Plant Pathology (2001) 50, 270–279

Genetic relationships and isozyme variability in the *Heterodera avenae* complex determined by isoelectrofocusing

M. F. Andrés[a]*†, M. D. Romero[a], M. J. Montes[b] and A. Delibes[b]

[a]*Departamento de Protección Vegetal, Centro de Ciencias Medioambientales, Consejo Superior de Investigaciones Científicas (CSIC), Serrano 115 dpdo, 28006 Madrid; and* [b]*Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, Av. Complutense sn, 28040 Madrid, Spain*

Isozyme variability was assessed among the principal species of the cereal cyst nematode complex to complete and enhance the information provided by classical nematode systematics, in order to clarify inter- and intraspecific relationships within this complex. Twenty populations of cereal cyst nematodes (*Heterodera avenae*, *H. filipjevi*, *H. latipons* and *H. mani*) were compared by means of five different isoenzymatic systems (esterase, malate dehydrogenase, phosphoglucoisomerase, phosphoglucomutase and superoxide dismutase) using isoelectrofocusing (IEF) on the electrophoretic separation. The results are in agreement with previous morphological and biochemical characterizations, which established genetic diversity between the Gotland strain and *H. avenae* and identified the Gotland strain with *H. filipjevi*. Populations from Israel, all included in the *H. avenae* group, exhibited well-defined intraspecific dissimilarity. The highest degree of polymorphism was found in the *H. avenae* group for all five enzymatic systems studied. The *H. mani* population was also included in the *H. avenae* group by these isozyme analyses. Malate dehydrogenase, phosphoglucoisomerase and phosphoglucomutase isozymes, fractionated for the first time by IEF in the cereal cyst nematode complex, displayed a higher level of polymorphism than using conventional electrophoresis. Isoelectric focusing has proved to be a useful tool for detecting genetic diversity within and among species of the cereal cyst nematode complex and for taxonomic purposes.

Keywords: cereal cyst nematode, *Heterodera avenae* complex, isoelectrofocusing, isozymes, taxonomy

Introduction

The cereal cyst nematode complex, or *Heterodera avenae* complex, is formed by a group of *Heterodera* species and forms of subspecific rank, all parasites of cereals or grasses. These nematodes sometimes share the same habitat, and their taxonomic status is, in some cases, difficult to determine. The group is presently formed (apart from species not described or *inquirendae*) by 11 species: *H. arenaria*, *H. aucklandica*, *H. avenae*, *H. bifenestra*, *H. filipjevi*, *H. hordecalis*, *H. iri*, *H. latipons*, *H. mani*, *H. spinicauda* and *H. turcomanica*. Some of these species can be easily distinguished by morphological features, but differences are not always well defined in the group formed by *Heterodera avenae sensu stricto*, *H. aucklandica*, *H. iri*, *H. filipjevi* and *H. mani*. Populations that for many years were known as 'race 3', 'pathotype 3' or 'Gotland strain' of *H. avenae* are presently considered as belonging to *H. filipjevi* (Rumpenhorst *et al.*, 1996).

Protein electrophoresis was the first technique applied to identify and establish genetic variability of plant nematodes (Dickson *et al.*, 1971; Hussey *et al.*, 1972; Stone & Williams, 1974; Bossis, 1991; Bossis & Rivoal, 1996). Isozymes and their mobility variants (allozymes) separated on the basis of molecular weight or isoelectric point (isoelectrofocusing) have also been used in nematode identification (Esbenshade & Triantaphyllou, 1985, 1990; Pais & Abrantes, 1989; Ibrahim & Rowe, 1995; Molinari *et al.*, 1996; Andrés *et al.* 2000). Both techniques have also been applied to clarify the relationships within the *H. avenae* complex (Bergé *et al.*, 1981; Gillois *et al.*, 1981; Rumpenhorst, 1985; Ferris *et al.*, 1989, 1994; Romero *et al.*, 1996; Subbotin *et al.*, 1996).

Molecular biology techniques, such as RFLP, RAPD and rDNA-RFLP, offer a number of new approaches in nematode identification (Caswell-Chen *et al.*, 1992; Powers & Harris, 1993; Castagnone-Sereno *et al.*, 1994;

*To whom correspondence should be addressed.

†E-mail: mafay@ccma.csic.es

Accepted 24 September 2000.

Ferris *et al.*, 1994; López-Braña *et al.*, 1996; Sturhan & Rumpenhorst, 1996; Bekal *et al.*, 1997; Subbotin *et al.*, 1999).

The isoelectrofocusing (IEF) method is used in this paper to fractionate five different isozyme systems with the aim of contributing to the study of genetic variation among populations of *H. avenae* from different geographical origins and clarifying the relationships within the *Heterodera avenae* complex.

Materials and methods

Biological materials

Twenty populations of the cereal cyst nematode complex, from different geographical origins, were used in this study. The populations had been morphologically characterized as four different species (Table 1).

Single seedlings of susceptible wheat (*Triticum aestivum*) cv. Capa were planted in pots filled with 100 cm^3 sterilized soil and placed in a controlled growth chamber (16°C, 16 h light). After two days, they were inoculated with three cysts per pot, which had been previously kept in water at 5°C until second-stage juveniles (J_2) began to emerge. A minimum of 15 pots was used to reproduce each population. About three months later, white females were collected from the roots and frozen at −80°C, in groups of 20, until used in enzyme assays. The *H. avenae* populations 8 (from Israel) and 13 (from Switzerland) reproduced scantily and therefore could not be tested for all enzyme systems.

Electrophoresis and enzyme visualization

Five enzymatic activities were studied by isoelectric focusing (IEF) on a Flat Bed apparatus FBE 3000 (Pharmacia, Uppsala, Sweden): EST (esterase E.C.3.1.1.1), MDH (malate dehydrogenase E.C.1.1.1.37), PGI (phosphoglucoisomerase E.C.5.3.1.9), PGM (phosphoglucomutase E.C.2.7.51) and SOD (superoxide dismutase E.C.1.15.1.1).

