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Isozyme Phenotypes for the Identification of *Meloidogyne* Species[1]

P. R. ESBENSHADE AND A. C. TRIANTAPHYLLOU[2]

Abstract: Extensive studies during the last 20 years have demonstrated that enzyme phenotypes, especially those of esterases, are species-specific for *Meloidogyne* and can be used as reliable taxonomic characters for identification of most major and several minor species of this genus. Recent progress in electrophoretic procedures and advanced computer technology have made available automated electrophoretic apparati that can process very thin polyacrylamide slab gels on which the phenotypes of two or more enzymes can be revealed from the protein extract of a single *Meloidogyne* female. Presently, such apparati facilitate objective species identification. They also are convenient for performing routine field surveys to determine the relative distribution of major *Meloidogyne* species, conducting population dynamics studies in the field and in microplots, and testing the purity of greenhouse cultures.
Key words: electrophoresis, identification, isozyme, *Meloidogyne*, taxonomy.

All root-knot nematodes are presently described in the large genus *Meloidogyne* which comprises about 70 species (12). Species characterization is based primarily on morphological features of second-stage juveniles, males, and females. Information about host range and host specificity is also included in the original descriptions of some species. In practice, identification of the most common and the most agriculturally important species is often attempted by microscopic examination of perineal patterns of adult females and by conducting differential host tests. Precise and reliable morphological identification of species is a formidable task even for well-qualified taxonomists with expertise in the genus *Meloidogyne* (4,9).

An explanation for the difficulties encountered in characterizing and identifying *Meloidogyne* species has been provided by extensive cytogenetic studies of about 600 populations from many parts of the world (13). Such studies have demonstrated that the most common *Meloidogyne* species do not constitute true biological species; instead, they represent discrete, predominantly parthenogenetic forms derived along different lines of evolution. Variation in mode of reproduction, from amphimictic to facultatively and obligatorily parthenogenetic, and in degree of ploidy, ranging from haploidy to various levels of polyploidy (somatic chromosome numbers of 14 to 74), illustrates the cytogenetic complexity of the genus *Meloidogyne*.

Extensive enzymatic studies have demonstrated that the major species of *Meloidogyne* can be differentiated by species-specific enzyme phenotypes which can be revealed by polyacrylamide-gel electrophoresis (5,8,11). Furthermore, recent progress in electrophoretic procedures have made possible, and also practical, the detection of the phenotype of one, two, and even more enzymes from the protein extract of a single *Meloidogyne* female. These events have opened the way for efficient use of enzyme phenotypes as diagnostic characters in identification of *Meloidogyne* species. Presently and until more convenient species and (or) race-specific molecular probes (DNA probes, monoclonal antibodies, etc.) become available, evaluation of enzyme phenotypes can be used as a rapid, practical, and nonsubjective method of identifying most major and some minor species of *Meloidogyne*.

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[2] Laboratory Research Specialist and Professor, Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614. Present address of first author: Lancaster Laboratories, Inc., 2425 New Holland Pike, Lancaster, PA 17601.

Historical Review

Comparative biochemical studies involving soluble proteins and enzymes have been conducted during the last 20 years in a continous effort to find practical and objective means to differentiate *Meloidogyne* species. The first demonstration that some enzymes may be species-specific and could be used in the identification of *Meloidogyne* species was the graduate work of D. W. Dickson (3). This was immediately followed by the work of R. S. Hussey (10). In these early works, both conducted at North Carolina State University, a crude protein extract from hundreds of adult females of a given *Meloidogyne* species was subjected to polyacrylamide-gel electrophoresis and appropriately stained to reveal an enzyme's phenotype. Esterase, malate dehydrogenase, and α-glycerophosphate dehydrogenase phenotypes gave a strong indication of being useful in the identification of the four most common species of *Meloidogyne*, i.e., *M. incognita, M. arenaria, M. javanica,* and *M. hapla.* These studies were conducted with a small number of populations from each species and, therefore, could not guarantee that the established relationships between enzyme phenotypes and *Meloidogyne* species would hold true should additional populations of the same and other species be studied in the future.

