# Biochemical Identification of the Two Races of Radopholus similis by Starch Gel Electrophoresis

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*Abstract:* Analysis of genetic variation between the banana and the citrus races of *Radopholus similis* by starch gel electrophoresis demonstrated that 7 of 16 enzyme-encoding loci could be used for their diagnostic separation. The two races are closely related and share approximately 75% of the enzymes evaluated. The level of dissimilarities of inherited bands indicates that no gene flow occurs between the races. Aldolase, $\alpha + \beta$ esterase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, and phosphoglucose isomerase are diagnostic markers of the races. *Key words:* isozyme, nematode races, burrowing nematode, genetics, biosystematics.

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Biochemical techniques, including gel electrophoresis, are potential methods available for defining systematic relationships of nematodes (13). Electrophoretic techniques are especially useful for obtaining estimates of genetic variation within and between populations. These techniques are particularly suitable for collecting such data because they allow for the separation and identification of specific soluble enzymes and nonenzymatic proteins (3).

Many soluble enzymatic proteins have multiple forms, with similar or identical substrate affinities, called isozymes (19). Comparisons of isozyme patterns obtained from the electrophoresis of proteins of mass homogenates or from individuals within a population can provide a measure of the similarity between different populations by analysis of shared or different band mobilities (15). The enzyme banding patterns provide information also about the genetic makeup of populations. Some enzymes appear to evolve more rapidly than the nonenzymatic proteins (20); thus, differences between closely related species and populations are more likely to be detected using them.

Starch gel electrophoresis has rarely been employed in biochemical systematic studies of nematodes (4,7,8). Our objectives in this study were 1) to determine the practicality of starch gel electrophoresis in identification of the banana and citrus race of *Radopholus similis,* 2) to provide estimates of the degree of population divergence between the races, and 3) to help understand the genetic makeup of the populations included in this study.

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## MATERIALS AND METHODS

*Nematode populations:* Six populations of the citrus race and ten populations of the banana race of *Radopholus similis* (Cobb) Thorne were investigated. The designations and sources of each of the two races were Hawaii I, *Anthurium,* Hawaii; Orlando I, *Citrus,* Orlando, Florida; Orlando II, *Citrus,* Orlando, Florida; Polk I, *Citrus,* Polk County, Florida; Polk II, *Citrus,* Polk County, Florida; Dade I, *Musa,* Dade County, Florida; Tapachula, *Musa,* Tapachula, Mexico; Guatemala, *Musa,* unknown, Guatemala; Guaruma, *Musa,* La Lima, Honduras; Coto, *Musa,* Coto, Costa Rica; Armuelles, *Musa,* Armuelles, Panama; Changuinola, *Musa,* Changuinola, Panama; Machala, *Musa,* Machala, Ecuador; and Hawaii II, *Musa,* Hawaii. One citrus race and one banana race population characterized previously by Huettel and Dickson (10) were analyzed also and designated Orlando and Florida, respectively.

*Preparation of nematodes for electrophoresis:* Nematodes (mixed life stages ca. 90% $L_4$ or young adults of both sexes) were removed from carrot cultures by gently washing the sides of the culture jars or tubes with water (11). The nematodes were placed into a sterilized graduated centrifuge tube and centrifuged for 1 min (1,610 *g*). The supernatant was decanted to 0.5 ml and the sample was added to 240 $\mu$l microcentrifuge tubes in 50 $\mu$l aliquots (ca. 1,500–3,000 nematodes).

The nematode samples were centrifuged at ca. 1,200 *g* for 1 min in a Beckman Microfuge; the supernatant was removed and 200 $\mu$l of buffer (12) was added. The sample was centrifuged again and all but ca. 10 $\mu$l of buffer was removed. Samples were stored at –87 C in an ultra-low freezer for up to 4 months with no loss of enzymatic activity.

