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Use of Enzyme Phenotypes for Identification of *Meloidogyne* Species[1]

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Abstract: Enzyme phenotypes were obtained for 291 populations from 16 species of *Meloidogyne* originating from 65 countries. Soluble proteins from macerates of individual egg-laying females were separated by electrophoresis in 0.7-mm-thick polyacrylamide gels. Enzymes investigated were nonspecific esterases, malate dehydrogenase, superoxide dismutase, and glutamate-oxaloacetate transaminase. Esterases were polymorphic and most useful in identification of major species. About 94% of the populations of *M. hapla,* 98% of *M. incognita,* and 100% of *M. javanica* could be identified to species on the basis of esterase phenotypes alone. About 84% of the populations of *M. arenaria* exhibited three distinct phenotypes. Two of them were highly species specific (accuracy of identification 98–100%). The third, and least prevalent, phenotype occurred also in two other species. Another 12 less common *Meloidogyne* species, of which only one or a few populations of each were studied, exhibited a variety of esterase phenotypes, some of which may prove to be species specific. Superoxide dismutase phenotypes similarly were helpful in the characterization of certain species; however, the same phenotype was often observed in more than one species. The remaining two enzymes, with few exceptions, proved to be less useful for identification of *Meloidogyne* species. Multienzyme phenotypes represented by two or more enzymes often offered biochemical profiles more valuable for definitive characterization of *Meloidogyne* species than single enzymes.

Key words: electrophoresis, isozymes, root-knot nematodes, taxonomy, biosystematics.

Enzyme phenotypes were first described for *Meloidogyne* species in 1971 (8). For demonstrating enzymes, soluble proteins, extracted by macerating whole nematodes in buffer solutions, are separated electrophoretically on starch or polyacrylamide gels. The gels are then stained for specific enzymes.

In early studies of nematode enzymes, disc electrophoresis was employed (8, 11, 15, 16). The extracts of many nematodes were combined in a single gel for identification of the phenotype of a particular nematode population. In later studies, micro-techniques developed for the extraction and electrophoresis of small amounts of soluble proteins (13,18,20,22) were adapted for the study of single *Meloidogyne* females in micro-gels (2,5). This method was an improvement over the earlier system. However, a problem common to both methods was the need to compare phenotypes obtained from different gels. Such comparisons are often difficult because of the observed variation in electrophoretic mobility of the same enzyme in different gels.

Recent utilization of a thin-slab technique for polyacrylamide gel electrophoresis has offered the most satisfactory method of comparing enzyme patterns of *Meloidogyne* species (6,19). By the thin-slab technique, the enzyme phenotype of individual females from the same or different nematode populations can be determined precisely, and comparisons of as many as 20–25 females can be performed on the same gel. Identification and comparison of enzyme phenotypes on thin-slab gels proved to be so reliable that the method can be useful in identification of root-knot nematodes.

The present paper describes a simplified version of the thin-slab method of polyacrylamide gel electrophoresis especially adapted for routine enzyme studies of *Meloidogyne* species. It also reports the findings of an extensive survey of populations of root-knot nematodes from many parts of the world, studied with regard to four enzymes, and evaluates the usefulness of such

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information for identification of *Meloidogyne* species.

Materials and Methods

We examined 291 populations of various *Meloidogyne* species originating from 65 countries and 20 states in the United States. Species identification was based on morphology of various life stages (10,28), perineal pattern and host range (9,25), cytology, and mode of reproduction (26). Nematodes were propagated on 'Rutgers' tomato in the greenhouse at 25–30 C. Some nematode species that do not infect tomato were propagated on their respective hosts; e.g., *M. carolinensis* on blueberry, *M. graminicola* on canary grass, *M. naasi* on wheat, *M. oryzae* on rice, *M. platani* on sycamore, *M. querciana* on oak, several unidentified populations from coffee on coffee, and the rice nematodes on rice.

Protein extraction: Young females that had deposited small, white egg masses were teased from the host roots and placed in a drop of a solution containing 20% sucrose and 2% Triton X-100. One or two nematodes were transferred with a fine pipette into an extraction tube. The extraction tube was made from a microhematocrit tube previously washed with acetone and heat-sealed at one end (5). The nematodes were macerated in the extraction tube with a small glass pestle. The pestle was constructed by heating a glass rod and drawing out the center portion to a thickness of about ⅔ the inside diameter of the microhematocrit tube. The rod was broken in the center to produce two pestles. Small balls were formed at the broken ends by reheating the tips. The balls were carefully sanded to produce an abrasive surface with close tolerance to the inside walls and the bottom of the extraction tubes. Sanding of the balls was guided by frequent observations under the stereoscope at 10 × magnification to assure perfect fit of the pestle inside the extraction tubes.

The nematodes were macerated in a minimal amount of liquid, and then additional sucrose-Triton solution was added to bring the total volume to about 10 microliters. The macerates were frozen immediately after extraction; they could be stored in the freezer (−10 to −15 C) for 4 months without noticeable decrease in enzyme activity. Nematodes to be assayed for malate dehydrogenase activity were extracted in a similar manner with a Tris buffer (27).

In order to facilitate comparisons of esterase phenotypes between gels, population U.S.A., VA-86 of *M. hapla*, whose major esterase band had a relative migration rate (Rm) of approximately half that of the Bromophenol Blue dye, was included in all gels as an internal standard. The relative migration of the bands in any particular gel were adjusted using the *M. hapla* band as a reference. Similarly, the same population of *M. hapla* and also several populations of other species, with characteristic and well-defined phenotypes, were always used as reference phenotypes in each one of the gels stained for the other three enzymes.

In case of uncertainty regarding the purity of a population, 10–20 single-female extracts were examined on the same gel. Mixed populations containing individuals of different species, or different enzymatic forms of the same species, were thus identified and subsequently purified through single egg-mass isolations.

Electrophoresis: Shortly before electrophoresis, the frozen samples were thawed and centrifuged at 13,000 *g* for 15 minutes in a microhematocrit centrifuge maintained at 4 C. After centrifugation each tube was broken just below the upper layer formed by the lipid portion of the extract. The clear aqueous phase in the lower part of the tube was then used directly for electrophoresis.

Electrophoresis was carried out anodally at 8 C in 125-mm × 175-mm polyacrylamide gel slabs, 0.7 mm thick, in a Pharmacia GE-2/4 apparatus (Pharmacia Fine Chemicals, Piscataway, New Jersey). The separating and stacking gels were homogeneous 7% and 4% polyacrylamide, respectively (7). The bridge buffer was Tris/glycine pH 8.3 (7,21). Gels to be stained for esterases were prepared with one-half the amount of Tris in the separating gel (19).

