the soybean cyst nematode by PCR using a species specific primer combination.

Materials and methods

Nematode populations

A total of 53 populations of *H. glycines* and several other cyst forming nematode species were used in this study (Table 1). All populations were identified by both morphology and PCR-ITS-RFLPs as described by Subbotin *et al.* (2000). For identification of *H. glycines* juveniles in combination with other species, mixtures of different free-living and plant parasitic nematodes were prepared. Soil inhabiting nematodes belonging to the orders Tylenchida, Dorylaimida and Rhabditida were extracted from soil samples collected in Belgian grasslands using the Baermann funnel method; *Pratylenchus* individuals were obtained from carrot disc cultures maintained at the Crop Protection Department, CLO, Merelbeke, Belgium. To prepare species mixtures, single *H. glycines* juveniles were ground in Eppendorf tubes containing 18, 36, 54 or 72 specimens of soil inhabiting nematodes or 75, 150, 225 or 300 specimens of *Pratylenchus*. Tubes containing different nematode numbers without *H. glycines* were used as controls.

DNA extraction

One or several cysts, or single juveniles alone or in mixture with other nematode species were transferred into Eppendorf tubes 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 microhomogeniser Vibro Mixer (Zürich, Switzerland) for 2.5 to 3 min. Two microlitres proteinase K (600 μg/ml) (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.

Duplex PCR

Two microlitres of the DNA suspension were used as a template for PCR. To these were added 18 μl of the PCR mixture containing: 2.0 μl 10× Qiagen PCR buffer, 1.6 μl 25 mM $MgCl_2$, 0.8 μl 10 mM deoxynucleotides, 0.2 μl each primers (1.0 μg/μl) (synthesised by Eurogentec, Merelbeke, Belgium), 0.2 μl *Taq* DNA Polymerase (5 U/μl) (*Taq* PCR Core Kit, Qiagen, Hilden, Germany) and 12.6 μl distilled water.

Four primers were used in the PCR reaction. The first set contained the universal D3A (5′-GACCCGTCTTGAAACACGGA-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (Al-Banna *et al.*, 1997; Thomas *et al.*, 1997), which amplify the D3 expansion region of the large subunit ribosomal gene (28S rDNA gene). The second set included a species specific primer GlyF1 (5′-TTACGGACCGTAACTCAA-3′) and a universal primer rDNA2 (5′-TTTCACTCGCCGTTACTAAGG-3′) which, in combination, amplify a fragment of the ITS2 region and a part of the 28S gene (Fig. 1). Amplification was performed in a MJ Research PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA). The PCR programme consisted of 4 min 94°C; 35 cycles of 30 s 94°C, 30 s 55°C and 1.5 min 72°C; then 10 min 72°C. A negative control containing the PCR mixture without any DNA template was also run. Seven microlitres of each amplified sample were analysed by electrophoresis in a 0.8% horizontal TBE buffered agarose gel (100 V, 40 min). Products visualised with ethidium bromide were photographed under UV-light. Duplex PCR were repeated at least twice with the same sample to verify the result. The experiments for *H. glycines* single juvenile identification in samples with or without soil inhabiting nematodes were repeated three times.

Results and discussion

For the development of the duplex PCR two sets of primers were selected; the first one for verifying the success of the PCR and the second one for specific detection of *H. glycines*. The first set included two universal primers for amplifying the D3 expansion region of the 28S gene which indicates the presence of template nematode DNA in the sample and, as a consequence, the quality of performance of the PCR. These primers were shown to be very useful for the amplification of a 28S gene fragment of many nematode species (Al-Banna *et al.*, 1997; Thomas *et al.*, 1997). This amplicon can be used as a control fragment as it does not vary significantly in length among nematode taxa: in *Caenorhabditis elegans* it is 362 bp long (Thomas *et al.*, 1997) and in *Pratylenchus* spp. (Al-Banna

Table 1. *Cyst nematode populations studied and PCR amplified products obtained during testing of the specific primer combinations.*

