Molecular Ecology (2000) **9**, 1223–1232

Rapid identification of cyst (*Heterodera* spp., *Globodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformational polymorphism (PCR-SSCP) in cultures and field samples

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Abstract

Cyst and root-knot nematodes show high levels of gross morphological similarity. This presents difficulties for the study of their ecology in natural ecosystems. In this study, cyst and root-knot nematode species, as well as some ectoparasitic nematode species, were identified using the second internal transcribed spacer (ITS2) sequence variation detected by polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP). The ITS2 region was sufficiently variable within the taxa investigated to allow species to be separated on the basis of minor sequence variation. The PCR primers used in this study were effective for 12 species with three genera within the Heteroderinae (*Globodera pallida*, *G. rostochiensis*, *Heterodera arenaria*/*avenae*, *H. ciceri*, *H. daverti*, *H. hordecalis*, *H. mani*, *H. schachtii*, *H. trifolii*, *Meloidogyne ardenensis*, *M. duytsi* and *M. maritima*). However, pathotypes of *Globodera pallida* and *G. rostochiensis* could not be distinguished. The method was tested at two coastal dune locations in The Netherlands (one in the lime-poor dunes of the north and one in calcareous dunes of the south) to determine the population structure of cyst nematodes. At each site, cyst nematodes were associated with three plant species: two plant species on the foredune (*Elymus farctus* and *Ammophila arenaria*) and one plant species occurring further inland (*Calamagrostis epigejos*). Two species of cyst nematodes, *H. arenaria* and *H. hordecalis*, were found. *H. arenaria* associated with vigorous *A. arenaria* and *H. hordecalis* in association with degenerating *A. arenaria and C. epigejos*. The field survey demonstrated that in coastal dunes abiotic factors may be the important affecting the distribution of cyst nematodes.

Keywords: *Globodera*, *Heterodera*, identification, *Meloidogyne*, nematode, PCR-SSCP

Received 23 November 1999; revision received 18 March 2000; accepted 23 March 2000

Introduction

Nematodes are an important biotic component of the rhizosphere (Nickle 1991). Plant parasitic nematodes are well-known pests in agroecosystems and are also thought to exert an important influence on the structure and stability of natural plant communities (Stanton 1988; Van der Putten & Van der Stoel 1998). Precise identification of the components of natural plant parasitic nematode communities is a prerequisite for these studies.

Several classical techniques have been used for nematode identification including host range tests and the use of morphological characters. Despite having specialized ecological functions, the overall morphology of many nematode taxa is conservative, especially as larvae and variation frequently range across species divisions. This is particularly true of closely related species with high morphological similarity, such as *Globodera rostochiensis* and *G. pallida* (Morgan Golden 1986). The use of morphology

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has, therefore, been augmented by techniques based on molecular characters which generally result in simple band patterns that are easily interpreted by nonspecialists.

Polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) (Orita *et al*. 1989) has considerable advantages over many other molecular techniques used for taxonomic characterization. A major advantage is that taxonomic differentiation utilizes the sum of all nucleotide sequence variation *between* PCR primers sites, rather than the taxon-specific annealing of primers or differences in restriction sites (often revealing no differences unless a suite of several enzymes is tested). The design of PCR primers can, therefore, be based on conserved regions and allows a single primer pair to differentiate species across more than one genus, combining the advantages of PCR with a sensitivity (over defined regions) that is on a par with DNA sequencing (Hayashi & Yandell 1993). The technique relies on differences in the mobility of single-stranded PCR amplicons in nondenaturing polyacrylamide gels. The length, position and extent of self complimentary base pairing affect the conformation taken up by the molecules and thus their electrophoretic mobility. Single base differences between amplicons can affect the tertiary conformation of the molecules and allow differentiation. This effect is enhanced by minor length polymorphisms and increasing amounts of sequence variation. SSCP patterns are highly reproducible between gels and generate two markers from each DNA sequence present, enabling identification to take place on the basis of minor nucleotide differences across several hundred bases of sequence, but without recourse to sequencing (Lessa & Applebaum 1993). The target region in this study was the second internal transcribed spacer (ITS2) of the ribosomal RNA (rRNA) gene clusters. Ribosomal genes are used most frequently for taxonomic work as they are present in all organisms and sequence data are available which enable phylogenetic affiliations to be rooted in a background of related taxa. The ITS2 was chosen over the small subunit rRNA genes because it was likely to show less sequence conservation and thus enable the discrimination of closely related species on this basis.

PCR-SSCP has been exploited for the identification of fungi (Simon *et al*. 1993; Clapp 1999), bacteria (Lee *et al*. 1996; Schwieger & Tebbe 1998) and carabid ground beetles (Boge *et al*. 1994). Gasser (1997), Gasser & Monti (1997) and Gasser *et al*. (1997) applied PCR-SSCP to distinguish veterinary parasites, but its potential for application to free-living and plant parasitic nematode communities has not been investigated. Cyst and root-knot nematodes are major agronomic pests (Lamberti & Taylor 1986) and may also be involved in natural soil pathogen complexes such as those occurring in coastal foredunes (De Rooij-Van der Goes *et al*. 1995). The aims of this study were to determine the suitability of PCR-SSCP for the identification of cyst and root-knot nematodes and to carry out an initial study in field populations directed at identifying the plant associations of *Heterodera* spp. The results of this preliminary field investigation were exploited and extended in a subsequent field investigation (Van der Stoel & Van der Putten submitted).