Enzyme extraction was carried out as described by Esbenshade & Triantaphyllou (1985), with 20 females per 60 μL extraction solution, and the same homogenates were used for two enzymatic tests. Different activities were detected in the five enzyme systems thus different quantities from these extracts were applied for each enzyme test. A maximum of 25 μL was loaded for PGM isozyme, which showed the lowest activity. Extracts were stored at −80°C without loss of activity of specific isozymes. At least four different extractions were run by IEF in order to confirm the reproducibility of each isozyme pattern.

IEF was performed on 0·25-mm-thick, 125-mm-wide polyacrylamide gels (5%C, 3%T) (Hhjerten, 1962)

Table 1 Populations of cereal cyst nematodes used in this study

	Species	Geographic origin	Country	Source
1	*H. latipons*	Unknown	Germany	D. Sturhan, Biologische Bundesanstalt Institut für Nematologie, Münster
2	*H. mani*	Hamminkeln	Germany	D. Sturhan, Biologische Bundesanstalt Institut für Nematologie, Münster
3	*H. avenae*	Santa Olalla	Spain	M. D. Romero, Consejo Superior de Investigaciones Científicas, Madrid
4	*H. avenae*	El Arahal	Spain	M. D. Romero, Consejo Superior de Investigaciones Científicas, Madrid
5	*H. avenae*[a]	Belló	Spain	A. Valdeolivas, Consejo Superior de Investigaciones Científicas, Madrid
6	*H. avenae*	Arganda	Spain	M. D. Romero, Consejo Superior de Investigaciones Científicas, Madrid
7	*H. avenae*	Nir Oz	Israel	Mishael Mor, The Volcani Center, Bet-Dagan
8	*H. avenae*[b]*	Bet She'an	Israel	Mishael Mor, The Volcani Center, Bet-Dagan
9	Under discussion*	Sa'ad	Israel	Mishael Mor, The Volcani Center, Bet-Dagan
10	*H. avenae* (Fr 1)	Villasavary	France	R. Rivoal, Institut National de le Recherche Agronomique, Rennes
11	*H. avenae* (Fr 4) *	St. Cristophe le Jajolet	France	R. Rivoal, Institut National de le Recherche Agronomique, Rennes
12	*H. avenae*	Aberystwyth	UK	R. Cook, Institute of Grassland and Environmental Research, Wales
13	*H. avenae*[c]*	Changins	Switzerland	R. Valloton, Station Fédérale de Recherches Agronomiques, Changins
14	*H. avenae*	Taaken	Germany	D. Sturhan, Biologische Bundesanstalt Institut für Nematologie, Münster
15	*H. filipjevi*	Pushkin	Russia	S. A. Subbotin, Institute of Parasitology, Moscow
16	*H. avenae* (Gotland)	Harene	Sweden	A. Ireholm, Swedish University of Agricultural Sciences, Alnarp
17	*H. filipjevi**	Dorot	Israel	Mishael Mor, The Volcani Center, Bet-Dagan
18	*H. filipjevi*	Baimak	Russia	S. A. Subbotin, Institute of Parasitology, Moscow
19	*H. filipjevi*	Torralba de Calatrava	Spain	M. D. Romero, Consejo Superior de Investigaciones Científicas, Madrid
20	*H. avenae* (Gotland)	Etelhem	Sweden	A. Ireholm, Swedish University of Agricultural Sciences, Alnarp

[a]This population shares morphological characteristics with the Gotland race.
[b]Tested for EST only.
[c]Tested for SOD only.
All populations have been used in previous studies except those with *, in which the identification corresponds to the source.

containing ampholytes (Servalyt 3–7 for all enzymes except MDH, for which Servalyt 4–6 was used) and 10% glycerol. Electrode solutions were 1 M NaOH (cathode) and 0·1 M H_2SO_4 (anode). Gels were pre-focused for 500 volts h^{-1} and samples were loaded upon the gel using a silicone rubber tape with 3 mm wide wells laid approximately 10 mm from the anode. Gels were run at constant power (1 W cm^{-1}) and focusing was finished at 4000 volts h^{-1}. Isoelectric points (pIs) were ascertained by measuring pH with a surface electrode and the gels were then immediately stained.

EST and MDH activities were visualized using the method described by Esbenshade & Triantaphyllou (1985), PGI and PGM according to Huettel *et al.* (1983) and SOD following Neuman & Hart (1986). For all enzymes except EST, staining used the Agar Overlay Method were carried out as follows: an appropriate amount of low-melting agar was weighed out to provide a 1% concentration in the final staining solution. The agar was added to 3/4 of the staining solution buffer and this mixture heated to boiling. The agar solution was allowed to cool to 45°C. The other reagents of the staining solution and 1/4 of buffer were stirred in an ultrasonic bath at 2–4°C and then added to the agar and the mixture stirred thoroughly. The agar mixture was then poured over the gel in a staining dish to cover the entire surface. After the agar overlay solidified, the gel was incubated at 37°C for as long as required for band development (usually 30 min). Although the patterns can generally be seen quite well through the agar, this may be removed if desired when the gel is interpreted. After staining, the gels were fixed in 10% acetic acid. The zymograms were recorded as soon as bands became evident, because the bands tended to diffuse. Only enzyme profiles resulting from the same assay (same electrophoresis gel, volume of sample in each lane and reaction stain) were compared.

Data analysis

Data matrices from the enzyme patterns were formed by recording the presence or absence (1 or 0) of a particular band. Only reproducible and clearly marked bands were considered further, while faint bands were ignored. The computer program STATISTICAL version 4·5 was used for the analysis of the electrophoretic data. The dissimilarity matrices (Euclidean distance) were used to generate dendrograms by Ward's method (STATSOFT, 1994).

