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Comparison of Populations of *Pratylenchus brachyurus* Based on Isozyme Phenotypes[1]

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Abstract: Enzymes from females of five *Pratylenchus brachyurus* populations and one *P. scribneri* population were analyzed by isoelectric focusing electrophoresis. Of the 18 enzyme systems investigated, only malate dehydrogenase (MDH), phosphoglucomutase (PGM), and phosphoglucose isomerase (PGI) were detected from all five *P. brachyurus* populations and *P. scribneri.* Faint bands were detected for isocitrate dehydrogenase and phosphogluconate dehydrogenase from one *P. brachyurus* population. Three distinct phenotypic groups were found in the MDH and PGM systems for *P. brachyurus* populations, but only a single electromorph was detected for PGI. Multiple electromorphs for MDH, PGM, and PGI were detected for *P. scribneri*; there was no similarity among these patterns with those from *P. brachyurus.* No phenotypic differences in PGI were observed between females and mixed juveniles of one population of *P. brachyurus.*

Key words: electrophoresis, interspecific variation, intraspecific variation, isocitrate dehydrogenase, isoelectric focusing, isozyme, lesion nematode, malate dehydrogenase, phosphoglucomutase, phosphogluconate dehydrogenase, phosphoglucose isomerase, *Pratylenchus brachyurus, P. scribneri.*

The genus *Pratylenchus* is stenomorphic because of the small number of diagnostic characters at the species level and the intraspecific variability of some of these characters (11,13,18). Recent reappraisals or compendia list the number of valid species in the genus as 49 (11), 63 (13), or 60 (18). Modern biochemical systematic techniques (16) are now available that may help our understanding of the intraspecific and interspecific variability within the genus. Some enzyme phenotypes in some nematode species appear to be descriptive (6,9,10), although a range of genetic variability also occurs (7,19).

Refinements and miniaturization of electrophoretic techniques permit analysis of proteins from a single pyriform female in the Heteroderoidea (1,3). Most plant-parasitic nematodes, however, are microscopic vermiform animals. Detection with electrophoretic techniques of enzyme activity from a single vermiform nematode is not currently possible. Such analysis requires mass homogenates of hundreds or thousands of nematodes and generally includes mixed life stages (17). The principal problems in isolating proteins from single or a few vermiform nematodes are macerating the nematodes and protecting the released proteins from degradation. Techniques to macerate a single pyriform female have been developed (8), but these techniques will not work with vermiform nematodes.

Isoelectric focusing electrophoresis (IEF) is sensitive enough to detect enzyme activity from a single *Heterodera glycines* Ichinohe female (14) and to detect intraspecific variability among 12 *H. glycines* populations (20). This technique is a useful tool for studying protein polymorphism and genetic diversity among nematode populations (20).

Our objectives were 1) to compare five populations of *Pratylenchus brachyurus* (Godfrey) Filipjev & Schuurmans-Stekhoven from various geographical regions and hosts and one *P. scribneri* Steiner population by analyzing 18 enzyme systems by IEF and 2) to determine similarities between mixed juvenile stages and females of one population of *P. brachyurus.*

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Materials and Methods

The designations and sources of the five populations of *P. brachyurus* were as follows: 101 from corn (*Zea mays* L. cv. Pioneer 304C) and 102 from peanut (*Arachis hypogaea* L. cv. Florunner), Alachua County, Florida; 103 from Florunner peanut, Tift County, Georgia; 105 from soybean (*Glycine max* L. cv. Forrest), Nash County, North Carolina; and 108 from citrus (*Citrus* sp.), Polk County, Florida. The identification of each species was verified by morphometric studies using light microscopy.

Each population was isolated from field soil and placed on root explant cultures of corn (*Zea mays* L. cv. Iochief) grown in plastic petri dishes containing Gamborg's B-5 medium without auxins or cytokinins (Gibco, Grand Island, New York) and maintained in the dark at 29 C (15). The *P. scribneri* population on corn root explants was obtained from USDA ARS, Beltsville, Maryland. Nematodes were extracted from the culture dishes by removing a block of agar with roots and placing it in a modified Baermann funnel containing water (5).

