

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

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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 observées 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 dendrogrammes ne concordent pas avec ceux obtenus par d'autres techniques. L'utilisation des isoestérasases 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; Dalmaso & 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. Nema-

todes 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.

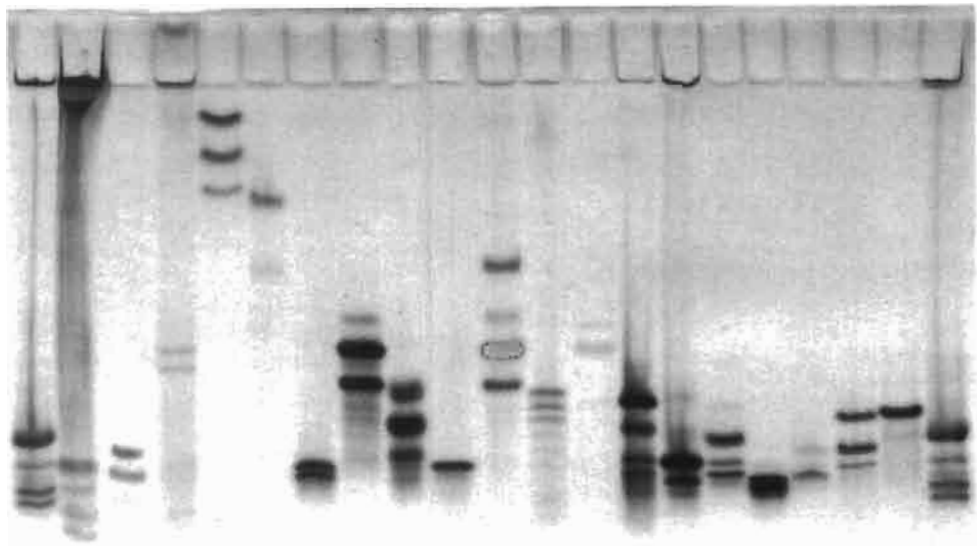
ISOELECTRIC FOCUSING

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 H₃PO₄ 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	
<i>H. arenaria</i>	Cooper, 1955	British Isles	<i>Agropyron repens</i>
<i>H. avenae</i>	Wollenweber, 1924	Sweden	<i>Hordeum vulgare</i>
<i>H. bifenestra</i>	Cooper, 1955	Sweden	<i>Poa</i> sp.
<i>H. cajani</i>	Koshy, 1967	India	<i>Cajanus cajan</i>
<i>H. carotae</i>	Jones, 1950	RES*	<i>Daucus carota</i>
<i>H. cruciferae</i>	Franklin, 1945	RES*	<i>Brassica oleracea</i>
<i>H. cynodontis</i>	Shahina & Maqbool, 1989	Pakistan	<i>Cynodon dactylon</i>
<i>H. elachista</i>	Oshima, 1974	Japan	<i>Oryza sativa</i>
<i>H. fici</i>	Kirjanova, 1954	Russia	<i>Ficus elastica</i>
<i>H. glycines</i>	Ichinohe, 1952	North America	<i>Glycine max</i>
<i>H. goettingiana</i>	Leibsch, 1892	RES*	<i>Pisum sativum</i>
<i>H. hordecalis</i>	Andersson, 1972	Sweden	<i>Hordeum vulgare</i>
<i>H. mani</i>	Mathews, 1971	Wales	<i>Poa</i> sp.
<i>H. oryzae</i>	Luc & Berdon-Brizuela, 1961	Côte d'Ivoire	<i>Oryza sativa</i>
<i>H. sacchari</i>	Luc & Merny, 1963	Côte d'Ivoire	<i>Oryza sativa</i>
<i>H. sorghi</i>	Jain <i>et al.</i> , 1982	India	<i>Sorghum vulgare</i>
<i>H. schachtii</i>	Schmidt, 1871	England	<i>Beta vulgaris</i>
<i>H. trifolii</i>	Goffart, 1932	RES*	<i>Trifolium hybridum</i>
<i>H. zaeae</i>	Koshy <i>et al.</i> , 1971	Pakistan	<i>Zea mays</i>

* Rothamsted Experimental Station.



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. zaeae* 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. bifenebra*, *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

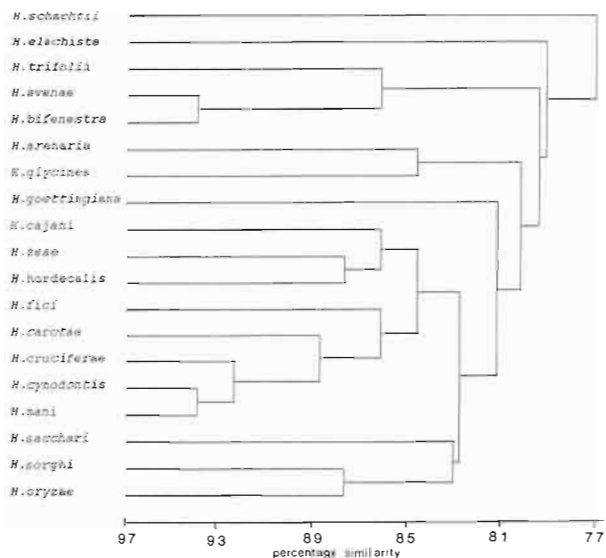


Fig. 2. Dendrogram showing the clustering and the percentage distance between the clusters of nineteen species of *Heterodera* derived from esterase banding patterns analyzed by isoelectric focusing.