Species	Population origin	Plant-host	Race	Length of PCR fragment	
				181 bp	~345 bp
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	3	+	+
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	4	+	+
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	14	+	+
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	6	+	+
H. glycines	Furu, Heilongjiang province, China	*Glycine max*	3	+	+
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Anda county, Heilongjiang province, China	*Glycine max*	6	+	+
H. glycines	Haerbin, Heilongjiang province, China	*Glycine max*	3	+	+
H. glycines	Jiamusi, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Jiamusi, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Jiamusi, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Baoquanlin, Heilongjiang province, China	*Glycine max*	3	+	+
H. glycines	Zhongbei town of Anda county, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Zhongbei school, Anda county, Heilongjiang province, China	*Glycine max*	n.t.	+	+
H. glycines	Peixiang, Jiangsu province, China	*Glycine max*	4	+	+
H. glycines	Pinganlin, Gongzhuling, Jilin province, China	*Glycine max*	n.t.	+	+
H. glycines	Kalunhu, Gongzhuling, Jilin province, China	*Glycine max*	n.t.	+	+
H. glycines	Qingjiatun, Gongzhuling, Jilin province, China	*Glycine max*	n.t.	+	+
H. glycines	Changyi county, Shandong province, China	*Glycine max*	7	+	+
H. glycines	Laixi county, Shandong province, China	*Glycine max*	1	+	+
H. glycines	Heze, Shandong province, China	*Glycine max*	4	+	+
H. glycines	Jiaoxiang, Shandong province, China	*Glycine max*	7	+	+
H. glycines	Weifan, Shandong province, China	*Glycine max*	7	+	+
H. glycines	Gaomi, Shandong province, China	*Glycine max*	7	+	+
H. glycines	Liangcheng, Inner Mongonia, China	*Glycine max*	n.t.	+	+
H. glycines	Wulanhaote, Inner Mongonia, China	*Glycine max*	n.t.	+	+
H. glycines	Shengyang, Liaoning province, China	*Glycine max*	3	+	+
H. glycines	Kaifen, Henan province, China	*Glycine max*	7	+	+
H. glycines	Shanqiu, Henan province, China	*Glycine max*	4	+	+
H. glycines	Kenli, Henan province, China	*Glycine max*	4	+	+
H. glycines	Changyuan, Henan province, China	*Glycine max*	4	+	+
H. glycines	Huaxiang, Henan province, China	*Glycine max*	7	+	+
H. glycines	Wenxiang, Henan province, China	*Glycine max*	7	+	+
H. glycines	Mongcheng, Anhui province, China	*Glycine max*	5	+	+
H. glycines	Xuxiang, Anhui province, China	*Glycine max*	4	+	+
H. glycines	Dansan, Anhui province, China	*Glycine max*	4	+	+
H. glycines	Zhangjiakou, Hebei province, China	*Glycine max*	n.t.	+	+
H. glycines	Zuxiang, Shanxi province, China	*Glycine max*	4	+	+
H. glycines	Yanggao, Shanxi province, China	*Glycine max*	4	+	+
H. glycines	Taigu, Shanxi province, China	*Glycine max*	4	+	+
H. glycines	Fanzhi, Shanxi province, China	*Glycine max*	4	+	+
H. glycines	Tongzhou, Beijing, China	*Glycine max*	n.t.	+	+
H. glycines	Dongbeiwng farm, Beijing, China	*Glycine max*	n.t.	+	+
H. glycines	IPP farm, Beijing, China	*Glycine max*	4	+	+
H. glycines	Baicheng, Jiling, China	*Glycine max*	n.t.	+	+
H. glycines	Jinxiang, China	*Glycine max*	4	+	+
H. glycines	Blagoveshchensk, Amur region, Russia	*Glycine max*	n.t.	+	+
H. glycines	Primorsky territory, Russia	*Glycine max*	n.t.	+	+
H. glycines	Arkansas, USA	*Glycine max*	14	+	+
H. glycines	North Carolina, USA	*Glycine max*	2	+	+
H. glycines	USA	*Glycine max*	n.t.	+	+
H. glycines	Brazil	*Glycine max*	1	+	+
H. schachtii	Goettingen, Germany	*Beta vulgaris*		–	+
H. schachtii	Kitzingen, Germany	*Beta vulgaris*		–	+
H. schachtii	Schladen, Germany	*Beta vulgaris*		–	+
H. schachtii	Gingelam, Belgium	*Beta vulgaris*		–	+
H. schachtii	Hermée, Belgium	*Beta vulgaris*		–	+
H. trifolii	Germany	*Beta vulgaris*		–	+
H. trifolii	Moscow region, Russia	*Trifolium repens*		–	+
H. medicaginis	Stavropol region, Russia	*Medicago sativa*		–	+
H. ciceri	Italy	*Cicer* sp.		–	+
H. cajani	India	*Cajanus cajan*		–	+
H. sp.1	Knokke, Belgium	*Atriplex litoralis*		–	+
H. sp.2	Germany	*Rumex* sp.		–	+
H. avenae	Pingu county, Beijing, China	*Triticum* sp.		–	+
H. cruciferae	The Netherlands	*Brassica* sp.		–	+
G. pallida	Germany	*Solanum tuberosum*		–	+