The top chamber of the electrophoretic apparatus was filled with buffer. Nematode extracts were then loaded into the wells in the stacking gel with a fine pipette that could be inserted into the bottom of each well. When all the samples had been loaded, a drop of Bromophenol Blue dye so-

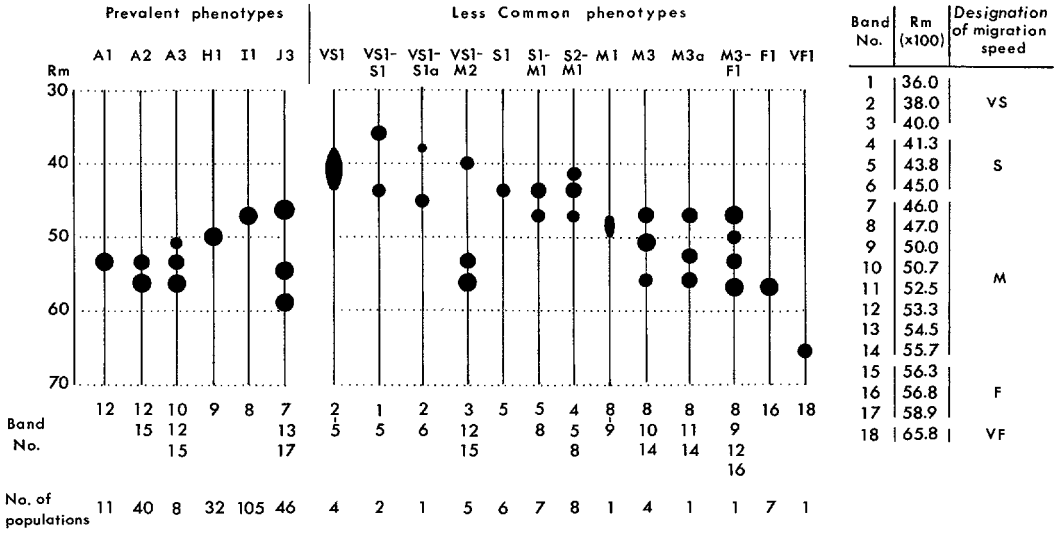

FIG. 1. Esterase phenotypes observed in a study of 291 populations of 16 *Meloidogyne* spp. Each phenotype is designated by a letter suggestive of the species it specifies (A = *arenaria*, H = *hapla*, I = *incognita*, J = *javanica*) or the rate of migration of the major bands (VS = very slow, S = slow, M = medium, F = fast, VF = very fast) and a number indicative of the number of bands present. For further explanation, see text.

lution (1 mg/ml) was added to the top chamber and electrophoresis was begun. The buffer was not circulated in the top chamber for the first 10 minutes to allow the Bromophenol Blue marker dye to enter the top of the gel. After a band of blue dye was observed in the gel, the buffer was allowed to circulate through the top chamber. Voltage was maintained at 80 volts for the first 30 minutes and at 200 volts for the remainder of the separation period. Electrophoresis was carried out for approximately 4 hours until the Bromophenol Blue dye had migrated 10 cm.

Enzyme staining: The gels were stained for specific enzymes according to methods previously published for esterases with the substrate α-naphthyl acetate (15), malate dehydrogenase (8,14), glutamate-oxaloacetate transaminase (12,14), and superoxide dismutase (1,5,12).

Since our main objective was to survey for easily detectable enzyme phenotypes that could be of high taxonomic value, only major bands of enzymatic activity were considered in this study. Minor bands occurred for most of the enzymes studied, but they were inconsistent and often undetectable in extracts from single females.

Influence of host plant on enzyme phenotype: In order to clarify whether the host plant on which the nematode is propagated has an effect on the phenotype of the enzyme under study, two populations each from the four common species of *Meloidogyne* were propagated on the following host cultivars: 'California Wonder' pepper, 'Carnation Flowered Orange Bowl' tagetes, 'N.C. 95' tobacco, 'Rutgers' tomato, and 'Charleston Gray' watermelon. Protein extracts from females of a given population propagated under the same greenhouse conditions on all these host plants were compared on the same gel for each enzyme.

RESULTS

Esterases (Est): Eighteen major bands of nonspecific esterase activity (electrophoretic forms) were detected in the 291 populations of root-knot nematodes. These bands were designated numerically, from 1 to 18, in order of increasing migration to the anode (Figs. 1, 2). Furthermore, 19 esterase phenotypes were recognized and characterized on the basis of single bands or combinations of several bands (Fig. 1).

Prevalent esterase phenotypes

Six of the nineteen esterase phenotypes detected in this study were more or less species specific. Thus, a distinct phenotype was associated with most of the populations of each of the major species, *Meloidogyne*

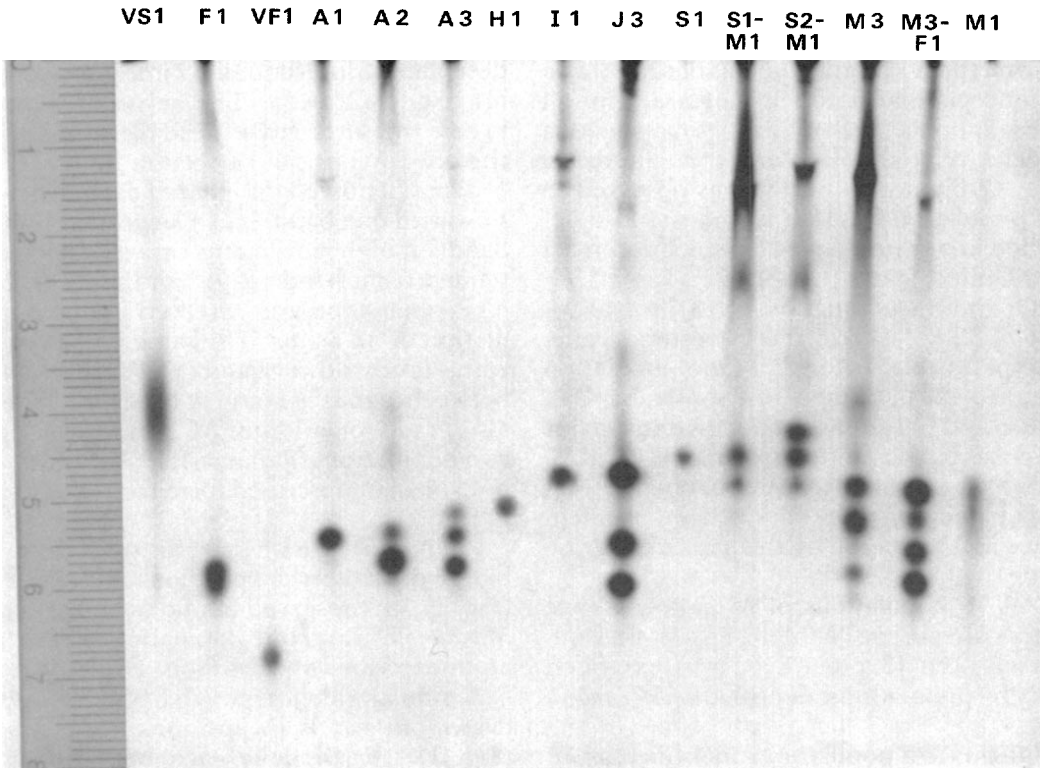

FIG. 2. Photograph of a 0.7-mm-thick polyacrylamide gel slab illustrating 15 of the 19 esterase phenotypes detected in 16 *Meloidogyne* spp. (α-naphthyl acetate substrate). For explanation of phenotype designations and additional information, see legend of Figure 1.

