Fundam. appl. Nematol., 1995, **18** (2), 189-196

Use of isoelectric focusing and polyacrylamide gel electrophoresis of nonspecific esterase phenotypes for the identification of cyst nematodes *Heterodera* species

Said K. IBRAHIM and Janet A. ROWE

Entomology and Nematology Department, IACR, Rothamsted Experimental Station, Harpenden, Hertfordshire, AL5 2JQ, UK.

Accepted for publication 4 June 1994.

Summary – Nineteen species of *Heterodera* from diverse geographic origins were examined for characteristic differences in their esterase banding patterns using isoelectric focusing and native gel electrophoresis. Twenty-six major bands of esterase activity were detected. Some bands were common to some species, while others were species specific. *Heterodera elachista* had distinct species-specific phenotypes. Comparisons between all *Heterodera* species using cluster analysis were performed in all possible combinations to examine the coefficients of similarity. Dendrograms were constructed but groupings obtained do not agree with those obtained by other methods. The use of esterase isoenzymes to differentiate species is a simple, rapid, inexpensive and reproducible approach.

Résumé – ***Identification de nématodes à kystes du genre* Heterodera *par leur phénotype estérasique non spécifique par électrophorèse sur gel de polyacrylamide et par la technique du point isoélectrique*** – Les différences spécifiques dans les profils estérasiques de dix-neuf espèces d'*Heterodera* d'origine géographique variée ont été observée à l'aide des techniques du point isoélectrique et de l'électrophorèse en gel natif. Vingt-six bandes majeures d'activité estérasique ont été détectées. Quelques bandes sont communes à plusieurs espèces, alors que d'autres sont particulières à certaines espèces. *Heterodera elachista* montre des phénotypes distincts et spécifiques. Les comparaisons entre les espèces d'*Heterodera* ont été réalisées par l'analyse en grappe, à tous les niveaux, afin de préciser les coefficients de similarité. Les groupements révélés par les dendogrammes ne concordent pas avec ceux obtenus par d'autres techniques. L'utilisation des isoestérases pour séparer les espèces constitue une approche simple, rapide, peu coûteuse et donnant des résultats reproductibles.

Key-words : Cyst nematodes, *Heterodera,* IEF, electrophoresis, esterase.

The genus *Heterodera* is one of the most widely distributed and economically important of the plant-parasitic nematode genera containing over 60 species which have been described from soils and roots of a wide variety of agricultural crops all over the world (Stone, 1986). Their taxonomy is based traditionally on morphological and biometric characters (Mulvey, 1957, 1972; Hesling, 1965; Golden, 1986). During the last two decades, the biology and morphological classification of the cyst-forming nematodes have been studied intensively by many workers and new species and revisions in the classification have been described (Mulvey, 1972; Stone, 1977). Morphological similarities between species of *Heterodera* makes their identification difficult. The current characters used for taxonomic identification of *Heterodera* spp. are the cone structures (Oostenbrink & Den Ouden, 1954; Cooper, 1955) and other characters defined by various workers (Goodey, 1959; Golden & Raski, 1977; Rao & Jayaprakash, 1978). Mulvey (1972) classified cyst nematode species into five groups. The criteria used were based on the cyst size (groups I and II), fenestral shape (groups II and III), vulval slit length (groups III and IV), and presence or absence of bullae and underbridge (groups IV and V). The validity of the groups depends mainly on the consistency of these characters within each group. However, some of these characters are variable even within a species and are not reliable (Sharma & Swarup, 1983; Nobbs *et al.,* 1992).

Biochemical approaches such as gel electrophoresis for separating enzymes and protein profiles, have shown great potential for assisting in identification and characterization of interspecific variation among plant parasitic nematodes (Dickson *et al.,* 1971; Hussey & Krusberg, 1971; Dalmasso & Bergé, 1978, 1983; Lawson *et al.,* 1984; Pozdol & Noel, 1984; Bakker & Bouwman-Smits, 1988). Esterase analysis is one of the most effective techniques for comparing organisms and this enzyme has proved of value in the separation of several nematode species that are difficult to distinguish morphologically, e.g. *Meloidogyne* (Hussey *et al.,* 1972; Janati *et al.,* 1982; Fargette, 1987; Esbenshade & Triantaphyllou, 1985, 1987, 1990; Pais & Abrantes, 1989; Ibrahim & Perry, 1993), *Radopholus similis* (Huettel *et al.,* 1983), *Aphelenchoides* (Ibrahim *et al.,* 1994; Hooper & Ibrahim,

1994), *Ditylenchus* (Dickson *et al.*, 1971), *Bursaphelenchus* (de Guiran *et al.*, 1985) and *Heterodera* (Ganguly *et al.*, 1992; Nobbs *et al.*, 1992).

Effective management of plant parasitic nematodes often depends on reliable, quick and simple screening techniques that enable growers to select resistant cultivars through accurate characterisation of field infestations. We examined nonspecific esterases of nineteen species of *Heterodera* from different geographical regions using IEF and native polyacrylamide gel electrophoresis (PAGE) to evaluate their systematic relationships.

Materials and methods

Nematodes

The designations, sources and hosts of the *Heterodera* species used in this study are listed in Table 1. *Meloidogyne javanica,* included for comparative purposes was cultured on tomato *(Lycopersicon esculentum)* cv. Pixie. All the nematode species were kept in glasshouse conditions (16-27 °C, average relative humidity 80 % and day length of 16 h) at Rothamsted Experimental Station.

