Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region

S. Bekal, J.P. Gauthier, and R. Rivoal

Abstract: This study examined the restriction polymorphism (RFLP) of the nuclear ribosomal DNA in *Heterodera avenae, H. filipjevi, H. mani, H. latipons*, and the taxonomically unclear Gotland strain in order to establish a molecular characterization and phylogenetic relationships in the complex of cereal cyst nematodes (CCN). The internal transcribed spacer (ITS) and 5.8S rDNA were amplified by PCR from a single female or a cyst of 27 different geographic isolates of the CCN complex and one population of *H. schachtii*, used as outgroup. The amplified product was 1.2 kb long and 14 of 15 enzymes produced restriction fragments for each isolate. Relationships between populations were determined from UPGMA analysis based on distance values calculated from RFLP data. Digestions with *Taq*I clearly differentiated *H. avenae*, *H. latipons*, and a group composed of *H. filipjevi* and the Gotland strain. Six endonucleases (*Hae*III, *Hin*fI, *Ita*I, *Pst*I, *Taq*I, and *Tru*9I) produced the same restriction pattern with *H. filipjevi* and the Gotland strain, and both were clearly separated from *H. avenae* with *Pst*I. Restriction sites have revealed a mixture of the species *H. latipons* and *H. avenae*, and possible infraspecific variation in *H. avenae*. The inferred phylogenetic relationships of species in the CCN complex are in agreement with their morphological characterization.

Key words: cereal cyst nematodes, *Heterodera avenae*, PCR, RFLP, ribosomal diversity.

Résumé : L'étude a examiné les profils de restriction de l'ADN ribosomique nucléaire chez *Heterodera avenae, H. filipjevi, H. mani, H. latipons* et la race Gotland dont la classification est indéterminée pour établir une caractérisation moléculaire et les relations phylogénétiques dans le complexe des nématodes à kystes des céréales (CCN). Les régions intercalaires transcrites ITS et le gène 5,8S ont été amplifiés par PCR à partir de femelles blanches ou de kystes individuels de 27 populations d'origine géographique distincte pour le complexe (CCN) et d'une population d'*H. schachtii*. Le produit amplifié est d'une longueur de 1,2 kb et 14 sur 15 enzymes testées ont donné un profil de restriction pour chaque isolat. Les relations entre les populations ont été déterminées avec l'analyse UPGMA basée sur les distances génétiques calculées à partir des sites de restriction. Les digestions avec *Taq*I différencient clairement *H. avenae, H. latipons* et un groupe comprenant *H. filipjevi* et la race Gotland. Six endonucleases (*Hae*III, *Hin*fI, *Ita*I, *Pst*I, *Taq*I and *Tru*9I) produisent le même profil de restriction pour *H. filipjevi* et la race Gotland et les deux entités sont clairement differenciées d' *H. avenae* avec *Pst*I. Les sites de restriction ont révélé un mélange d'espèces entre *H. latipons* et *H. avenae* ainsi qu'une possible variation infraspécifique chez *H. avenae*. Les relations génétiques dans le complexe de nématodes à kystes des céréales sont en accord avec la caractérisation morphologique.

Mots clés : nématodes à kystes des céréales, *Heterodera avenae*, PCR, PLFR, diversité ribosomique.

Introduction

In the genus *Heterodera*, the cereal cyst nematodes represent a complex of species and populations that may reflect coevolution with a large range of wild and cultivated Gramineae species (Stone and Hill 1982). This complex includes at least nine formal species: *Heterodera avenae* Wollenweber, *Heterodera bifenestra* Cooper, *Heterodera filipjevi* Madzhidov, *Heterodera hordecalis* Andersson, *Heterodera iri* Matthews, *Heterodera latipons* Franklin, *Heterodera mani* Mathews, *Heterodera spinicauda* Wouts et al., and *Heterodera turcomanica* Kirjanova & Shagalina (Wouts et al. 1995). Several of these species are economically important and *H. avenae* limits cereal production in Europe, Australia, and North America (Ritter 1982; Rivoal and Cook 1993). *Heterodera avenae* also shows a wide distribution and a large genetic infraspecific variability, including many pathotypes (Andersen and Andersen 1982). Recently, on the basis of protein patterns revealed by two-dimensional polyacrylamide gel electrophoretic (2-D PAGE) analysis, isolates of the Gotland strain, also called pathotype 3, and *H. avenae* sensu stricto were shown to have a genetic distance generally found in interspecific relationships (Ferris et al. 1994*a*; Bossis and Rivoal 1996).

Several morphological studies have identified criteria for characterization of these species. The main taxonomic characters are the size and shape of cyst, the vulval slit length, the

Corresponding Editor: D. Zickler.

Received September 2, 1996. Accepted March 18, 1997.

S. Bekal, J.P. Gauthier, and R. Rivoal.[1] Institut national de la recherche agronomique, Centre de recherches de Rennes, Laboratoire de zoologie, B.P. 29 35653 Le Rheu Cédex, France.

[1] Author to whom all correspondence should be addressed (e-mail: rivoal@rennes.inra.fr).

Table 1. Origin of the cereal and beet cyst nematode populations used in this study.