hapla, M. incognita, and *M. javanica.* Three phenotypes were associated with most of the populations of *M. arenaria.* Because of such associations, these phenotypes were designated with a letter suggesting the particular nematode species, followed by a number indicating the number of major bands of enzymatic activity. The following phenotype-nematode species associations were recognized: H1 for *M. hapla,* I1 for *M. incognita,* J3 for *M. javanica,* and A1, A2, and A3 for *M. arenaria* (Figs. 1, 2). These six phenotypes were observed in 242 (83%) of the 291 populations studied. The remaining 49 (17%) populations exhibited a range of phenotypes which were designated in a more conventional manner; i.e., according to the relative rates of migration of their major bands, as will be explained later (Fig. 1).

Eighty-four percent of the populations identified as *M. arenaria* on the basis of a combination of taxonomic criteria gave one of the three phenotypes designated as characteristic of this species; i.e., A1, A2, or A3 (Fig. 1). The A3 phenotype included only triploid (3n ~ 54) populations, considered to be the most typical of *M. arenaria* (Table 1). The A1 and A2 phenotypes included populations of all chromosomal forms; i.e., the triploid, the diploid (2n ~ 36), and an intermediate hypotriploid form which has somatic chromosome numbers ranging from 40 to 48 (A. C. Triantaphyllou, unpubl.). Some populations of the diploid and the intermediate chromosomal forms were less typical of *M. arenaria,* but they could be classified into this species without much difficulty. However, 10 populations were definitely atypical of the species and were listed separately as "*M. arenaria* (atypical populations)" (Table 1). The latter populations were also different from *M. arenaria* in esterase patterns (Table 1).

The A3 phenotype was observed only in populations of *M. arenaria.* However, the A1 and A2 phenotypes appeared also in a

few populations of other species. Thus, in addition to the seven populations of *M. arenaria,* the A1 phenotype was observed also in one population of *M. hapla* and in all three populations of *M. microcephala,* a species related to *M. arenaria.* Similarly, the A2 phenotype which was observed in 39 populations of *M. arenaria* was also detected in one unidentified population from the United States (CA-E1071; Table 1).

Of the 34 populations identified as *M. hapla,* 32 (94%) had the H1 esterase phenotype (Table 1; Fig. 1). One population (France-461), studied previously by Dalmasso and Bergé (6), did not give any major esterase bands, and another one (U.S.A., MN-520) exhibited the A1 phenotype. This last population would have been misidentified as *M. arenaria* on the basis of esterase pattern alone.

All 46 populations of *M. javanica* gave the J3 phenotype (Table 1; Figs. 1, 2). Conversely, the J3 phenotype was exhibited only by populations identified as *M. javanica.*

Of the 106 populations identified as *M. incognita,* 105 gave the I1 phenotype (Table 1; Fig. 1). One population (India-E675) gave a different phenotype not characteristic of any particular species. The nature of the latter phenotype will be explained in the following section.

Rare esterase phenotypes

In addition to the six prevalent species-specific esterase phenotypes described previously, another 13 phenotypes were detected in 46 of the 291 populations studied. These phenotypes occurred primarily in less prevalent *Meloidogyne* species and in some populations which could not be identified to species. Each phenotype is characterized precisely in Figure 1 by its major band or bands, their relative migration rates, and by the assigned band numbers. Furthermore, for convenience the phenotypes are named and referred to, in the text and the tables, by one or more letters suggestive of the rate of migration of their bands (VS = very slow, for bands #1–3; S = slow, for bands #4–6; M = medium, for bands #7–14; F = fast, for bands #15–17; and VF = very fast, for bands #18 or faster) accompanied by a number indicative of the number of bands present. In only two occasions, where two phenotypes with similar banding patterns would have had the same name, were the phenotypes designated differentially with a small letter; e.g., M3, M3a. The relationship between the alternative designations of all the phenotypes can be seen in Figure 1.

The phenotype designated as VS1 (very slow with one band) had a large drawn-out band of high enzymatic activity that extended from band #2 to band #5 (Fig. 1). This phenotype was observed in one population of *M. oryzae.* The same phenotype, with slightly slower band (VS1), extending between bands #1 and #4, was detected also in a population of *M. graminicola* and two populations (Panama-E30, Costa Rica-552) of an undescribed form from rice (Table 1).

Phenotype VS1-S1, with two narrow bands of activity in positions #1 and #5 (Fig. 1), was observed in one population of *M. enterolobii* and one population (E180) of an undescribed species from Puerto Rico.

A similar phenotype VS1-S1a had two drawn-out bands in positions #2 and #6 (Fig. 1). Occasionally each one of these bands resolved into two distinct bands. This phenotype was observed in only one population of *M. carolinensis.*

Phenotype VS1-M2 was observed in five populations (India-E662, E670; Mexico-273; U.S.A., NC-477; and U.S.A., TX-532) that could not be identified to species. The M2 bands (#12 and #15) of this phenotype appear to have similar rates of migration as the A2 bands of *M. arenaria* (Fig. 1).

The phenotype designated as S1 (slow, with one band) was observed in all four populations of *M. chitwoodi,* in one population of *M. incognita,* and in the single population of *M. platani* studied (Table 1). The S1 phenotype expressed by the *M. chitwoodi* populations differed slightly from that of the other species in that its single band was somewhat drawn out and weakly stained.

The slow band (#5) of the S1-M1 phenotype is similar to the single band of the S1 phenotype, whereas the fast band (#8) has a migration rate approximately the same as the I1 band of the *M. incognita* phenotype. This phenotype was observed in six atypical populations of *M. arenaria* and one population (U.S.A., AR-E944) that could not be identified to species and probably represents a new form.

The S2-M1 phenotype includes the two

TABLE 1. Enzyme phenotypes and chromosome numbers of 16 *Meloidogyne* species and several unidentified forms.