*Preparation and loading of nematode extracts for starch gel electrophoresis:* Approximately 30 $\mu$l of diluted Poulik buffer (16) was added to the microcentrifuge tube with 30 mg of 0.1 mm glass beads. Each sample was homogenized for ca. 1 min with a Teflon homogenizer fitted to a motor operated by a variable transformer. One or two wicks of precut number 1 Whatman chromatography paper, either 0.6 cm × 1.0 cm or 0.4 cm × 1.0 cm, were placed in each tube.

*Electrophoresis:* Horizontal starch gel electrophoresis was conducted as described from Bush and Huettel (2). Two different buffers were used in the electrode buffer trays: 1) Tris-citrate buffer pH 7.0 (0.13 M Trizma base and 0.043 M citric acid). 2) Tris-EDTA-borate, pH 7.0 (0.5 M Trizma base, 0.016 M ethylenediamine tetraacetic acid [EDTA] and 0.65 M boric acid) (19). Sites of enzyme activity were detected following electrophoresis by immersing the gels in the following reaction mixtures (2, 18):

- Acid phosphatase-(ACPH). 100 mg $MgCl_2$, 100 mg $MnCl_2$, 100 mg polyvinylpyrrolidone (PVP), 300 mg NaCL, 160 mg $\alpha$-naphthyl acid phosphate ($\alpha$-NAP), and 40 mg fast blue (FB) in 100 ml tris-maleate buffer.
- Adenylate kinase-(ADK). 90 mg glucose, 21 mg $MgCl_2$, 25 mg nicotinamide adenine dinucleotide phosphate (NADP), 25 mg adenosine 3′5′-cyclic monophosphate (AMP), 6 mg 3-(4,5-dimethylthiozil-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 80 units hexokinase (HEX), and 3 mg phenazine methosulfate (PMS) in 100 ml dehydrogenase buffer (DH).
- Alcohol dehydrogenase-(ADH). 20 mg nitro blue tetrazolium (NBT), 25 mg $\beta$-nicotinamide adenine dinucleotide (NAD), 1 mg PMS, and 4 ml 2-propanol in 100 ml DH buffer.
- Aldolase-(ALD). 50 mg NAD, 30 mg NBT, 156 mg tetrasodium-fructose-1,6-diphosphate (Fl,6P), 100 units glyceraldehyde-3-phosphate (G3P), and 1 mg PMS in 100 ml DH buffer.
- Alkaline phosphatase-(ADH). 100 mg $MgCl_2$, 100 mg $MnCl_2$, 100 mg NaCl, 160 mg $\alpha$-NAP, and 40 mg FB in 100 ml Poulik buffer (16).
- $\alpha$ + $\beta$-Esterase-($\alpha$ + $\beta$-EST). 15 mg $\alpha$-naphthyl acetate ($\alpha$-NA), 15 mg $\beta$-napthyl acetate ($\beta$-NA), 40 mg fast garnet (FG), and 5 ml 1-propanol in 100 ml phosphate buffer (2).
- Fumarase-(FUM). 75 mg NAD, 20 mg NBT, 200 units malic dehydrogenase, 200 mg fumaric acid (FUM), and 1 mg PMS in 100 ml DH buffer.
- Glucose-6-Phosphate dehydrogenase-

(G6PDH). 70 mg NADP, 100 mg $MgCl_2$, 200 mg glucose-6-phosphate (G6P), 50 mg NBT, and 1 mg PMS in DH buffer.

