

JOURNAL OF NEMATOLOGY

VOLUME 3

JANUARY 1971

NUMBER 1

Dehydrogenases, Acid and Alkaline Phosphatases, and Esterases for Chemotaxonomy of Selected *Meloidogyne*, *Ditylenchus*, *Heterodera* and *Aphelenchus* spp.¹

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Abstract: Various taxonomically useful profiles of four dehydrogenases (lactate, malate, glucose-6-phosphate, and α -glycerophosphate) and three hydrolases (acid and alkaline phosphatase and esterase) were detected in whole nematode homogenates of *Meloidogyne javanica*, *M. hapla*, *M. incognita*, *M. arenaria*, *Ditylenchus dipsaci*, *D. trifomis*, *Heterodera glycines*, and *Aphelenchus avenae*. The enzyme profiles were stable in populations cultured on several different hosts. A tentative enzymically-determined phylogeny of *Meloidogyne* is given. **Key Words:** Enzymes, Disc-electrophoresis, Chemotaxonomy, Phylogeny, *Meloidogyne*, *Ditylenchus*, *Heterodera*, *Aphelenchus*.

Comparative biochemical composition data supplementing morphological differences in nematodes may be taxonomically valuable. Consistent disc-electrophoretic protein profiles characteristic of four *Meloidogyne*, two *Ditylenchus*, one *Heterodera* and one *Aphelenchus* species have been obtained (10).

Recent investigations of specific enzyme complements of several kinds of organisms suggest these may provide a more sensitive chemotaxonomic tool than general protein analyses. Several investigators have reported esterases to be particularly useful in distinguishing species (4, 7, 19, 24, 26, 28).

Hydrogenases and dehydrogenases of numerous bacterial species have also been reported to have definitive electrophoretic band profiles (1, 6, 37). Nealson and Garber (25) and Berchev and Izmirov (5) reported certain enzyme profiles provide an acceptable biochemical method for defining fungal taxa.

The objectives of this investigation were to use disc-electrophoresis to: (i) compare the specific enzyme profiles of selected *Meloidogyne* spp. with those of several other nematode genera; (ii) determine whether specific enzyme profiles of *Meloidogyne* remain stable and characteristic for each species; (iii) determine whether the hosts on which *Meloidogyne* is cultured affect these profiles; (iv) determine the specific enzyme profiles of different developmental stages of *Meloidogyne*; (v) determine whether these profiles might serve in conjunction with other criteria for identification of *Meloidogyne*; and (vi) apply the enzyme data obtained to the phylogeny of selected *Meloidogyne* spp.

Received for publication, 10 December 1969.

¹ Portion of a Ph.D. thesis submitted by the senior author to North Carolina State University, Raleigh, North Carolina 27607. Journal Series Paper No. 3040 of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

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MATERIALS AND METHODS

Details of the nematode species and populations, methods of rearing and recovery, and protein extract preparation were described in a previous paper (10). Larvae and egg masses of *M. incognita* (Kofoid and White) Chitwood were compared in this study with young adult females of this species.

M. incognita (116-Tai) larvae were mist-chamber extracted from 50-day-old infected tomato roots. The harvests of three consecutive days were each washed eight times on 270, 325, 400-mesh nested sieves, concentrated and disinfected 15 min with 0.5% chlorhexidine acetate (Hibitane®, Ayerst Laboratories, 685 Third Avenue, New York, N.Y. 10017) on an 8 μ Millipore filter, washed with 1 liter sterile distilled water, Baermann extracted (4-ply tissue) for 24 hr, and stored at 5 C.

Egg masses of *M. incognita* (116-Tai) were obtained from 80 forty-five-day-old, greenhouse-grown (15.2-cm clay pots, 24–30 C) tomato plants originally inoculated with six-to-ten nematode egg masses at transplant. After harvesting, the roots were enzymatically softened (10), washed on 40-mesh and 60-mesh nested sieves, and the separated egg masses transferred to small counting dishes from which remaining debris was removed by pipette. They were then treated 15 min with 0.5% Hibitane, weighed and immediately homogenized in liquid nitrogen. Larvae were homogenized in a similar manner.

ENZYME REACTION MIXTURE: Sites of enzyme activity were detected following electrophoretic separation by immersing the polyacrylamide gels in reaction mixtures. Lactate dehydrogenase (LDH) malate dehydrogenase (MDH), and α -glycerophosphate dehydrogenase (GDH) activities were demonstrated on polyacrylamide gels by nitro blue tetrazolium (NBT) deposition according to Gilbert and Goldberg (12).

Glucose-6-phosphate dehydrogenase (G-6-PDH) was detected by the addition of 0.2 M D-glucose-6-phosphate disodium salt to 0.1 M Tris-HCl (pH 7.5), 0.3 mg/ml nicotinamide adenine dinucleotide phosphate (NADP), and 0.1 ml of 10% solution $MgCl_2$.

Acid phosphatase (AcP), alkaline phosphatase (AlkP) and esterase activity were located on the gels by deposition of an insoluble azo dye as described by Lawrence *et al.* (17). Slight modifications were made in some of the reaction mixtures. A Tris-citrate buffer containing 0.076 M Tris-HCl, and 0.0055 M citric acid (pH 8.8) was used for AlkP tests. Gels to be tested for AcP activity were washed five times in 0.2 M acetate buffer (pH 4.6) before introducing the reaction mixture. Gels to be tested for esterase activity were first placed in 0.2 M phosphate buffer (pH 7.0). Half of this solution was decanted before adding the substrate solution. Fast blue RR was the staining dye in all hydrolytic enzyme tests. Incubation of gels was carried out at 22–25 C in the dark for 5 to 30 min.

The sites of enzyme activity were analyzed with a Canalco model D, microdensitometer. Electrophoretic mobility (E_t) for each enzyme band was calculated as the ratio of its movement to that of the tracking dye.