Species	Location	Country	Isolate number	Source[a]
Heterodera avenae	Sidi Hosni	Algeria	E43	F. Labdelli, INA, El Harrach, Algeria
	Tiaret	Algeria	E41	F. Labdelli
	Dahmouni	Algeria	E42	F. Labdelli
	Villasavary	France	Fr1	R. Rivoal, INRA, Le Rheu, France
	St. Georges du Bois	France	Fr2	R. Rivoal
	Argentan	France	Fr3	R. Rivoal
	Nuisement/Coole	France	Fr4	R. Rivoal
	Santa Olalla	Spain	E48	M. Romero, CSIC, Madrid, Spain
	Meknès	Morocco	E46	G. Caubel, INRA, Le Rheu, France
	Nir Oz	Israel	E57	Y. Spiegel, The Volcani Center, Bet Dagan, Israel
	South Australia	Australia	E50	J.M. Fisher, University of Adelaide, Australia
	Hadco	Saudi Arabia	E92	R. Cook, IGER, Aberystwyth, Great Britain
	Taaken	Germany	E86	D. Sturhan, Institut für Nematologie, Münster, Germany
	Najafgarh	India	E83	K.K. Kaushal, IARI, New Dehli, India
Gotland strain	Etelhem	Sweden	E55	A. Ireholm, Swedish University of Agricultural Sciences, Alnarp, Sweden
	Torralba de Calatrava	Spain	E49	M. Romero
	Karnobat	Bulgaria	A26	D. Stoyanov, Plant Protection Institute, Kostinbrod, Bulgaria
Heterodera filipjevi	Puschkin	Russia	E88	A.A. Subbotin, Academy of Sciences, Moscow, Russia
	Merkez	Turkey	E89	D. Sturhan
Heterodera mani	Hamminkeln	Germany	E87	D. Sturhan
	Morfa Maur (Np409)	Great Britain	A35	R. Cook
	Morfa Maur (Np414)	Great Britain	A36	R. Cook
	St. Germain	France	E54	H. Marzin, LNPV, Nématologie, Le Rheu, France
Heterodera latipons	Gilat	Israel	E69	Y. Spiegel
	Breda	Syria	E100	U. Scholz, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany
	Boueider	Syria	E101	U. Scholz
	Sétif	Algeria	E110	A. Mokabli, INA, El Harrach, Algeria
Heterodera schachtii	Laon	France	ESh	G. Caubel

[a]INA, Institut national agronomique; INRA, Institut national de la recherche agronomique; CSIC, Consejo Superior de Investigaciones Cientificas; IGER, Institute of Grassland and Environmental Research; IARI, Indian Agricultural Research Institute, India; LNPV, Laboratoire national de la protection des végétaux.

fenestral shape and the presence or absence of bullae and underbridge in the vulval cone, and the length of second stage juveniles and the shape of their stylet knobs (Mulvey 1972; Mulvey and Golden 1983; Wouts and Weischer 1977; Cook 1982). However, the great variability of these characters makes taxonomic discrimination inconsistent and time consuming. Confusion could also arise from mixed species populations as these nematodes have similar host plants.

The molecular approach has consistently improved identification and systematics in plant parasitic nematodes through evaluation of genetic polymorphism expressed through native or denaturing proteins and extrachromosomal or nuclear DNA (Burrows 1990; Abad 1994). The analysis of nuclear ribosomal DNA has been investigated in systematic studies of numerous organisms and is suited for separation of populations at or below the species level (Vrain et al. 1992; Ferris et al. 1994*b*). The rDNA cistron is repeated and highly conserved, allowing the use of heterologous probes or primers in species where the genetic knowledge of the genome is very poor. It contains two internal transcribed spacer regions (ITS1 and ITS2) located between the 18S and 26S genes, and is thought to be more variable (Ibrahim et al. 1995; Chen 1992). Nasmith et al. (1996) have described the advantages of combined PCR–RFLP (restriction fragment length polymorphism) analysis of the rDNA locus using minute amounts of biological organisms.

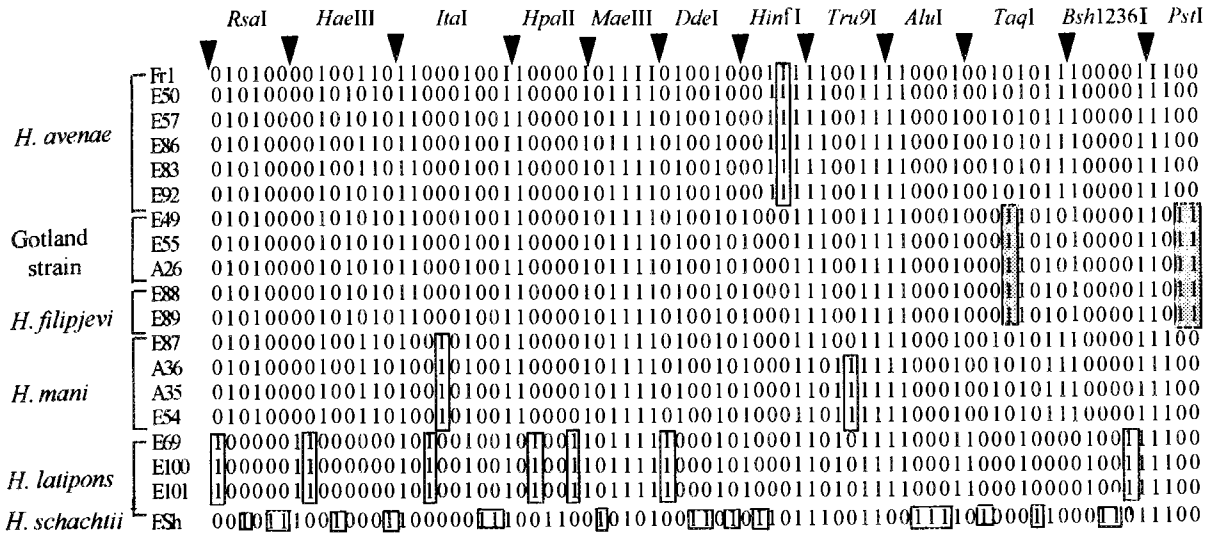
Using PCR to amplify ITS1 and ITS2, and endonucleases to produce RFLPs, we aimed to characterize molecular variation and to infer phylogenetic relationships among a range of populations of the four main species, *H. avenae*, *H. filipjevi*, *H. latipons*, and *H. mani*, and to explore their relationships to the Gotland strain.