n. t.: pathotype not tested; + : presence of amplified fragment; – : absence of amplified fragment. Races of *H. glycines* based on Riggs and Schmitt (1988) scheme reported according to personal communications from D. Peng (Chinese populations), E.L. Davis (North Carolinian population), R.T. Robbins (Arkansas population) and R.C.V. Terente (Brazilian population).

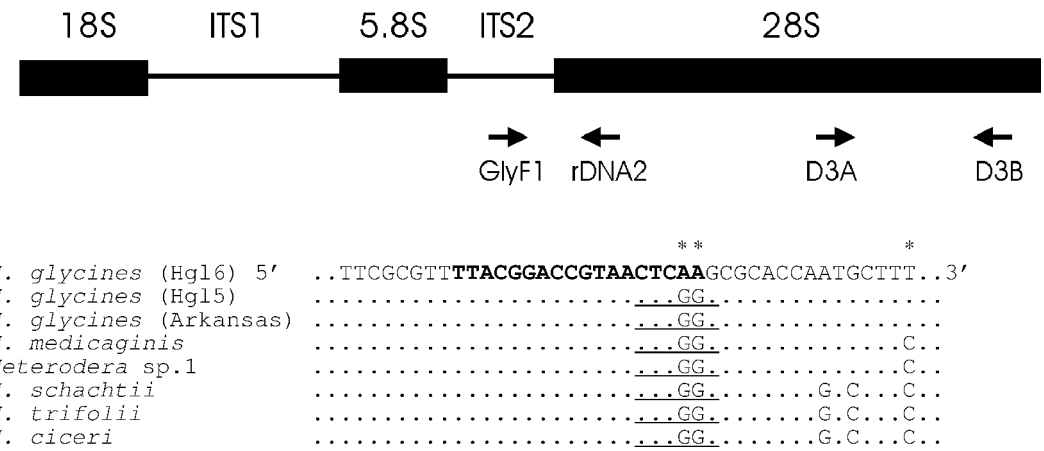

Fig. 1. *Schematic drawing showing positions of four primers in the rDNA genes used in the duplex PCR for identification of* Heterodera glycines *and ITS2 fragment alignment used for development of the species specific primer. The fragments of the ITS2 sequence of the two clones (Hgl) correspond to two ITS haplotypes of* H. glycines *according to Zheng et al. (2000). Position of GlyF1 primer is indicated in bold font, restriction site recognised by* Ava*I (C/YCGRG) is underlined and asterisks show nucleotide positions distinguishing* H. glycines *from other* schachtii*-group species.*

et al., 1997) and cyst forming nematode species (Subbotin *et al.*, unpubl.) its length is approximately 345 bp.