For each species, white females were extracted from the pot cultures using a fluidising column (Trudgill *et al.*, 1973). Females from each species were hand-picked with forceps and placed in a cavity glass block. Nematodes were washed with distilled water several times until they were free of debris. Approximately twenty females were transferred into a centrifuge tube containing 300 µl distilled water and frozen at – 80 °C until needed.

Enzyme extraction

Thirty minutes before electrophoresis, the frozen females were removed from the freezer and thawed but retained in an ice bath whenever possible throughout the subsequent procedures. A 40 µl solution of extraction buffer, containing 20 % glycerol, 2 % Triton X-100 and 0.01 g bromophenol blue was added to each tube. The nematodes were homogenized in the extraction tube with a small plastic pestle and centrifuged at 14 000 r.p.m. for 10 min at 4 °C. The clarified supernatant was introduced immediately into the electrophoresis cell.

Isoelectric focusing

Esterase isoenzymes were separated with an LKB Multiphor equipment utilizing a self-regulating power supply, using ampholine (pH 4-7). The cathode and anode electrode strips were soaked in 1 M NaOH and 1 M H_3PO_4 respectively, and were placed directly onto the ends of the gel and two platinum wires connected from the power supply were then laid on top of these strips. The gel was placed on a platform which was also a cooling stage, kept at 4 °C, and prefocused for 30 min before 20 µl samples of nematode extracts were applied

Table 1. *Sources of the* Heterodera *species.*

Species		Location	Host
H. arenaria	Cooper, 1955	British Isles	*Agropyron repens*
H. avenae	Wollenweber, 1924	Sweden	*Hordeum vulgare*
H. bifenestra	Cooper, 1955	Sweden	*Poa* sp.
H. cajani	Koshy, 1967	India	*Cajanus cajan*
H. carotae	Jones, 1950	RES*	*Daucus carota*
H. cruciferae	Franklin, 1945	RES*	*Brassica oleracea*
H. cynodontis	Shahina & Maqbool, 1989	Pakistan	*Cynodon dactylon*
H. elachista	Oshima, 1974	Japan	*Oryza sativa*
H. fici	Kirjanova, 1954	Russia	*Ficus elastica*
H. glycines	Ichinohe, 1952	North America	*Glycine max*
H. goettingiana	Leibscher, 1892	RES*	*Pisum sativum*
H. hordecalis	Andersson, 1972	Sweden	*Hordeum vulgare*
H. mani	Mathews, 1971	Wales	*Poa* sp.
H. oryzae	Luc & Berdon-Brizuela, 1961	Côte d'Ivoire	*Oryza sativa*
H. sacchari	Luc & Merny, 1963	Côte d'Ivoire	*Oryza sativa*
H. sorghi	Jain *et al.*, 1982	India	*Sorghum vulgare*
H. schachtii	Schmidt, 1871	England	*Beta vulgaris*
H. trifolii	Goffart, 1932	RES*	*Trifolium hybridum*
H. zeae	Koshy *et al.*, 1971	Pakistan	*Zea mays*

* Rothamsted Experimental Station.

on the well surface of the gel. Electrofocusing was achieved in 3 h with a constant power of 800 V. At the end of the running time the power supply was temporarily disconnected and readings were taken at 1 cm intervals from anode to cathode using a surface pH electrode (Pye-Unicam). Finally, the gel was refocused for a further 10 min to improve the definition of the bands.

POLYACRYLAMIDE GEL ELECTROPHORESIS

The procedures used for gel electrophoresis and enzyme detection have been described previously (Ibrahim & Perry, 1993). In brief, the esterase isoenzymes present in the nematode extracts were separated by native polyacrylamide gel electrophoresis. A 3 % acrylamide stacking gel and 7 % acrylamide separation gel were used. Samples of 20-25 µl were injected into the electrophoresis cell. The buffer system was essentially that of Laemmli (1970), except that Triton X-100 was substituted for dodecyl sulfate (SDS). A constant current of 15 mA was applied until the marker dye reached the stacking gel, the current was then increased to 20 mA. When the marker dye reached the bottom of the separating gel, the power was turned off and the gel removed.

ENZYME STAINING

Esterase activity was determined by incubating the gels at 37 °C for 15-30 min in a solution of 100 mg Fast Blue RR Salt, 50 µg α,ß-naphthyl acetate and 50 µg α-naphthyl butyrate dissolved in 5 ml acetone made up to 100 ml with 0.2M Tris-Chloride buffer pH 6.6. The solution was filtered through Whatman No. 1 filter paper and used immediately. The reaction was stopped by adding 10 % acetic acid. Relative electrophoretic mobility (Rm) for each enzyme band was calculated as the ratio of its movement to that of the marker dye.

CLUSTER ANALYSIS

For each of the nineteen *Heterodera* species, the esterase banding patterns were scored as " 0 " (absent) or " 1 " (present). A matrix was then produced with nematode species as rows and the esterase bands as columns. These data were analysed using Genstat 5 (Payne *et al.*, 1987) to give the similarity matrix and for cluster analysis.