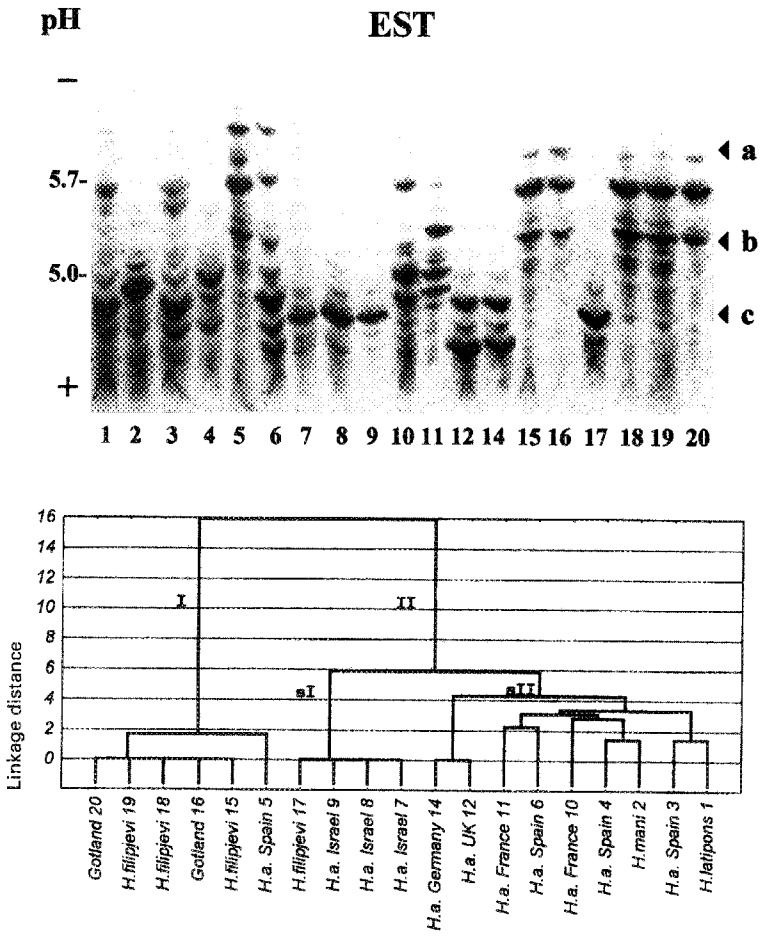

Figure 1 Upper panel: esterase zymograms obtained by IEF (3–7 ampholytes range) of 19 populations of the *Heterodera avenae* complex labelled by arabic numbers. Letters a to c show the EST enzyme bands that differentiate population groups. Lower panel: dendrogram showing the clustering performed by Ward's method and the percentage distance based on EST isozyme data. Roman numerals indicate the different groups and subgroups (preceded by 's') established by cluster analysis.

Results

The five isoenzymatic systems studied (EST, PGM, PGI, MDH and SOD) by means of IEF were consistently visualized and clear patterns were detected for all populations of the *Heterodera avenae* complex analysed. Better results were obtained by loading the samples approximately 1–2 cm from the anode rather than from the cathode and by using the Agar Overlay Method to stain, because the agar gel prevented the reactant from diffusing rapidly away from the site of the enzyme bands. The esterase patterns were very similar with and without agar, and consequently this method was discarded.

The five enzyme systems used in this study revealed a total of 45 different markers (electrophoretic bands): 13 for EST (Fig. 1), four for PGM (Fig. 2), 13 for PGI (Fig. 3), eight for MDH (Fig. 4) and seven for SOD (Fig. 5) isozymes.

A high degree of polymorphism in the populations was detected by EST zymograms. Based on three differential well-spaced isozyme bands, two main groups were established for this enzyme (Fig. 1). Group I, which included *H. filipjevi* (15, 18), Gotland strain (16, 19, 20) and Spanish (5) populations, was characterized by the presence of two isozyme bands (a and b). Group II was divided into two subgroups: the four populations from Israel (7, 8, 9, 17) formed subgroup I with the presence of the major c band and absence of a and b bands; subgroup II was heterogeneous, with at least six distinctive profiles detected.

The PGM zymograms showed high similarity among all populations (Fig. 2). *H. filipjevi*, Gotland strain and Spanish (5) populations formed a differentiated group based only on the absence of the a band.

The PGI isoenzymatic patterns identified two main isozyme groups (Fig. 3). Group I was identified by the presence of two close bands (a and b), and within this group *H. filipjevi* and Gotland strain populations formed a distinct subgroup with a common characteristic band pattern. Group II, characterized by the absence of a and b bands, presented enough variation to allow six different isozyme profiles to be distinguished.