The second set of primers contained both a species specific primer and a universal primer. For the design of the specific primer, nucleotide sequences of the ITS region from several populations of *H glycines*, *H. ciceri*, *H. medicaginis*, *H. trifolii* (including the sugar beet race), *H. schachtii*, *H. cajani*, an undescribed *Heterodera* sp.1 infecting *Atriplex litoralis* L. and other cyst forming nematode species, were aligned (data not shown). The sequence of the *H. glycines* population from Arkansas (GenBank AF274390) obtained from our unpublished study and sequences of two ITS haplotypes of the Chinese population published by Zheng *et al.* (2000) were taken for this alignment. Zheng *et al.* (2000) showed the presence of two haplotypes of the ITS region in the genome of *H. glycines*, which could be distinguished by *Ava*I. In the alignment, the sequence of *H. glycines* was very similar to that of *H. medicaginis* and *Heterodera* sp.1. We found only four nucleotide positions distinguishing *H. glycines* from these two species. Two point nucleotide mutations, which were common for all ITS haplotypes, occurred in ITS1 and ITS2, respectively. Two nucleotide differences were typical for one ITS haplotype only and occurred in the *Ava*I restriction site of ITS2 (Fig. 1). Based on this information several putative specific PCR primers differentiating these positions were manually designed and various combinations of primers and PCR conditions were primarily tested. The putative diagnostic primers based upon one nucleotide difference in combination with universal primers amplified DNA from other cyst-forming species and were excluded from further tests (data not shown). The species specific primer GlyF1 and the universal primer rDNA2 (Fig. 1), amplified a fragment (181 bp) of the ITS2-28S gene of one haplotype and showed promising results. The position of the specific primer GlyF1 corresponds to bp 935-952 on the sequence alignment of the Chinese *H. glycines* and *H. avenae* populations as presented by Zheng *et al.* (2000). This primer combination was chosen for further testing.

Duplex PCR with these two sets of primers were performed on both single and multiple cyst samples from 53 populations of *H. glycines* and several populations of other cyst forming nematode species. All the *H. glycines* populations yielded two distinct fragments (181 and 345 bp), whereas all the other species produced only one (345 bp) (Fig. 2). No distinct additional unspecific amplification products were observed under 345 bp.

To test the sensitivity of this method, we tried to detect a single *H. glycines* second stage juvenile (of populations from Baicheng, Jiling, China, and from North Carolina, USA) either alone or in a mixture with different proportions of soil inhabiting nematodes or *Pratylenchus* spp. The PCR with all samples containing a single second stage juvenile showed two distinct DNA bands; *H. glycines* free samples yielded only one fragment (Fig. 3). We observed a decrease in the intensity of the species specific band in samples in which *H. glycines*

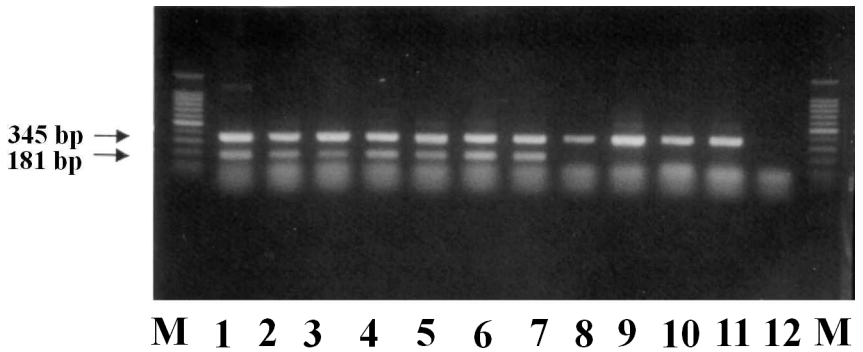

Fig. 2. *Duplex PCR with GlyF1 species specific primer. The amplified products are obtained from single cyst samples. Lane M: 100 bp DNA ladder (Promega, USA); Lane 1-7:* Heterodera glycines *(1: Amur region, Russia; 2: Arkansas, USA; 3: North Carolina, USA; 4: Anda county, Heilongjiang province, China, race 6; 5: Shengyang, Liaoning province, China; 6: IPP farm, Beijing, China; 7: Zhangjiakou, Hebei province, China); Lane 8:* H. schachtii *(Schladen, Germany); Lane 9:* H. ciceri*; Lane 10:* H. medicaginis*; Lane 11:* H. cajani*; Lane 12: sample without nematode DNA.*