Results

ISOELECTRIC FOCUSING

Separations of multiple forms of nonspecific esterase isoenzymes by PAGE were repeated five times for each species and were reproducible (Fig. 1, 3). Within the pH (4-7) range, a total of 27 isoenzymes were observed as sharply defined bands in the isoelectric points (pIs) range of 4.8-7.1 (Fig. 1).

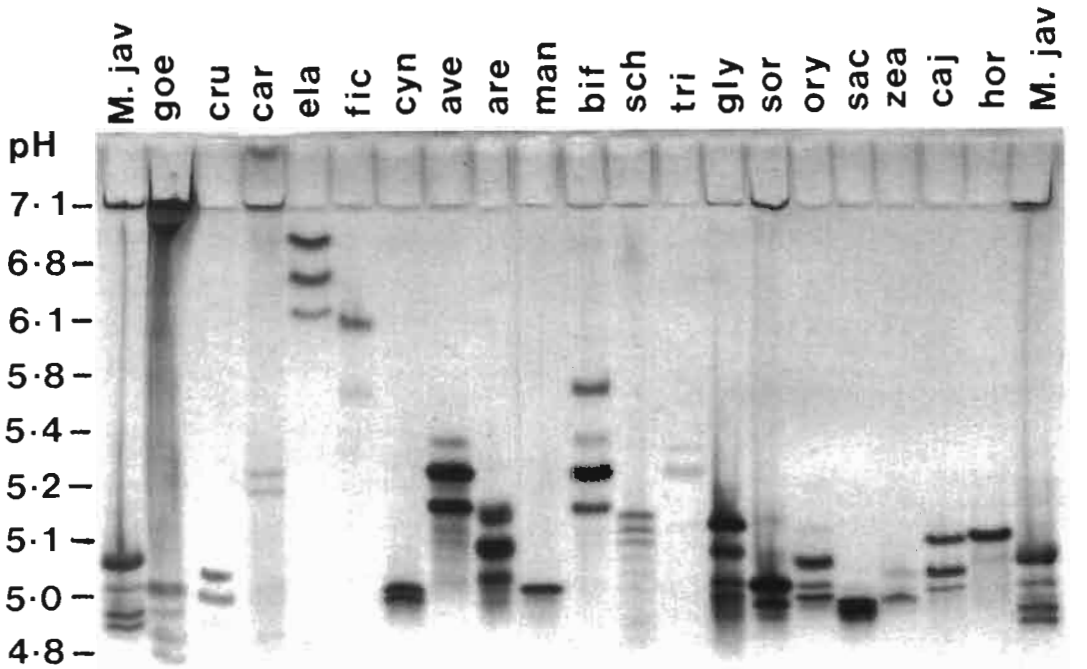

Fig. 1. *Comparative nonspecific esterase profiles obtained by isoelectric focusing of* Heterodera *spp :* Heterodera goettingiana *(H. goe),* H. cruciferae *(H. cru),* H. carotae *(H. car),* H. elachista *(H. ela),* H. fici *(H. fici),* H. cynodontis *(H. cyn),* H. avenae *(H. ave),* H. arenaria *(H. are),* H. mani *(H. man),* H. bifenestra *(H. bif),* H. schachtii *(H. sch.),* H. trifolii *(H. tri),* H. glycines *(H. gly),* H. sorghi *(H. sor),* H. oryzae *(H. ory),* H. sacchari *(H. sac),* H. zeae *(H. zea),* H. cajani *(H. caj),* H. hordicalis *(H. hor),* Meloidogyne javanica *(M. jav). pH range 4-7.*

Meloidogyne javanica was included throughout this study as a marker because it has a very stable and consistent esterase banding pattern (Esbenshade & Triantaphyllou, 1990; Ibrahim & Perry, 1993). In pair-wise comparisons for all the species, the vast majority of esterase bands could be scored unambiguously as shared or species-specific. Although equal numbers of females were used for each run, the esterase activity was different from one species to another as indicated by differences in banding intensity (Fig. 1). White females gave very strong and stable esterase phenotypes, whereas cysts gave smeared and unclear banding patterns (Ibrahim, unpubl.).

The esterase phenotypes obtained with IEF electrophoresis supported the grouping of the nineteen species of *Heterodera* into six clusters based on the occurrence of the number of bands and their position (Fig. 2). Cluster I contained the species *H. oryzae, H. sorghi,* and *H. sacchari* with four bands. Cluster II was divided into two subgroups a and b: a) contained *H. mani, H. cynodontis, H. cruciferae, H. carotae,* and *H. fici* with two bands, while b) contained *H. hordecalis, H. zeae* and *H. cajani* with three bands. Cluster III contained *H. goettingiana* which had four bands. Cluster IV contained *H. glycines* and *H. arenaria* with three to five bands. Cluster V contained *H. bifenestra, H. avenae* and *H. trifolii* with two to five bands. Cluster VI contained *H. elachista* and cluster VII, *H. schachtii,* both with three bands. The *H. elachista* group shows the most distinctly different esterase phenotypes with three distinct bands at pI values of 6.84, 6.17 and 6.1. The *Heterodera goettingiana* had four bands at pI = 5.0, 4.85, 4.8 and 4.75; the last two bands