ELECTROPHORETIC SYSTEMATICS IN MELOIDOGYNE: A computer method employing the set-theory approach to taxonomy developed by Moore and Goodman (21) and Moore *et al.* (22) was used to construct a phylogenetic classification of populations of *Meloidogyne* spp. based on information derived from electrophoretic studies of seven enzymes. This system utilized the number of non-matching electrophoretic enzyme bands between two populations and is based on the assumption that an equal number of non-matching bands implies equal distance from common ancestry, while a greater num-

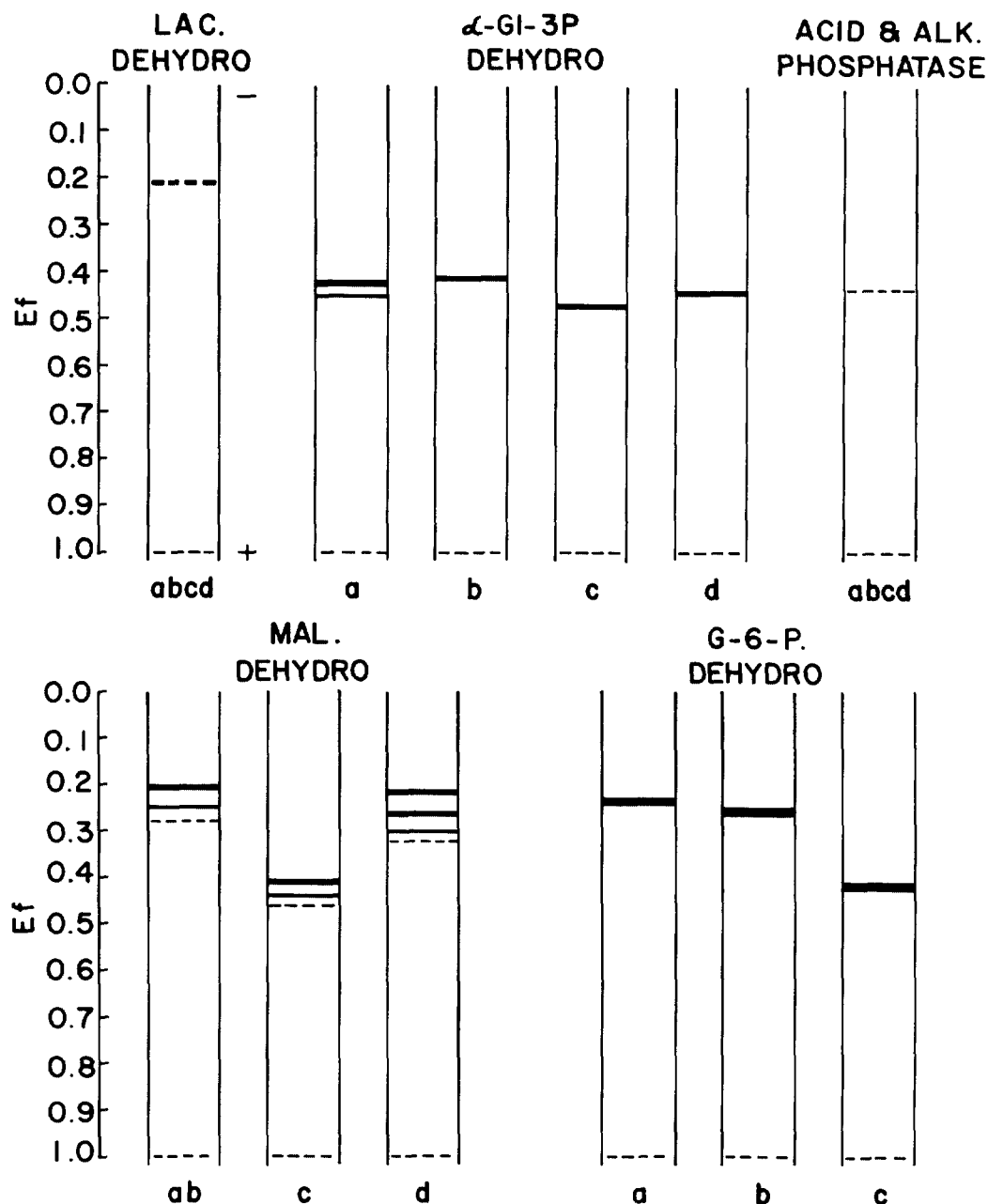


FIG. 1. Electrophoretograms of *Meloidogyne* spp. demonstrating bands of enzyme activity. **a** = *M. javanica* isolates 7-NC, 12-Rhod, 112-Aust, 217-Iv. Coast; **b** = *M. incognita* isolates 84-NC, 116-Tai, 159-Iv. Coast; **c** = *M. hapla* isolates 106-NC, 23-Engl, 268-Okla; **d** = *M. arenaria* isolates 64-NC.

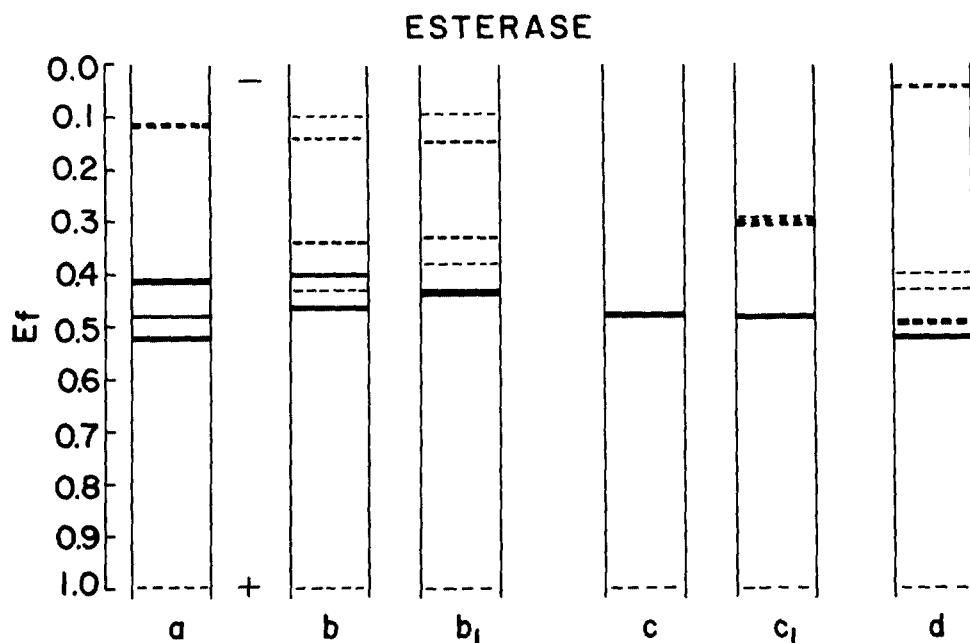


FIG. 2. Electrophoretograms of *Meloidogyne* spp. demonstrating bands of esterase activity. **a** = *M. javanica* isolates 7-NC, 12-Rhod, 112-Aust, 217-Iv. Coast; **b** = *M. incognita* isolate 84-NC. **b₁** = 116-Tai, 159-Iv. Coast; **c** = *M. hapla* isolates 106-NC, 23-Engl. **c₁** = 268-Okla; **d** = *M. arenaria* isolate 64-NC.

ber of non-matching bands implies greater distance from common ancestry. E_r values, which were averages of several runs were compared. If two E_r values were within 99% confidence limits (E_r 0.20 mm), it was assumed they probably represent homologous molecular structures and that the set-theoretical approach is valid.

COLORIMETRIC ASSAY FOR ESTERASE ACTIVITY: Indophenyl acetate (N-(4'-acetoxyphenyl-*p*-quinoneimine)) substrate was used to determine acetylcholinesterase (AChE) activity (16). Absorbance was measured with a Beckman DB-G spectrophotometer at 625 $m\mu$ after 30 min incubation at 37 C. Specific enzyme activity was reported as absorbancy change in O.D. units per min per mg protein $\times 10^{-3}$.