Materials and methods

Nematodes: identified species

Three *Globodera* and eight *Heterodera* species from cultures, together with three species of *Meloidogyne* from the Haringvliet (51°52′ N 4°04′ E) field locality were investigated in this study (Table 1). Twenty individual cysts were analysed from each pathotype of *G. pallida* and *G. rostochiensis* in addition to extractions from multiple cysts and individual larvae. Five cysts of *G. tabacum* were also analysed. Similarly, 5–15 individual cysts were analysed for each cultured *Heterodera* species (total 58). DNA was extracted from egg masses (total $n = 6$) and larvae (total $n = 106$) of the *Meloidogyne* species from maritime dune locations. *M. maritima*, *M. duytsi* and *M. ardenensis* were associated with *Ammophila arenaria*, *Elymus farctus* and a *Salix* sp., respectively, and identified microscopically by Henk Duyts.

Nematodes: field samples

Comparisons were made between nematode populations occurring at three locations along the coast of The Netherlands in this pilot study using one-way analysis of variance. Based on an earlier field study at six locations along the Dutch coast, two field sites were chosen, one at Texel (53°07′ N 4°45′ E; lime poor) and one at Haringvliet (51°52′ N 4°04′ E; calcareous). A third site, Walcheren (51°35′ N 3°32′ E; calcareous) was included to obtain additional information on the population density of cysts and larvae. Eight soil samples (1 kg each), subsequently combined, were collected from the rhizosphere of a series of dominant plant species: *Elymus farctus*, *Ammophila arenaria*, *Festuca rubra* ssp. *arenaria*, *Carex arenaria*, *Elymus athericus* and *Calamagrostis epigejos*. Soil was sampled from both vigorous and degenerating stands of *A. arenaria*. Cysts were extracted by flotation and collected by sieving before selection for molecular analysis.

DNA extraction

The extraction protocol for individual cysts was based on that of Caswell-Chen *et al*. (1992). However, the volumes were doubled because more diluted DNA was acceptable for the PCR. Nematode larvae were picked individually into 10 μL sterile water on glass slides and disrupted

Table 1 List of nematode isolates and samples used in this study

	Culture code	Host plant	Origin
Globodera pallida Pa2	D-381	*Solanum tuberosum*	PD
G. pallida Pa2	D-475	*S. tuberosum*	PD
G. pallida Pa3	E-412	*S. tuberosum*	PD
G. rostochiensis Ro1	A-50	*S. tuberosum*	J. v. Bezooijen
G. rostochiensis Ro1	A-56	*S. tuberosum*	PD
G. rostochiensis Ro3	B-140	*S. tuberosum*	PD
G. rostochiensis Ro4	F539	*S. tuberosum*	PD
G. rostochiensis Ro5	G1526	*S. tuberosum*	PD
G. tabacum	C-6876	*Nicotiana* sp.	PD
Heterodera trifolii	A1-1	*Trifolium repens*	PD
H. ciceri	Pot 30	*Phaseolus vulgaris*	PD
H. avenae	Field	—	D. Sturhan
H. arenaria	Field	*Ammophila arenaria*	J. A. Rowe
H. mani	Field	—	D. Sturhan
H. daverti	LU68	*T. repens*	PD
H. schachtii	Pot 7	*Brassica* sp.	PD
H. hordecalis	—	*Hordeum vulgare*	S. Andersson
Meloidogyne ardenensis	Field	*Salix* sp.	Haringvliet
M. maritima	Field	*A. arenaria*	Haringvliet
M. duytsi	Field	*Elymus farctus*	Haringvliet
Heterodera sp.	Field	*Calamagrostis epigejos*	Haringvliet
Heterodera sp.	Field	*A. arenaria* (vig.)	Haringvliet
Heterodera sp.	Field	*A. arenaria* (deg.)	Haringvliet
Heterodera sp.	Field	*Calamagrostis epigejos*	Texel
Heterodera sp.	Field	*A. arenaria* (deg.)	Texel
Heterodera sp.	Field	*A. arenaria* (vig.)	Texel
Heterodera sp.	Field	*Ammophila arenaria* (deg.)	Texel

— indicates no culture is available. PD refers to the Nederlands Plantenziektenkundige Dienst, Wageningen, The Netherlands. *H. avenae* and *H. mani* were isolated from sites at Grafenreuth and Hamminkeln, Germany, respectively, and provided by Dr D. Sturhan, Biologische Bundesanstalt, Institut fur Nematologie und Wirbeltierkunde, Toppheideweg 80, D-48161, Munster, Germany. *H. arenaria* was provided by J.A. Rowe, Department of Entomoloy and Nematology, Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, UK. *H. hordecalis* was provided by Dr S. Andersson, National Swedish Institute for Plant Protection, S-230 47, Åkarp, Sweden. *G. rostochiensis* A-50 was collected from Mierenbos and provided by Jan van Bezooijen, Department of Nematology, Wageningen Agricultural University, Wageningen, The Netherlands.

manually with a needle. The larval fragments were then diluted in 45 µL sterilized water of which 5 µL was used in the subsequent PCR. Extractions of both cysts and larvae were flash-frozen in liquid nitrogen and stored at −20 °C until required.