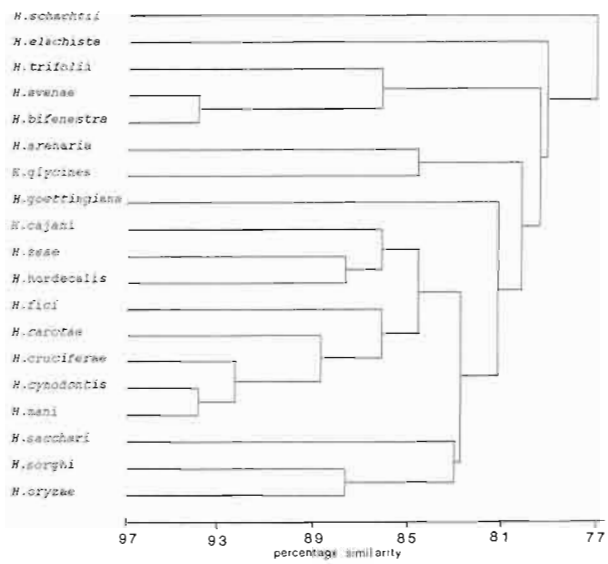

Fig. 2. *Dendrogram showing the clustering and the percentage distance between the clusters of nineteen species of* Heterodera *derived from esterase banding patterns analyzed by isoelectric focusing.*

were species specific (Fig. 1). The *H. schachtii* was also different from all the others with four bands at pI = 5.14, 5.12, 5.09 and 5.07.

The greatest similarity among the species (94 %) was between *H. mani* and *H. cynodontis* in the cluster II and between *H. bifenestra* and *H. avenae* in cluster VI. The first two species can be differentiated from each other by the position of the bands, *H. mani* had one strong band at pI = 5.01 and a weak band at pI = 5.0 while *H. cynodontis* showed two strong bands at pI = 5.01 and 4.89. *Heterodera bifenestra* and *H. avenae* were also very similar, with the same bands at pI values 5.27, 5.22 and 5.15 but differed from each other by a single band at pI = 5.47 in *H. bifenestra.* All the remaining species could be differentiated from each other by at least one or more bands (Fig. 1).

Native polyacrylamide gel electrophoresis

Separation of multiple forms of nonspecific esterase isozymes by native gel electrophoresis was consistent and highly reproducible for all species tested (Fig. 3). Each nematode species investigated had a characteristic esterase pattern. Electrophoresis of native enzymes clearly distinguished all the species either by the number of bands present or by their relative mobility. In total, 26 bands were resolved for the eighteen *Heterodera* spp. tested. *Heterodera hordecalis* was not included in this experiment because of lack of material. All the species were run on one gel for accurate comparison, for better presentation the species were split into two gels run at the same time (Fig. 3 A, B).

The cluster analysis from native gel electrophoresis grouped the 18 species of *Heterodera* into five clusters (Fig. 4). Two species *H. mani* and *H. avenae* were very similar, but both species had slightly different banding patterns (Fig. 3). *Heterodera mani* had two strong bands at Rm = 0.37 and 0.40, while *H. avenae* had three strong bands at Rm = 0.37, 0.40 and 0.43. *Heterodera carotae* and *H. cajani* were in the same cluster, but the two species could be differentiated from each other by their banding patterns : *H. cajani* had three clear bands at Rm = 0.42, 0.45 and 0.48, while *H. carotae* had smeared banding patterns on most gels, but visible bands were present at the same position on the gels (Fig. 3). *Heterodera trifolii* and *H. bifenestra* were clustered together, however, both species had two bands with different relative mobilities (0.27, 0.29 and 0.30, 0.34, respectively).

Heterodera goettingiana and *H. elachista* were markedly distinct from each other and from the remaining species (Fig. 3). Some bands were common to more than one species, but others were species-specific or absent. A common band at Rm = 0.37 was shared between *H. avenae, H. fici, H. arenaria* and *H. mani* (Fig. 3). The clusters obtained from IEF and native gel electrophoresis were combined, which resulted in the grouping of all the species into five clusters (Fig. 5).