AChE activity in the nematode protein homogenate was further substantiated by in-

vestigating inhibition of indophenyl acetate hydrolysis by eserine. The sulfate salt of eserine at concentrations of 1.2×10^{-2} , 1.2×10^{-3} , 1.2×10^{-4} and 1.2×10^{-5} M was incubated in the nematode homogenate 30 min at 37 C before the substrate was added.

Hestrin's colorimetric method as modified by Simpson *et al.* (31) was also used to determine cholinesterase (ChE) activity. Acetylthiocholine iodide (0.004 M) substrate in 0.067 M potassium phosphate buffer, pH 7.2 was used. The activity was expressed as μ moles acetylcholine hydrolyzed per 30 min per mg protein.

DETECTION OF ESTERASE ACTIVITY WITH SEVERAL SUBSTRATES: Specificity of the esterases determined for *Meloidogyne* spp. were tested using naphthol AS acetate, naphthol AS-D acetate, indoxyl acetate, thiocholine, and α -naphthyl acetate as sub-

strates (32). Reagents and procedures with modifications are described below:

Naphthyl AS acetate and Naphthol AS-D acetate.—0.1 M potassium phosphate buffer pH 7.0, 25 ml; Fast Blue RR, 25 mg; and either 2.5% naphthol AS acetate or 1.0% naphthol AS-D acetate in acetone, 0.5 ml.

Dibromoindoxyphenyl acetate and Indoxyphenyl acetate.—0.1 M potassium phosphate buffer pH 8.0, 25 ml; 1.0% 2,6-dibromoindoxyphenyl acetate in absolute ethyl alcohol, 1.0 ml, or 6.2×10^{-2} M indophenyl acetate in absolute ethyl alcohol, 0.25 ml.

Acetylthiocholine and Butyrylthiocholine.—Substrate base: 1.0 M acetate buffer pH 5.3, 25 ml; copper sulfate, 0.3 g; glycine, 0.375 g; MgCl_2 , 1.0 g; water to 200 ml. Add 20 mg of acetylthiocholine iodide or 25 mg butyrylthiocholine iodide dissolved in a few drops of water to 20 ml of substrate base. Incubate gels in the above solution up to 5 hr, rinse and add 10 ml 1.0% solution of ammonium sulfide.

Indoxyl acetate.—0.1 M Tris (hydroxymethyl) aminomethane pH 8.5, 10 ml; 5.0% 5-bromoindoxyl acetate in absolute ethyl alcohol, 2.5 ml; 0.05 M potassium ferricyanide, 5 ml; 0.05 M potassium ferrocyanide, 5 ml.

All gels tested except those employing the thiocholine methods were incubated at 22–25°C for 5–30 min depending on the enzyme activity of the preparation.

Sodium fluoride reported to inhibit simple esterases, at 2×10^{-3} M (14) and 7.5×10^{-2} M (23), and eserine reported to inhibit ChE at 1×10^{-5} M (8) were used in conjunction with the α -naphthyl acetate method in further analyses of the esterases of *Meloidogyne* spp. In addition 10^{-5} M Iso-OMPA (Tetra-isopropyl-pyrophosphoramidate) a selective pseudocholinesterase (PChE) inhibitor (27) and 10^{-5} M eserine were employed with the thiocholine methods. Gels were pretreated in appropriate buffers containing these inhib-

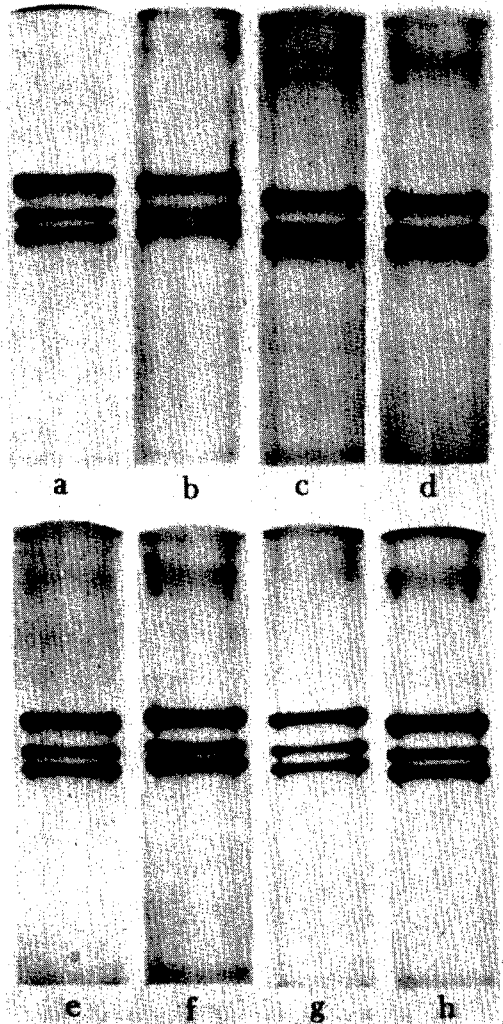


FIG. 3. Profiles of esterase enzymes from individual extracts and mixtures of homogenates of populations of *Meloidogyne javanica*. a. 7-NC; b. 112-Aust; c. 217-Iv. Coast; d. 12-Rhod; e. 112-Aust + 12-Rhod; f. 12-Rhod + 217-Iv. Coast; g. 217-Iv. Coast + 112-Aust; h. 217-Iv. Coast + 112-Aust + 12-Rhod.

itors prior to incubation in the reaction mixture which also contained the inhibitor.