Materials and methods

Nematode preparation

The populations originated from 15 countries in different geographical and climatic areas of the world. Details of nematode species and population location and numbers are given in Table 1. Special attention was paid to infraspecific variation in *H. avenae* by including 14 populations. The other populations were the Gotland strain (3 populations), *H. filipjevi* (2), *H. mani* (4), *H. latipons* (4). One population of the beet cyst nematode *Heterodera shachtii* Schmidt was used as an outgroup. The nematode populations were analyzed directly from either cysts or white females produced on wheat, cv. Arminda,

Fig. 1. Polymorphic RFLP markers of the ribosomal ITS region observed for 19 *Heterodera* populations inferred from *Rsa*I, *Hae*III, *Ita*I, *Hpa*II, *Mae*III, *Dde*I, *Hin*fI, *Tru*9I, *Alu*I, *Taq*I, *Bsh*1236I, and *Pst*I. The presence of a band is coded by a 1 and its absence by a 0. The white boxes correspond to specific markers; grey boxes denote markers common to both *H. filipjevi* and the Gotland strain.



cultivated in a sand–kaolin mixture in a controlled growth chamber (16 ± 1°C, 18 h light) (Jahier et al. 1996). White females were washed from roots under tap water and recovered on a 250 µm aperture sieve. They were then picked under a stereomicroscope and stored at −70°C. On average, 30 cysts or white females were individually analyzed for each population.

DNA extraction

Total genomic DNA was extracted using a modified version of the method described by De Jong et al. (1989) and Caswell-Chen et al. (1992) and then amplified by PCR. Each nematode was washed in distilled water, squashed in 90 µL of lysis buffer (0.1 M Tris–HCL, 50 mM EDTA, 1% SDS, plus 0.17 M NaCl (pH 8.0), and set overnight with 10 µL of proteinase K (5 µg/µL) at 37°C. The DNA was purified with phenol – chloroform – isoamyl alcohol (25:24:1), precipitated with isopropanol (90%), washed with ethanol (70%), and then dried and resuspended in 5.3 µL Tris–EDTA buffer (pH 8.0). The DNA yields, evaluated by spectrodensitometry, were approximately 2.5 ng per female. DNA samples were stored at 4°C prior to amplification.

PCR

A pair of 21-mer primers localized at the extremities of the 18S and 26S ribosomal genes originally isolated from a *Xiphinema* library by Vrain et al.(1992), 5′-TTG-ATT-ACG-TCC-CTG-CCC-TTT-3′ and 5′-TTT-CAT-TCG-CCG-TTA-CTA-AGG-3′, were used to amplify the two internal transcribed regions, ITS1 and ITS2, and the 5.8S gene. These primers were synthesized by Eurogentec (Centre hospitalier régional universitaire, Laboratoire de biologie moléculaire, Angers, France).

Amplifications were carried out in 50 µL reaction volumes, containing 10× Taq buffer (Appligene), 0.44 mM $MgCl_2$, 0.25 µM each primer, 0.1 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 U Taq DNA polymerase, and 2.5 ng single white female or cyst DNA. Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler 480 programmed for 1 cycle of 30 s at 94°C; and 40 cycles of 1 min at 94°C, 1 min at 72°C, and 1 min at 55°C; followed by 5 min at 72°C. An aliquot of 10 µL from each reaction product was resolved by electrophoresis on 1.5% agarose gel in 1× TAE buffer (working solution from Sigma), stained with ethidium bromide, and visualized with UV light (260 nm).

Restriction endonuclease digestion

Fifteen restriction endonucleases (Boehringer Mannheim) were used: *Alu*I, *Bsh*1236I, *Dde*I, *Hae*III, *Hin*fI, *Hpa*II, *Ita*I, *Mae*I, *Mae*III, *Nde*II, *Pst*I, *Rsa*I, *Sau*3A, *Taq*I, and *Tru*9I. Digestions were carried out overnight in reaction mixtures containing 0.2 U of the enzyme and 10 µL of the amplification product, according to the manufacturer's recommendations. The DNA fragments were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide (0.5 µg/µL), and visualized under UV light. The length of each fragment was estimated using DNA markers (Boehringer Mannheim). There were four replicate digestions for each enzyme and isolate.