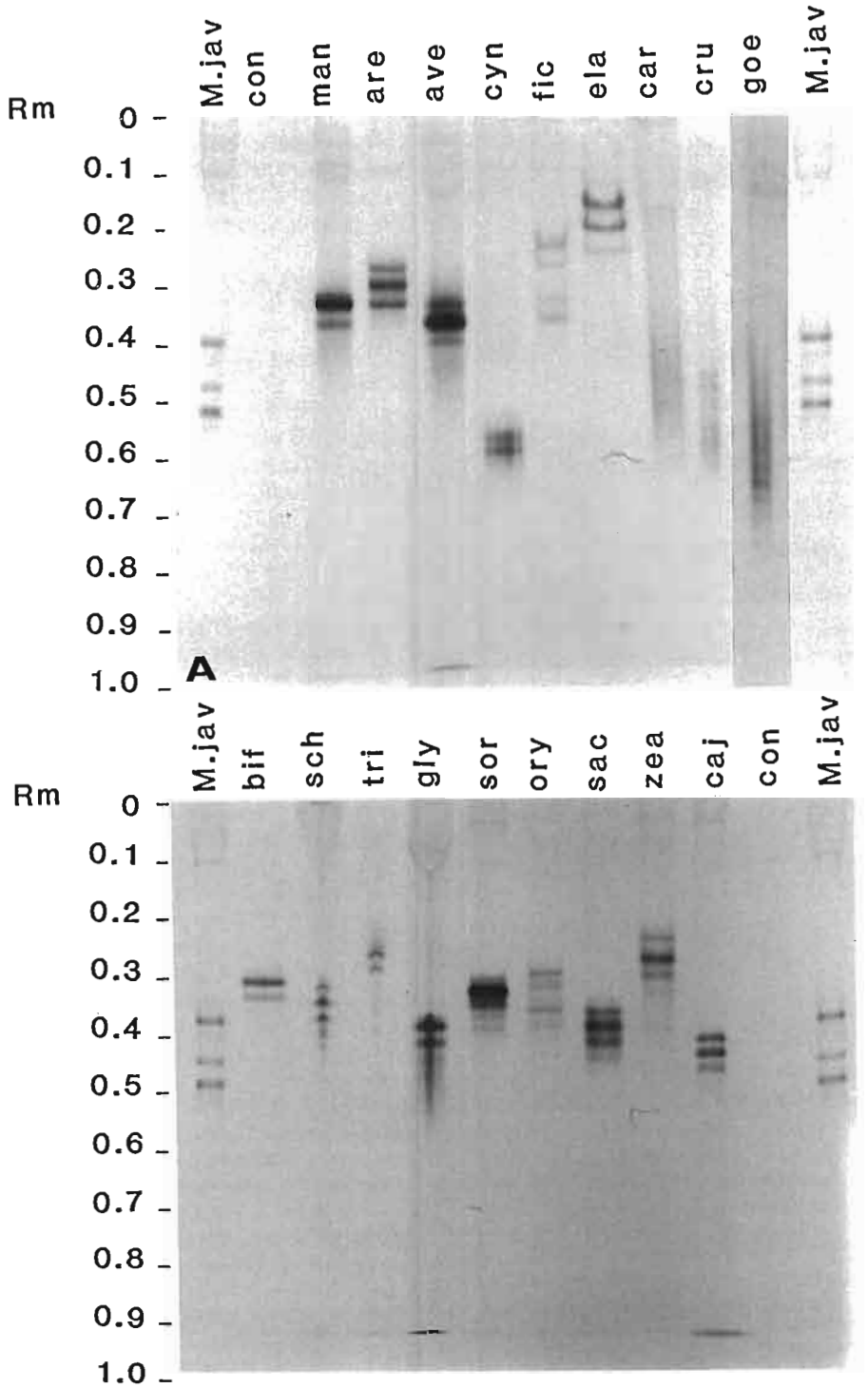

Fig. 3. *Comparative nonspecific esterase profiles obtained by (7 %) native gel electrophoresis (Key to species as for Fig. 1. Con : serves as negative control without nematode extracts.*

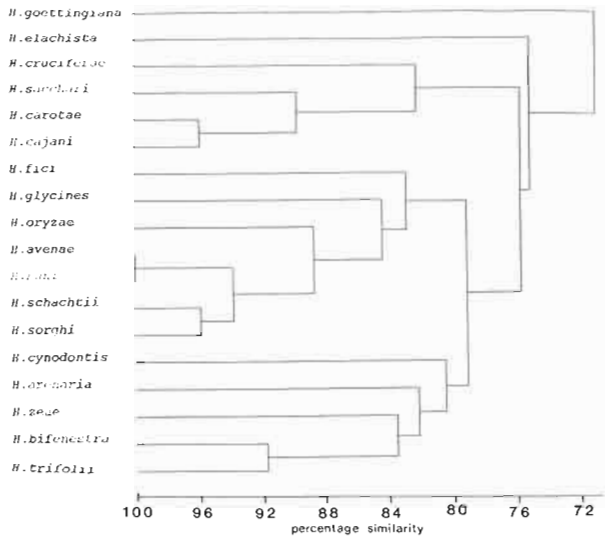

Fig. 4. *Dendrogram showing the clustering and the percentage distance between the clusters of eighteen species of* Heterodera *derived from esterase banding patterns analyzed by native gel electrophoresis.*

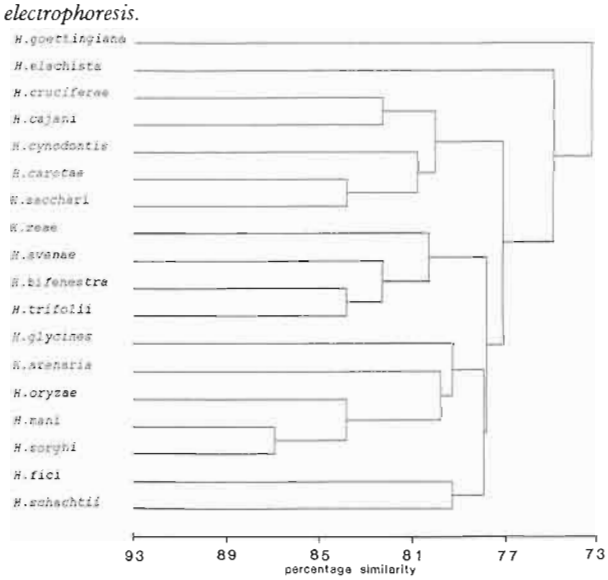

Fig. 5. *Dendrogram showing the clustering and the percentage distance between the clusters of eighteen species of* Heterodera *derived from esterase banding patterns analyzed by isoelectric focusing and native gel electrophoresis.*

Discussion

In this study two types of gel were used in an attempt to determine species-specific esterase phenotypes of the nineteen *Heterodera* species. Polyacrylamide isolectric focusing and native gel electrophoresis of female homogenates and subsequent esterase staining were successful in separating all nineteen *Heterodera* species, many of which had not previously been characterised biochemically.