- $\alpha$-Glycerophosphate dehydrogenase-($\alpha$-GDH). 200 mg $\alpha$-glycerophosphate, 25 mg NAD, 20 mg NBT, 1 mg PMS in 100 ml DH buffer.
- Isocitrate dehydrogenase-(IDH). 20 mg NBT, 20 mg NADP, 100 mg $MgCl_2$, 200 mg isocitric acid (IDH), and 1 mg PMS in 100 ml DH buffer.
- Lactate dehydrogenase-(LDH). 50 mg NAD, 20 mg NBT, 10 ml sodium lactate (LDH), and 2 mg PMS in 100 ml DH buffer.
- Malate dehydrogenase-(MDH). 20 mg NBT, 10 mg NADP, 25 mg malic acid, 100 mg $MgCl_2$, and 1 mg PMS in 100 ml DH buffer.
- 6-Phosphogluconate dehydrogenase-(6PDH). 20 mg NADP, 30 mg NBT, 20 mg trisodium-6-phosphogluconate (PDH), and 2 mg PMS in 100 ml DH buffer.
- Phosphoglucose isomerase-(PGI). 100 mg $MgCl_2$, 25 mg fructose-6-phosphate (F6P), 15 mg NADP, 20 mg MTT, 25 units G6PD, and 6 mg PMS in 100 ml DH buffer.
- Phosphoglucomutase-(PGM). 85 mg $\alpha$-D-glucose-1-phosphate (G1P), 5 mg NADP, 5 mg MTT, 1 mg PMS, and 100 mg $MgCl_2$, and 80 units G6PD in 100 ml of DH buffer.
- Stain buffers. 1) DH buffer (0.5 M Tris-HCl buffer pH 8.0, diluted 1:3 with distilled water). 2) Poulik buffer. 3) 0.1 M Tris–0.1 M maleic acid buffer, pH 8.2. 4) 0.1 M phosphate buffer pH 8.0 (2).

Different buffers were required to give good resolution of different enzymes. Incorrect buffer and/or pH resulted in band smearing. The optimum buffer or buffers and coenzyme combinations for the enzymes studies are listed in Table 1.

Incubation of gels was carried out at 37 C in the dark for 1–24 hours, depending upon the reaction. When the desired staining intensity was attained, the gels were removed from the reaction mixture and rinsed twice in distilled water. They were fixed for at least 12 hours in a solution of five parts methanol, five parts distilled water, and one part glacial acetic acid. The gels were immediately photographed, wrapped in plastic food wrap, and stored in a refrigerator.

*Population analysis:* Coefficients of similarity (CS) were calculated based on the number of band patterns observed on the gels (14). If a band was observed in the same position in both the citrus and banana races, it was given the same number (i.e., PGI-1). Different band positions were numbered consecutively from the origin (i.e., EST-1, EST-2). To determine the CS value, the number of bands determined to be common in both races was divided by the total number of bands detected in both races.

## RESULTS

*Isozymes:* All isozymes resolved migrated anodally on the gels except for one isozyme of MDH which migrated cathodally. For convenience, the bands of each enzyme are numbered consecutively beginning with the one nearest the origin. Each scorable isozyme pattern listed in Table 2 is discussed separately.

- Aldolase. One isozyme, 12 mm from the origin, was detected in all populations of the banana race; another band with a distinctly different mobility rate, 10 mm

Table 1. Buffers and enzyme combinations that gave the best results on starch gels.

| Buffers | Enzymes |
|---|---|
| Tris-citrate (TC), pH 7.0 | acid phosphatase, alcohol dehydrogenase, alkaline dehydrogenase, $\alpha$ + $\beta$ esterase, $\alpha$-glyceraldehyde dehydrogenase, lactate dehydrogenase, malate dehydrogenase, malic enzyme, phosphoglucomutase |
| Tris-EDTA-borate (TVB), pH 7.0 | adenylate kinase, fumarase, phosphoglucose isomerase |
| Tris-EDTA-borate (TVB), pH 7.0 and NADP | glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, 6-phosphoglucose dehydrogenase |
| Tris-EDTA-borate (TVB), pH 7.0 and NAD | aldolase, glyceraldehyde-3-phosphate dehydrogenase |

Table 2. Isozymes surveyed by starch gel electrophoresis from mass homogenates of ten populations of the banana race and six populations of the citrus race of *Radopholus similis*.