A second period of important contributions in this line of research followed a few years later when A. Dalmasso and J. Berge of the Nematode Research Station at Antibes, France, expanded their enzymatic studies to a larger number of *Meloidogyne* populations of more diverse origin and representing more species (1,2). These same investigators also miniaturized the electrophoretic system by employing acrylamide gels cast in thin glass tubes, originally of 2 mm or more internal diameter (i.d.), but eventually using hematocrit tubes of only 1.1 mm i.d. Such miniaturization of the gels allowed the recording of an enzyme's phenotype from protein extracts of fewer adult females per gel, usually two or three, and occasionally from extracts of single females. Still, in this system reseachers had to evaluate many gels in order to establish the phenotype of a given enzyme because there was substantial variation in the relative electrophoretic mobility of an enzyme from one gel to another.

By 1980 thin-slab gel electrophoresis had gained wide acceptance over disc electrophoresis. The enzyme phenotype of a single *Meloidogyne* female could be revealed consistently on slabs 0.7 mm thick, and the phenotypes of many females could be compared on the same gel. This method, originally tested in Antibes, was subsequently deployed more extensively at North Carolina State University. A collaborative research effort by the two laboratories provided the strongest evidence that enzyme phenotypes, especially those of esterases, could be utilized as characters for species identification in the genus *Meloidogyne* (11). Some phenotypes were definitely species specific and could have been named after the species they represented. Nevertheless, it was decided at that time not to name them until more populations of each species could be studied.

An extensive effort to study additional populations was made later in collaboration and with the support of the International *Meloidogyne* Project. Approximately 300 populations, originating from 65 countries from various continents and representing 16 *Meloidogyne* species, were studied with regard to four enzyme systems, i.e., nonspecific esterases, malate dehydrogenase, superoxide dismutase, and glutamate-oxaloacetate transaminase (5). Esterases, as in previous studies, were found to be the most useful in the 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. Two additional esterase phenotypes could identify *M. arenaria* with 98–100% accuracy. Phenotypes of two or more enzymes, when considered in combination, could provide even more definitive characterization of *Meloidogyne* species.

In these studies, only major bands of enzymatic activity were considered. Minor bands, which had been recorded earlier in anticipation of being race-specific, were judged as too variable for an objective evaluation and were ignored. In general, the prospect for using enzymatic data for identification of *Meloidogyne* species at that time was so bright that a strong recommendation was issued to other researchers in the field to use the method. The procedure for carrying out electrophoresis was simple and feasible for routine work in any nematology laboratory equipped with even elementary facilities for electrophoretic work. Still, acceptance of this biochemical approach in other laboratories had to wait until further simplification of the electrophoretic procedure could be made. This happened recently when automated electrophoretic systems using precast and ready-to-use slab gels became available commercially. Several nematology labs are now equipped, or in the process of being equipped, with such electrophoretic apparati for routine identification of *Meloidogyne* species.

Present Status

The electrophoretic procedure employed in the most recent studies (6–8,11) which demonstrated the usefulness of enzyme phenotypes for identification of *Meloidogyne* species, has been described in detail (6). Although it is relatively cumbersome and time consuming, it is the most accurate and reliable system for initial investigations to determine the specific electrophoretic conditions under which the activity of a specific enzyme can be demonstrated. Automated electrophoretic apparati, which became available during the last 3 years, are much more convenient for routine work on enzymes that have already been studied by the previous method. The automated system (PhastSystem by Pharmacia) that we have used for routine work in our laboratory is a miniaturized system that uses very thin (0.4 mm) polyacrylamide gels precast in 4.3 × 5-cm slabs. It can run homogeneous, gradient, and isoelectric focusing gels. The entire procedure (i.e., temperature of the separation bed, application of the samples, and accumulated volthours of electric current for each step) is all computer controlled. Once the computer is programmed for these variables, the program is maintained in memory and can be recalled for each subsequent run. Actual time for electrophoresis is about 30 minutes. Enzyme phenotypes can be observed immediately after staining, which requires about 5 minutes for malate dehydrogenase or 1 hour for esterases. Therefore, results from 24 female nematodes can be obtained in less than 2 hours from the time the females are collected from infected plant roots.