A controlled vacuum aspirator (12) was used to pipet 250 females or 450 juveniles in deionized water from a dish into a depression slide previously coated with Repel-Silane (LKB, Bromma, Sweden) to produce a hydrophobic surface. After the nematodes settled to the bottom, the volume of water was reduced to about 20 µl by pipetting out excess water. Nematodes and water were sucked into a 25-µl capillary tube and used immediately or stored overnight at 5 C. The contents of each capillary tube were placed into a small well previously cut in the ground-glass surface of a Wheaton microculture slide (Thomas Scientific, Swedesboro, NJ) (Fig. 1A). Four wells with straight sides, flat bottoms, and beveled lips were cut into the slide. To cut the wells, a starter hole was made with a 1.6-mm drill bit then a 1.75-mm flat-headed miniscrewdriver, each attached to a 9.5-mm drill. Each well was drilled ca. 1.5 mm deep, and the lips were beveled with a 2.8-mm drill bit. The dimensions of each well were ca. 2.0 mm wide at the bottom, 2.5 mm wide at the surface, and 1.5 mm deep (Fig. 1B).

The surface of the slide, excluding the wells, was coated with Repel-Silane to obtain a hydrophobic surface that prevented the spreading of the droplet from the capillary tube and allowed the nematodes to fall to the bottom of the well. Nematodes that did not fall were pushed into the well with a hair attached to the end of a needle. The glass slide was then placed in a plastic sandwich box (Fig. 1B) containing an ice block, which cooled the nematodes during maceration, and a mirror directly under the glass slide, which enhanced visibility. The entire process was done under the dissecting microscope at 30× magnification.

After placing the nematodes in the well, most of the water was removed with a 1-ml disposable syringe with the needle located near the edge of the well. The remaining water was removed with filter paper (Whatman No. 2). The nematodes, which remained attached to the walls of the well by a thin film of water, were easily macerated for 30 seconds with a precooled ground-glass rod made to fit the well snugly. After maceration, 2 µl solution containing 20% sucrose (w/v) and 2% (v/v) Triton X-100 (8) was added to the well and the contents were mixed thoroughly. Finally, the contents of the well were absorbed onto a 5- × 10-mm filter paper (LKB, Bromma, Sweden) and applied directly to the surface of the isoelectric focusing gel.

Precast 24.5- × 10.5-cm polyacrylamide gels with a concentration that consisted of T = 5%, C = 3%, and 2.4% ampholytes with a pH range of 3.5–9.5 were used. The LKB (Pharmacia LKB Biotechnology, Piscataway, NJ) ultrophor system was used with 1 M H_3PO_4 wicked across the anode and 1 M NaOH wicked across the cathode (21). During isoelectric focusing, the gels were maintained at a constant temperature of 5 C and run at a constant voltage. The power was applied from an LKB macrodrive 5 constant power supply set at 1,500 volts, 50 milliamps, and 30 watts. The pre-