PCR

Forward and reverse primers were designed for the second ITS ribosomal RNA spacer region (ITS2) based upon all available Heteroderinae sequences: *CysNFwd*1 (5′-GATCGATGAAGAACGCAGC), *CysNRvs*1 (5′-TCCTC-CGCTAAATGATATG), respectively. The ITS2 was chosen as it was expected to show interspecific variation and sequence data were available for several species in the literature and sequence databases. The expected amplicon sizes based on available sequence information were in the following ranges and varied according to genus: *Globodera*, 394 bp; *Meloidogyne*, 292–298 bp; *Heterodera*, 392–401 bp. All amplifications were carried out in a volume of 20 µL using 5 µL DNA extraction, 20 µM dNTP, 0.4 U DNA polymerase (DynaZyme™, Finnzymes) and 20 pmol of each primer. Amplifications were carried out in a PTC-200 thermocycler (MJ-Research) with heated lid and did not require an oil overlay. Product quality was checked by agarose gel electrophoresis in 2% gels, stained with ethidium bromide. The PCR parameters used were as follows: 96 °C for 55 s, 53 °C for 55 s, 72 °C for 45 s, 10 cycles; next 20 cycles, anneal temperature reduced to 51 °C and extension time increased to 2 min; final 15 cycles, anneal temperature reduced to 50 °C and extension time increased to 3 min.

SSCP

Optimization (maximizing band separation) of PCR-SSCP is empirical (Orita *et al*. 1989) because the conformations adopted by PCR amplicons cannot be predicted in advance, even if sequence data are available. Therefore, although different conditions were tried, such as altered gel concentration and running temperature, no significant benefit could be gained across all species tested. It should be stressed that, in this application, the technique is designed to profile unknown samples where the optimal conditions for a particular sequence are unknown and cannot be met in a single gel. Therefore, we advocate that a compromise set of conditions is used. This may not be optimal for an individual sequence but in general worked well. The conditions described for this study enabled the differentiation of all species investigated with different ITS2 sequence. MDE (0.5×, FMC BioProducts, Biozym) nondenaturing polyacrylamide gels were poured on a wide H03 system (Pharmacia) as recommended by the manufacturer. The TBE running buffer was cooled to 4 °C before sample application. Two microlitres of PCR product was combined with 8 μL of denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) followed by 3 min denaturation at 94 °C. The samples were snap cooled on ice before loading and running at 6 W for 20 000 Volt.hours (Vh). Electrophoresis was carried out at 4 °C. Bands were detected by silver staining or by incorporation of ^{32}P-labelled dATP following established procedures. PCR-SSCP gels were highly reproducible under the conditions described.

Sequencing

To address the possibility that the additional bands of equal intensity seen in some species arose from the presence of multiple ITS2 sequences, silver-stained bands were excised from a dried polyacrylamide gel, resuspended in water and reamplified by PCR. Four bands were excised from the profile of a *H. arenaria* cyst and three from a cyst of *H. hordecalis* (Fig. 4). The double-stranded products were cloned into pGem-T and two recombinants sequenced in both directions using an ALF sequencer (Pharmacia). Sequences, excluding the primer sites, were aligned with published sequences using Clustal W (http://www2.ebi.ac.uk/clustalw/) (Thompson *et al*. 1994, 1997) with some manual adjustment using JALVIEW. A distance matrix with Kimura's two-parameter correction for multiple substitutions (Kimura 1980) was used to construct a Neighbour-Joining tree (Saitou & Nei 1987).