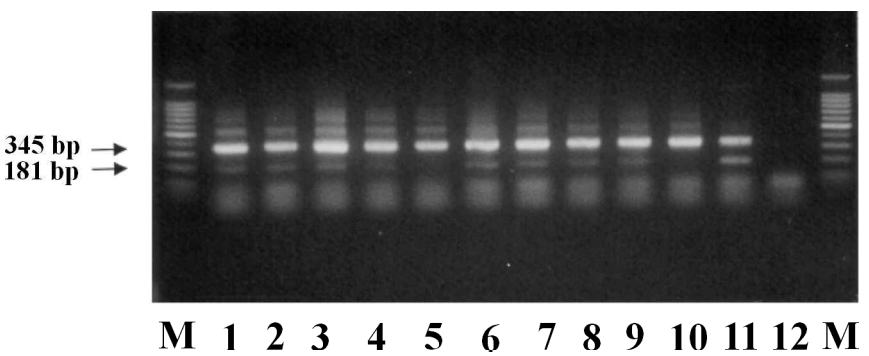

Fig. 3. *Duplex PCR with GlyF1 species specific primer. The amplified products are obtained from samples containing a single second stage juvenile of* Heterodera glycines *alone or in mixture with other soil nematodes. Lane M: 100 bp DNA ladder (Promega, USA); Lanes 1-4: a single juvenile in mixture with 18, 36, 54, 72 specimens of soil inhabiting nematodes, respectively; Lane 5: 72 soil inhabiting nematodes without a* H. glycines *second stage juvenile; Lanes 6-9: a single second stage juvenile mixed with 75, 150, 225, 300 specimens of* Pratylenchus *spp., respectively; Lane 10: 300* Pratylenchus *individuals without a* H. glycines *second stage juvenile; Lane 11: a single* H. glycines *second stage juvenile; Lane 12: sample without nematode DNA.*

was mixed with other nematodes. This intensity, however, did not significantly depend on the number of other nematodes. The sensitivity of this method proved to be much higher than one second stage juvenile as in our analyses we used the equivalent of one tenth of the DNA of a single juvenile.

Species specific primers need to be designed based on the broad knowledge of sequence divergence of the target DNA region in many populations of the same species and in closely related species. This knowledge allows both the detection of populations with small differences in sequences, and avoids the amplification of an identical specific fragment in other species. This technique is particularly important for regulatory services where the information on the species identity is limited.

Two ITS haplotypes may be present in different proportions in rDNA clusters of the genome of *H. glycines* populations (Zheng *et al.*, 2000). Our species specific primer amplifies only a fragment of one of the haplotypes. Nevertheless, these primers identified all the *H. glycines* populations tested and originating from Asia, USA and Brazil. However, further testing of American populations is needed, as well as tests with other nematode species to verify the robustness of our method in routine identifications of *H. glycines.*

Acknowledgements

The first author is grateful for the support of a NATO Research Fellowship and the Russian Foundation of Fundamental Researches (Grant N° 99-04-48332). The authors thank J. Rowe (UK), R. Robbins (USA), E. Davis (USA), L. Waeyenberge (Belgium), N. Vovlas (Italy), A. Eroshenko (Russia), D. Sturhan (Germany), R.C.V. Tenente (Brazil) and N.A. Kostjuk (Russia) for providing nematode material.

References

AL-BANNA, L., WILLIAMSON, V. & GARDNER, S.L. (1997). Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Molecular Phylogenetic and Evolution* 7, 94-102.

BALDWIN, J.G. & MUNDO-OCAMPO, M. (1991). Heteroderinae, cyst- and noncyst-forming nematodes. In: Nickle, W.R. (Ed.). *Manual of agricultural nematology*. New York, NY, USA, Marcel Dekker, pp. 275-362.

BEKAL, S., GAUTHIER, J.P. & RIVOAL, R. (1997). Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. *Genome* 40, 479-486.

BESAL, E.A., POWERS, T.O., RADICE, A.D. & SANDALL, L. (1988). A DNA hybridization probe for the detection of soybean cyst nematode. *Phytopathology* 78, 1136-1139.

BRIDGE, P.D., ARORA, D.K., REDDY, C.A. & ELANDER, R.P. (1998). *Applications of PCR in mycology*. Wallingford, UK, CAB International, 357 pp.