Statistical analysis

The patterns of DNA bands originating from RFLP within ITS fragments were compiled in a binary matrix (Fig. 1) coded as "1" or "0" when the band was present or absent, respectively. Only the polymorphic patterns inferred from endonuclease digestions were included in the matrix. However, eight populations of *H. avenae* were excluded from the matrix, as they showed the same RFLP patterns typical for the Fr1 isolate. The relationships among species and populations were studied using Nei's genetic distance, *d* (Nei 1979). Cluster analysis with the unweighted pair-group method using arithmetic averages (UPGMA) was applied using the NEIGHBOR program. Bootstrap analysis (Felsenstein 1985), using 1000 bootstrapped data sets, was performed to determine statistical consistency of the classification. Both analysis were achieved with PHYLIP software (J. Felsenstein 1993).[2]

Results

Extraction, amplification, and digestion

DNA was successfully extracted from white females and from cysts, provided these were full of eggs and had been moistened in water for 24 h. PCR amplification of the entire ITS

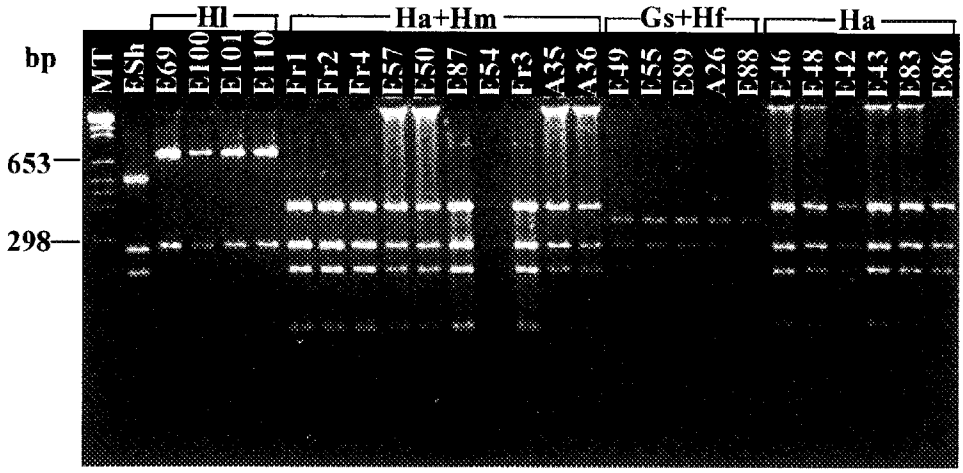
[2] J. Felsenstein. 1993. PHYLIP (phylogeny inference package). Version 3.5 c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Table 2. Associations of cereal and beet cyst nematode species according to their RFLP patterns in nuclear ribosomal DNA.

Restriction endonucleases	Species grouping			
*Hae*III, *Taq*I	Ha + Hm	Hl	Hf + Gs	ESh
*Hin*fI	Ha	Hl + Hf + Gs		ESh
*Ita*I, *Tru*9I	Ha + Hf + Gs	Hl	Hm	ESh
*Pst*I	Ha + Hl + Hm + ESh	Hf + Gs		

Note: Ha, *H. avenae*; Gs, Gotland strain; Hf, *H. filipjevi*; Hl, *H. latipons*; Hm, *H. mani*; and ESh, *H. schachtii*.

Fig. 2. Agarose gels of amplified DNA digested with *Taq*I for different populations of *H. avenae* (Ha), *H. filipjevi* (Hf), the Gotland strain, (Gs), *H. latipons* (Hl), *H. mani* (Hm), and *H. schachtii* (ESh). For isolate designations see Table 1; MT, DNA size marker.



region, including the 5.8S rDNA gene plus flanking areas of the 18S and 26S genes, generated only one fragment approximately 1.2 kb long. No variation in this fragment length was observed over all populations and species of cyst nematodes.

RFLP

The amplified rDNA region of all cyst nematode populations was digested by all endonucleases tested, except *Mae*I. Four to nine fragments were generated depending on the isolate and restriction enzyme. The polymorphism exhibited a clear differentiation between cereal cyst nematode species (Fig. 1).

Phenetic and phylogenetic analysis

The polymorphism that was recorded differentiated between groups of nematode populations and species (Table 2). The restriction patterns following digestion with *Taq*I (Fig. 2) and *Hae*III (Fig.1) clearly differentiated the populations into 3 groups: *H. avenae* plus *H. mani*; *H. latipons*; and *H. filipjevi* plus the Gotland strain; with *H. schachtii* as the outgroup. *Hin*fI differentiated *H. avenae* sensu stricto populations at two restriction sites, while a single site was observed for other groups (Fig.1). *Tru*9I (Fig. 3) and *Ita*I generated RFLP allowing a clear differentiation of *H. mani* (populations A35, A36, and E54) from *H. avenae*, *H. filipjevi*, and the Gotland strain isolates. With six endonucleases (*Hae*III, *Hin*fI, *Ita*I, *Pst*I, *Taq*I, and *Tru*9I), populations of the Gotland strain (E49, E55, and A26) produced the same RFLP pattern as the populations of *H. filipjevi* (E88 and E89). The differentiation of both *H. filipjevi* and Gotland strain populations from *H. avenae* was demonstrated by RFLP generated with *Pst*I (Fig. 4).

The genetic distance (d), calculated from the restriction data, ranged from 0.028 to 0.583 for the different populations in the CCN complex. The smaller genetic distances resulted from comparisons of *H. avenae* populations with E92 (Saudi Arabia), E50 (Australia), and E57 (Israel). The genetic distance increased progressively when *H. avenae* was compared with *H. filipjevi*, including the Gotland strain population (d = 0.159), *H. mani* (d = 0.114), and *H. latipons* (d = 0.403). The genetic dissimilarity between the grouping *H. filipjevi*, including the Gotland strain, and *H. mani* or *H. latipons* was 0.147 and 0.393, respectively. The greatest genetic distance, that between *H. schachtii* and the different graminaceous cyst nematodes, was expected, because it had been chosen as an outgroup species.