| Enzyme | Locus | Banana | Citrus |
|---|---|---|---|
| acid phosphatase | ACPH | 1* | 1 |
| adenylate kinase | ADK | 1 | 1 |
| alcohol dehydrogenase | ADH | 1 | 1 |
| aldolase | ALD | | 1 |
| | | 2 | |
| alkaline phosphatase | APH | 1 | 1 |
| $\alpha + \beta$ esterase | $\alpha + \beta$ EST | 1 | 1 |
| | | 2 | 2 |
| | | 3 | |
| fumarase | FUM | 1 | 1 |
| glucose-6-phosphate dehydrogenase | G-6-PD | 1 | 1 |
| | | | 2 |
| $\alpha$-glyceraldehyde dehydrogenase | $\alpha$-GDH | 1 | 1 |
| isocitrate dehydrogenase | IDH | 1 | 1 |
| | | 2 | 2 |
| | | 3 | |
| lactate dehydrogenase | LDH | | 1 |
| | | 2 | |
| malic enzyme | ME | 1 | 1 |
| malate dehydrogenase | MDH | 1 | 1 |
| | | | 2 |
| phosphoglucose isomerase | PGI | 1 | 1 |
| | | 2 | 2 |
| phosphoglucomutase | PGM | 1 | 1 |
| | | 2 | 2 |
| 6-phosphoglucose dehydrogenase | 6-PDH | 1 | 1 |

*Each band observed on the gel was assigned a number. Different band positions were numbered consecutively from the origin. Bands with the same number for a particular enzyme were observed in the same relative positions. Weak and inconsistent bands were not included in the table.

from the origin, was found in all populations of the citrus race (Fig. 1 A).

• $\alpha + \beta$ Esterase. Generally, two isozymes were found in populations of the banana and citrus races. A third weak band was resolved in the banana race as well as a very weak fourth band in about 25% of these samples. The bands were spaced 10, 15, 20, and 25 mm from the origin (Fig. 1 B).

• Glucose-6-Phosphate dehydrogenase. Two isozymes were detected from the populations of the citrus race, whereas only one isozyme was found in the populations of the banana race. Bands one and two were 5 and 10 mm from the origin, respectively (Fig. 1 C).

• Isocitrate dehydrogenase. Three isozymes were present, respectively, in populations of the citrus and banana races. The first band of each race was located 5 mm from the origin. The second band for each race was 12 mm from band one (17 mm from the origin), whereas band three of the citrus and banana races was 22 mm and 27 mm from the origin, respectively (Fig. 1 D).

• Lactate dehydrogenase. One isozyme, 10 mm from the origin, was observed in all populations of the citrus race, whereas a second isozyme was observed in all populations of the banana race (15 mm from the origin) (Fig. 1 E).

• Malate dehydrogenase. Three isozymes were detected, one that migrated cathodally and two that migrated anodally. The cathodally migrating isozyme band was identical in populations of the citrus and banana races. A single anodally migrating band was detected 5 mm from the origin in populations of the banana race. Two bands, one at 5 mm and one at 10 mm, were resolved from populations of the citrus race (Fig. 1 F).

• Phosphoglucomutase. Two isozyme bands, 10 mm and 15 mm, from the origin were observed for both races (Fig. 1 G).