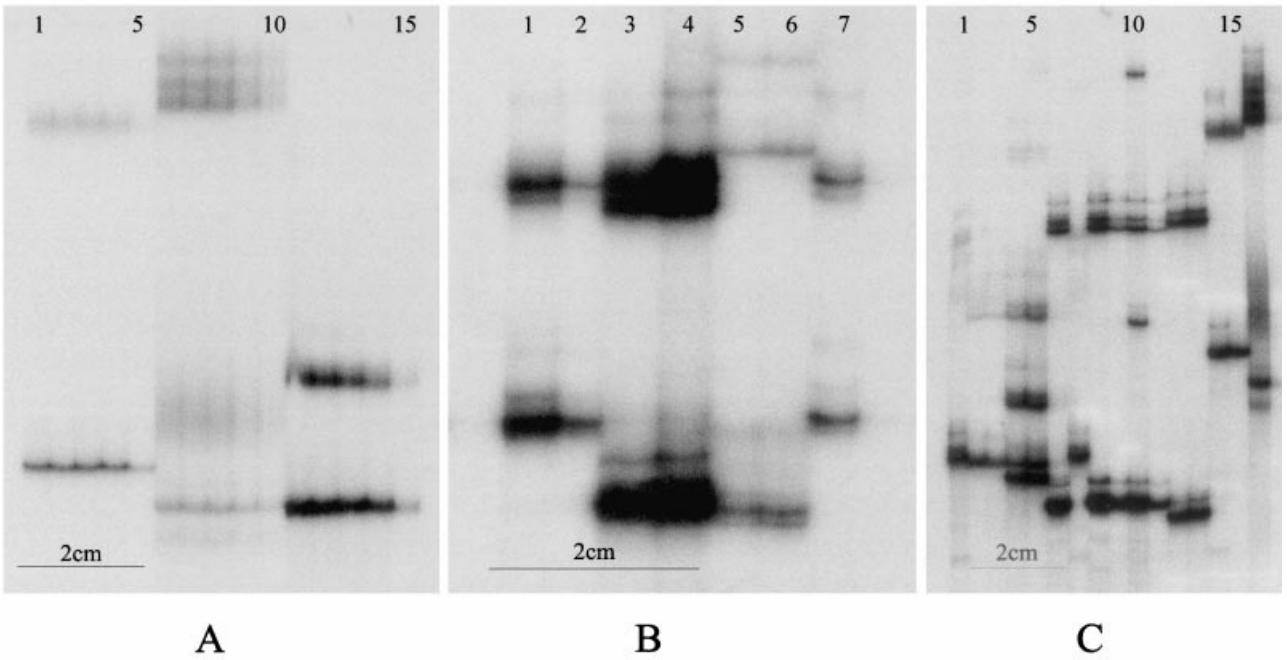

Fig. 1 PCR-single-strand conformational polymorphism (PCR-SSCP) profiles of ITS2 regions from a selection of nematode species included in this study. (a) *Globodera* spp. Lanes 1–5: *G. tabacum* (C-6876); lanes 6–10: *G. rostochiensis* (A-50, A-56, B-140, F-539 and G-1526); lanes 11–15: *G. pallida* (D-381, D-475, E-412, J2 larvae D-381, single J2 larva D-381). (b) *Meloidogyne* spp. Lanes 1, 2 and 7: *M. maritima*; lanes 3 and 4: *M. ardenensis*; lanes 5 and 6: *M. duytsi*. (c) *Heterodera* spp. Lanes 1 and 7: *H. ciceri* (Pot 30); lanes 2 and 3: *H. trifolii* (A1-1); lanes 4 and 5: *H. daverti* (LU68); lanes 6, 8 and 9: *H. avenae* (D. Sturhan); lanes 10 and 11: *H. arenaria* (J. Rowe); lanes 12 and 13: *H. mani* (D. Sturhan); lanes 14 and 15: *Globodera tabacum* (C-6876); lane 16: *G. rostochiensis* (A-50).

The ClustalW.dnd file was displayed using Treeview (Page 1996). The tree was rooted using *Meloidogyne* spp. sequences as an outgroup.

Results and discussion

Globodera

Figure 1a shows representative SSCP profiles obtained from the three *Globodera* species in this study. SSCP profiles were identical between different populations within each species. The lack of ITS2 variation (Thiéry & Mugniéry 1996) did not allow pathotypes to be distinguished, presumably reflecting sequence identity in this region. Both *G. tabacum* (lanes 1–5) and *G. pallida* (lanes 11–15) profiles were composed of two bands, however, the *G. rostochiensis* (lanes 6–10) profile was consistently comprised of multiple bands. These could be due to the formation of metastable (secondary) conformations by this strand (see below) but could also be due to the presence of multiple sequences in the ITS2 region. Identical profiles were obtained from single juveniles for all three species (results not shown). The profiles obtained from *Globodera* were distinct and easily distinguished from those of the *Heterodera* species (Fig. 1c). This allowed the relative mobilities of *Globodera* and *Heterodera* to be compared directly and demonstrates pattern reproducibility between gels and samples.