The dendrogram (Fig. 5) constructed from Nei's distances with UPGMA analysis revealed four main clusters and an entity A grouping linked specifically to the *H. avenae* cluster. Cluster I contained all populations of *H. avenae* originating from different countries. Cluster II contained four populations of *H. mani*. Cluster III contained populations of both

Fig. 3. Agarose gels of amplified DNA digested with *Tru*9I for different populations of *H. avenae* (Ha), *H. filipjevi* (Hf), the Gotland strain (Gs), and *H. mani* (Hm). For isolate designations see Table 1; MT, DNA size marker.

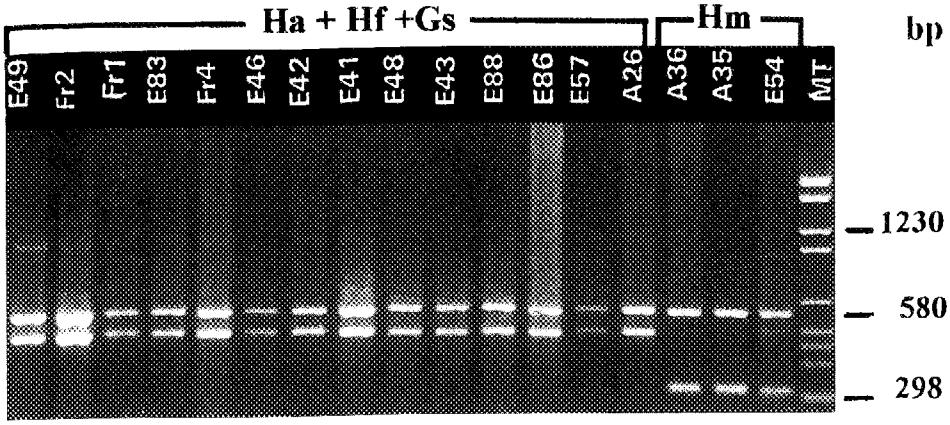
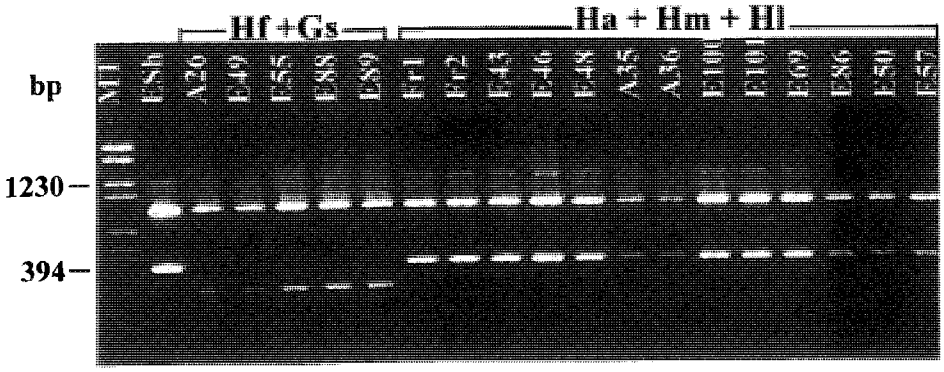


Fig. 4. Agarose gels of amplified DNA digested with *Pst*I for populations of *H. avenae* (Ha), *H. filipjevi* (Hf), *the* Gotland strain (Gs), *H. latipons* (Hl), *H. mani* (Hm), and *H. schachtii* (ESh). For isolate designations see Table 1; MT, DNA size marker.



H. filipjevi and the Gotland strain. Cluster IV grouped three populations of *H. latipons*. The entity A grouping contained populations originally recognized as *H. avenae* originating from Australia (E50), Israel (E57), and Saudi Arabia (E92).