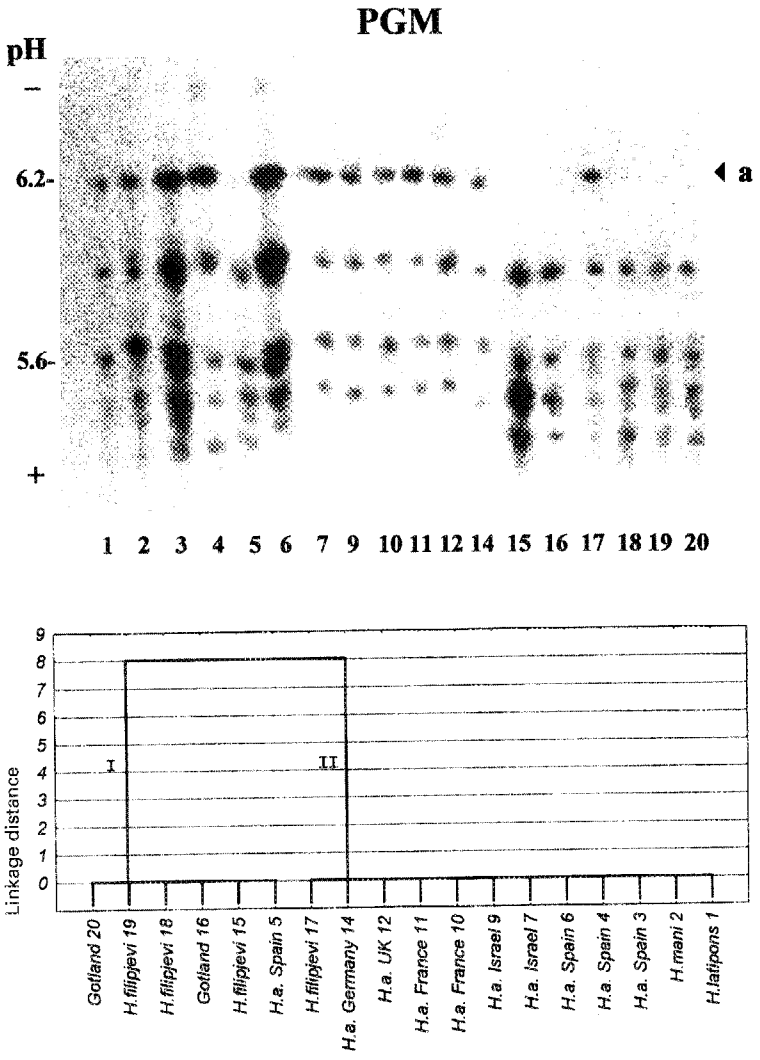

Figure 2 Upper panel: phosphoglucomutase zymograms obtained by IEF (3–7 ampholytes range) of 18 populations of the *Heterodera avenae* complex labelled by arabic numbers. The letter a shows the PGM enzyme band that differentiates population groups. Lower panel: dendrogram showing the clustering performed by Ward's method and the percentage distance based on PGM isozyme data. Roman numerals indicate the different groups established by cluster analysis.

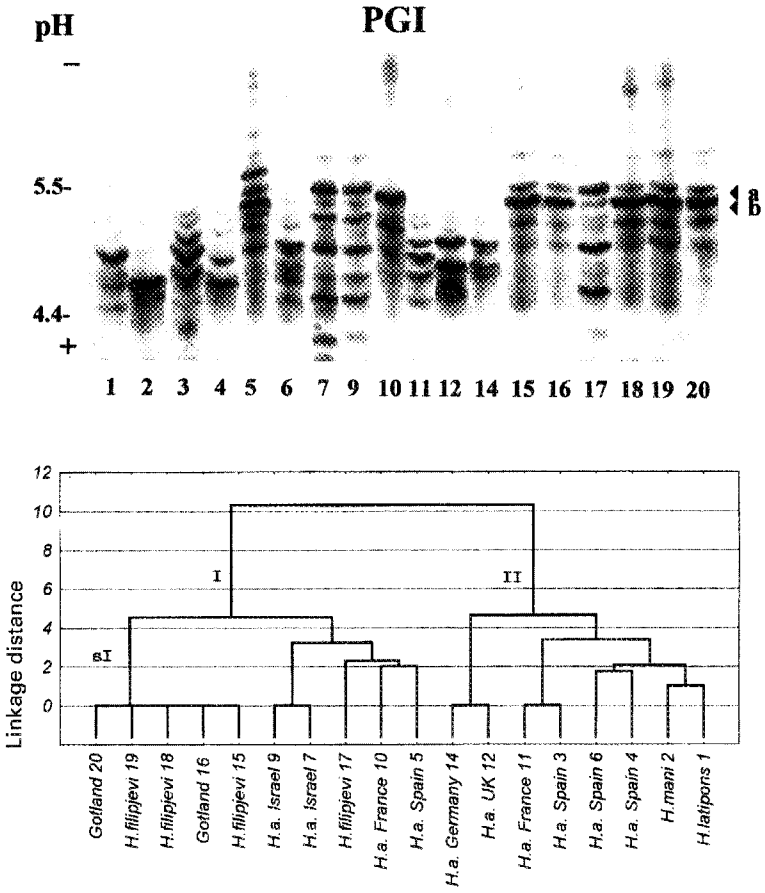

Figure 3 Upper panel: phosphoglucoisomerase zymograms obtained by IEF (3–7 ampholytes range) of 18 populations of the *Heterodera avenae* complex labelled by arabic numbers. Letters a and b show the PGI enzyme bands that differentiate population groups. Lower panel: dendrogram showing the clustering performed by Ward's method and the percentage distance based on PGI isozyme data. Roman numerals indicate the different groups and subgroups (preceded by 's') established by cluster analysis.

The MDH patterns (Fig. 4) showed many electrophoretic bands; however, a set of them (e), with very close pIs, was found to be common to all populations. Based on the presence of the b and d and the absence of the a and c isozyme bands, *H. filipjevi*, Gotland strain and Spanish (5) populations formed a different group resembling that established by means of EST isozymes.

Colourless bands indicating SOD activities, formed against a blue background (Fig. 5), clearly differentiated Group I formed by *H. filipjevi* and Gotland strain populations, which displayed only one band (c). Based on two differential isozyme bands, two distinct subgroups were detected among remaining populations (Group II). Subgroup I was identified by the absence of the *a* band and included pattern 1 with b and c bands and pattern 2 with only the b band. Subgroup II, characterized by the presence of the c band, showed more variability than subgroup I.

Cluster analysis on isozyme data from EST, PGM, PGI, MDH and SOD (Fig. 6) patterns grouped the 18 *H. avenae* complex populations into two major clusters. *H. filipjevi* (15, 18), Gotland strain (16, 19, 20) and Spanish (5) populations formed a differentiated cluster (I). Cluster II included the *H. avenae*, *H. latipons* and *H. mani* populations, and was divided into two subclusters. Populations from Israel (7, 9) formed a subcluster and the remaining populations (3, 4, 6, 10, 11, 12, 14, 17) and the species *H. latipons* and *H. mani* were grouped in a broad subcluster, which reflects its enzymatic pattern variation.

Discussion

The main purpose of this study was to contribute to the understanding of the relationships among the principal species of the cereal cyst nematode complex. The results confirm the usefulness of isozyme fractionation by isoelectrofocusing for investigating enzyme polymorphism and genetic diversity within nematodes species, as other authors have demonstrated for *H. glycines* (Radice *et al.*, 1988) and *Pratylenchus* spp. (Payan & Dickson, 1990; Andrés *et al.* 2000).