Nematode species and populations*	Enzyme phenotypes†				Chromosome number‡
	Est	Mdh	Sod	Got	
Prevalent species					
M. arenaria					
Chile—E603, E604, E1019; El Salvador—E444; Korea—E16; Peru—E505B; Taiwan—E13	A1	N1	JA2	N1	36–52
Argentina—559, E1141; Belize—E620; Brazil—E432; China—E1033; Colombia—392, E195, E280, E321; Ecuador—E255; Guadeloupe—E5; Iran—E790A; Ivory Coast—E553; Jamaica—E900; Korea—E467, E1076; Portugal—E685; Surinam—E304; Uruguay—E782	A2	N1	JA2	N1	37–54
Australia—E482	A2	N1	A4	N1	~ 54
China—E938; Colombia—256; Korea—E908B; Nigeria—413; U.S.A., CA—576, E1123; GA—E1145; NC—56, 568A; TX—523, 529; VA—54	A2	N3	A4	N1	~ 54
U.S.A., GA—436	A2	N3	A4	H2	~ 54
U.S.A., TX—533	A2	N3b	A4	N1	~ 49
Nigeria—E1028; Surinam—E839; W. Samoa—E927, E929	A2	N3	JA2	N3	42–54
W. Samoa—E930	A2	N1	JA2	N3	~ 43
Argentina—E334, E335, E857; Chile—288; Nigeria—E729; Turkey—E455; U.S.A., GA—E1084	A3	N1	A4	N1	52–54
Uruguay—E819	A3	N1	JA2	N1	52–54
M. arenaria (atypical populations)					
Nigeria—E82, E608	S1–M1	N1	I2	N1	37–43
Ivory Coast—E551; Philippines—501, 502; W. Samoa—E928	S1–M1	N3	I2	N1	36–42
Australia—446, E481; Fiji—E947	S2–M1	N1	I2	N1	36–42
El Salvador—E445	M3–F1	N1	JA2	N1	~ 42
M. hapla					
Argentina—E859; Canada—42; Chile—230; China—E1047, E1049, E1051; England—23; France—465, 466; Germany—E122; Holland—E284; Italy—E817; Korea—E15, E24, E468, E907, E908A; Libya—E848; Taiwan—E289, E404; U.S.A., FL—E869; GA—554; IA—E1052; MD—66; MI—595; NC—6, 14, 48, 482, 594A; SD—572; VA—86	H1	H1	H1	H2	13–17 (n) 30–48
France—461	null	H1	H1	H2	17 (n)
U.S.A., MN—520	A1	H1	H1	H2	14 (n)
M. incognita					
Argentina—E337, E599; Australia—E484; Bangladesh—E969; Belgium—188; Bermuda—E463, E487; Brazil—E199, E253, E797; Canary Isl.—E931A, E932A, E933A, E950; Chile—E573A; China—E934, E940, E1106, E1107; Colombia—E879, E880; Dominican Republic—E1153; Egypt—E228, E894, E1054, E1055; El Salvador—E26, E324; Ghana—E88, E89, E170; Greece—E406; Guatemala—E918; India—E639, E640, E645, E646, E649, E651, E653, E671, E672, E681; Indonesia—569, E512, E853A; Iraq—E996B; Ivory Coast—E543, E544, E557; Jamaica—E520, E863, E901; Japan—E916; Malaysia—E810; Nigeria—E152, E156, E507, E509, E651, E691; Pakistan—E1034; Peru—E502, E504, E505A, E579, E830; Philippines—E216, E909; Portugal—E1144; Puerto Rico—E301; Scotland—596, 597; Seychelles—E1080; Senegal—E139; Surinam—E836; Thailand—E159; Trinidad—E269, E313; Uruguay—E105; Zimbabwe—	I1	N1	I2	N1	32–46

TABLE 1. Continued.

Nematode species and populations*	Enzyme phenotypes†				Chromosome number‡
	Est	Mdh	Sod	Got	
E316; U.S.A., AL—108, 553; AR—E954; CA—E1089, E1135; IN—E681; KS—543; LA—450; NC—68, 71, 246, 401, 403, 424, 557, 594B, E589; NM—E1103; SC—282; TN—63; TX—527, 534, E1042					
Bermuda—E490	I1	N3b	I2	N1	~ 42
India—E675	S1	N1	I2	N1	42–45
M. javanica					
Argentina—E621, E897, E899, E1038; Brazil—548, 549, 563, E93, E121; Canary Isl.—E931B, E932B, E933B; Chile—E534, E535; China—E936; Colombia—E856; Cyprus—E394; Egypt—E826, E892, E893; Fiji—E402; Greece—E878; Iran—E631, E793; Iraq—E996A, E1035: Jamaica—E866; Malawi—E1009; Morocco—E977, E981, E984; Nepal—E986; Nigeria—E1003; Portugal—591, E905; Seychelles—E1081; Sudan—E948; Syria—E903; Thailand—E991; Uruguay—E825, E896; Zimbabwe—E318, E1143; U.S.A., NC—7	J3	N1	JA2	N1	41–48
Bangladesh—E972; Korea—E1078	J3	N3	JA2	N1	42–46
Less prevalent species					
M. carolinensis					
U.S.A., NC—E873	VS1–S1a	H1	H1a	N1	18 (n)
M. chitwoodi					
Argentina—E526; Holland—E282; U.S.A., ID—E459, E460	S1	N1a	H1	H2	14–18 (n)
M. cruciani					
St. Croix—614	M3a	N1	JA2	N1	42–44
M. enterolobii					
China—E939	VS1–S1	N1a	I2	N3	~ 46
M. graminicola					
U.S.A., LA—232	VS1	N1a	H1	N1	18 (n)
M. hispanica					
Fiji—E73; Korea—E19; Portugal—E428; Spain—468	S2–M1	N1	I2	N1	33–36
M. microcephala					
Chile—E574, E602; Thailand—E116	A1	N1	JA2	N1	36–40
M. microtyla					
Canada—425	M1	H1	H1	H2	19 (n)
M. naasi					
England—245	VF1	N1a	H1	H2	18 (n)
M. oryzae					
Surinam—E303	VS1	N1a	H1	N1	52–54
M. platani					
U.S.A., NC—471	S1	N1a	I2	H2	42–44
M. querciana					
U.S.A., VA—166	F1	N3a	H1	H2	30–32
Unidentified populations					
From coffee					
Brazil—E189, E190, E191, E252; Peru—189; Surinam—E192	F1	N1	N2	N1	50–56
From rice					
Costa Rica—552	VS1	N1	I2	N1	36

TABLE 1. Continued.

Nematode species and populations*	Enzyme phenotypes†				Chromosome number‡
	Est	Mdh	Sod	Got	
Panama—E30	VS1	N3	H1a	N3	36
From various hosts					
Puerto Rico—E180	VS1–S1	N3c	I2	N3	44–45
Mexico—273	VS1–M2	N3c	I2	N3	30–35
India—E662, E670; U.S.A., NC—477, TX—532	VS1–M2	N1	I2	N1	34–46
U.S.A., AR—E944	S1–M1	N1a	I2	N1	35
U.S.A., AR—E860	S2–M1	N1a	I2	N1	35
Argentina—E595; Bolivia—E354; Ecuador—E536; Turkey—E923	M3	N1	N1	N1	40–46
U.S.A., CA—E1071	A2	N5	I2	H2	35

* Nematode populations are designated with the name of the country of origin and a number, and correspond to the designations used in the International *Meloidogyne* Project collection.