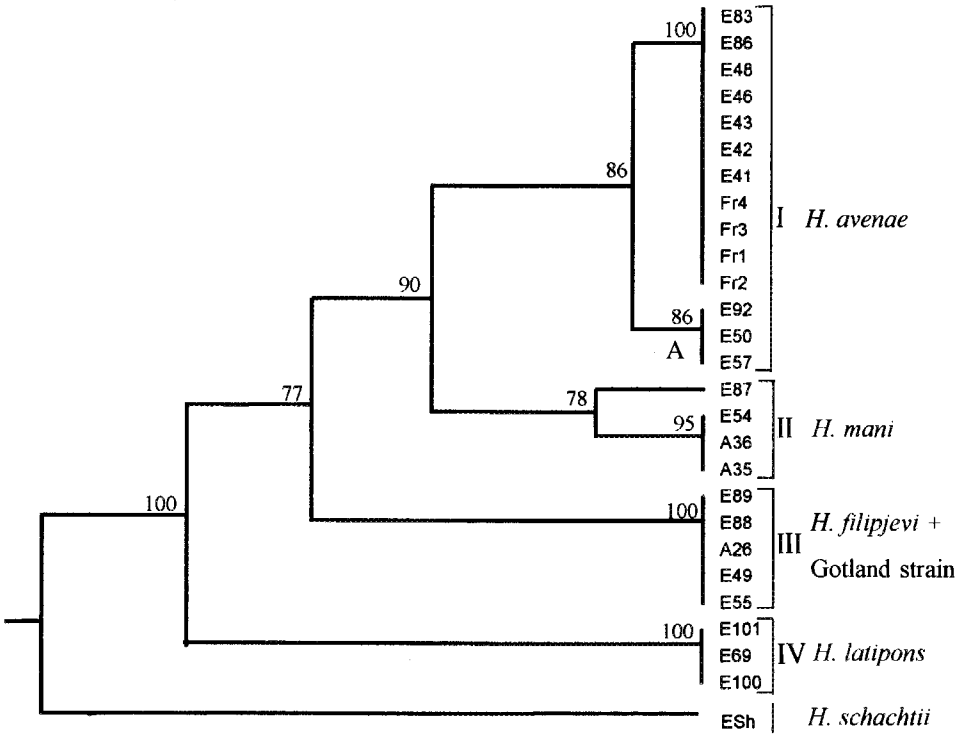
Bootstrap analysis confirmed clusters I, III, and IV with 100% confidence between populations within each grouping, and 95% confidence was demonstrated between populations within cluster II. The entity A grouping exhibited a lower stability (86%), probably resulting from a heterogeneous composition of populations. Connections between the four main clusters showed probabilities ranging from 77 to 100%. The genetic dissimilarity between *H. schachtii* and the farthest CCN, *H. latipons*, was demonstrated with 100 % confidence.

Discussion

The principal concern of this study was to clarify the taxonomic status among the main species of cyst nematodes encountered on cereal and wild graminaceous species. As already observed for three species of the stem nematode *Ditylenchus* (Wendt et al. 1993), the results confirm the usefulness of RFLPs of amplified nuclear ribosomal DNA for verifying the relationships among populations from a complex of species. On the basis of the partial sequences obtained from ITS DNA by PCR, Ferris et al. (1993) demonstrated that ITS1 and ITS2 were highly conserved among three cyst nematode species of the *H. schachtii* group. Full sequence data showed large differences between *H. avenae* sensu stricto, the Gotland strain, and *H. schachtii* (Ferris et al. 1994*b*).

Compared with other phylogenetic studies with RFLPs of DNA, the number of restriction endonucleases used here was low. Chosen on the basis of results with species of the potato cyst nematode (PCN) complex (Thiery and Mugniery 1996), the restriction endonucleases (except for only one, *Mae*I) revealed a sound level of molecular polymorphism useful for differentiating species or populations. Each of these graminaceous cyst nematode entities was differentiated with at least two endonucleases and no fragment polymorphism was observed between *H. filipjevi* and the Gotland strain. The 800-bp band obtained after digestion with *Pst*I was common to all samples tested, including very distinct species like *H. avenae* and *H. schachtii*, and confirmed results previously published (Lasserre et al. 1996). In contrast, the second restriction site revealed with this restriction enzyme was specific to *H. filipjevi*, including the Gotland strain, and differentiated this grouping from *H. avenae* sensu stricto (Fig. 4).

Fig. 5. UPGMA dendrogram constructed from Nei's genetic distance estimates for 27 populations of cereal cyst nematodes and the beet cyst nematode, *H. schachtii*. Bootstrap values (%) based on 1000 resamplings are given on appropriate clusters. See Table 1 for population numbers.



Our results established clear genetic dissimilarities between *H. avenae*, *H. filipjevi*, and *H. latipons*. They distinguished *H. mani*, which also shows the most ambiguous morphological differences, from *H. avenae* (Cook 1982). They also confirm the genetic differentiation of the Gotland strain populations from *H. avenae*, already established from 2-D PAGE protein patterns (Ferris et al. 1994*a*; Bossis and Rivoal 1996) and the complete sequence of the two ITSs and the 5.8S rDNA gene (Ferris et al. 1994*b*). López-Braña et al. (1996) used the RAPD (random amplified polymorphic DNA) technique to separate *H. avenae* from Gotland strain populations originating from Spain (Torralba de Calatrava = E49) and Sweden (Etelhem = E55). But the relatedness of the Gotland populations to *H. filipjevi* was unexpected, and constitutes valuable new information about phylogenetic relationships in graminaceous cyst nematodes. No genetic comparisons between the two groups of populations has previously been made. However, Valdeolivas and Romero (1990) demonstrated that morphological measurements of Gotland strain populations, such as the Spanish Torralba de Calatrava population, agreed with the description of *H. filipjevi* (Madzhidov 1981). Moreover, morphological studies and isoelectric focusing (IEF) of protein extracts demonstrated that in the former USSR, cereal cyst nematodes are essentially represented by *H. filipjevi* (Subbotin et al. 1996). Nevertheless, additional information on the potential for hybridization between *H. filipjevi* and the Gotland strain would be useful to confirm their species relationship.

The rDNA restriction site data have revealed increasing genetic distances between *H. avenae, H. filipjevi*, and *H. latipons*, which agrees with specific morphological characters (Cook 1982). In this complex of nematodes, congruence of genetic evolution with morphological changes had already been evoked from previous 2-D PAGE protein data (Bossis and Rivoal 1996). *Heterodera mani* also differed substantially from the other graminaceous cyst species. However, the E87 isolate, originally recognized as *H. mani*, differed significantly from other *H. mani* populations; but Rumpenhorst (1985) has also observed that populations of *H. mani* can differ from each other in their IEF protein patterns.