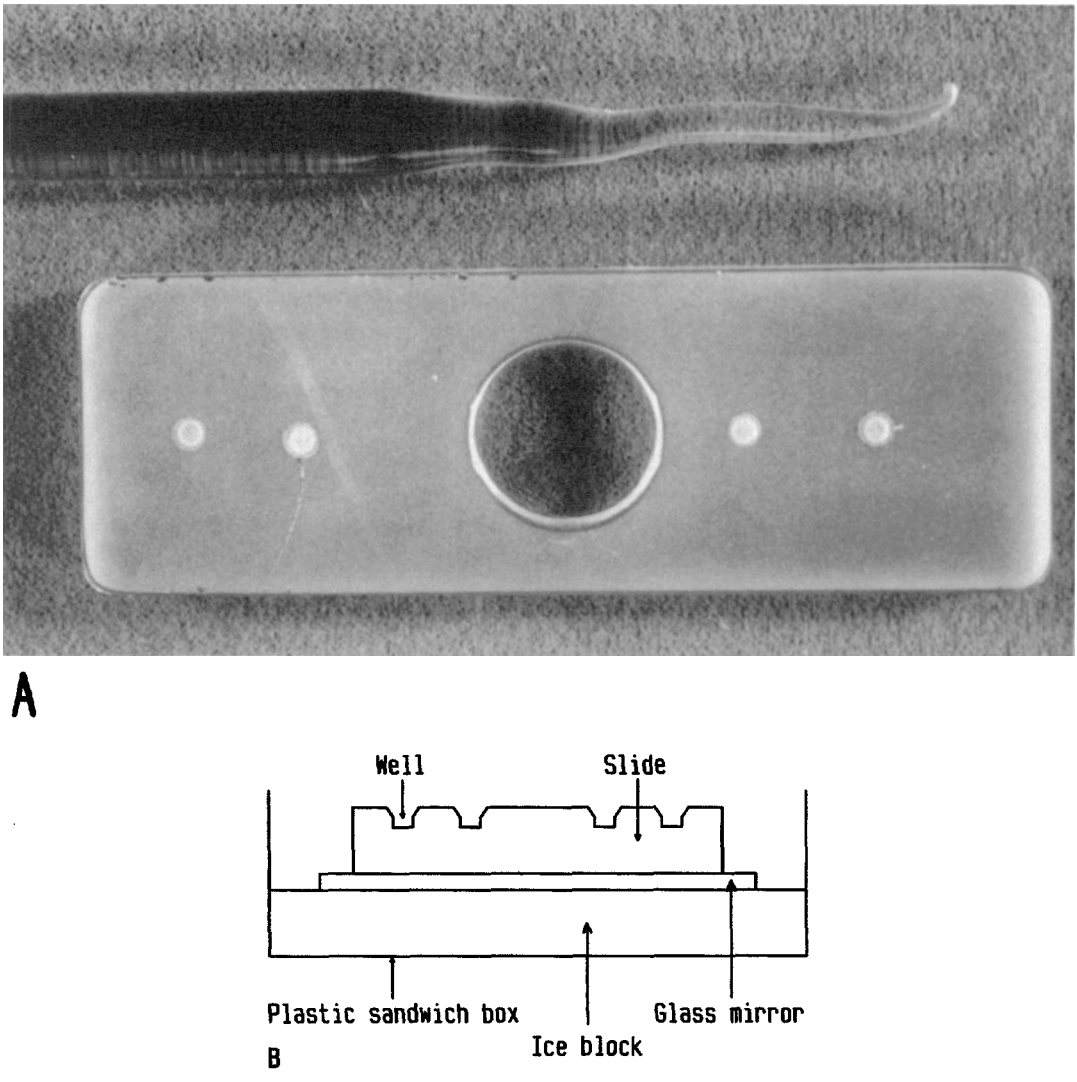

FIG. 1. Protein extraction equipment. A) Wheaton microculture slide showing the small wells and the glass rod constructed to macerate vermiform nematodes for protein extraction. B) Cross section of the microscope slide (not to scale) used for macerating vermiform nematodes. The dimensions of each well are ca. 2.0 mm wide at the bottom, 2.5 mm wide at the surface (beveled), and 1.5 mm deep. The slide was placed over a glass mirror that lay over an ice block contained in a plastic sandwich box.

cast gels were cut generally into smaller sections for runs with 5–10 nematode preparations. The settings of the power supply were reduced proportionally to the size of the gel. Each gel was prefocused for 10 minutes, nematode samples were applied in duplicate, and the gel was run for 1.5 hours. The pH gradient of each gel was determined immediately following a run by measuring the pH at 0.5-cm intervals from the anodic to the cathodic end using a surface electrode.

The gels were immersed in buffer (the same buffer used in the stain mixture) for 3 minutes to remove excess electrolytes from the gel surface. The gels were then submerged into the enzyme reaction mixtures in a plastic box lined previously with plastic film and incubated in the dark at 25 C until bands appeared. The enzymes used and the references to the stain and reaction mixtures are in Table 1. Each enzyme system was evaluated initially in protein preparations from females of *P. brachyurus* 101

TABLE 1. Enzymes examined, activity, and references to stains and reaction mixtures used in isoelectric focusing of *Pratylenchus brachyurus* mass homogenates.

	Enzyme commission number	Activity	Reference
Oxidoreductases			
Aldehyde oxidase	1.2.3.1	−	(24)
α-Glycerophosphate dehydrogenase	1.2.1.12	−	(2)
Isocitrate dehydrogenase	1.1.1.42	+	(2)
Malate dehydrogenase	1.1.1.37	+	(2)
Malic enzyme	1.1.1.40	−	(23)
Octanol dehydrogenase	1.1.1.73	−	(23)
Phosphogluconate dehydrogenase	1.1.1.44	+	(24)
Superoxide dismutase	1.15.1.1	−	(24)
Xanthine dehydrogenase	1.2.1.37	−	(23)
Transferases			
Glutamate oxaloacetate transaminase	2.6.1.1	−	(24)
Hexokinase	2.7.1.1	−	(24)
Phosphoglucomutase	2.7.5.1	+	(24)
Hydrolases			
Acid phosphatase	3.1.3.2	−	(24)
Alkaline phosphatase	3.1.3.1	−	(24)
Esterase	3.1.1.8	−	(2)
Lyase			
Fumarase	4.2.1.2	−	(2)
Isomerases			
Mannose phosphate isomerase	5.3.1.8	−	(23)
Phosphoglucose isomerase	5.3.1.9	+	(24)