Polymorphism was detected in all systems analysed, especially in MDH, EST and PGI isozymes, and the highest degree was found in the *H. avenae* group. IEF isozyme patterns provided a higher numbers of bands in each system than conventional electrophoresis, thus increasing the level of polymorphism that could be detected and allowing more intra- and interspecific differences to be observed. SOD, MDH, EST and PGM isozymes had previously been fractionated by native polyacrylamide gel electrophoresis to compare two Spanish populations of *H. avenae*, giving patterns with only two bands as maximum (Romero *et al.*,

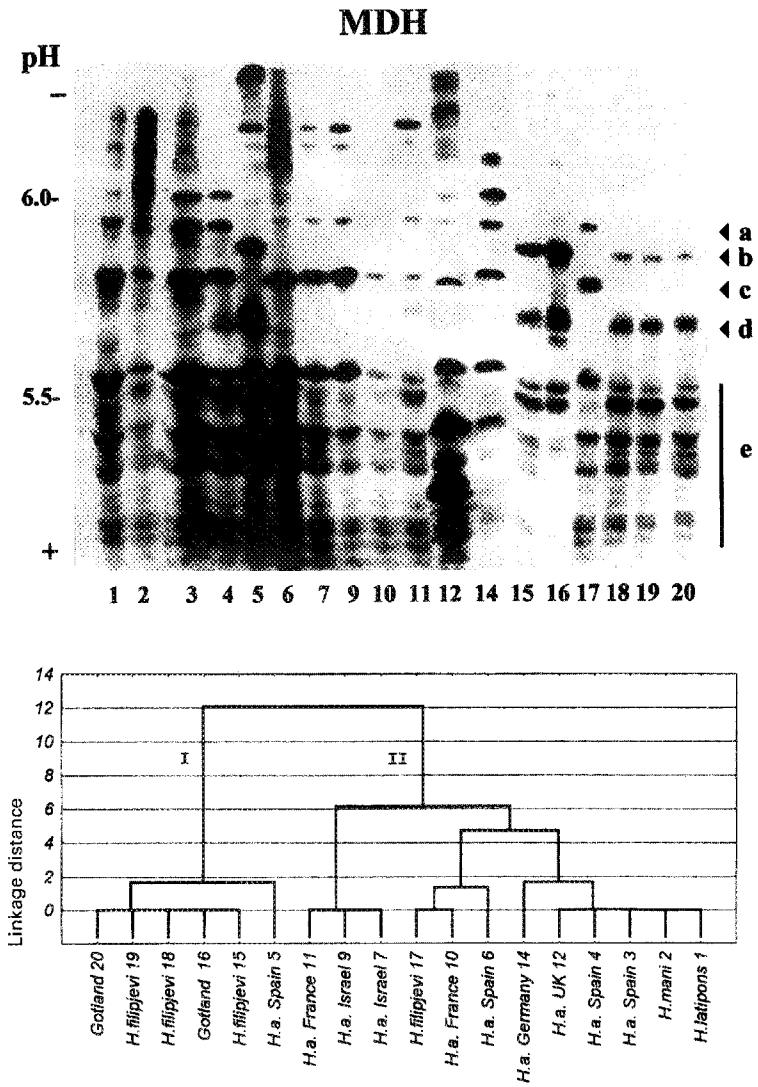

Figure 4 Upper panel: malate dehydrogenase zymograms obtained by IEF (4–6 ampholytes range) of 18 populations of the *Heterodera avenae* complex labelled by arabic numbers. Letters a to e show the MDH enzyme bands that differentiate population groups. Lower panel: dendrogram showing the clustering performed by Ward's method and the percentage distance based on MDH isozyme data. Roman numerals indicate the different groups established by cluster analysis.

1996). MDH, PGI and PGM isozymes have not previously been fractionated by IEF in the cereal cyst nematode complex. This is also the first time in *Heterodera* studies that the overlay technique, which increases the resolution for isozyme staining, has been used.

Analysis of the EST isozyme system by IEF as well as by conventional electrophoresis allowed the identification and separation of 19 *Heterodera* species (Nobbs *et al.*, 1992; Ibrahim & Rowe, 1995). However, these studies were restricted to analysis of interspecific variability, using only one population for each species. The present findings indicated that *H. filipjevi* (except population 17), Gotland strain and Spanish (5) populations could be identified by a specific EST phenotype. Likewise, a common characteristic EST pattern clearly distinguished *H. avenae* populations from Israel and those from other geographic origins. The level of variation in EST patterns observed here among *H. avenae sensu stricto* populations is as great as among other species (*H. latipons* and *H. mani*).

In the present study, PGM exhibited the least degree of polymorphism. However, the group formed by *H. filipjevi* (except population 17), Gotland strain and *H. avenae* (5) populations was clearly identified by the absence of only one allozyme. The remaining populations (including *H. latipons* and *H. mani*) showed a common phenotype.

The PGI and SOD enzyme systems showed the highest level of genetic variability among *H. avenae sensu stricto*, *H. latipons* and *H. mani* populations from various geographic locations. Nevertheless, both isozyme systems clearly differentiated a group formed by *H. filipjevi* (except population 17) and Gotland strain populations. Radice *et al.* (1988) had previously used PGI enzyme patterns to investigate intraspecific genetic variability in *H. glycines* and Molinari *et al.* (1996) had identified six *Heterodera* species by means of SOD isozyme patterns, although only one population of *H. avenae* was used.