Wendt et al. (1993) also demonstrated that the rDNA digestions revealed infraspecific variation differentiating the polyploid giant race in stem nematode *Ditylenchus dipsaci* Filipjev. Infraspecific variation in *H. avenae* could be assessed with *Hae*III, which differentiates the populations E57, E50, and E92 originating from Israel, South Australia, and Saudi Arabia, respectively (Fig. 1). The feasibility of such an infraspecific variabilily for the three isolates could be ascertained, because for the nematodes analyzed, RFLPs were classified as the Gotland strain pattern with *Hae*III and as *H. avenae* for the others. E50, E57, and E92 (R. Cook, personal communication) partially overcome the resistance gene *Cre*1 from wheat cv. Loros, as does the Gotland strain (Andersen and Andersen 1982; Mor et al. 1992). For the South Australian population E50, the RFLP result was inconsistent with the 2-D PAGE protein data, which placed this isolate very close to two French populations of *H. avenae* (Bossis and Rivoal 1996). But Ferris et al. (1995) had assessed that, for nematodes, the relationships between 2-D PAGE protein patterns and rDNA ITSs are presently unclear. Nevertheless, the ITSs are known to be untranslated and consequently not related to virulence traits. It might also be hypothesized that the infraspecific dissimilarity exhibited by the E57, E50, and

E92 isolates of *H. avenae* sensu stricto could result from a common evolutionary process in diverse populations that could have been introduced afterwards in different countries or continents. It has been hypothesized that the most likely site of origin of *H. avenae* is with cereals in the Middle East, and that the nematode could have been introduced to Australia from northern Europe (J.M. Fisher, personal communication). For a given restriction endonuclease, the RFLP patterns of isolate E110, when compared with all others, were related to either *H. latipons* or *H. avenae*, according to the individual cyst analyzed. This observation might result from a mixture of *H. avenae* and *H. latipons*, which supposition was further established morphologically by A. Mokabli (personal communication). For this reason, the E110 isolate was excluded from the UPGMA analysis.

As previously demonstrated for migratory or encysted nematodes (Vrain et al. 1992; Ferris et al. 1993; Thiéry and Mugniéry 1996), the polymorphism in rDNA provides a powerful tool for reliable diagnostics and for analysis of evolutionary relationships between species in the cereal cyst nematode complex. According to our experience with the E110 isolate, RFLPs could be useful for detecting mixtures of species when restriction enzymes are used with amplified DNA extracted from a bulk of cysts and white females. A further application of this technique could be for identifying populations at the infraspecific level (pathotypes) by using more restriction endonucleases on the same and other extrachromosomal (mitochondrial) DNA (De Giorgi et al. 1994). This molecular approach would be worth repeating with less conserved nuclear DNA such as repeated sequences (Stratford et al. 1992). The present work is part of a research program that aims to improve our insight into the relationships between the graminaceous cyst nematodes, based on morphobiometrics, genetics, and virulence on bread and durum wheats.

Acknowledgements

The authors thank the people who assisted with the acquisition of the nematodes used in this study, and S. Baumann, R. Cook, J. Mallard, and J.C. Simon for reading and commenting on the manuscript.

References

Abad, P. 1994. Repetitive DNA in plant-parasitic nematodes: use for interspecific and intraspecific identification. *In* Advances in molecular plant nematology. *Edited by* F. Lamberti, C. De Giorgi, and D.McK. Bird. Plenum Press, New York. pp. 141–158.

Andersen, S., and Andersen, K. 1982. Suggestions for determination and terminology of pathotypes and genes for resistance in cyst-forming nematodes, especially *Heterodera avenae*. EPPO (Eur. Mediterr. Plant Prot. Organ.) Bull. **12**: 379–386.

Bossis, M., and Rivoal, R. 1996. Protein variability in cereal cyst nematodes from different geographic regions assessed by two-dimensional gel electrophoresis. Fundam. Appl. Nematol. **19**: 25–34.

Burrows, P.R. 1990. The use of DNA to identify plant parasitic nematodes. Nematological Abstracts, Vol. 59. Commonwealth Agricultural Bureaux International, Wallingford, U.K. pp. 1–8.

Caswell-Chen, E.P., Williamsom, V.M., and Wu, F.F. 1992. Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* population. J. Nematol. **24**: 343–351.

Chen, W. 1992. Restriction fragment length polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. Phytopathology, **82**: 1467–1472.

Cook, R. 1982. Cereal and grass hosts of some graminaceous cyst nematodes. EPPO (Eur. Mediterr. Plant Prot. Organ.) Bull. **12**: 399–411.

De Giorgi, C., Sialer, M.E., and Lamberti, F. 1994. The use of mtDNA for the identification of plant parasitic nematodes. *In* Advances in molecular plant nematology. *Edited by* F. Lamberti, C. De Giorgi, and D.McK. Bird. Plenum Press, New York. pp. 109–118.

De Jong, A.J., Bakker, J., Roos, M., and Gommers, F.J. 1989. Repetitive DNA and hybridization patterns demonstrate extensive variability between the sibling species *Globodera rostochiensis* and *G. pallida*. Parasitology, **99**: 133–138.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, **39**: 783–791.

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

Ferris, V.R., Faghihi, J., Ireholm, A., and Ferris, J.M. 1994*a*. Two-dimensional protein patterns of cereal cyst nematodes. Phytopathology, **79**: 927–933.