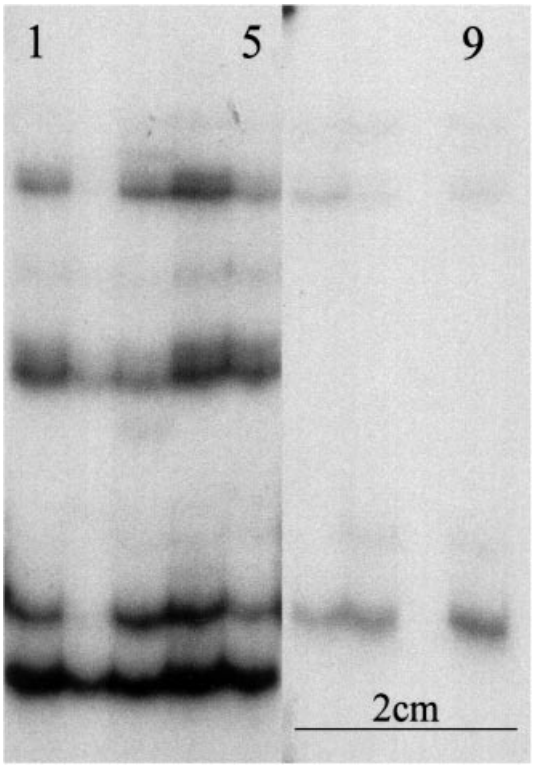

Fig. 2 PCR-SSCP profiles of ITS2 regions from cysts of *Heterodera daverti* and *H. trifolii*. Lanes 1–5: *H. daverti* (LU68); lanes 6, 7 and 9: *H. trifolii* (A1–1). Lane 2 shows a *H. daverti* cyst without the '*trifolii*' bands.

Meloidogyne

PCR-SSCP profiles of the ITS2 allowed the three *Meloidogyne* spp. in this study to be distinguished (Fig. 1b). The sympatrically occurring dune species (*M. maritima* and *M. duytsi*) were separated easily (Fig. 1b, lanes 1, 2 and 7; lanes 5 and 6). The PCR products from these species stained to a similar intensity with ethidium bromide after agarose gel electrophoresis but *M. ardenensis* showed a much stronger SSCP signal after radiolabelling (Fig. 1b, lanes 3 and 4). This is may be due to the *M. ardenensis* ITS2 being more AT rich than those of the other species.

Heterodera

The identity of PCR-SSCP profiles within species reflected sequence conservation of the ITS2 region which has been noted in sequencing studies (Ferris *et al*. 1993, 1994, 1995; Thiéry & Mugniéry 1996; Bekal *et al*. 1997). The sequence of the ITS2 is more conserved than the ITS1 (Ferris *et al*. 1993, 1994, 1995) but there is sufficient interspecific variation to make it a prime target for species differentiation. All the *Heterodera* species investigated (Figs 1c, 2 and 4) could be differentiated on the basis of ITS2 PCR-SSCP with the exception of *H. avenae* (Fig. 1c, lanes 6, 8 and 9) and *H. arenaria* (Fig. 1c, lanes 10 and 11). The inability to separate these species reflects their close taxonomic relationship and almost certain sequence identity in the ITS2 spacer region. This supports restriction enzyme data showing that there are no enzymes capable of separating European populations of *H. arenaria* and *H. avenae* in this region (Subbotin *et al*. 1999). *H. arenaria* was originally described as *H. major* var. *arenaria* (Cooper 1955), although '*avenae*' was later preferred to '*major*' (Cooper 1968), before being raised to a full species by Kirjanova & Krall (1971). Because of its larger size, *H. arenaria* may be a polyploid of *H. avenae*, however, no difference in ploidy level has yet been detected (G. Karssen & J. G. van der Beek, personal communication). A single cyst of *H. arenaria* (Fig. 1c, lane 10) showed two strong additional bands in its SSCP profile. The origin of these are unknown but possibly come from a parasitizing nematode within the cyst. The SSCP profile of *H. avenae* is consistently different from that of *H. mani* (Fig. 1c, lanes 12 and 13) and does not support the synonomy of *H. mani* with *H. avenae* proposed by Ebsary (1991).

The SSCP profiles of *H. ciceri* (Fig. 1c, lanes 1 and 7) and *H. trifolii* (Fig. 1c, lanes 2 and 3, Fig. 2, lanes 6, 7 and 9) tended to be less intense than those of other species, although a diagnostic pattern could be identified for each. The single-stranded bands from *H. ciceri* may be superimposed under

the conditions used. This could be determined by end-labelling of single primers and comparing the relative mobilities of the individual bands. The SSCP profiles of *H. trifolii* cysts were less intense, particularly when visualized using radiolabelling as opposed to silver-staining (not shown). In the latter case two bands of equal intensity were obtained. It is probable that the method of labelling is responsible and the high signal generated by the lower band due to it being proportionally richer in labelled bases. *H. daverti* cysts (Fig. 2, lanes 1–5) consistently showed four bands, two being shared with *H. trifolii* (Fig. 2, lanes 6, 7 and 9). There were, however, exceptions in which individual *H. daverti* cysts did not have the two bands diagnostic of *H. trifolii* (Fig. 2, lane 2). It would appear that many cysts of this isolate contained larvae with two distinct sequences or a mixture of larvae. Hybridization between nematode species is not an uncommon event and frequently results in the production of viable interspecific hybrids (Mulvey 1958; Mugniery 1979; Ferris & Ferris 1992; Thiéry *et al*. 1996). *H. daverti* and *H. trifolii* are closely related with most members of the *H. trifolii* complex being described as nonsexual species or members of a parthenogenic species complex (Mulvey 1958; Triantaphyllou & Hirschmann 1978; Sikora & Maas 1986). *H. daverti* has also been described as a sexual form of *H. trifolii* (Wouts & Sturhan 1978) and could be expected to produce viable offspring with *H. trifolii*. Cysts containing hybrid progeny containing sequences from both species may have been observed in this study. SSCP, as well as allowing identification to species, may also allow the parentage and frequency of interspecific hybridization to be studied in nematodes.