RESULTS

ENZYME PROFILES OF MELOIDOGYNE SPP. AND SEVERAL OTHER NEMATODES: Multiple

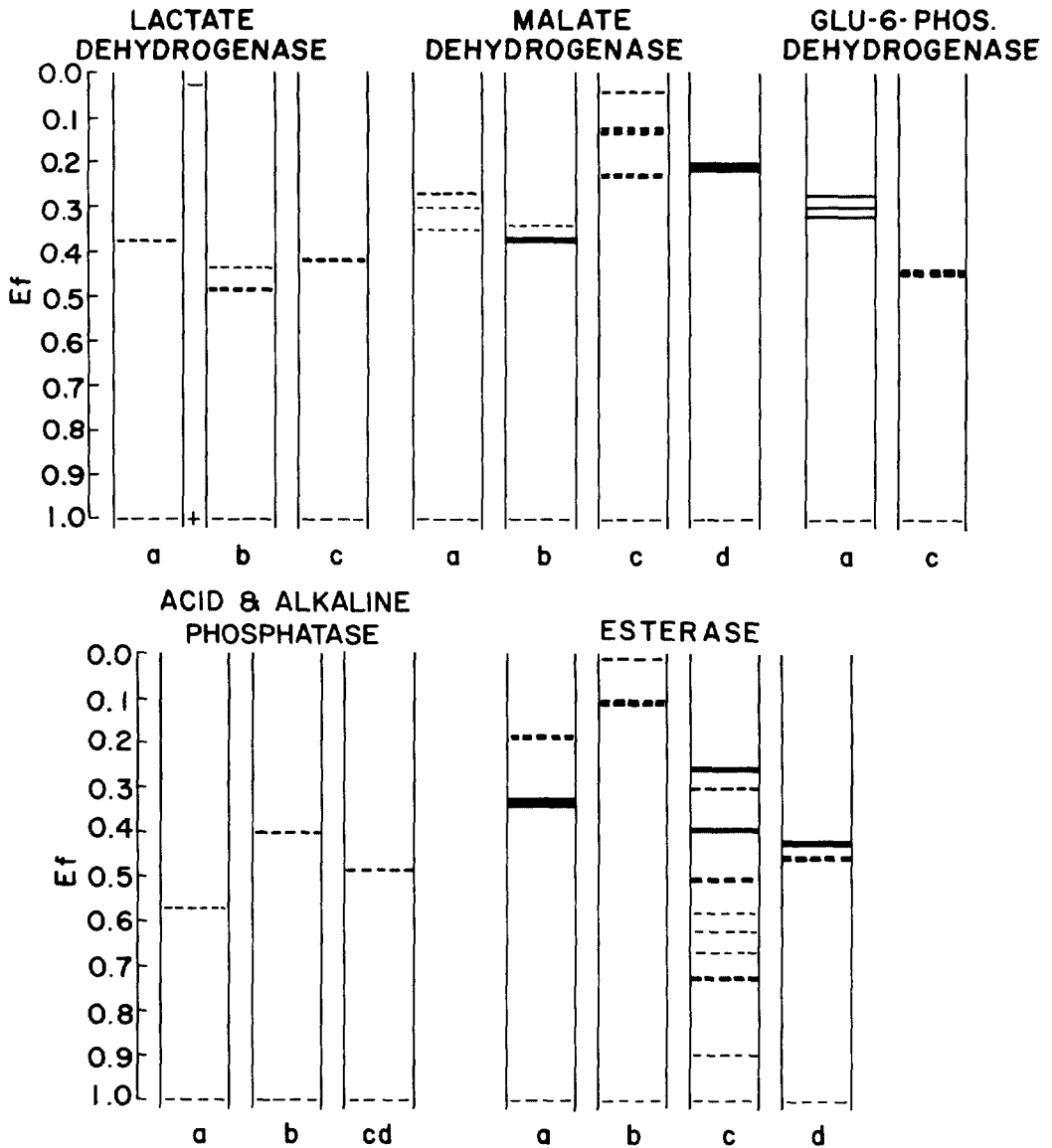


FIG. 4. Electrophoretograms of four nematode genera demonstrating bands of enzyme activity. a. *Ditylenchus trifomis*; b. *D. dipsaci*; c. *Aphelenchus avenae*; d. *Heterodera glycines*.

bands of MDH and esterase were detected for all populations of *Meloidogyne* spp., but only a single band of LDH, G-6-PDH, AcP and AlkP was demonstrated (Fig. 1 and 2). Two GDH bands were resolved for *M. javanica*, but only a single band for other

Meloidogyne spp. The relative activity at each site is represented by shading in the electrophoretograms. The intensity of the staining reaction on the gel surface is related to enzymic activity. The E_f values, as determined from densitometric tracing, were

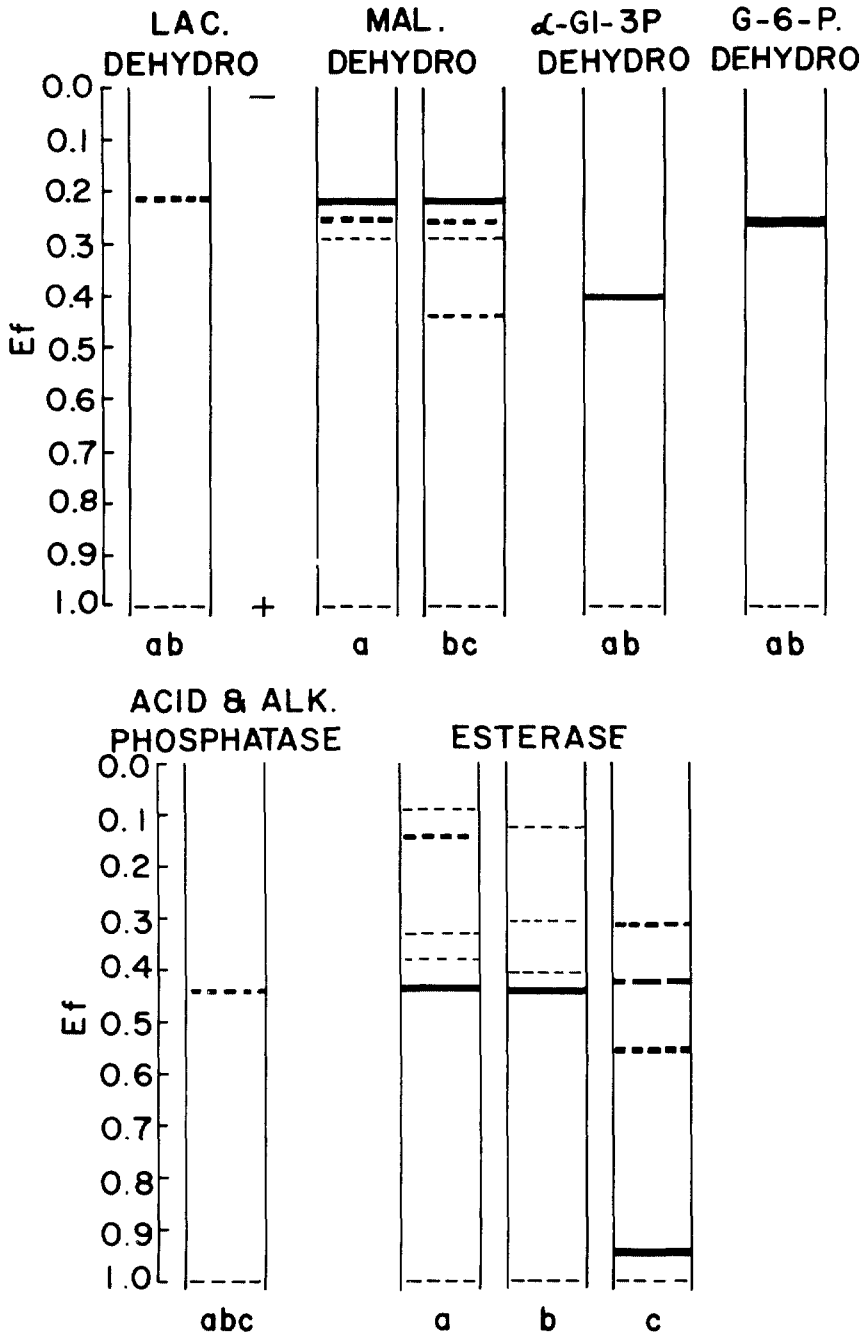


FIG. 5. Electrophoretograms of three developmental stages of *Meloidogyne incognita* (116-Tai) demonstrating bands of enzyme activity. a. female stage; b. larval stage; c. egg stage.

highly reproducible when tested under standardized conditions.