+ activity detected; − no activity detected.

or 105, and all runs were repeated 4–20 times. The isoelectric point (pI) of each electromorph (i.e., a single resolved band) was determined by plotting the distance migrated by the electromorph on the regression line fitted to the pH gradient of the gel.

A paired affinity (Pa) index (4) was calculated for all populations to compare shared electromorphs. The index is the number of shared bands divided by the total number of bands.

RESULTS

Of the 18 enzyme systems studied, only malate dehydrogenase (MDH), phosphoglucomutase (PGM), and phosphoglucose isomerase (PGI) were consistently detected and highly reproducible from all five populations of *P. brachyurus* and *P. scribneri* (Fig. 2A–C). Isocitrate dehydrogenase (ICD) and phosphogluconate dehydrogenase (PGD) showed faint bands and were not reproducible for *P. brachyurus* 105; therefore they were not tested with the other populations.

The MDH system revealed three distinct phenotypic groups for *P. brachyurus* populations and a different pattern for *P. scribneri* (Fig. 2A). Two electromorphs were resolved for populations 101 and 105 at pH 5.48 and 5.97. Populations 103 and 108 shared the two electromorphs from group one but had two others at pH 6.35 and 6.78. Population 102 contained eight electromorphs. Two were shared with all four populations, and two others were shared with populations 103 and 108 at pH 6.35 and 6.78. The remaining four electromorphs at pH 5.91, 6.16, 6.41, and 6.53 were unique to population 102. *Pratylenchus scribneri* had two electromorphs at pH 7.28 and 7.65.

Three distinct phenotypes were detected in the PGM system for *P. brachyurus* with each differing from the *P. scribneri* phe-