Esterase phenotypes are the most instructive for identification of *Meloidogyne* species. Identification of major species—such as *M. incognita, M. arenaria, M. hapla,* and *M. javanica*—and some less common species—such as *M. chitwoodi, M. naasi,* etc.—can be made on the basis of esterase phenotype alone (Fig. 1). Evaluation of esterase phenotypes is simple, especially when *M. javanica* is used as the control in a central lane of each electrophoretic gel. In some instances, identification may not be definitive because of the similarity of the phenotypes of two species. For example, the esterase phenotypes of *M. hapla* and *M. incognita* consisting of a single major band of slightly different electrophoretic mobility (47% for *M. incognita,* 49–50% for *M. hapla*) may be confused. Staining the same gel a second time for the enzyme malate dehydrogenase differentiates these species as their malate dehydrogenase phenotypes are strikingly different (Figs. 1, 2).

Analogous procedures, which take advantage of phenotype differences for a second or a third enzyme, can and should be employed in differentiating other species combinations with similar or identical esterase phenotypes. Table 1 of Esbenshade and Triantaphyllou (5) can be used as a primary guide for such *Meloidogyne* species–enzymes combinations. Additional enzymes that in our limited survey have been reliable in differentiating certain *Meloido-*

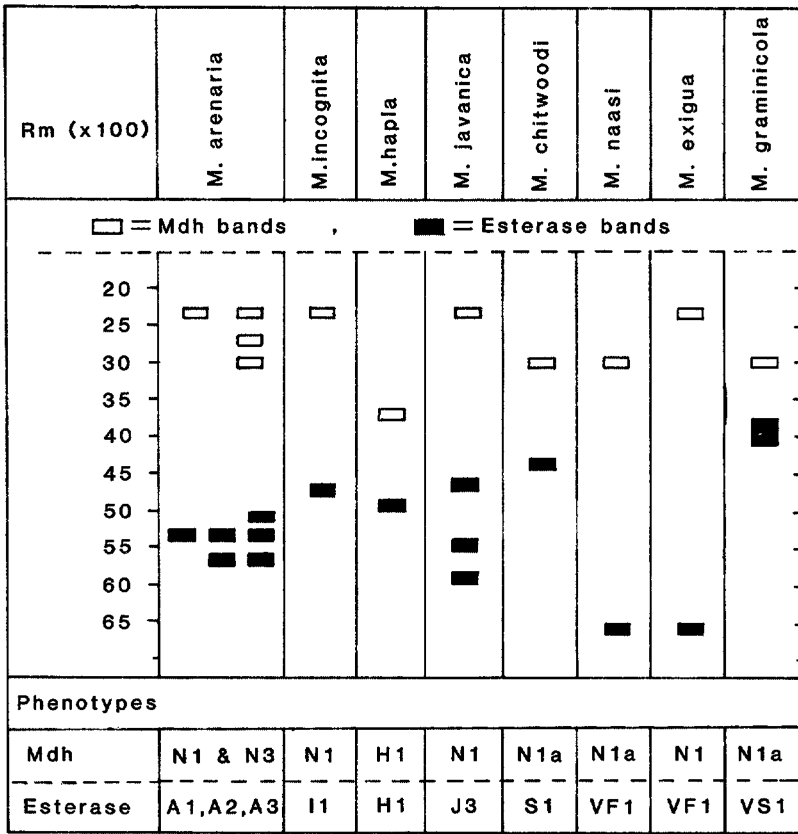

FIG. 1. Species-specific phenotypes of malate dehydrogenase (Mdh) and esterases helpful in differentiating eight species of *Meloidogyne*. On the same polyacrylamide gel (10–15% gradient), Mdh bands are bluish gray, those of esterases are black. Phenotype designations correspond to those of Esbenshade and Triantaphyllou (5).

gyne species in pair-wise comparisons include glucose phosphate isomerase (for differentiating *M. graminicola* from *M. chitwoodi, M. naasi,* and *M. graminis; M. hapla* from *M. microtyla,* and *M. incognita* from *M. microcephala*), fumarate hydratase (for differentiating *M. naasi* from *M. chitwoodi*), hexokinase, and triose phosphate isomerase (7).

Since data about many minor *Meloidogyne* species are based on the study of only one or a few populations, the enzyme phenotype survey must be extended to many more populations to establish the stability of an enzyme's phenotype within each species. As more laboratories deploy this diagnostic tool, more data will become available to be compiled for use by other laboratories. Still, a reliable diagnosis of a species through conventional taxonomic criteria, possibly including scanning electron microscope, host range, cytogenetic, and other data, will be necessary before enzyme phenotype data are permanently assigned to a species. Providing enzymatic phenotypes for incorrectly identified nematodes will only lead to confusion. In view of the difficulties of identifying root-knot nematodes, it may be advisable to seek verification of the identification to species from a lab with expertise in the genus *Meloidogyne*.