Over sixty species of *Heterodera* have been identified and there is considerable controversy about their taxonomy. Most of the earlier biochemical studies are restricted to a limited number of species, and in some cases separate gels were used for each species (Ganguly *et al.*, 1992) making comparison difficult.

The results presented here for *H. avenae, H. carotae, H. cruciferae, H. glycines, H. schachtii* and *H. trifolii* correlate well with previous electrophoretic studies of soluble protein, isozymes phenotypes and molecular DNA and indicate that these species are well differentiated (Dickson *et al.*, 1971; Pozdol & Noel, 1984; Radice *et al.*, 1988 *a, b;* Caswell-Chen *et al.*, 1992; Ferris *et al.*, 1993).

The results of this study using esterase phenotype to differentiate between *H. avenae, H. cajani* and *H. sorghi* agree with the results of Ganguly *et al.* (1992) in the number of bands for *H. avenae* and *H. cajani*, but disagree in the position of the bands detected for *H. sorghi*. In the present study *H. sorghi* had one strong band and two weak bands at Rm = 0.31, 0.33, 0.38 in the middle of the gel, while Ganguly *et al.*, (1992) found that *H. sorghi* had six bands at Rf values 0.042, 0.071, 0.1, 0.42, 0.48, 0.7 at the top, middle and bottom of the gel, suggesting that different phenotypes could be present in this species. Slight variation in the electrophoretic patterns may be expected only in the Rm values of minor and fainter bands which could be as a result of different techniques and/or laboratory conditions (Pozdol & Noel, 1984) but not in the position and number of the main bands. The results obtained by both techniques (IEF and native gel electrophoresis) were consistent throughout this study, and also the microscopic examination confirmed the identification of *H. sorghi*. Further studies are required to determine the intraspecific variation of esterase phenotypes of this species and other pathotypes within *Heterodera* groups.

Cluster analysis of the results from IEF of nonspecific esterase banding patterns enabled the nineteen *Heterodera* species to be grouped by their banding patterns into seven groups. These groups can also be detected by the native gel electrophoresis, but are not as well defined as in the IEF gels. Dendrogram for each method gave different groupings (cf. Figs 2 and 4).

The phylogenetic classification derived from cluster analysis suggests that some *Heterodera* species are very closely related. The subgroups established during cluster analysis do not correlate fully with the host plants or geographical origin of the nineteen *Heterodera* species.

Earlier attempts at grouping species of the genus *Heterodera* have been based on morphological characters (Mulvey, 1972; Stone, 1975; Ferris, 1979), host ranges (Krall & Krall, 1978), 2-D PAGE protein patterns (Bakker & Bouwman-Smits, 1988) or molecular analysis (Ferris *et al.*, 1993). In the present work the cluster analysis based on the esterase phenotypes obtained by IEF resulted in seven groups and the greatest similarity

was between *H. mani* and *H. cynodontis* in group III and between *H. avenae* and *H. bifenestra* in group VI. Analysis of the results obtained from the native gel gave five groups, the highest similarity being between *H. avenae* and *H. mani* in group II. Although Ebsary (1991) considered *H. mani* to be a junior synonym of *H. avenae*, the present work show clear differences in the banding patterns of the two species.

Based on morphological studies *H. avenae* and *H. mani* were very similar (Mathews, 1971), but could be separated by the presence of an underbridge in *H. mani*. Native gel for the two species were very similar but both had slightly different banding patterns. However, with IEF both species exhibited different esterase phenotypes. Based on the combined results from both techniques, the cluster analysis showed slightly different groupings, with the greatest similarity between *H. mani* and *H. sorghi*. Although the results demonstrate the close relationship among the *Heterodera* species, *H. goettingiana, H. elachista* and *H. schachtii* were always in separate groups.

Although almost the same number of bands were detected on the gel by both electrophoretic techniques for each species, results of the cluster analysis differed slightly between techniques. This may be explained by the different basis of each technique. In IEF, the proteins are carried in with pH ampholyte until they reach zero net charge (isoelectric point, pI). By contrast, PAGE separates proteins on the basis of their molecular weight rather than on their charge. Analysing combined data from the two techniques may not be useful in grouping *Heterodera* species; thus, each technique should be examined separately. Based on 2-D PAGE protein patterns Bakker (1987) divided *Heterodera* species into three outgroups, *H. schachtii* and *H. glycines* in group I, *H. cruciferae* and *H. goettingiana,* in group II, *H. mani, H. humuli* and *H. fici* in group III. In this study *H. mani* and *H. fici* fell in one group, while *H. schachtii* and *H. glycines* were in separate groups. It is possible that the cluster analysis would provide a better grouping between subgroups or species if other enzymes were included. However, increasing the number of populations analyzed with a wide range of enzymes or using molecular DNA probes would perhaps not simplify the problem, which probably can be resolved effectively only by comparing species using a stable and reliable character (Esbenshade & Triantaphyllou, 1987). The IEF and native gel electrophoresis techniques clearly separated all the *Heterodera* species tested, some of which are difficult to separate by standard morphometrics. A combination of morphological, biochemical and molecular techniques is sometimes necessary for the accurate identification of some species within this group.