Cysts obtained from a culture of *H. schachtii* showed two distinct SSCP profiles which represented both *H. schachtii* and *H. avenae* (results not shown). On further investigation it emerged that the culture had been isolated from an area in which *H. schachtii* was abundant, rather than initiated with pure identified material.

Field samples

There was no overall effect of collection site on the distribution of cysts and larvae. Variations in the host plant associations of *Heterodera* species were seen between the sites but no consistent pattern was observed. At all locations, *Heterodera* cysts and larvae were found mainly associated with *E. farctus*, *A. arenaria* and *C. epigejos* (Fig. 3). Although not statistically significant ($P = 0.173$), there tended to be more cysts/gram root associated with *E. farctus* and *A. arenaria* than any other plant. Similarly, cysts/L soil showed a tendency to be greatest in rhizosphere soil of *E. farctus*, *A. arenaria* and *C. epigejos* ($P = 0.113$). The number of larvae/L of soil was, however, significantly ($P = 0.039$) greater from the rhizosphere of *E. farctus* and *A. arenaria*

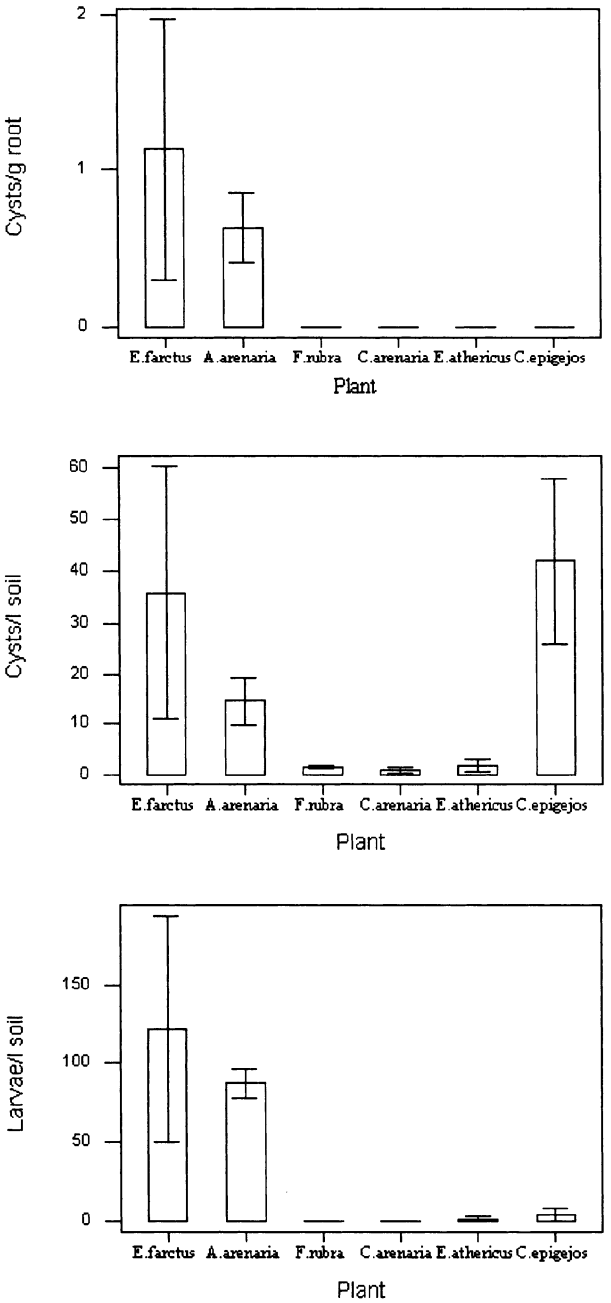

Fig. 3 Numbers and distribution of cysts and larvae of *Heterodera* spp. associated with coastal sand dune plants. Plant species occurring earliest in the dune succession, left to right. Bars represent the standard error of each sample mean.

than in that of other plants. Based on this survey, it was decided to omit the second calcareous dune site (Walcheren) from the SSCP analysis because it was similar to that of Haringvliet.

H. arenaria/avenae and *H. hordecalis* cysts, identified by PCR-SSCP, occurred at both sites investigated in this study. The identification of field cysts by PCR-SSCP