† For explanation of phenotype designations corresponding to each enzyme (Est = esterase, Mdh = malate dehydrogenase, Sod = superoxide dismutase, Got = glutamate-oxaloacetate transaminase), see legends of Figures 1 and 3.

‡ Plain numbers indicate somatic (2n) chromosome numbers and imply reproduction by mitotic parthenogenesis. Numbers designated as (n) refer to haploid chromosome numbers and imply reproduction by cross-fertilization or meiotic parthenogenesis. A range in chromosome numbers (e.g., 36–52) applies to the entire group of populations of that category. Individual populations usually have a definite chromosome number or show a small range of ± 1 chromosomes.

§ This is a new species to be described (Hedwig Hirshmann, pers. comm.). Population "Spain—468" is the same population referred to as Seville population by Dalmasso and Bergé (6).

bands of the S1-M1 phenotype and an additional slower (#4), heavily staining band (Figs. 1, 2). It was detected in three atypical populations of *M. arenaria*, one unidentified population from the United States (AR-E860), and four populations of a species that will be described as *M. hispanica* (Hedwig Hirschmann, pers. comm.).

A phenotype designated as M1 was observed only in the single population of *M. microtyla* studied. The M1 band was drawn out and covered the area between bands #8 and #9 (Figs. 1, 2).

The M3 phenotype with three bands in positions #8, #10, and #14 was observed in three populations from South America and one population from Turkey (E923), which could not be identified to species (Table 1).

A second phenotype, M3a, with three bands (#8, #11, and #14) of medium migration rate was observed only in one population of *M. cruciani.*

The M3-F1 phenotype (Figs. 1, 2) was encountered in a single atypical population of *M. arenaria.*

The F1 phenotype is characterized by one large band in position #16. Occasionally, this large band resolved into two closely spaced bands. This phenotype was associated with six populations of an undescribed species isolated from coffee and one population of *M. querciana* (Table 1).

The VF1 phenotype, with the fastest esterase band (#18) observed in this study, occurred in only one population of *M. naasi* (Figs. 1, 2).

Malate dehydrogenase (Mdh): Eight bands of Mdh activity were detected among the 291 populations of *Meloidogyne* studied. These bands were designated numerically from 1 to 8, according to increasing electrophoretic mobility toward the anode (Fig. 3). An additional very slow band with variable intensity of enzymatic activity was observed in most populations, but not consistently (Fig. 4, arrows). Apparently, its expression in a given population depended on various factors, such as extraction procedures, handling of the samples following extraction, and others. We have disregarded this band because it seems to have no taxonomic value. Possibly this band represents the mitochondrial Mdh.

Eight distinct phenotypes were recognized on the basis of particular bands or band combinations, as illustrated in Figure 3. Only one of these phenotypes was species

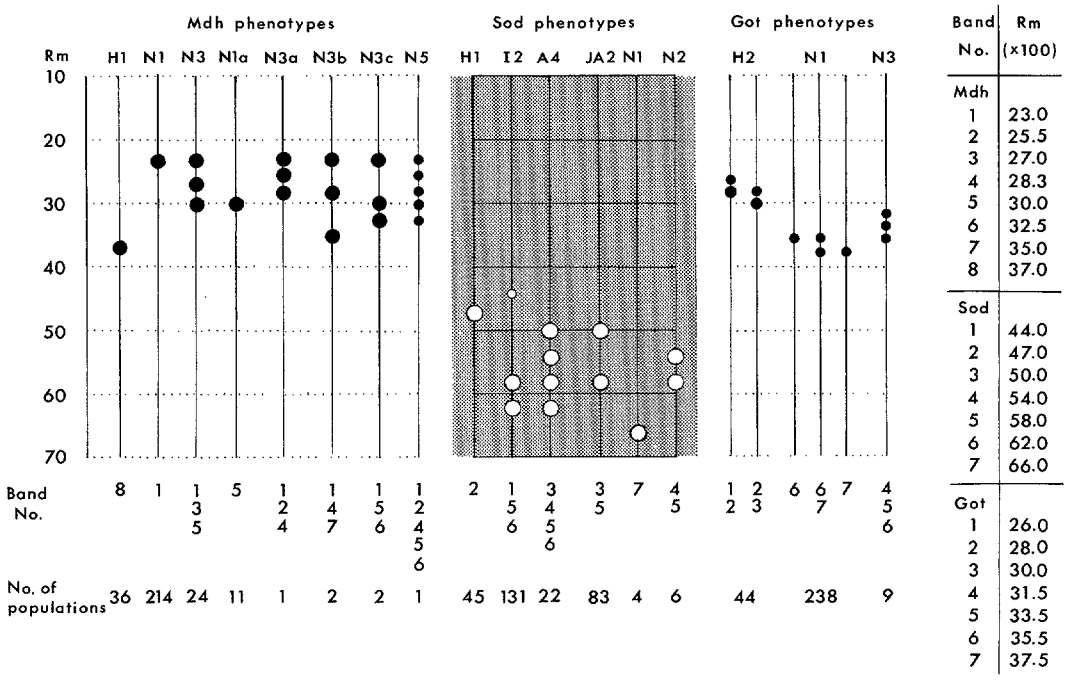

FIG. 3. Phenotypes of malate dehydrogenase (Mdh), superoxide dismutase (Sod), and glutamate-oxaloacetate transaminase (Got) observed in a study of 291 populations of 16 *Meloidogyne* spp. Phenotypes considered to be specific for one or more of the major species are designated with a letter or letters indicative of the species they specify (A = *arenaria*, H = *hapla*, I = *incognita*, J = *javanica*). Phenotypes not characteristic of any particular species are designated with N, for nonspecific. The number following the letter designation indicates the number of major bands of that phenotype. In phenotype I2 of Sod, band #1 is illustrated but is not considered in the designation because it has no taxonomic value.

specific. It was designated H1, for "*M. hapla* phenotype," with one major band (#8) which had the highest rate of migration of all bands observed. All 34 populations of *M. hapla* studied had this phenotype, and none of the populations of the other common species exhibited this phenotype (Table 1). However two rare species, *M. carolinensis* and *M. microtyla* (one population of each was studied), also had this phenotype and could not be distinguished from *M. hapla* on this basis.

The remaining seven phenotypes were not species specific and were designated, for convenience, by the letter N, standing for "non-specific phenotype," and a number indicating the number of bands of activity. Phenotypes with the same number of bands were differentially designated with small letters; e.g., N1, N1a, etc.

In addition to the H1 phenotype, two other phenotypes (N1 and N3) were very common but occurred in populations of several species and therefore were not characteristic of any particular species. The N1 phenotype, with band #1, was the most common, representing about 83% of the non-*M. hapla* populations. It occurred in 42 populations of *M. arenaria*, 105 populations of *M. incognita*, 44 populations of *M. javanica*, 6 populations from an undescribed species from coffee, 4 populations of *M. hispanica*, 3 populations of *M. microcephala*, 1 population of *M. cruciani*, and 9 unidentified populations (Table 1).