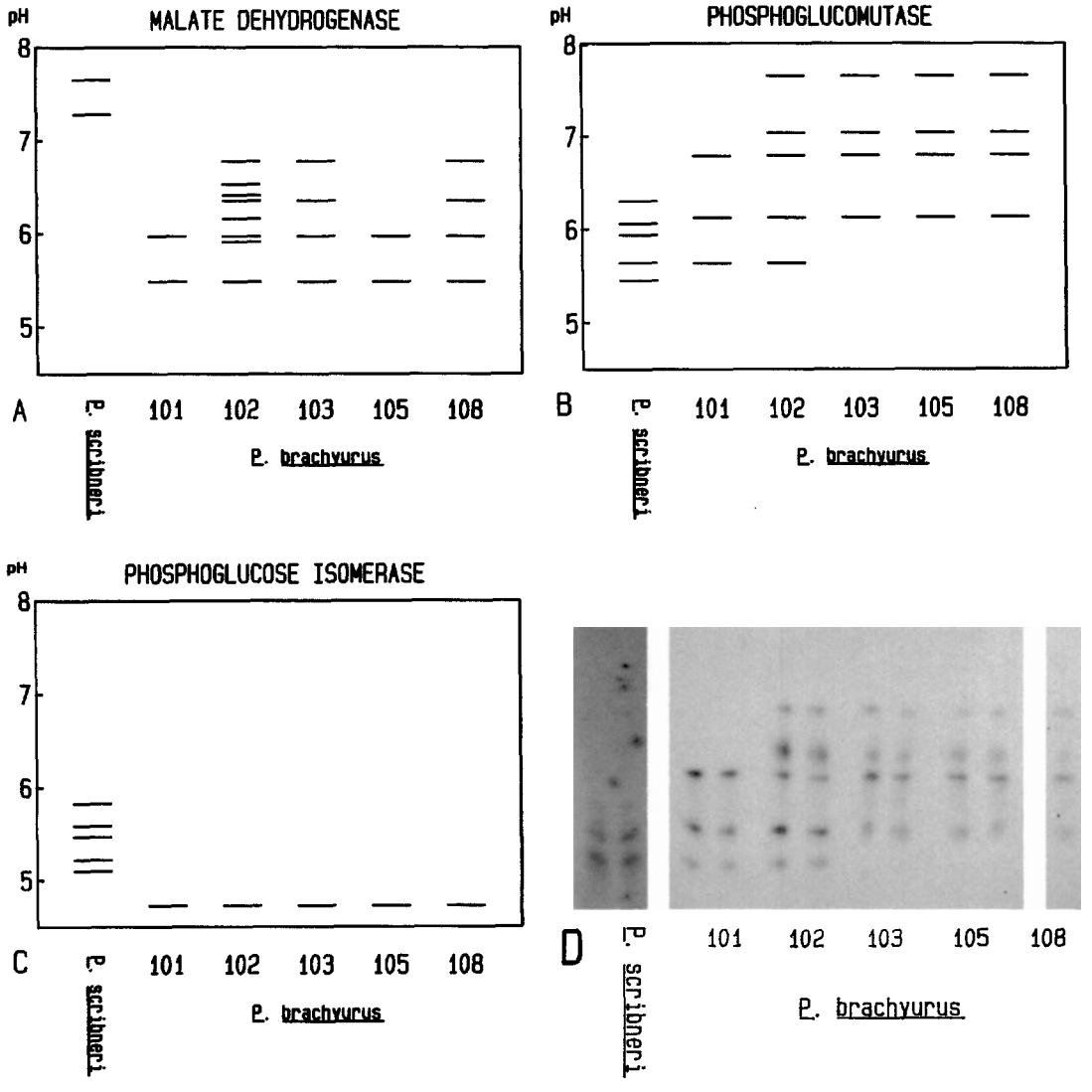

FIG. 2. A–D) Diagrammatic sketches (A–C) and a photograph (D) of isozyme patterns following isoelectric focusing of crude protein homogenates from 250 females from each of five *Pratylenchus brachyurus* populations and one *P. scribneri* population. Population origins: 101—'Pioneer 304C' corn, Alachua County, Florida; 102—'Florunner' peanut, Alachua County, Florida; 103—'Florunner' peanut, Tift County, Georgia; 105—'Forrest' soybean, Nash County, North Carolina; 108—*Citrus* sp., Polk County, Florida. *Pratylenchus scribneri* from USDA ARS, Beltsville, Maryland. A) Malate dehydrogenase. B) Phosphoglucomutase. C) Phosphoglucose isomerase. D) Phosphoglucomutase isomerase.

notype (Fig. 2B, D). Population 101 had three electromorphs located at pH 5.64, 6.13, and 6.8. Populations 103, 105, and 108 shared two electromorphs with populations 101 (pH 6.13 and 6.8) but had two others located at pH 7.04 and 7.65. Population 102 contained five electromorphs. Two were shared with all populations (pH 6.13 and 6.8), one with population 101 (pH 5.64), and two with the other three populations (pH 7.04 and 7.65). *Pratylenchus scribneri* showed five electromorphs at pH 5.46, 5.65, 5.95, 6.07, and 6.31. The PGI system exhibited only one electromorph at pH 4.73 for all *P. brachyurus* populations, whereas five electromorphs were resolved for *P. scribneri* at pH 5.1, 5.22, 5.47, 5.59, and 5.83 (Fig. 2C).

Population 105 showed one faint band at pH 7.0 in the ICD system and one at pH

5.6 in the PGD system. When comparing females with mixed juvenile stages of population 105 using the PGI system, no phenotypic differences were observed. In addition, females of all *P. brachyurus* populations, except 108, extracted from greenhouse cultures of Harvester snap bean exhibited MDH patterns identical to those obtained from females extracted from corn root explant cultures. Population 108 was not tested.

Based on the enzyme systems tested, the populations of *P. brachyurus* with the most similarity were 103 from Georgia and 108 from Florida (Table 2). Their Pa index was 100%. These two populations had the same phenotypes in all enzyme systems. On the other hand, the populations with the least similarity among the enzyme systems tested were 101 and 102 from Florida with a Pa index of 43%. Population 105 from North Carolina was more closely related to 103 and 108 than to 101 or 102.

TABLE 2. Paired affinity indices† (%) of five *Pratylenchus brachyurus* populations based on phenotypes of malate dehydrogenase, phosphoglucomutase, and phosphoglucose isomerase.