MDH zymograms gave the greatest range of bands, many of which were intense and close to each other,

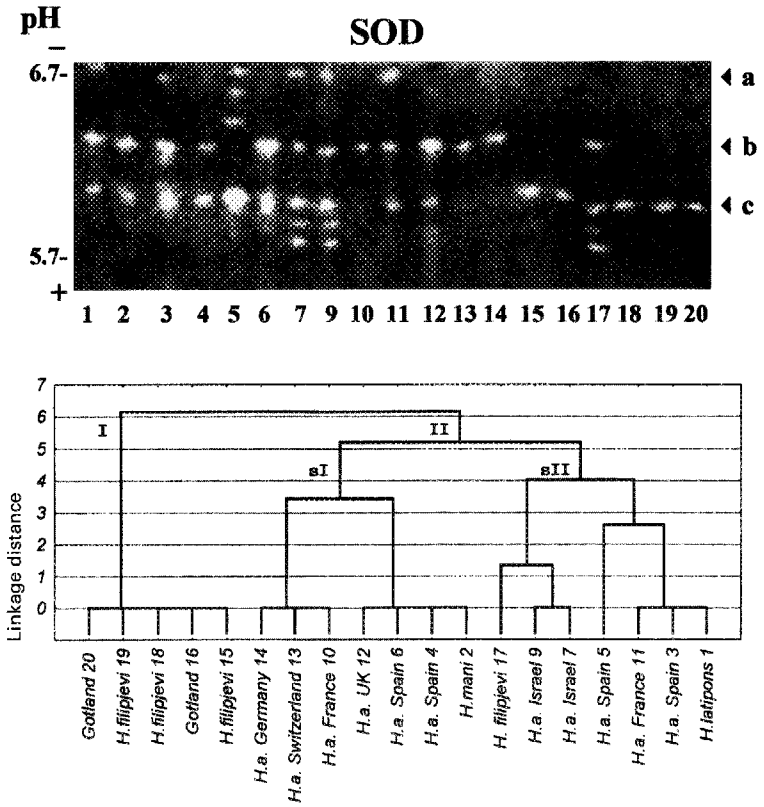

Figure 5 Upper panel: superoxide dismutase zymograms obtained by IEF (3–7 ampholytes range) of 19 populations of the *Heterodera avenae* complex labelled by arabic numbers. Letters a to c show the SOD enzyme bands that differentiate population groups. Lower panel: dendrogram showing the clustering performed by Ward's method and the percentage distance based on SOD isozyme data. Roman numerals indicate the different groups and subgroups (preceded by 's') established by cluster analysis.

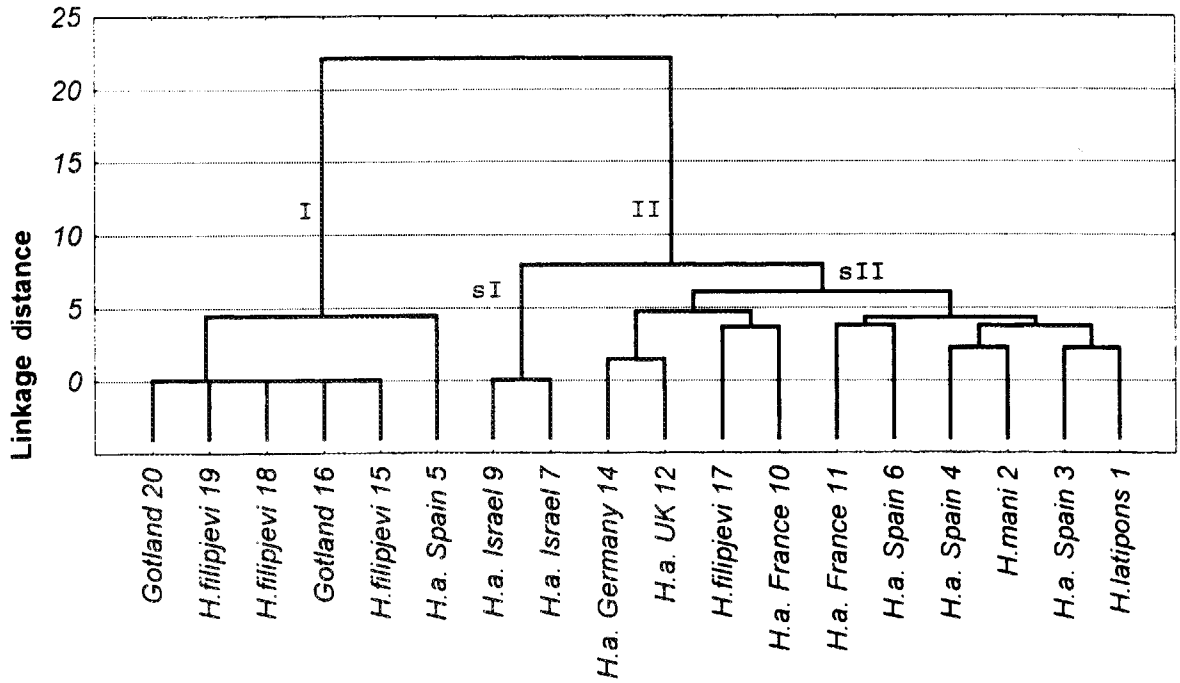

Figure 6 Dendrogram showing the clustering performed by Ward's method and the percentage distance between the clusters of 18 *H. avenae* complex populations, labelled by arabic numbers, derived from EST, MDH, PGI, PGM and SOD banding patterns analysed by IEF. Roman numerals indicate the different groups and subgroups (preceded by 's') established by cluster analysis.

making the evaluation of intraspecific MDH isozyme variability difficult. Nevertheless it is possible to differentiate a specific MDH pattern for the group constituted by *H. filipjevi*, Gotland strain and Spanish (5) populations.

Cluster analysis of 18 populations based on IEF-enzyme phenotypes of the five systems established clearly two main clusters. Cluster I contained *H. filipjevi* (except population 17), Gotland strain and Spanish (5) populations. This result confirms the genetic differentiation of Gotland strain populations from *H. avenae s. str.*, which had previously been demonstrated by biochemical (Ferris *et al.*, 1989; Bossis & Rivoal, 1996; Romero *et al.*, 1996) and molecular biology techniques (Ferris *et al.*, 1994; López-Braña *et al.*, 1996). Likewise, the present results identified Gotland strain with *H. filipjevi* populations, which would be in agreement with some previous (Valdeolivas & Romero, 1990) and recent (Sturhan & Rumpenhorst, 1996; Bekal *et al.*, 1997; Subbotin *et al.*, 1999) investigations. The presence of *H. filipjevi* (populations 19) in the Central Region of Spain is clearly in discordance with 'the main centre of distribution' of this species (Eastern European–Oriental region) hypothesized by Subbotin *et al.* (1999). Based on morphological characteristics, population 17 from Israel had been previously identified as belonging to the *H. filipjevi* group (Table 1); however, according to the enzyme characterization carried out in the present study, it belongs to the *H. avenae* group. Comparing these results with those of previous studies, the assignment of population 5 turned out to be uncertain. Morphological features (Valdeolivas, 1987) and IEF protein pattern (Sturhan & Rumpenhorst, 1996) showed that this population was close to Gotland race and *H. avenae sensu stricto*, respectively. However, molecular characterization (López-Braña *et al.*, 1996) and the data reported here suggest that population 5 is distinguishable from *H. avenae sensu stricto* and should be included in the *H. filipjevi* group.