The N3 phenotype, with bands #1, #3, and #5, was observed in 9% of the non-*M. hapla* populations. It was detected in 21 populations of *M. arenaria*, 2 populations of *M. javanica*, and 1 population of an undescribed species from rice.

Phenotype N1a, with one band (#5) of medium electrophoretic mobility, was expressed in only two unidentified populations (U.S.A., AR-E860, AR-E944), four populations of *M. chitwoodi*, and one pop-

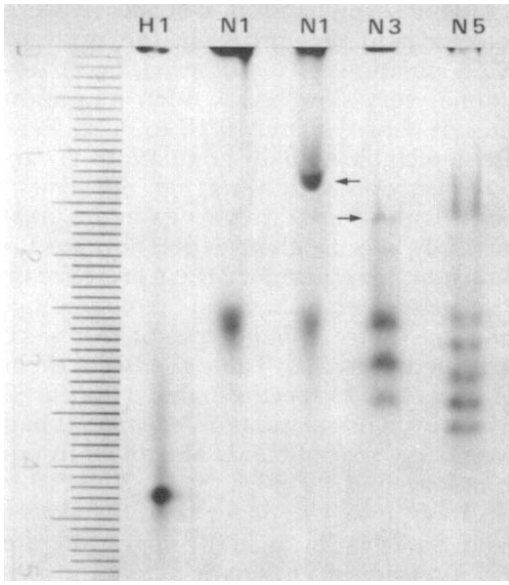

FIG. 4. Four of the eight malate dehydrogenase phenotypes detected in 16 *Meloidogyne* spp. For explanation of phenotype designations see legend of Figure 3 and text. Arrows point to a slow band that was not considered in phenotype designations.

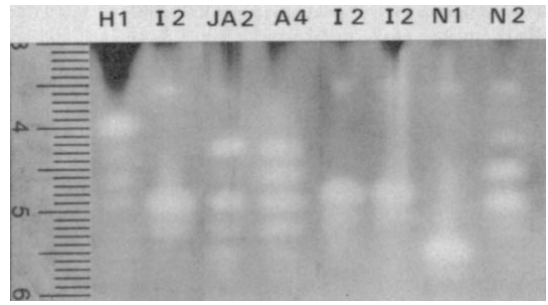

FIG. 5. Superoxide dismutase phenotypes detected in 16 *Meloidogyne* spp. For explanation of phenotype designations, see legend of Figure 3 and text.

ulation each of *M. enterolobii, M. graminicola, M. naasi, M. oryzae,* and *M. platani.*

The remaining four Mdh phenotypes were very rare, as they were encountered in only one or two populations each. Thus, phenotype N3a occurred in a single population of *M. querciana,* phenotype N3b in one population each of *M. arenaria* and *M. incognita,* and phenotypes N3c and N5 occurred in two and one populations, respectively, none of which could be identified to species (Figs. 3, 4; Table 1).

Superoxide dismutase (Sod): Six phenotypes of Sod were identified in the 291 populations of various *Meloidogyne* species (Figs. 3, 5; Table 1). Four of them were encountered frequently; the other two were observed in only 3% of the populations investigated.

Because of the negative staining procedure employed for demonstration of Sod activity, individual bands of activity and overall phenotypes were not always easily detectable from extracts of single females. Repeated runs were necessary for verification of the data presented here. A total of seven bands of activity were detected and were numbered from 1 to 7 in order of migration rate toward the anode. All seven bands were probably present in each of the six recognized phenotypes, but only a few of the bands were sufficiently intense to be clearly detectable in extracts of single females (major bands). The other bands were relatively weak (minor) and could be detected only when extracts of three or more females were processed as one sample. Band #1 was found to be insensitive to 1 millimolar potassium cyanide, whereas bands #2–7 were inhibited completely at this concentration. It is suspected that band #1 represents mitochondrial Sod (1,23). Its presence and intensity were influenced by extraction procedures and had no taxonomic value. Still, band #1 was often detectable in phenotype I2 (Fig. 3). Similarly, band #7 was detectable as a weak or moderately intense band in many phenotypes and appeared to lack taxonomic value. Its presence or absence was not considered in our phenotype designations except in one phenotype (N1) in which band #7 was the only major detectable band. Some phenotypes were characteristic of some major nematode species. For this reason, such phenotypes were designated with one or two letters suggesting their species-specificity, followed by a number indicating the number of major bands present.

Phenotype H1, indicative of *M. hapla,* had one strong band (#2) which was not present as a major band in any of the other phenotypes (Figs. 3, 5). The same phenotype usually had weak bands in positions #3, #4, and #5. Each of the 34 populations of *M. hapla* studied had the H1 phenotype, and 76% of the populations with this phenotype were identified as *M. hapla.* Of the

remaining populations, four were identified as *M. chitwoodi* and the other included one population each of *M. carolinensis, M. graminicola, M. microtyla, M. naasi, M. oryzae, M. querciana,* and an undescribed form from rice (Panama-E30). A strong band #3, in addition to band #2, was also frequently detected in *M. carolinensis* and *M. oryzae.*

Phenotype I2, indicative of *M. incognita,* contained two strong bands in positions #5 and #6. Band #7 was also moderately strong in this phenotype, and band #1 was often detectable as a minor band. All of the populations of *M. incognita* studied had this phenotype, and 81% of the populations with this phenotype were *M. incognita.*

Phenotype A4 had four major bands (#3–6) with slightly lower enzymatic activity than the major bands of the other phenotypes. Band #7 was also detectable frequently in the A4 phenotype. This phenotype was observed only in populations of *M. arenaria.* However, only 22 of the 64 populations of *M. arenaria* studied exhibited this phenotype.

Most of the other populations of *M. arenaria* (51%), as well as all populations of *M. javanica,* exhibited a phenotype designated as JA2 (Figs. 3, 5). This phenotype, with major bands #3 and #5, is similar to the A4 phenotype, but with much weaker bands in positions #4 and #6. The #7 band is also easily detectable in this phenotype. About 55% of the populations exhibiting this phenotype were identified as *M. javanica* and 40% as *M. arenaria.* Only four other populations, one of *M. cruciani* and three of *M. microcephala,* had the same phenotype.

In addition to the above four phenotypes, which were the most prevalent, another two Sod phenotypes were encountered in a few populations from various species. Phenotype N1, with one major band of activity in position #7, was encountered in three biochemically similar, unidentified populations from South America and one from Turkey (Table 1). As mentioned previously, this was the only phenotype in which band #7 was the sole major band. Phenotype N2, with major bands #4 and #5, was observed in only six populations of an undescribed species from coffee.