APPLICATIONS

If an automated electrophoretic apparatus is available in the laboratory, determination of the esterase phenotypes of a small number of females may be the best first step in the species identification of a *Meloidogyne* population. Since such an ap-

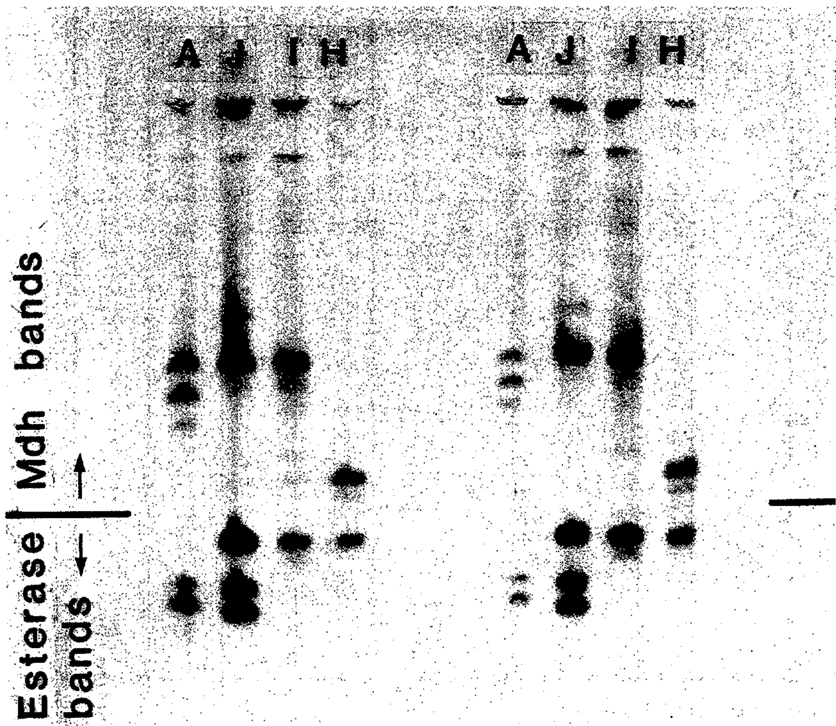

Fig. 2. A polyacrylamide gel (PhastSystem) showing the esterase and malate dehydrogenase (Mdh) phenotypes of single females of four major *Meloidogyne* species. A = *M. arenaria.* J = *M. javanica.* I = *M. incognita.* H = *M. hapla.*

paratus may accommodate two gels, 12 or 24 females (12 females per gel) can be studied in each electrophoretic run. Such a study will reveal whether the population is pure (all females have the same esterase phenotype) or the population is mixed, consisting of females of two or more species (two or more species-specific esterase phenotypes present). In most cases, when the species involved are among those with characteristic, species-specific esterase phenotypes (Fig. 1), identification of the species is easy and reliable. Examination of additional taxonomic characters to further confirm the identification may be undertaken subsequently. In some cases, when two or more species have the same esterase phenotype (e.g., *M. naasi* and *M. exigua*, Fig. 1) staining for a second enzyme becomes necessary for species identification. In such cases, malate dehydrogenase is a very instructive and easily detected secondary enzyme. A gel can be stained for 5 minutes for malate dehydrogenase, rinsed for 1 minute, and then stained for an additional 45 minutes for esterases. Bands of both enzymes are preserved on the same gel, which can be kept as a permanent record (Fig. 2).

Electrophoretic screening will be very helpful for testing greenhouse cultures for purity prior to conducting detailed and labor-intensive studies on host specificity, plant resistance, molecular diagnostics, etc. Similarly, this approach can be very useful in nematode population-dynamics studies. It can provide an estimate of the relative proportions of different *Meloidogyne* species in field or microplot populations at various intervals within a crop rotation scheme. Presently, and until more practical molecular probes become available, the use of

the esterase and malate dehydrogenase phenotypes as the only diagnostic character for species identification may prove to be the most rapid and efficient method for carrying out extensive field surveys to determine the frequency and relative distribution of the major *Meloidogyne* species. Unfortunately, this method provides no information about the host races of these nematodes.

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