Cluster II contained two groups, and included *H. avenae* populations from Israel in Group I. A similar intraspecific differentiation was found by RFLP analysis in the same *H. avenae* population originating from Israel (7) and in two other populations from South Australia and Saudi Arabia (Bekal *et al.*, 1997). It has been hypothesized that 'the intraspecific dissimilarity exhibited by the *H. avenae sensu stricto* populations group, could result from a common evolutionary process in diverse populations that could have been introduced afterwards in different countries or continents' (Bekal *et al.*, 1997). Group II revealed intraspecific variation in *H. avenae sensu stricto*, indicated by the high degree of polymorphism observed in the EST, PGI, MDH and SOD enzymatic systems. The substantial genetic differences shown by all populations belonging to *H. avenae sensu stricto* have also been demonstrated by 2D-PAGE protein (Bossis & Rivoal, 1996) and rDNA-RFLP (Bekal *et al.*, 1997; Subbotin *et al.*, 1999) analyses, which, according to Subbotin *et al.* (1999), confirms that a great number of genetic population types are included in the *H. avenae sensu stricto* group. Furthermore, the level of variation in enzyme patterns among *H. avenae* populations is similar to that among the other species populations (*H. latipons* and *H. mani*). The data obtained in this study by means of enzyme pattern analysis of *H. latipons* were inconsistent with the morphological (Franklin, 1969; Romero, 1980; Cook, 1982) and molecular (Bekal *et al.*, 1997; Subbotin *et al.*, 1999) characterizations of this species. Contamination in this specific case should not be discounted because all of the available specimens of *H. latipons* were used for the isozyme analysis and could not be compared morphologically afterwards. Based on morphological studies, *H. avenae* and *H. mani* looked very much alike (Mathews, 1971; Meagher, 1974; Romero, 1977; Cook, 1982); even Ebsary (1991) considered *H. mani* as a junior synonym of *H. avenae*. Ibrahim & Rowe (1995) also demonstrated, by means of conventional electrophoresis of esterases, a high similarity between *H. avenae* and *H. mani*. However, both species exhibited different IEF esterase phenotypes (Ibrahim & Rowe, 1995) and rDNA-RFLP patterns (Bekal *et al.*, 1997). Furthermore, intraspecific variation among *H. mani* populations has been demonstrated by means of both IEF protein (Rumpenhorst, 1985) and rDNA-RFLP (Bekal *et al.*, 1997) patterns. Taking into account these considerations, it might be concluded that, by IEF enzyme patterns, the *H. mani* population is not significantly differentiated from *H. avenae sensu stricto* populations. However, it would be interesting to extend this study by analysing *H. mani* populations from diverse geographical origins.

In summary, isoelectrofocusing in conjunction with certain enzyme-staining systems was successful in detecting interspecific variability, as well as being sensitive enough to detect polymorphism within *H. avenae sensu stricto* populations. This relatively cheap and simple technique allows the study of enzyme polymorphism and genetic diversity within and among species of the cereal cyst nematode complex. The generally good agreement found between morphological identifications and those produced using IEF-isozyme patterns also favours the applicability of this technique for taxonomic purposes.

Acknowledgements

The authors thank all persons who kindly supplied nematode populations, as listed in Table 1. Thanks are also given to A. Duce, M. Duque, G. Gago and J. Garcia for their technical assistance, and A. Redondo and I. López-Braña for critical reading of the manuscript. This work was supported by grant AGF98-1057-CO4 from the Comision Interministerial de Ciencia y Tecnologia of Spain.

References

Andrés MF, Pinochet J, Hernández-Dorego A, Delibes A, 2000. Detection and analysis of inter- and intraspecific diversity of

Pratylenchus species using isozyme markers. *Plant Pathology* **49**, 640–9.

Bekal S, Gauthier JP, Rivoal R, 1997. Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribe spacer region. *Genome* **40**, 479–86.

Bergé JB, Dalmasso A, Person F, Rivoal R, Thomas D, 1981. Isoestérases chez le nematode *Heterodera avenae*. I – Polymorphisme chez différentes races françaises. *Revue de Nématologie* **4**, 99–105.

Bossis M, 1991. Discrimination entre *Heterodera carotae* et *H. cruciferae* par l'électrophorese (SDS PAGE) de protéines solubles extraites à partir de kystes individualisés. *Revue de Nématologie* **14**, 325–7.

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

Castagnone-Sereno Ph, Vanlerberghe-Marutti F, Leroy F, 1994. Genetic polymorphism between and within *Meloidogyne* species detected with RAPD markers. *Genome* **37**, 904–9.

Caswell-Chen EP, Williamson VM, Wu FF, 1992. Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* populations. *Journal of Nematology* **24**, 343–51.

Cook R, 1982. Cereal and grass hosts of some gramineous cyst nematodes. *EPPO Bulletin* **12**, 399–411.

Dickson DW, Huisingh D, Sasser JN, 1971. Dehydrogenases, acid and alkaline phosphatases and esterases for chemotaxonomy of selected *Meloidogyne, Ditylenchus, Heterodera* and *Aphelenchus* spp. *Journal of Nematology* **3**, 1–16.

Ebsary BA, 1991. *Catalog of the Order Tylenchida*. Ottawa, Canada: Research Branch, Agriculture Canada, publication no. 1989/B.