Glutamate-oxaloacetate transaminase (Got): A total of seven bands of Got activity were detected and recognized in the study of 291 populations of *Meloidogyne* (Fig. 3). Additional very slow bands, with migration rates of approximately 0.18 to 0.22, were also detectable in many populations in various electrophoretic runs (Fig. 6, arrows). These bands were disregarded in the present study because their expression was inconsistent and therefore their presence or absence seemed to have no taxonomic significance. Three phenotypes of Got were identified (Figs. 3, 6; Table 1). One of them (H2) was species specific in that it appeared primarily in populations of *M. hapla.* It had two closely spaced bands of activity in positions #1 and #2 (Figs. 3, 6). The faster band was always stronger than the slower band. Some variation in the migration rate and intensity of enzymatic activity in this phenotype depended on the age of the females. In extracts from young females that had deposited 10–30 eggs, the bands migrated faster and occupied positions #2 and #3. Furthermore, these bands were stronger than the bands from extracts of older females.

All 34 populations of *M. hapla* examined had the H2 phenotype, and 77% of the populations with this phenotype were *M. hapla.* Four populations of *M. chitwoodi* also had the H2 phenotype but with distinctly lower enzymatic activity. The H2 phenotype appeared also in one population each of *M. arenaria, M. microtyla, M. naasi, M. platani, M. querciana,* and an unidentified form (E1071) from California.

The second and most prevalent phenotype of Got was encountered in 93% of the non-*M. hapla* populations evaluated. It was designated as N1, for nonspecific phenotype with one band (Figs. 3, 6). In most populations, the single band (#6) of this phenotype was stable with regard to its migration rate. However, in 14 populations, this band often was accompanied by another slightly faster band, or the faster band (#7) was the only one present. Such variation was observed in each of the 14 populations in different electrophoretic runs. The N1 phenotype was detected in 58 populations of *M. arenaria,* 106 of *M. incognita,* 46 of *M. javanica,* 4 of *M. hispanica,* 3 of *M. microcephala,* 1 population each of *M. carolinensis, M. cruciani, M. gramini-*

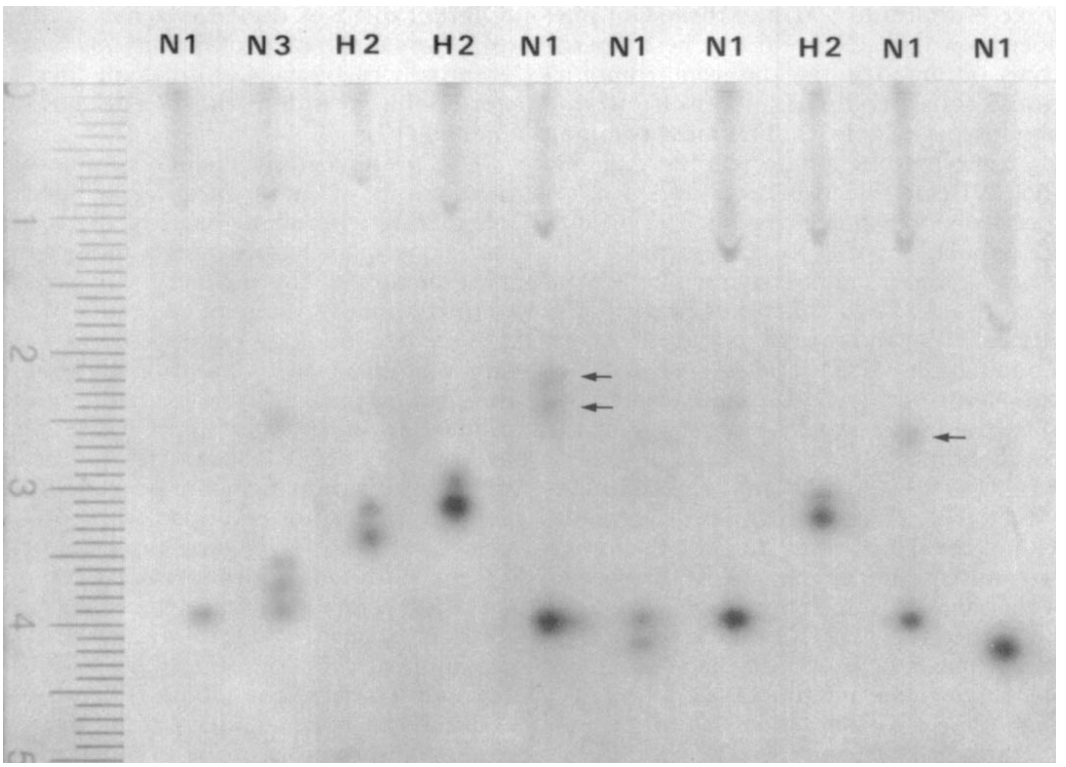

FIG. 6. Glutamate-oxaloacetate transaminase phenotypes detected in 16 *Meloidogyne* spp. For explanation of phenotype designations, see legend of Figure 3 and text. Variation observed within phenotype H2 was influenced by the age of the females. Variation in phenotype N1 appeared to be influenced by extraction and electrophoretic conditions (see text). Arrows indicated slow bands that were observed in some gels. These bands were not considered in the designations of phenotypes because of their inconsistent appearance.

cola, and *M. oryzae,* and in 19 unidentified populations from coffee, rice, and various other hosts (Table 1).

The third, and least, common phenotype of Got was designated as N3 for nonspecific phenotype with three bands of activity in positions #4, #5, and #6 (Fig. 3). The fastest band of this phenotype had the same rate of migration as the band of the N1 phenotype. The other two bands had slower migration rates. This phenotype was observed in five populations of *M. arenaria,* one population of *M. enterolobii,* and three unidentified populations from various countries of Central America (Table 1).

Effect of the host plant on enzyme phenotype: Females of a given nematode population propagated on different host plants (pepper, tagetes, tobacco, tomato, and watermelon) had the same phenotypes of Est, Mdh, Sod, and Got. The number of major bands of activity was constant for each nematode-enzyme combination, independent of the host plant on which the nematode was reared. Similarly, there was no additional variation in the rate of migration of the major bands that could be attributed to the influence of the host plant.

Evaluation of multienzyme phenotypes: In Table 1, populations of a species with the same combination of phenotypes for the four enzymes studied (multienzyme phenotype) were grouped together. The number of such groups within a species may indicate the extent of the overall enzymatic diversification in that species. Furthermore, the relative number of populations with the same multienzyme phenotype may suggest the taxonomic significance of that phenotype, provided the same phenotype does not appear in other species.