There was little difference in the enzymic forms in each population of *M. javanica*. This was borne out when extracts of four populations were analyzed by mixing the homogenates. The esterase profiles of the mixture could not be distinguished from any of the four profiles individually (Fig. 3). Similar results were also obtained for mixtures analyzed for MDH activity.

The enzyme bands in three populations of *M. incognita* were very similar, however, a slightly different esterase profile was detected for *M. incognita* (84-NC) (Fig. 2). Mixed protein extracts of *M. incognita* (116-Tai) and (159-Iv. Coast) gave the same profiles for MDH and esterase activity as did each extract when tested alone.

The three populations of *M. hapla* exhibited a striking similarity of enzymic forms. Only 268-Okla showed a slight difference in esterase activity (Fig. 2). The migration of MDH, GDH, G-6-PDH, and esterase for populations of *M. hapla* was markedly different from the migration of these same enzymes in other *Meloidogyne* spp. (Fig. 1 and 2).

Unique enzyme profiles were detected for MDH and esterases from a single population of *M. arenaria* (Fig. 1 and 2). Four MDH bands were detected, each having a higher relative activity than those of the other species of *Meloidogyne*. Five bands of esterase activity were found but no G-6-PDH activity was detected.

To ascertain the effect of different methods of homogenization on enzyme profiles, *M. javanica* (7-NC) females were disrupted by the following techniques: (i) glass-tissue homogenizer; (ii) liquid nitrogen treatment; and (iii) French pressure cell. The profiles for all seven enzymes except GDH were identical. Two GDH bands were resolved in extracts prepared in a glass-tissue

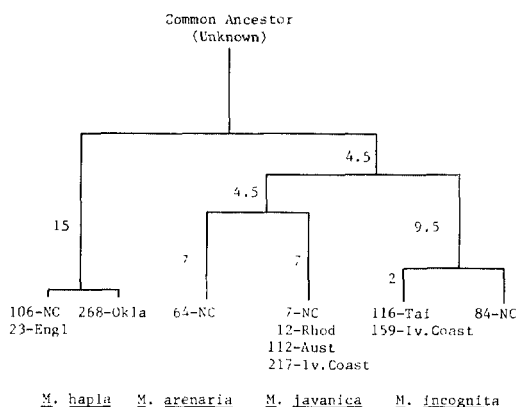


FIG. 6. Phylogenetic tree resulting from a comparison of E_t values of several enzymes from populations of *Meloidogyne*. The numbers designate the average number of non-matching enzyme bands which presumably have arisen since the two lineages split apart.

homogenizer and under liquid nitrogen; however, only one band was resolved after the French pressure cell was used.

In all tests, AcP and AlkP had similar electrophoretic mobility indicating molecular size and charge apparently were the same. Although gels to be tested for AcP activity were washed with buffer at pH 4.5 and were incubated six hours in this buffer to lower the internal pH of the gel before adding the substrate, the enzyme was still detected at the same E_t as for AlkP.

The E_t values for enzymes of extracts of *M. javanica* (7-NC) isolated at different times from four hosts, (tomato, tobacco, cucumber and wheat) were essentially identical. Similar results were obtained for other species of *Meloidogyne*.

There were few similarities between the enzyme profiles of *D. trifurmis*, *D. dipsaci*, *A. avenae* and *H. glycines* (Fig. 4) and none was noted between these nematodes and *Meloidogyne*. In preliminary tests with *H. glycines*, protein extracts left on ice for 12 hr turned very dark brown suggesting high polyphenol oxidase activity. In view of the report by Ellenby and Smith (11) that

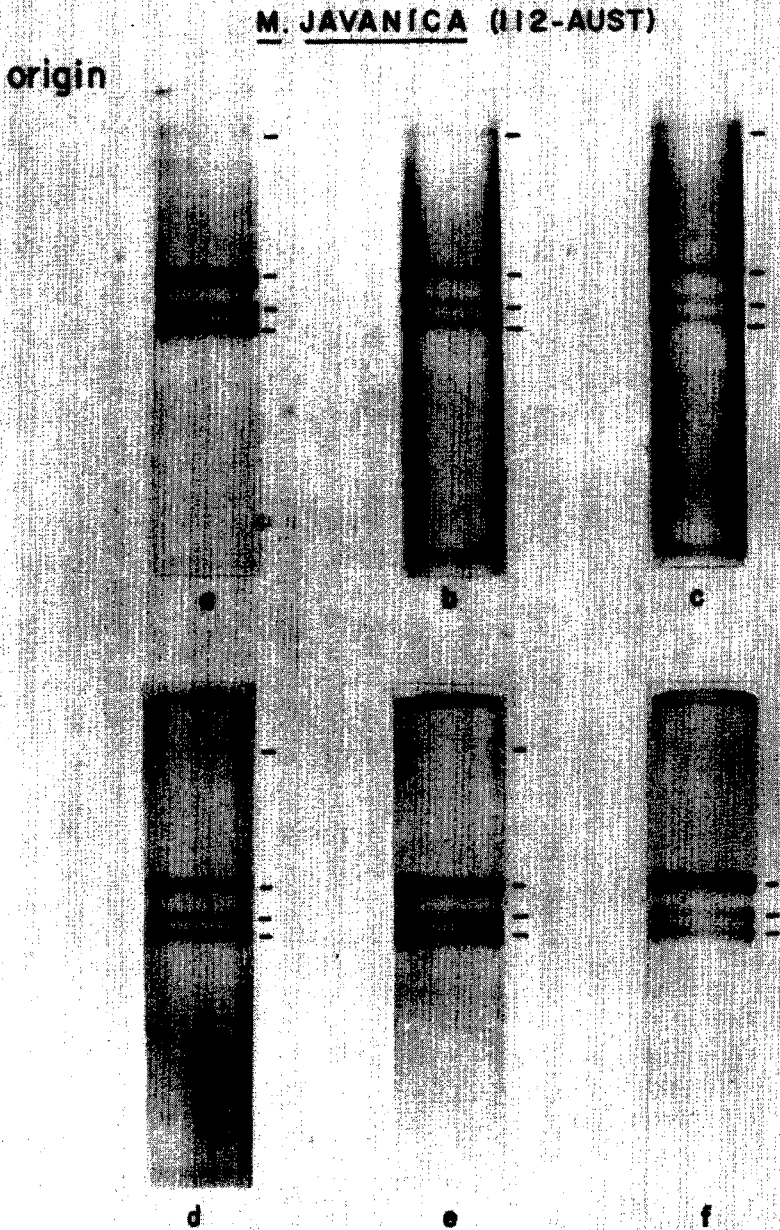


FIG. 7. Effect of different substrates and inhibitors on esterase profile of *Meloidogyne javanica* (112-Aust). **a.** α -naphthyl acetate; **b.** naphthol AS acetate; **c.** naphthol AS-D acetate; **d.** sodium fluoride 7.5×10^{-3} M; **e.** sodium fluoride 2×10^{-5} M; **f.** eserine 10^{-6} M. (α -naphthyl acetate was used as substrate for **d**, **e** and **f**.)