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. bifenebra* 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 bifenebra* 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. bifenebra*. 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. bifenebra* 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).

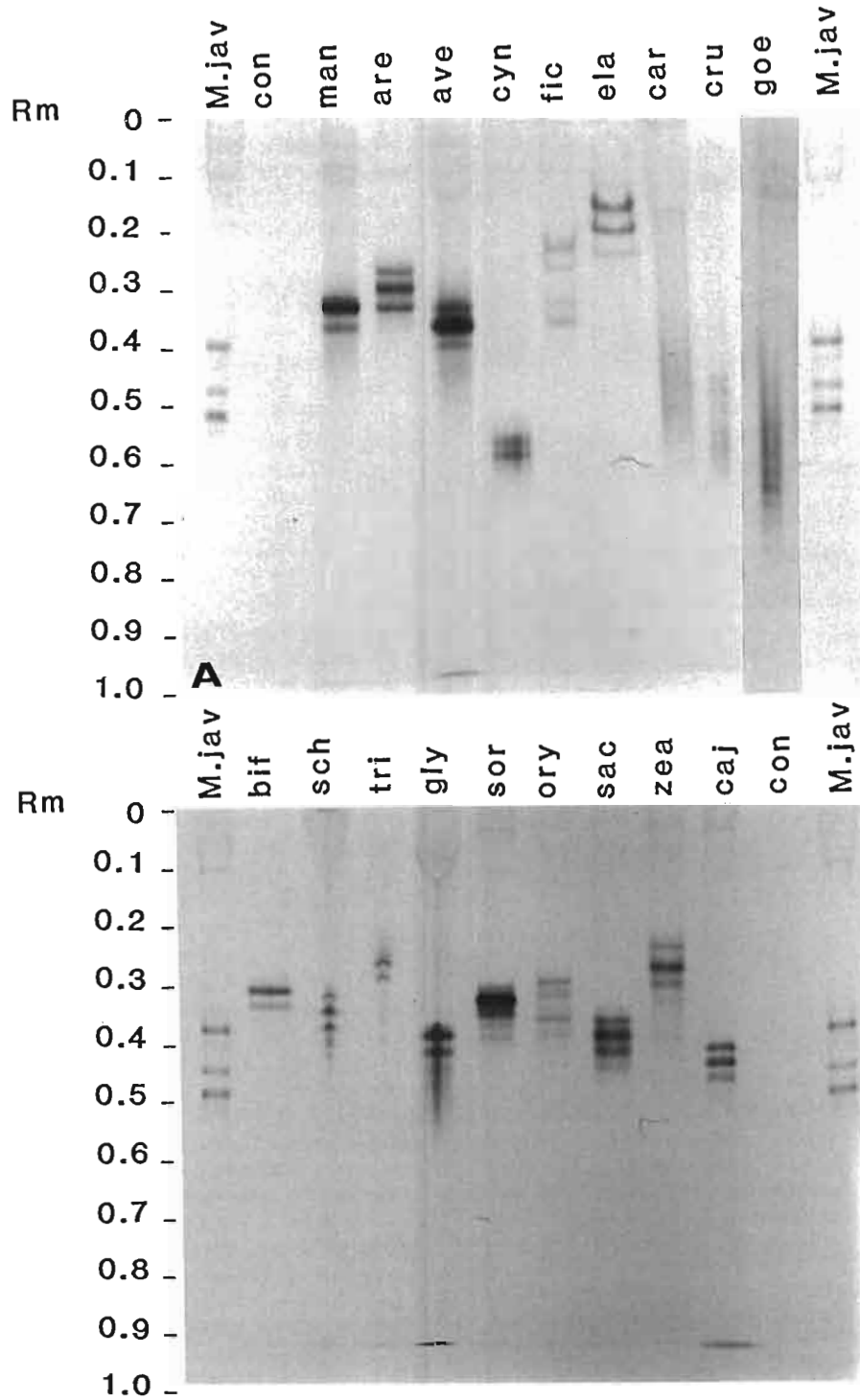


Fig. 3. Comparative nonspecific esterase profiles obtained by (7%) native gel electrophoresis (Key to species as for Fig. 1. Con : serves as negative control without nematode extracts.