Acknowledgement

The authors are grateful to Dr. Roland Perry and Mr. David Hooper for reading the manuscript and to Dr. Paul Burrows for helpful comments. The senior author thanks the Leverhulme Trust for financial support.

References

BAKKER, J. (1987). *Phylogenetic value of evolutionary conservative proteins resolved by two dimensional gel electrophoresis.* Ph. D. Thesis, Agricultural University, Wageningen, the Netherlands, 159 p.

BAKKER, J. & BOUWMAN-SMITS, L. (1988). Contrasting rates of protein and morphological evolution in cyst nematode species. *Phytopathology,* 78 : 900-904.

CASWELL-CHEN, E. P., WILLIAMSON, V. M. & WU, F. F. (1992). Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* populations. *J. Nematol.,* 24 : 343-351.

COOPER, B. A. (1955). A preliminary key to British species of *Heterodera* for use in soil examination. *In :* Kevan, D. K. McE. (Ed.) *Soil zoology,* London, Butterworths : 269-280.

DALMASSO, A. & BERGÉ, J. B. (1978). Molecular polymorphism and phylogenetic relationships in some *Meloidogyne* spp. Application to the taxonomy of *Meloidogyne. J. Nematol.,* 10 : 323-332.

DALMASSO, A. & BERGÉ, J. B. (1983). Enzyme polymorphism and the concept of parthenogenetic species, exemplified by *Meloidogyne. In :* Stone, A. R., Platt, H. M. & Khalil, L. (Eds). *Concepts in nematode systematics.* London & New York, Academic Press : 187-196.

DICKSON, D. W., HUISINGH, D. & SASSER, J. N. (1971). Dehydrogenases, acid and alkaline phosphatases, and esterases for chemotaxonomy of selected *Meloidogyne, Ditylenchus* and *Aphelenchus* spp. *J. Nematol.,* 3 : 1-16.

EBSARY, B. A. (1991). *Catalog of the order Tylenchida.* Res. Branch Agric. Canada, Publ. 1989 / B. 1991, 196 p.

ESBENSHADE, P. R. & TRIANTAPHYLLOU, A. C. (1985). Use of enzyme phenotypes for identification of *Meloidogyne* species. *J. Nematol.,* 17 : 6-20.

ESBENSHADE, P. R. & TRIANTAPHYLLOU, A. C. (1987). Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda : Tylenchida). *J. Nematol.,* 19 : 8-18.

ESBENSHADE, P. R. & TRIANTAPHYLLOU, A. C. (1990). Isozyme phenotypes for the identification of *Meloidogyne* species. *J. Nematol.,* 22 : 10-15.

FARGETTE, M. (1987). Use of the esterase phenotype in the taxonomy of the genus *Meloidogyne.* 1. Stability of the esterase phenotype. *Revue Nématol.,* 10 : 39-43.

FERRIS, V. R. (1979). Cladistic approaches in the study of soil and plant parasitic nematodes. *Am. Zoologist,* 19 : 1195-1215.

FERRIS, V. R., FERRIS, J. M. & FAGHIHI, J. (1993). Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundam. appl. Nematol.,* 16 : 177-184.

GANGULY, A. K., DASGUPTA, D. R. & RAJASEKHAR, S. P. (1992). b-Esterase variation in three common species of *Heterodera. Indian J. Nematol.,* 20 (1990) : 113-114.

GOLDEN, A. M. (1986). Morphology and identification of cyst nematodes. *In :* Lamberti, F. & Taylor, C. E. (Eds) *Cyst nematodes.* London & New York, Plenum Press : 23-45.

GOLDEN, A. M. & RASKI, D. J. (1977). *Heterodera thornei* n.sp. (Nematoda : Heteroderidae) and a review of related species, *J. Nematol.*, 9 : 93-112.

GOODEY, J. B. (1959). Data to be considered, observed and where possible, reported upon when presenting descriptions of new species. *Nematologica*, 4 : 211-216.

DE GUIRAN, G., LEE, M. J., DALMASSO, A. & BONGIOVANNI, M. (1985). Preliminary attempt to differentiate pinewood nematodes *Bursaphelenchus* spp. by enzyme electrophoresis. *Revue Nématol.*, 8 : 85-92.

HESLING, J. J. (1965). *Heterodera :* Morphology and identification. *In :* Southey, J. F. (Ed.) *Plant nematology*, London, M.A.F.F. Techn. Bull. 7 : 103-130.

HOOPER, D. J. & IBRAHIM, S. K. (1994). *Aphelenchoides nechaleos* n.sp. and *A. paranechaleos* n.sp. (Nematoda : Aphelenchoidea) from rice plants. *Fundam. appl. Nematol.*, 17 : 153-160.

HUETTEL, R. N., DICKSON, D. W. & KAPLAN, D. T. (1983). Biochemical identification of the two races of *Radopholus similis* by starch gel electrophoresis. *J. Nematol.*, 15 : 338-344.

HUSSEY, R. S. & KRUSBERG, L. R. (1971). Disc-electrophoretic patterns of enzymes and soluble proteins of *Ditylenchus dipsaci* and *D. triformis*. *J. Nematol.*, 3 : 79-84.