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

EROSHENKO, A.S., KAZACHENKO, I.P. & VOLKOVA, T.V. (1990). [Distribution and damage induced by the soybean cyst nematode in the Primorsky territory]. *Zaschita rastenii na Dalnem Vostoke*. Vladivostok, Russia DVO AN SSSR, pp. 90-100.

EVANS, K. & ROWE, J.A. (1998). Distribution and economic importance. In: S.B. Sharma (Ed.). *The cyst nematodes*. Dordrecht, The Netherlands, Kluwer Academic Publishers, pp. 1-30.

FULLAONDO, A., BARRENA, E., VIRIBAY, M., BARRENA, I., SALAZAR, A. & RITTER, E. (1999). Identification of potato cyst nematode species *Globodera rostochiensis* and *G. pallida* by PCR using specific primer combinations. *Nematology* 1, 157-163.

GRANEY, L.S.O. & MILLER, L.I. (1982). Comparative morphological studies of *Heterodera schachtii* and *H. glycines*. In: Riggs, R.D. (Ed.). *Nematology in the Southern region of the United States*, 1982. Fayetteville, AR, USA, Southern Cooperative Series Bulletin 276, pp. 96-107.

LIU, X.Z., LI, J.Q. & ZHANG, D.S. (1997). History and status of soybean cyst nematode in China. *International Journal of Nematology* 7, 18-25.

LOUWS, F.J., RADEMAKER, J.L.W. & DE BRUIJN, F.J. (1999). The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Annual Review of Phytopathology* 37, 81-125.

MULHOLLAND, V., CARDE, L., O'DONNELL, K.L., FLEMING, C.C. & POWERS, T.O. (1996). Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. In: Marshall, G. (Ed.). *Proceedings of diagnostics in crop production symposium*. Farnham, UK, British Crop Production Council, pp. 247-252.

NOEL, G.R. (1985). The soybean cyst nematodes. In: Lamberti, F. & Taylor, C.E. (Eds). *Cyst nematodes*. New York, NY, USA, Plenum Press, pp. 257-268.

ORUI, Y. (1997). Discrimination of *Globodera rostochiensis* and four *Heterodera* species (Nematoda: Heteroderidae) by PCR-RFLP analysis. *Japanese Journal of Nematology* 27, 67-75.

PETERSEN, D.J., ZIJLSTRA, C., WISHART, J., BLOK, V. & 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.

RIGGS, R.D. & NIBLACK, T.L. (1993). Nematode pest of oilseed crops and grain legumes. In: Evans, K., Trudgill, D.L. & Webster, J.M. (Eds). *Plant parasitic nematodes in temperate agriculture*. Wallingford, UK, CAB International, pp. 209-258.

RIGGS, R.D. & SCHMITT, D.P. (1988). Complete characterization of the race scheme for *Heterodera glycines*. *Journal of Nematology* 9, 34-39.

SETTERQUIST, R.A., SMITH, G.K., JONES, R. & 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.

SIKORA, R.A. & MAAS, P.W.TH. (1985). An analysis of the *Heterodera trifolii* complex and other species in the *schachtii* group attacking legumes. In: Lamberti, F. & Taylor, C.E. (Eds). *Cyst nematodes*. New York, NY, USA, Plenum Press, pp. 293-312.

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

SUBBOTIN, S.A., WAEYENBERGE, L., MOLOKANOVA, I.A. & MOENS, M. (1999). Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. *Nematology* 1, 195-207.

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

SZALANSKI, A., SUI, D.D., HARRIS, T.S. & 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. & MUGNIÉRY, D. (1996). Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of solanaceous plants. *Fundamental and Applied Nematology* 19, 471-479.

THOMAS, W.K., VIDA, J.T., FRISSE, L.M., MUNDO, M. & BALDWIN, J.G. (1997). DNA sequences from formalin-fixed nematodes: integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29, 250-254.

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

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

ZHENG, J., SUBBOTIN, S.A., WAEYENBERGE, L. & MOENS, M. (2000). Molecular characterisation of Chinese *H. glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. *Russian Journal of Nematology* 8, 109-133.