Esbenshade PR, Triantaphyllou AC, 1985. Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* **17**, 6–20.

Esbenshade PR, Triantaphyllou AC, 1990. Isozyme phenotypes for the identification of *Meloidogyne* species. *Journal of Nematology* **22**, 10–5.

Ferris VR, Faghihi J, Ireholm A, Ferris JM, 1989. Two dimensional protein patterns of cereal cyst nematodes. *Phytopathology* **79**, 927–33.

Ferris VR, Faghihi J, Ireholm A, Ferris JM, 1994. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *Journal of Nematology* **26**, 144–51.

Franklin MT, 1969. *Heterodera latipons* n.sp. a cereal-cyst nematode from the Mediterranean region. *Nematologica* **15**, 535–42.

Gillois M, Chevalet C, Thomas D, Dalmasso A, 1981. Isoestérases chez le nematode *Heterodera avenae*. II. Modelisation appliquée à la structure génétique de deux populations. *Revue de Nématologie* **4**, 107–14.

Hhjerten S, 1962. Molecular sieve olnomatography on pslyacrylamide gels prepared according to a simplified method. *Archives of Biochemistry and Biophysics* (Suppl. 1), 147–51.

Huettel RN, Dickson DW, Kaplan DT, 1983. Biochemical identification of the two races of *Radopholus similis* by starch gel electrophoresis. *Journal of Nematology* **15**, 338–44.

Hussey RS, Sasser JN, Huisingh D, 1972. Disc electrophoretic studies on soluble proteins and enzymes of *Meloidogyne incognita* and *M. arenaria*. *Journal of Nematology* **4**, 183–9.

Ibrahim SK, Rowe J, 1995. Use of isoelectric focusing and polyacrylamide gel electrophoresis of nonspecific esterase phenotypes for the identification of cyst nematodes *Heterodera* species. *Fundamental and Applied Nematology* **18**, 189–96.

López-Braña I, Romero MD, Delibes A, 1996. Analysis of *Heterodera avenae* populations by the random amplified polymorphic DNA technique. *Genome* **39**, 118–22.

Mathews HJP, 1971.The new species of cyst nematode *Heterodera mani* n. sp. & *H. iri*. n. sp. from Northern Ireland. *Nematologica* **17**, 553–65.

Meagher JW, 1974. The morphology of the cereal cyst nematode (*Heterodera avenae*) in Australia. *Nematologica* **20**, 1–8.

Molinari S, Evans K, Rowe J, Russell M, 1996. Identification of *Heterodera* cysts by SOD isozyme electrophoresis profiles. *Annals of Applied Biology* **129**, 361–8.

Neuman PR, Hart GE, 1986. Genetic control of the mitocondrial form of superoxide dismutase in hexaploid wheat. *Biochemical Genetics* **24**, 435–46.

Nobbs J, Ibrahim SK, Rowe J, 1992. A morphological and biochemical comparison of the four cyst nematode species, *Heterodera elachista, H. oryzicola, H. oryzae* and *H. sacchari* (Nematoda: Heteroderidae) known to attack rice (*Oryza sativa*). *Fundamental and Applied Nematology* **15**, 551–62.

Pais CS, Abrantes IM, 1989. Esterase and malate dehydrogenase phenotypes in Portuguese populations of *Meloidogyne* species. *Journal of Nematology* **21**, 342–6.

Payan LA, Dickson DW, 1990. Comparison of populations of *Pratylenchus brachyurus* based on isozyme phenotypes. *Journal of Nematology* **22**, 538–45.

Powers TO, Harris TS, 1993. A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* **25**, 1–6.

Radice AD, Riggs RD, Huang HH, 1988. Detection of intraspecific diversity of *Heterodera glycines* using phenotypes. *Journal of Nematology* **20**, 29–39.

Romero MD, 1977. The morphology of *Heterodera avenae* in Spain. *Nematologia mediterranea* **3**, 291–7.

Romero MD, 1980. *Heterodera latipons* especie nueva para España. *Nematologia mediterranea* **8**, 95–8.

Romero MD, Andrés MF, López-Braña I, Delibes A, 1996. A pathogenic and biochemical comparison of two Spanish populations of the cereal cyst nematode. *Nematologia mediterranea* **24**, 235–44.

Rumpenhorst HJ, 1985. Vergleichende elektrophoretische Untersuchungen von Proteinem einiger Zystennematoden von Getreide und Grasern. *Mitteilungen aus der Biologischen Bundesanstalt für Land und Forstwirtschaft Berlin-Dahlen* 226, 64–74.

Rumpenhorst HJ, Elekcioglu IH, Sturhan D, Enneli S, 1996. The cereal cyst nematode *Heterodera filipjevi* in Turkey. *Nematologia mediterranea* **24**, 135–8.

Statsoft, 1994. *Statistical, Version 4.5 for the Windows Operating System, Reference for Statistical Procedures*. Tulsa, OK, USA: Statsoft.

Stone AR, Williams TD, 1974. The morphology and soluble protein electrophoresis of *Heterodera avenae* pathotypes. *Annals of Applied Biology* **76**, 231–6.

Sturhan D, Rumpenhorst HJ, 1996. Untersuchungen über den *Heterodera avenae* komplex. *Mitteilungen aus der Biologischen Bundesanstalt für Land und Forstwirtschaft Berlin-Dahlen* 317, 75–91.

Subbotin SA, Rumpenhorst HJ, Sturhan D, 1996. Morphological and electrophoretic studies on populations of the *Heterodera avenae* complex from the former USSR. *Russian Journal of Nematology* 4, 29–38.

Subbotin SA, Waeyenberge L, Molokanova IA, Moens M, 1999. Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. *Nematology* 1, 195–207.

Valdeolivas A, 1987. *Estudio Bioecológico y Variabilidad Intraespecífica del Nematodo delos Cereales* Heterodera avenae *Wollenweber, 1924 (Nematoda: Heteroderidae) en la España Mediterránea*. Madrid, Spain: Universidad Autonoma de Madrid, PhD thesis.

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