Heterodera cyst walls consisted of tanned protein resulting from polyphenol oxidase activity, we theorized that its oxidative action might alter dehydrogenase activity. Three approaches were used in an attempt to decrease oxidation: (i) ascorbic acid in extraction buffer was increased 20-fold; (ii) protein was extracted under a nitrogen atmosphere; and (iii) the protein was electrophoresed immediately after extraction. No LDH, GDH, or G-6-PDH activity was detected in any of the above tests. With increased amounts of ascorbic acid, a very broad zone of activity preceding the tracking dye was detected when gels were incubated in dehydrogenase reaction mixtures. A similar band was also found in gels incubated in control (no substrate) reaction mixtures. Apparently this zone represents non-specific staining caused by the increased ascorbic acid in the extraction buffer. AcP and AlkP bands were sharper and more distinct when the proteins had been extracted under nitrogen.

ENZYME PROFILES FOR THREE DEVELOPMENTAL STAGES OF *MELOIDOGYNE INCOGNITA*: Four MDH isoenzymes were found in the larval and egg stage and only three in the female stage of *M. incognita* (116-Tai) as illustrated in Fig. 5. MDH profiles were similar for eggs and larvae, with both having a fast-migrating fourth band. Esterase bands were similar for larvae and females except one slow-moving esterase in females was absent in larvae. The esterase profile of eggs had only two bands resembling those of the female and larval stages. In addition, the eggs contained an esterase band located at $E_r .94$. One band each of LDH, GDH, and G-6-PDH was found in the female and larval stage but not in the egg stage.

ELECTROPHORETIC SYSTEMATICS IN *MELOIDOGYNE*: The phylogeny in Fig. 6 was obtained by applying the set-theory methods to electrophoretic data on several enzymes

TABLE 1. Cholinesterase activity of selected populations of *Meloidogyne* spp.

Source	μ moles of ACh hydrolyzed/mg protein/ 80 min
<i>M. incognita</i> (84-NC)	0.32
<i>M. incognita</i> (116-Tai)	0.24
<i>M. javanica</i> (217-Iv. Coast)	0.25
<i>M. javanica</i> (7-NC)	0.31
<i>M. hapla</i> (106-NC)	0.03

of populations of *Meloidogyne*. The numbered stems in the figure designate the average number of nonmatching enzyme bands which presumably reflect genetic changes which have occurred since the two lineages diverged. Computer analysis of host response data produced a phylogenetic tree identical at the species level but differing slightly at the population level.

ESTERASES OF *MELOIDOGYNE* SPP.: Esterase activity studied using indophenyl acetate at fixed substrate concentration for extracts from *M. javanica* (217-Iv. Coast) and (7-NC) and *M. incognita* (84-NC) was 196, 102 and 212, respectively.

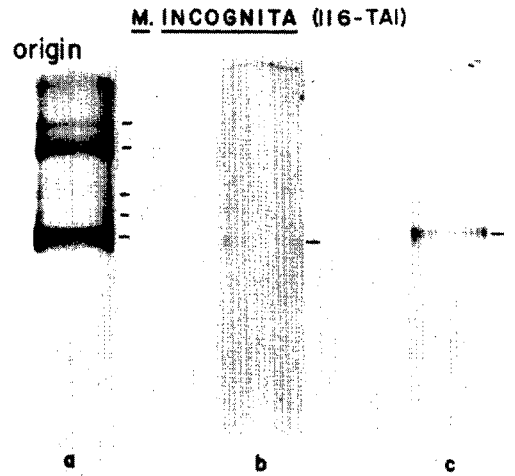


FIG. 8. Effect of different substrates on esterase profile of *M. incognita* (116-Tai). a. α -naphthyl acetate; b. naphthol AS acetate; c. naphthol AS-D acetate.