The present study revealed six esterase,

three Mdh, three Sod, and three Got phenotypes within *M. arenaria.* These phenotypes occurred in 14 different combinations, thus producing 14 multienzyme phenotypes (Table 1). The most common multienzyme phenotype was the A2, N1, JA2, N1 (for Est, Mdh, Sod, and Got, respectively) which occurred in 19 (30%) of the populations of *M. arenaria* studied. Two other common multienzyme phenotypes were the A2, N3, A4, N1 with 12 populations (19%) and the A3, N1, A4, N1 with 7 populations (11%). The A1, N1, JA2, N1 phenotype occurred in 7 populations of *M. arenaria,* but it was also observed in the populations of *M. microcephala.*

In *M. hapla,* a single multienzyme phenotype (H1, H1, H1, H2) was observed in 94% of the populations studied. The other two multienzyme phenotypes of this species occurred in only one population each. Similarly, the I1, N1, I2, N1 multienzyme phenotype occurred in 98% of the populations of *M. incognita* and the J3, N1, JA2, N1 phenotype was observed in 96% of the populations of *M. javanica.*

A single multienzyme phenotype (S2-M1, M1, I2, N1) occurred in all four populations of *M. hispanica* studied. The four populations of *M. chitwoodi* were similarly unified by a single multienzyme phenotype (S1, N1a, H1, H2). Six unidentified populations from coffee also had a single multienzyme phenotype. Because only one population was examined from each one of the remaining species, their multienzyme phenotypes cannot be evaluated at this time.

Discussion

Single enzyme, as well as multienzyme, phenotypes detected in the present study proved to be very useful characters in identification of the major *Meloidogyne* species and in characterization of new taxonomic forms. The same conclusion had been reached earlier by several investigators, either in exploratory work (8,16) or in more extensive investigations aiming at clarification of the biochemical diversity within the major species of *Meloidogyne* (5,6,19). Furthermore, similar studies have demonstrated the usefulness of enzyme phenotypes in the taxonomic characterization of several other nematodes. Thus, two morphologically similar species of *Caenorhabditis, C. elegans* and *C. briggsae,* were different in 22 of the 24 enzymes studied (3), whereas two races of *Radopholus similis,* eventually recognized as different species, were different in 8 of the 17 enzymes examined (17).

The present study included numerous populations of many *Meloidogyne* species originating from many parts of the world and, consequently, provides a more complete picture of the diversity with regard to the enzymes studied in the genus *Meloidogyne.* Methodology employed and evaluation of results were intentionally kept to the simplest level possible in an attempt to render this biochemical approach feasible for routine work in laboratories interested in reliable identification of root-knot nematodes but not equipped with sophisticated facilities for electrophoretic work.

This study confirmed previous reports that the esterase phenotype is the most useful biochemical character for identification of major *Meloidogyne* species (5,6,8,16,19). A single esterase phenotype (J3) was observed in all 46 populations of *M. javanica* studied and in none of the populations of other *Meloidogyne* species or undescribed forms. This observation strongly suggests that the esterase pattern is a very reliable character for identification of *M. javanica.* Similarly, 99% of the populations of *M. incognita* and 94% of the populations of *M. hapla* had esterase phenotypes characteristic for these species; i.e., I1 and H1, respectively. The same phenotypes were not observed in populations of any other *Meloidogyne* species or undescribed forms. *M. arenaria,* the most variable species morphologically (4) and cytologically (A. C. Triantaphyllou, unpubl.), was not firmly associated with any particular esterase phenotype. Nonetheless, 84% of the populations identified as *M. arenaria* had one of three esterase phenotypes designated as A1, A2, and A3 (Fig. 1). The A3 phenotype was specific for *M. arenaria,* as it did not appear in any other *Meloidogyne* species. The A1 and A2 phenotypes, however, were observed also in some other *Meloidogyne* forms (Table 1).

The rare esterase phenotypes, which occurred primarily in less prevalent *Meloidogyne* species, seemed not to be characteristic of any particular species. Each such phenotype was observed in several species or unidentified forms. However, some of

these phenotypes may prove to be specific for certain species, as all populations of some minor species appear to exhibit the same phenotype. Thus, four populations of *M. chitwoodi* from three continents had the same esterase phenotype (S1). Similarly, four populations of *M. hispanica* from widely separated parts of the world had a common esterase phenotype (S2-M1). Six populations of an undescribed species from coffee also had the same esterase phenotype (F1). Such phenotypes may appear in more than one *Meloidogyne* species, but they may be characteristic for those species and, therefore, useful in differentiating them from other species.

Next to the esterases, the Sod phenotypes showed the highest specificity in their association with certain species of *Meloidogyne*. Still, only the A4 Sod phenotype occurred exclusively in populations of *M. arenaria* and proved to be specific for this species. The other major phenotypes were partially specific for certain species; e.g., the H1 phenotype for *M. hapla*, the I2 phenotype for *M. incognita*, and the combination phenotype JA2 for most populations of *M. javanica* and *M. arenaria*. The latter phenotypes occurred also in other species or undescribed forms and, for this reason, were less useful as taxonomic characters. Furthermore, detection of Sod bands and determination of Sod phenotypes was not as easy as determination of esterase phenotypes.

Mdh and Got phenotypes were useful for differentiating *M. hapla* from the other major species and many of the less common species. However, *M. arenaria*, *M. incognita*, and *M. javanica* had similar Mdh and Got phenotypes and could not be differentiated on this basis.

Multienzyme phenotypes, formed by phenotypes of two or more enzymes, offered a biochemical profile or a variety of profiles for each species. These profiles often characterized *Meloidogyne* species more definitively than did the constituent single enzymes when considered individually. For example, a multienzyme profile of Est-A2, Sod-JA2 for esterase and Sod was more specific for *M. arenaria* than Est-A2 or Sod-JA2 considered separately. In most cases, however, the multienzyme phenotypes supplied limited, mostly supportive criteria for species characterization and identification. For most populations, the esterase phenotype, possibly in conjunction with the Sod phenotype, provided most of the biochemical information of taxonomic significance. This situation will probably change in the future if other, especially polymorphic, enzymes are detected and are considered in similar biochemical comparisons of *Meloidogyne* species.

For routine identification of the various phenotypes described in this paper, one should not rely heavily on the numerical expression of the migration rate of the various bands. The rate of migration (Rm) of proteins in a gel is highly dependent upon the conditions of protein extraction, sample storage, and electrophoretic conditions of separations (24). Such factors or conditions may vary among laboratories and from one electrophoretic run to the other in the same laboratory. For reliable identification of the phenotype of an unknown population, protein extracts from a pure population of a known species should always be included in the same gel where they can be compared directly. We recommend that pure populations of *M. hapla* and *M. javanica* be used as standard controls in each gel. Especially with reference to esterases, the phenotypes of these two species will serve as efficient guides for identification of all other phenotypes, often by simple visual evaluation of the gels (without actual measurement of Rm values).

With the present electrophoretic procedure, esterase phenotypes of as many as 20–25 individual females can be compared in the same gel. Egg masses can be saved and used later as inoculum to obtain progeny of the individual females. These features render this biochemical approach a valuable tool in *Meloidogyne* research. We have used this procedure in our laboratory to assess the purity or homogeneity of greenhouse cultures, to separate individual *Meloidogyne* species from multispecies field populations, and to initiate hybridization experiments using enzyme phenotypes as genetic markers. The same biochemical information can be used in many other areas of basic and applied research.

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