Ferris, V.R., Ferris, J.M., Faghihi, J., and Ireholm, A. 1994*b*. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. J. Nematol. **26**: 144–151.

Ferris, V.R., Miller, L.I., Faghihi, J., and Ferris, J.M. 1995. Ribosomal DNA comparisons of *Globodera* from two continents. J. Nematol. **27**: 273–283.

Ibrahim, S.K., Perry, R.N., Burrows, P.R., and Hooper, D.J. 1995. Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. J. Nematol. **26**: 412–421.

Jahier, J., Tanguy, A.M., Abelard, P., and Rivoal, R. 1996. Utilization of deletions to localize a gene for resistance to the cereal cyst nematode, *Heterodera avenae*, on an *Aegilops ventricosa* chromosome. Plant Breed. **115**: 282–284.

Lasserre, F., Gigault, F., Gauthier, J.P., Henry, J.P., Sandmeier, M., and Rivoal, R. 1996. Genetic variation in natural populations of the cereal cyst nematode (*Heterodera avenae* Woll.) submitted to resistant and susceptible cultivars of cereals. Theor. Appl. Genet. **93**: 1–8.

López-Braña, I., Romero, M.D., and Delibes, A. 1996. Analysis of *Heterodera avenae* populations by the random amplified polymorphic DNA technique. Genome, **39**: 118–122.

Madzhidov, A.R. 1981. New species *Bidera filipjevi* sp. nov. (Heteroderina: Tylenchida) from Tadzhikistan. [In Russian.] Izv. Akad. Nauk Tadzh. SSR Otd. Biol. Nauk, **83**: 40–44.

Mor, M., Cohn, E., and Spiegel, Y. 1992. Phenology, pathogenicity and pathotypes of cereal cyst nematodes, *Heterodera avenae* and *H. latipons* (Nematoda: Heteroderidae) in Israel. Nematologica, **38**: 494–501.

Mulvey, R.H. 1972. Identification of *Heterodera* cysts by terminal and cone top structures. Can. J. Zool. **50**: 1277–1292.

Mulvey, R.H., and Golden, A.M. 1983. An illustrated key to the cyst-forming genera and species of Heteroderidae in the western hemisphere with species morphometrics and distribution. J. Nematol. **15**: 1–59.

Nasmith, C.G., Speranzini, D., Jeng, R., and Hubbes, M. 1996. RFLP analysis of PCR amplified ITS and 26S ribosomal RNA genes of selected entomopathogenic nematodes (Sternernematidae, Heterorhadditidae). J. Nematol. **28**: 15–25.

Nei, M., and Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A., **76**: 5269–5273.

Ritter, M. 1982. Importance des nématodes à kystes des céréales. EPPO (Eur. Mediterr. Plant Prot. Organ.) Bull. **12**: 307–316.

Rivoal, R., and Cook, R. 1993. Nematode pests of cereals. *In* Plant parasitic nematodes in temperature agriculture. *Edited by* K. Evans, D.L. Trudgill, and J.M. Webster. Commonwealth Agricultural Bureaux International, Wallingford, U.K. pp. 259–303.

Rumpenhorst, H.J. 1985. Comparative electrophoretic studies on the proteins of some cyst-nematodes from cereals and grasses. [In German.] *In* Beiträge zur Nematodenforschung, Institut für Nematologie 25 Jahre in Münster-Gievenbeck: Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem. pp. 64–74.

Stone, A.R., and Hill, A.J. 1982. Some problems posed by the *Heterodera avenae* complex. EPPO (Eur. Mediterr. Plant Proc. Oregan.) Bull. **12**: 317–320.

Stratford, R., Shields, R., Goldsbrough, A.P., and Fleming, C. 1992. Analysis of repetitive DNA sequences from potato cyst nematodes and their use as diagnostic probes. Phytopathology, **82**: 881–886.

Subbotin, S.A., Rumpenhorst, H.J., and Sturhan, D. 1996. Morphological and electrophoretic studies on populations of the *Heterodera avenae* complex from the former USSR. Russ. J. Nematol. **4**: 29–38.

Thiéry, M., and Mugniéry, D. 1996. Interspecific spacer rDNA restriction fragment length polymorphism in *Globodera* species, parasites of Solanaceous plants. Fundam. Appl. Nematol. **5**: 471–479.

Valdeolivas, A., and Romero, M.D. 1990. Morphometric relationships of some members of the *Heterodera avenae* complex (Nematoda: Heteroderidae). Nematologica, **36**: 292–303.

Vrain, T.C., Wakarchuk, D.A., Lévesque A.C., and Hamilton, R.I. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundam. Appl. Nematol. **15**: 563–573.

Wendt, K.R., Vrain, T.C., and Webster, J.M. 1993. Separation of three species of *Ditylenchus* and some host races of *D. dipsaci* by restriction fragment length polymorphism. J. Nematol. **25**: 555–563.

Wouts, W.M., and Weischer, B. 1977. A classification of fifteen species of Heteroderinae commonly occurring in Western Europe based on the larval characteristics. Nematologica, **23**: 289–310.

Wouts, W.M., Schoemaker, A., Sturhan, D., and Burrows, R. 1995. *Heterodera spinicauda* sp.n. (Nematoda: Heteroderidae) from mud flats in the Netherlands, with a key to the species of the *H. avenae* group. Nematologica, **41**: 575–583.