HUSSEY, R. S., SASSER, J. N. & HUISING, D. (1972). Disc-electrophoretic studies of soluble proteins and enzymes of *Meloidogyne incognita* and *M. arenaria*. *J. Nematol.*, 4 : 183-189.

IBRAHIM, S. K. & PERRY, R. N. (1993). Use of esterase patterns of females and galled roots for the identification of species of *Meloidogyne*. *Fundam. appl. Nematol.*, 16 : 187-191.

IBRAHIM, S. K., PERRY, R. N. & HOOPER, D. J. (1994). Use of esterase and protein patterns to differentiate two new species of *Aphelenchoides* on rice from other species of *Aphelenchoides* and from *Ditylenchus angustus* and *D. myceliophagus*. *Nematologica*, 40 : 267-275.

JANATI, A., BERGÉ, J. B., TRIANTAPHYLLOU, A. C. & DALMASSO, A. (1982). Nouvelles données sur l'utilisation des isoestérases pour l'identification des *Meloidogyne*. *Revue Nématol.*, 5 : 147-154.

KRALL, E. & KRALL, H. (1978). [The revision of plant nematodes of the family Heteroderidae (Nematoda : Tylenchida) by using comparative ecological method of studing their phylogeny] *In : Prinsipy i methody izucheniya vzaimootnoshenii mezhdu parazitichenskimi nematodami i rasteniyami*, Akad. Nauk Estonoski SSR, Tartu : 39-56.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature*, 227 : 680-685.

LAWSON, E. C., CARTER, G. E. Jr. & LEWIS, S. A. (1984). Application of isoelectric focusing to the taxonomic identification of *Meloidogyne* spp. *J. Nematol.*, 16 : 91-96.

MATHEWS, H. J. P. (1971). Two new species of cyst nematode, *Heterodera mani* n.sp. and *Heterodera iri* n.sp. from Northern Ireland. *Nematologica*, 17 : 553-565.

MULVEY, R. H. (1957). Taxonomic value of the cone top and the underbridge in the cyst forming nematodes *Heterodera schachtii, H. schachtii* var. *trifolii* and *H. avenae* (Nematoda : Heteroderidae). *Can. J. Zool.*, 35 : 421-423.

MULVEY, R. H. (1972). Identification of *Heterodera* cysts by terminal and cone top structures. *Can. J. Zool.*, 50 : 1277-1292.

NOBBS, J. M., IBRAHIM, S. K. & ROWE, J. (1992). A morphological and biochemincal comparison of the four cyst nematode species, *Heterodera elachista, H. oryzicola, H. oryzae* and *H. sacchari* (Nematoda : Heteroderidae) known to attack rice *(Oryza sativa)*. *Fundam. appl. Nematol.*, 15 : 551-562.

OOSTENBRINK, M. & DEN OUDEN, H. (1954). De structuur van de kegeltop als taxonomisch kenmerk by *Heterodera*-soorten met citroenvormige cysten, *Tijdschr. PlZiekt.*, 60 : 146.

PAIS, C. S. & ABRANTES, I. M., DE O. (1989). Esterase and malate dehydrogenase phenotypes in Portuguese populations of *Meloidogyne* species. *J. Nematol.*, 21 : 342-346.

PAYNE, R. W. & Genstat 5 Committee (1987). *Genstat 5, Reference manual.* New York, Oxford University Press, 749 p.

POZDOL, R. F. & NOEL, G. R. (1984). Comparative electrophoretic analyses of soluble proteins from *Heterodera glycines* races 1-4 and three other *Heterodera* species. *J. Nematol.*, 16 : 332-340.

RADICE, A. D., POWERS, T. O. SANDALL, L. J. & RIGGS, R. D. (1988 *a*). Comparisons of mitochondrial DNA from the sibling species *Heterodera glycines* and *H. schachtii*. *J. Nematol.*, 20 : 443-450.

RADICE, A. D., RIGGS, R. D. & HUANG, F. H. (1988 *b*). Detection of intraspecific diversity of *Heterodera glycines* using isozyme phenotypes. *J. Nematol.*, 20 : 29-39.

RAO, Y. S. & JAYAPRAKASH, A. (1978). Role of thiamine hydrochloride as a hatching factor in larval emergence from cysts of *Heterodera oryzicola*. (Nematoda : Heteroderidae) on rice. *Curr. Sci.*, 47 : 635-636.

SHARMA, S. B. & SWARUP, G. (1983). Identification scheme for *Heterodera* species in India. *Indian J. Nematol.*, 13 : 171-180.

STONE, A. R. (1975). Head morphology of second-stage juveniles of some Heteroderidae (Nematoda : Tylenchoidea), *Nematologica*, 21 : 81-88.

STONE, A. R. (1977). Recent developments and some problems in the taxonomy of cyst nematodes, with a classification of the Heteroderoidea. *Nematologica*, 23 : 273-288.

STONE, A. R. (1986). Taxonomy and phylogeny of cyst nematodes. *In :* Lamberti, F. & Taylor, C. E. (Eds). *Cyst nematodes*. London & New York, Plenum Press : 1-21.

TRUDGILL, D. L., EVANS, K. & FAULKNER, G. (1973). A fluidising column for extracting nematodes from soil. *Nematologica*, 18 (1972) : 469-475.