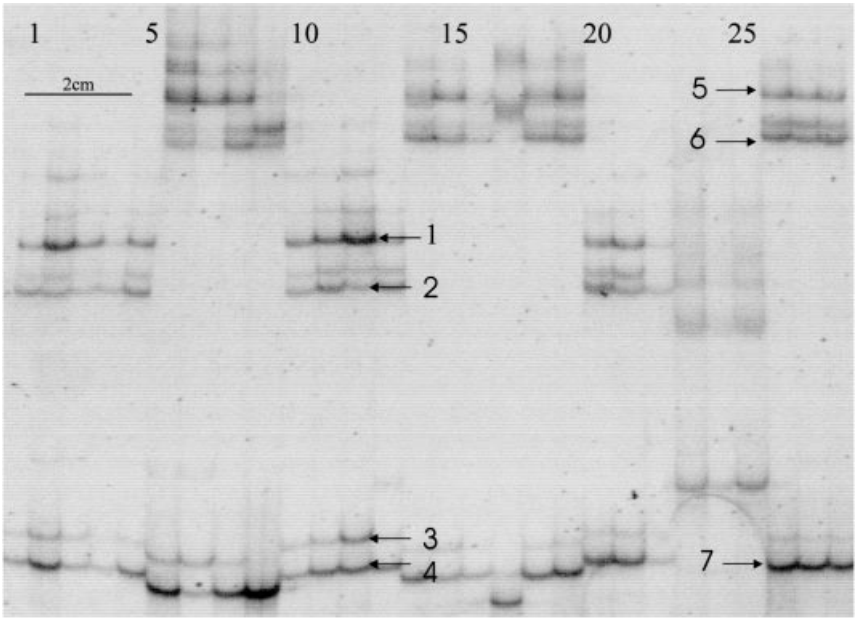

Fig. 4 An example of the PCR-SSCP profiles obtained from the ITS2 of field cysts collected from Texel and Haringvliet. Lanes 1–9, Texel field sites. Lane 1: cyst from vigorous *Ammophila arenaria*; lanes 2–5 cysts from degenerating stands of *A. arenaria*; lanes 6–9: cysts obtained from the root zone of *Calamagrostis epigejos*; lanes 10–19, Haringvliet field sites. Lanes 10–13: cysts obtained from the root zone of vigorous *A. arenaria*; lanes 14–19: cysts obtained from the root zone of degenerating *A. arenaria*. Lanes 20–28, control profiles. Lanes 20–22: *H. avenae* (D. Sturhan); lanes 23–25: *H. trifolii* (A1-1); lanes 26–28: *H. hordecalis* (S. Andersson). Arrows indicate bands excised and sequenced.

demonstrated differences in cyst nematode population structure between field sites and between host plant species (Fig. 4). At Haringvliet, distinct nematode populations were associated with vigorous and degenerating stands of *A. arenaria*. Degenerating stands of *A. arenaria* were favoured by *H. hordecalis*, whereas vigorous stands were populated by *H. arenaria/avenae*, the former considered the most likely (Cook 1982; Robinson *et al*. 1996). This differentiation was not marked at Texel, where *A. arenaria* was parasitized by *H. arenaria* alone, although *H. hordecalis* was still present, associated with *Calamagrostis epigejos* further back in the dune succession. This most likely reflects the different abiotic environments at these sites. The coastal dunes at Texel have a much larger area over which *A. arenaria* gradually becomes degenerate and the transition of vigour is therefore not as abrupt as at Haringvliet (Van der Putten *et al*. 1989). The extended vegetation succession at Texel also allows the spatial separation of *C. epigejos* from different stages of the *A. arenaria* degeneration sequence. At Haringvliet, the distance over which plant succession occurs is greatly reduced and degenerating stands of *A. arenaria* occur in close proximity to *C. epigejos*. The distribution of *H. arenaria/avenae* and *H. hordecalis* seems, therefore, to be correlated with abiotic environmental dynamics rather than particular host species. This initial study shows that *H. arenaria* may be better adapted to mobile dunes with regular influxes of wind-transported beach sand, but is succeeded by *H. hordecalis* in more stable areas, where sand deposition is lower. A single cyst originating from degenerating *A. arenaria* at Haringvliet (Fig. 4, lane 17) had a PCR-SSCP profile identical to *H. mani*.

Presence of extra bands

SSCP analysis of a PCR product, originating from a single cyst, was expected to give rise to two single bands, however, this seldom occurred. The majority of SSCP bands showed the presence of additional less intense bands. There are a number of possible reasons for their presence: samples may have been incompletely denatured or partially renatured prior to loading, there may be multiple sequences present (the result of hybridization, polymorphic PCR target sequences or heterozygous loci) or the single strands may have formed metastable conformers. Incomplete denaturation/partial renaturation were considered to be unlikely for several reasons. The foremost being that the presence of extra bands was reproducible between PCRs, different samples of the same species and between gels.

Because the denaturing conditions used in the PCR to obtain the samples for SSCP were 55 s at 96 °C, the time used to denature the samples (in a high percentage of formamide) for SSCP was considered adequate for complete denaturation and undenatured samples were found to migrate much faster through the gels in control experiments and were usually electrophoresed off the bottom. In addition, the denaturing and loading conditions were rigorously reproduced from gel to gel, with samples being cooled immediately after heat denaturation in wet ice and loaded rapidly through cold (4 °C) buffer. Faint additional bands were attributed to the presence of metastable conformers (Zehbe *et al*. Pharmacia Application Note 384). These are identical in sequence to those of the primary bands but have an alternative conformation which affects their mobility relative to the primary conformer. Metastable