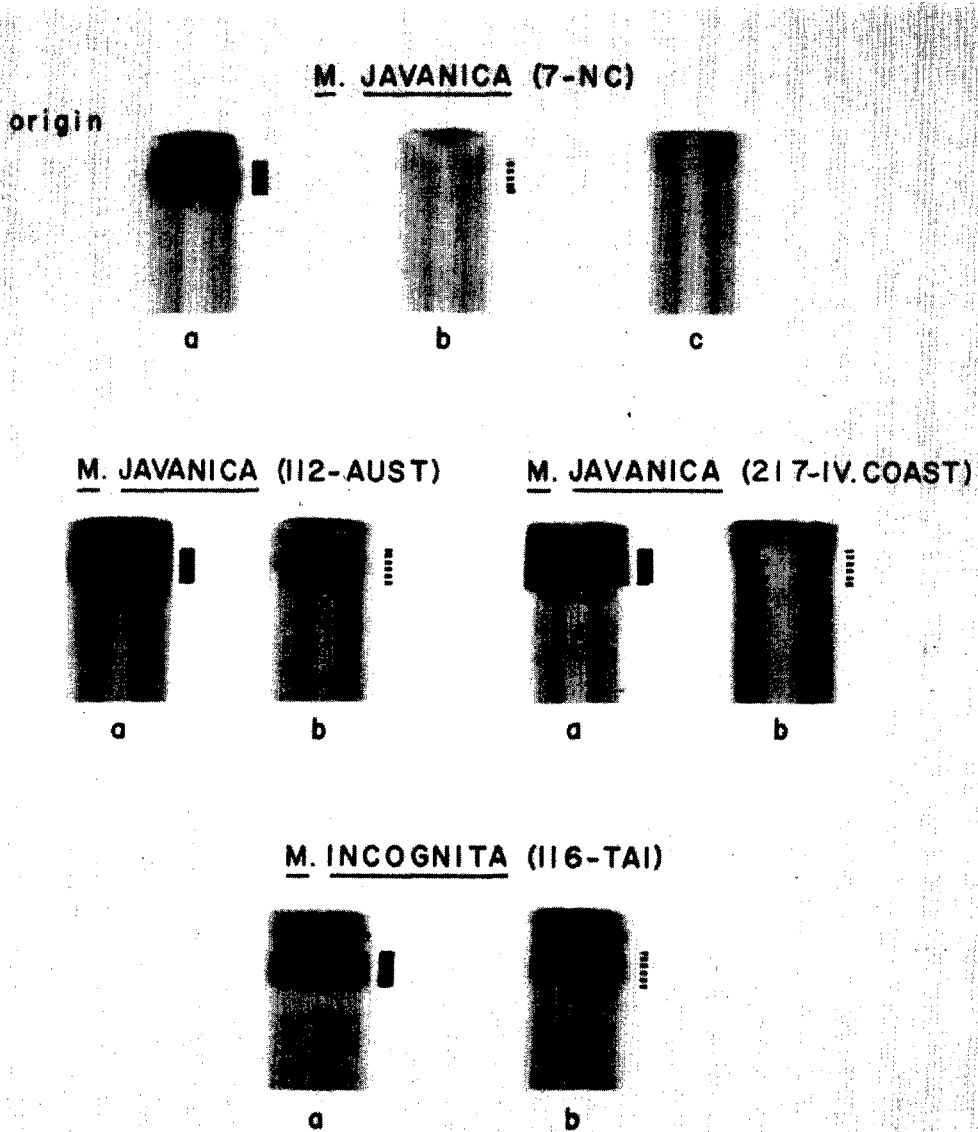


FIG. 9. Detection of cholinesterase activity for *Meloidogyne javanica* (7-NC), *M. javanica* (112-Aust), *M. javanica* (217-Iv. Coast) and *M. incognita* (116-Tai) following incubation with the substrates, acetylthiocholine and butyrylthiocholine. a. acetylthiocholine; b. butyrylthiocholine; c. eserine 10^{-8} M.

Hydrolysis of indophenyl acetate by extracts from *M. javanica* (7-NC) was inhibited by 10^{-2} M eserine ca. 93%, but that of *M. incognita* (84-NC) only 66%. The

more sensitive Hestrin colorimetric test for ChE revealed similar activities for *M. incognita* (84-NC) and (116-Tai) and *M. javanica* (217-Afr) and (7-NC) (Table 1),

but only very low activity for *M. hapla* (106-NC).

Reaction of esterases with different substrates is shown in Fig. 7. Results for *M. javanica* (7-NC) (12-Rhod) and (217-Iv. Coast) resembled those for *M. javanica* (112-Aust). Hydrolysis of indoxyl acetate, indophenyl acetate, and dibromo-indophenyl acetate produced a diffusible colored product and the slow-migrating band (Fig. 7) was not detected.

A striking difference in substrate specificity was revealed in *M. incognita* (116-Tai) and *M. hapla* (268-Okla). Only the fastest-migrating esterase hydrolyzed naphthol AS acetate or naphthol AS-D acetate (Fig. 8).

Sodium fluoride ($2 \times 10^{-3}M$ and $7.5 \times 10^{-2}M$) did not measurably affect esterase activity (Fig. 7, e); eserine at $2 \times 10^{-4}M$ slightly inhibited activity of all the esterases and completely inhibited the slow-moving band for four populations of *M. javanica* and *M. incognita* (116-Tai). Eserine at $10^{-5}M$ also inhibited the slow-moving band of *M. javanica* (Fig. 7).

With acetylthiocholine as a substrate, a strong band was formed at the cathodic end of the gel for *M. javanica* (7-NC), (112-Aust) and (217-Iv. Coast) and *M. incognita* (116-Tai) (Fig. 9) indicating AChE activity. Very slight activity was detected with butyrylthiocholine substrate which is rapidly hydrolyzed only by ChE (Fig. 9b). Eserine at $10^{-5}M$ completely inhibited the hydrolysis of acetylthiocholine (Fig. 9c), but isoOMPA had no effect.

DISCUSSION

Comparative disc-electrophoretic analyses for several specific enzymes show these can be used for taxonomic classification of nematodes. The finding of markedly different enzyme profiles supports previously reported evidence of generic and species-level protein composition differences (10) between the nematodes investigated in this study.

These methods can also be used to distinguish various species of *Meloidogyne*. Enzyme profiles of MDH, GDH, and esterase, can be used to differentiate between species of *Meloidogyne*. Esterase analysis provides a biochemical means of separating species in this genus. This is in agreement with findings of several investigators working with various microorganisms (7, 19, 24, 26, 28).

In addition, disc-electrophoretic analysis of enzymes revealed intraspecific differences within species of *Meloidogyne* collected from various geographical regions around the world. Differences in esterase profiles were found within populations of *M. incognita* and *M. hapla*. Therefore, to increase the confidence in identification of *Meloidogyne* spp. by enzyme profiles, other enzyme data should be included. In our system, MDH and GDH profiles gave valuable supporting evidence for separation of *Meloidogyne* spp.

This study revealed several closely-related multiple molecular forms of MDH, GDH, LDH, G-6-PDH and esterases in some of the nematodes. The term 'isoenzyme', will be used to describe multiple enzyme forms occurring in a single species. Isoenzymes found in this investigation have been reported by Wilkinson (36).

The finding of isoenzymes of MDH for *Meloidogyne* spp., *Ditylenchus* spp. and *A. avenae* was not surprising in view of the observation that a mitochondrial and 'supernatant' form of the enzyme is found in many organisms (9, 13, 33). In this study, however, the origin of these isoenzymes was not established.

Electrophoretic mobilities, substrate specificities and action of inhibitors indicates heterogeneous groups of esterases from species of *Meloidogyne*. Esterases from populations of *M. javanica* showed the broadest range of substrate specificities, whereas only one esterase of *M. incognita*

(116-Tai) hydrolyzed different substrates. This difference in substrate specificity suggests that the esterases of *M. incognita* may have different biochemical characteristics, and different metabolic roles. The difference in substrate specificity shown by esterase enzymes in *M. incognita* (116-Tai), suggests that the term isoenzyme should not be applied, whereas the esterases of *M. javanica* may be isoenzymes. In some cases multiple bands of esterases may indicate isoenzymes. Allen (2, 3) found a single gene involved in the synthesis of a set of esterases from protozoa.

GDH has also been reported to occur in a soluble and mitochondrial form (30), although in this study only one band was detected for *M. hapla*, *M. incognita* and *M. arenaria*. Two bands of GDH were found for *M. javanica*, although when nematodes were homogenized with the French pressure cell one band was found. The rather violent action of the French pressure cell may have denatured one form of the enzyme. Further work is needed to resolve this discrepancy.

A single band of LDH was found for *Meloidogyne* spp., *D. trifurmis* and *A. avenae*. Two bands of LDH were found for *D. dipsaci*. The presence of this enzyme suggests an active glycolytic pathway leading to the production of lactate; thus the nematodes could exist under partial anaerobic conditions. This enzyme was slow in showing on the gels and was weakly stained, suggesting it was not very active.