	Population‡				
	101	102	103	105	108
101		6/14 (43%)	5/10 (50%)	5/8 (62%)	5/10 (50%)
102			9/14 (64%)	7/14 (50%)	9/14 (64%)
103				7/9 (78%)	9/9 (100%)
105					7/9 (78%)

† Paired affinity index is the number of shared bands divided by the total number of bands.

‡ Population origins: 101—'Pioneer 304C' corn, Alachua County, Florida; 102—'Florunner' peanut, Alachua County, Florida; 103—'Florunner' peanut, Tift County, Georgia; 105—'Forrest' soybean, Nash County, North Carolina; 108—*Citrus* sp., Polk County, Florida.

DISCUSSION

The technique developed during this study allowed us to consistently detect enzyme patterns from protein homogenates from only 250 vermiform females. Fewer females can be used, but to standardize our methodology all our tests were performed with 250 females. Excellent resolution of PGI was obtained using 100 females or 450 mixed juveniles from *P. brachyurus* population 105 from North Carolina. Of the 18 enzyme systems studied, only the MDH, PGM, and PGI enzyme systems were resolved consistently. Although the ICD and PGD systems were detected, the bands were poorly resolved. Exhaustive attempts failed to detect esterases in any *P. brachyurus* population or *P. scribneri*, although excellent detection occurred repeatedly with single females of *Meloidogyne javanica* (Treub) Chitwood.

The poor detection obtained with ICD and PGD isozyme systems may be related to nematode age. It was subsequently found that nematodes must be used within 3 days of extraction from roots and must be physically active at the time of extraction for successful detection of enzyme phenotypes. Electromorphs were missing or faint when nematodes were stored in water for more than 4 days before use. Although the electromorphic staining intensity can be increased by using larger numbers of nematodes, our goal was to minimize the number of individuals required for unambiguous visualization. Unfortunately, these observations were discovered at a later date and were not fully evaluated in the ICD and PGD systems. The analyses conducted with MDH, PGI, and PGM were performed with freshly collected nematodes. The problems of extraction, selection, and collection of the nematodes in a small volume of water in addition to running the electrophoretic gel in the same day were solved by storing live nematodes overnight at 5 C in a capillary tube.

The MDH and PGM systems indicated distinct levels of intraspecific variability among *P. brachyurus* populations collected from various geographical locations and hosts, whereas interspecific variability was observed in these two systems and in the PGI system. *Pratylenchus brachyurus* is a monosexual species, reproduces by mitotic parthenogenesis, and has a relatively high chromosome number, 30–32, indicative of polyploidy (22). To understand the coding

of genes for the different enzyme systems in plant-parasitic nematodes, it is necessary to design studies with controlled single-pair crosses with identifiable genetic markers, as has been done with *H. glycines* (8). Such studies presently are impossible with a parthenogenetic species such as *P. brachyurus.*

Several interpretations are possible for the distinct phenotypic groups found in the MDH system in *P. brachyurus*: 1) The multiple bands may represent isozymes, i.e., gene products of slightly variant genes. There may be eight isozymes involved in population 102, whereas four similar or identical isozymes may be associated with populations 103 and 108 and two similar or identical isozymes with populations 101 and 105. It is possible that some of these isozymes are rare and either not present or not expressed in populations with fewer bands. 2) Since MDH is a dimeric enzyme, it is possible that the extraction procedure may cause the monomeric units to react enzymatically and break down. It is not known if some of these bands represent artifacts. 3) There may be different allelic forms of the same gene or genes. Some populations may be homozygous for one form, others homozygous for the other form, and still others with both forms are heterozygous.

Distinct phenotypic groups were found in the PGM systems in *P. brachyurus.* Also, in addition to the interpretations given for the MDH systems, it is possible that there were two distinct groups of populations with distinct phenotypes. One group included population 101, and the other group included populations 103, 105, and 108. Population 102 may contain a mixture of genes from the two groups. The only phenotype for *P. brachyurus* populations in the PGI system could be the product of a single locus.

Literature Cited

1. Bergé, J. B., and A. Dalmasso. 1975. Caracteristiques biochimiques de quelques populations de *Meloidogyne hapla* et *Meloidogyne* spp. Cahiers ORSTOM serie Biologie 10:263–271.

2. Bush, G. L., and R. N. Huettel. 1972. Starch gel electrophoresis of tephrid proteins: A manual of techniques. Population Genetics Project, Phase I. International Biological Programme, Working Group on Fruit Flies, University of Texas, Austin.