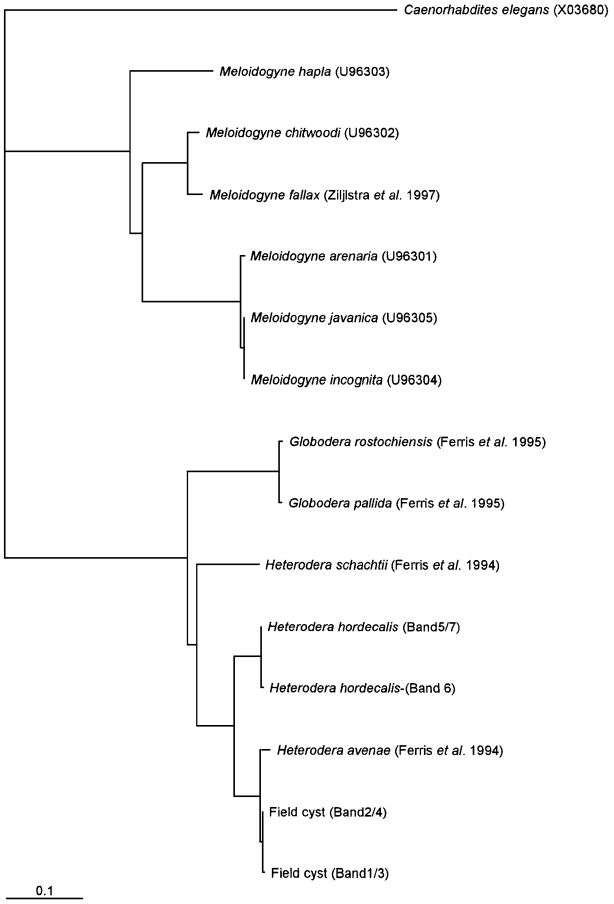

Fig. 5 Phylogenetic tree showing the relationships of the excised bands with sequences of related species. Accession nos for the bands are: field-collected *Heterodera arenaria*, bands 1/3 (AF239233), bands 2/4 (AF239234). *H. hordecalis*, bands (5/7 AF239235) and band 6 (AF239236).

confomers were, therefore, considered to be the most likely explanation for fainter bands but stronger bands merited further investigation.

The presence of multiple sequences was a distinct possibility, particularly where bands of equal intensity were observed. Analysis of ITS2 regions of *H. avenae* (Subbotin *et al*. 1999) revealed the presence of two ITS2 types (A and B). Type A being European and B from an Indian population. However, both types were detected in three French populations. To address the possibility of multiple ITS2 sequences indicated by additional PCR-SSCP bands, seven were excised from *H. avenae* and *H. hordecalis* SSCP patterns (indicated by arrows, Fig. 4), re-amplified and sequenced. A phylogenetic tree showing the relationship of these sequences in relation to ITS2 sequences of related nematodes, is shown in Fig. 5. The sequencing data indicated that two ITS2 sequences were present in both *H. arenaria* and *H. hordecalis* cysts. The difference was minor, a single base, but considering the overall conservation of ITS sequences in these nematodes reported previously, is nevertheless significant. The presence of multiple sequences has been inferred from the RFLP patterns of other nematodes (Zijlstra *et al*. 1995). The presence of additional bands did not, however, affect the ability of SSCP to effect an identification.

Conclusions

This work was initiated to develop a method that would enable the identification of larval and adult cyst and root-knot nematodes for ecological studies in natural ecosystems. It is clear from the success of the field investigation, that a single PCR primer pair used in conjunction with SSCP across a variable region has major diagnostic potential for nematodes. SSCP profiles were reproduced from different cysts and larvae of the same species, different PCR amplifications and on different gels. The primers were found to amplify well from all species tested within the three genera in this investigation in addition to *Rotylenchus* and *Filenchus* spp. (not shown). This suggests that the primers may be suitable for several genera allowing the application to be widened. Because the technique can utilize broad specificity primers it is likely that cryptic species could be detected if encountered. Band position alone allowed identification when nematodes of known identity were available for comparison, however, where profiles could not be matched to controls, bands could be excised and sequenced. PCR-SSCP is simpler to perform, broader in application and more economic in terms of time and resources than many other techniques. From the viewpoint of ecological investigation and plant protection, the use of this sensitive and highly discriminatory PCR-based technique allows rapid and routine identification of a broad range of species with minimal resources and development time.

Acknowledgements

This work was funded by the NIOO-CTO stimulation fund supporting J. P. Clapp from the KNAW, The Netherlands. Many thanks to Gerrit Karssen, Jan van Bezooijen, Janet Rowe, D. Sturhan and Henk Duyts for providing or helping to obtain the nematode isolates used in this study. NIOO publication number 2629.

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The Department of Plant–Micro-organism Interactions research programme focuses on the interactions between plant species and soil microorganisms in natural terrestrial ecosystems. The current work was carried out as part of 2-year postdoctoral research programme for J.P. Clapp and is being used to assist in the investigation of cyst nematode populations associated with marram grass in Dutch coastal dune ecosystems.