A single band of G-6-PDH was found for *M. javanica*, *M. hapla*, *M. incognita*, *D. trifurmis*, *D. dipsaci* and *A. avenae*. This enzyme, appeared as a broad intensely stained band. No G-6-PDH was found in *M. arenaria* or *H. glycines* while GDH was found 50% of the time. These enzymes were detected for populations of *M. javanica* and *M. hapla*. Vesell (35) showed that the concentration of enzymes applied prior to elec-

trophoresis may alter the isoenzyme profile. He found that with increased dilutions of LDH, the enzyme was altered until it failed to migrate into the gels. GDH and G-6-PDH may act similarly and thus at very low concentrations would be unsuitable for study by electrophoresis.

A single diffuse band of AcP and AlkP was resolved for all nematodes. The same enzyme appeared active at both an acid and alkaline pH; however, before this can be definitely established, further work coupling substrate affinities, effects of inhibitors and immunochemical reactions should be employed.

The need for defining the developmental state when characterizing *Meloidogyne* by disc-electrophoresis is well illustrated from the investigation of *M. incognita* (116-Tai). The enzyme profiles of the adult females were similar to those of larvae with the exception of MDH and esterase, in which there was a one band difference in each of the two enzymes. The similarity shown between adult females and larvae is not surprising in view of the fact that the larvae are fully differentiated except for reproductive structures. The additional esterase band found in females may be contributed by the female gonads. With the exception of AcP and AlkP, which appeared similar in all three of the nematode stages, no enzyme similarities were noted between the eggs and adult females. MDH profiles were similar for eggs and larvae.

It appears from the correspondence shown by two esterase bands (E_r 0.31, 0.34, and 0.44) in nematodes in three developmental stages that these enzymes may have been synthesized early during development. It is interesting to note that the esterase band tentatively identified as AChE was not present in eggs. The absence of this enzyme suggests that the nematode nervous system is not active until later in development.

Rohde (29) and Lee (18) have demonstrated ChE, probably AChE, in the nervous system of several plant-parasitic nematodes and *Ascaris lumbricoides*. Additional studies of shifts in several other enzyme systems may help clarify the ontogenetic sequence of enzyme synthesis during nematode development.

Enzyme E_r values for females of *Meloidogyne* isolated from four hosts, (tomato, tobacco, cucumber, and wheat) were essentially identical. Ishibashi (15) recently reported finding different profiles of esterases and acid phosphatases for *Meloidogyne* species isolated from different hosts or from host plants growing under different conditions. His results differ from ours and the difference may be attributed to the method used in isolating females of *Meloidogyne*. A freezing and thawing procedure as used by Ishibashi would alter all membranes of host and nematode, thus allowing possible contamination of the nematode protein by the host proteins. In our investigation, *Meloidogyne* females obtained from frozen roots gave different protein profiles than those obtained from unfrozen roots, and the method was discarded.

No relationships were found between enzyme profiles and chromosome numbers or mode of reproduction for *Meloidogyne*. Triantaphyllou (34) reported two chromosomal-reproductive forms of *M. hapla* which were designated as races. 'Race A' comprises populations that undergo regular meiosis, have a reduced chromosomal number of 15 to 17 and reproduce by amphimixis or meiotic parthenogenesis, or both. 'Race B' includes populations that reproduce by mitotic parthenogenesis and have a somatic chromosome number of 45. *M. hapla* (106-NC) and (268-Okla) each with 17 chromosomes and *M. hapla* (23-Engl) with 45 chromosomes would comprise 'race' A and 'race' B, respectively. The enzyme profiles

obtained for *M. hapla* (106-NC) and (23-Engl) were identical. *M. hapla* (268-Okla) had one more esterase band than the other two populations. Although Triantaphyllou suggests that a critical morphological comparison of these races may result in their recognition as distinct species, this work would not support such a separation. With regard to their enzyme profiles, these three populations of *M. hapla* constitute a uniform taxonomic unit exhibiting considerable differences from other species of *Meloidogyne*.

The chromosomal number of the populations of *M. javanica* varied from 43 to 48; however, no differences were detected in their enzyme profiles.

The phylogenetic classification derived according to the set-theoretical approach to taxonomy revealed that *M. javanica* and *M. arenaria* are more closely related to *M. incognita* than any of them are to *M. hapla*. It appears that two lineages diverged from a common ancestor, one giving rise to *M. hapla* and one to *M. javanica*, *M. arenaria* and *M. incognita*. The mathematical model used to draw the above conclusions establishes the relative time of separation of the two lineages, but not the era in which evolutionary divergence occurred. *M. hapla*, which has the greatest number of non-matching enzyme bands, may represent the most primitive member.

The greater degree of non-matching enzyme bands obtained for *M. hapla* suggests this organism diverged from the other species at an earlier date. This hypothesis is supported by Triantaphyllou (34) who suggested on the basis of cytological work and other criteria that *M. hapla* appears to be the most primitive species. He concluded that *M. hapla* (race A), which reproduces by facultative meiotic parthenogenesis, gave rise to pentaploid parthenogenic populations of *M. hapla* (race B) and tetraploid and hexaploid populations of *M. arenaria*. This latter

species is thought to have evolved from *M. hapla* (race A) because its chromosomal behavior during the growth period and during maturation of oocytes was also similar to that of *M. hapla* (race B). Other criteria, such as host response and morphology, were used to arrive at this conclusion. Such placement of *M. arenaria* is contradictory to our findings. However, since only one population was investigated, its interrelationship with other species cannot be definitely established. With this one exception, the phylogenetic tree is identical with Triantaphyllou's and supports the utility of comparative electrophoretic separation of specific enzymes to elucidate the phylogeny of *Meloidogyne*. However, in view of the small number of populations of each species investigated, the proposed scheme of interrelationships is only tentative.

The use of electrophoresis to study the phylogeny of *Meloidogyne* could enhance our understanding of the interrelationships of various members of this genus. Further studies should include other species and populations of *Meloidogyne* from the entire world.

No relationships were observed between the enzyme profiles and host response of any population of *Meloidogyne*. Macko *et al.* (20) similarly did not find any differences in enzyme profiles of dehydrogenases or esterases from avirulent and virulent races of *Puccinia graminis* var. *tritici*. On the other hand, peroxidase and polyphenol oxidase were different between the two races of rust. It would appear, therefore, that in an assessment of differences in pathogenicity between *Meloidogyne* spp. by electrophoresis one should study enzymes which at present are not known to be essential to the metabolic integrity of cells, but which may play key roles in pathogenesis, e.g., amylases, maltases, pectinases, cellulases, hemicellulases, proteases, lipases, nucleases and oxidases.

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