3. Dalmasso, A., and J. B. Bergé. 1978. Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* spp.: Application to the taxonomy of *Meloidogyne.* Journal of Nematology 10:323–332.

4. Ellison, W. L., R. E. Alston, and B. L. Turner. 1962. Methods of presentation of crude biochemical data for systematic purposes, with particular reference to the genus *Bahia* (Compositae). American Journal of Botany 49:599–604.

5. Endo, B. Y. 1959. Responses of root-lesion nematodes, *Pratylenchus brachyurus* and *P. zeae,* to various plants and soil types. Phytopathology 49:417–421.

6. Esbenshade, P. R., and A. C. Triantaphyllou. 1985. Use of enzyme phenotypes for identification of *Meloidogyne* species. Journal of Nematology 17:6–20.

7. Esbenshade, P. R., and A. C. Triantaphyllou. 1987. Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). Journal of Nematology 19:8–18.

8. Esbenshade, P. R., and A. C. Triantaphyllou. 1988. Genetic analysis of esterase polymorphism in the soybean cyst nematode, *Heterodera glycines.* Journal of Nematology 20:486–492.

9. Fargette, M. 1987. Use of the esterase phenotype in the taxonomy of the genus *Meloidogyne.* 1. Stability of the esterase phenotype. Revue de Nématologie 10:39–43.

10. Fargette, M. 1987. Use of the esterase phenotype in the taxonomy of the genus *Meloidogyne.* 2. Esterase phenotypes observed in West African populations and their characterization. Revue de Nématologie 10:45–56.

11. Frederick, J. J., and A. C. Tarjan. 1989. A compendium of the genus *Pratylenchus* Filipjev, 1936 (Nemata: Pratylenchidae). Revue de Nématologie 12: 243–256.

12. Ford, H. W. 1957. A source of controlled vacuum for pipetting nematodes. Plant Disease Reporter 14:89–90.

13. Handoo, Z. A., and A. M. Golden. 1989. A key and diagnostic compendium to the species of the genus *Pratylenchus* Filipjev, 1936 (lesion nematodes). Journal of Nematology 21:202–218.

14. Huettel, R. N. 1986. Analysis of phosphoglucose isomerase from single female soybean cyst nematodes, races 1, 3, 4, and 5 by isoelectric focusing. Journal of Nematology 18:613 (Abstr.).

15. Huettel, R. N., and R. V. Rebois. 1985. Culturing plant-parasitic nematodes using root explants. Pp. 155–158 *in* B. W. Zuckerman, W. F. Mai, and M. B. Harrison, eds. Plant nematology, laboratory manual. University of Massachusetts Agricultural Experiment Station, Amherst.

16. Hussey, R. S. 1979. Biochemical systematics of nematodes—a review. Helminthological Abstracts. Series B, Plant Nematology 48:141–148.

17. Hussey, R. S., and L. R. Krusberg. 1971. Disc-electrophoretic patterns of enzymes and soluble proteins of *Ditylenchus dipsaci* and *D. triformis.* Journal of Nematology 3:79–84.

18. Luc, M. 1987. A reappraisal of Tylenchida (nemata). 7. The family Pratylenchidae Thorne, 1949. Revue de Nématologie 10:203–218.

19. Pais, C. S., and I. M. de O. Abrantes. 1989. Esterase and malate dehydrogenase phenotypes in Portuguese populations of *Meloidogyne* species. Journal of Nematology 21:342–346.

20. Radice, A. D., R. D. Riggs, and F. H. Huang. 1988. Detection of intraspecific diversity of *Heterodera glycines* using isozyme phenotypes. Journal of Nematology 20:29–39.

21. Righetti, P. G. 1983. Isoelectric focusing: Theory, methodology and applications. Pp. 1–135 *in* T. S. Work and R. H. Burdon, eds. Laboratory techniques in biochemistry and molecular biology. Amsterdam: Elsevier Biomedical Press.

22. Roman, J., and A. C. Triantaphyllou. 1969. Gametogenesis and reproduction of seven species of *Pratylenchus*. Journal of Nematology 1:357–362.

23. Shaw, C., and R. Prasad. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. Biochemical Genetics 4:297–320.

24. Vallejos, C. E. 1983. Enzyme activity staining. Pp. 469–516 *in* S. D. Tanksley and T. J. Orton, eds. Isozymes in plant genetics and breeding. Part A. Amsterdam: Elsevier Scientific Publishing Company.