• Phosphoglucose isomerase. Two iso-

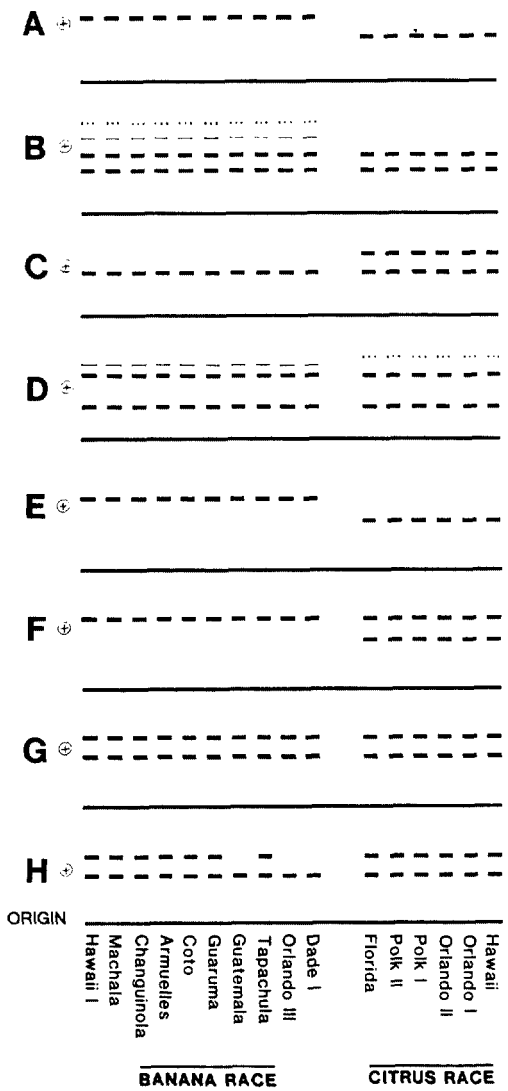


Fig. 1. Relative isozyme mobilities and band intensities of A) aldolase, B) $\alpha$ + $\beta$ esterase, C) glucose-6-phosphate dehydrogenase, D) isocitrate dehydrogenase, E) lactate dehydrogenase, F) malate dehydrogenase, G) phosphoglucomutase, and H) phosphoglucose isomerase from the banana and citrus races of *Radopholus similis*. (Diagrammatic sketches, not to scale.)

zyme bands were detected from all populations of the banana and citrus races except for Dade I, Orlando III, and Guatemala populations of the banana race. These populations had only one isozyme band. The distance between the first and second isozymes on the starch gels was 5 mm (Fig. 1 H). Band two appeared to be fainter in banana populations than in all the citrus populations.

• Fumarase, adenylate kinase, alcohol dehydrogenase, malic enzyme, acid phosphatase, alkaline phosphatase, 6-phosphoglucose dehydrogenase, and $\alpha$-glycerophosphate dehydrogenase. Only single bands, ca. 10 mm from the gel origin were resolved for each of these isozymes for all populations studies of both races.

Out of 24 enzyme-encoding loci investigated, 16 were resolved. Enzymes not detected included glutamate-oxaloacetate transaminase, hexanol dehydrogenase, hexokinase, manitol dehydrogenase, monamine oxidase, octanol dehydrogenase, sorbital dehydrogenase, and xanthine dehydrogenase. These may not be present in nematodes; however, it is more likely that they are present in small but undetectable amounts.

There was no detectable genetic variation among any of the citrus populations from Florida and the *Anthurium* populations from Hawaii. The lack of intraracial genetic variation among populations was found also within all the populations of the banana race with the exception of one enzyme locus. The Florida (Dade I and Orlando III) and the Guatemala banana populations had only one isozyme band for PGI; all other banana populations had two bands for this enzyme (Fig. 1 H).

*Coefficients of similarity:* The number and relative positions of the bands resolved on starch gels are given in Table 2. Within the banana race there was a total of 22 isozyme bands in each population except two populations from Florida and one population from Guatemala; these three populations lacked one PGI band. The other 21 bands were identical to other banana populations. The intraracial CS is, therefore, 21/22 or 0.95. The CS value of the citrus and banana race based on the number of shared bands is 0.69 (18/26) for all banana populations except Florida and Guatemala. In these populations the CS value is 0.65 (17/26). The mean CS value is 0.67.

## DISCUSSION

The analysis of enzymatic variation from 10 populations of the banana and 6 populations of the citrus race of *R. similis* showed that 7 of 16 enzyme-staining systems could be used diagnostically for identifica-

tion (Fig. 1). Since mass homogenates were used in these studies, each band observed on a single gel is referred to as an isozyme. This term is preferred, since it could not be determined if these bands were allelic variants produced by a single genetic locus. Alleles and their corresponding proteins, generally called electromorphs on gels, can be only distinguished by analysis of individuals (1).

Isozyme analysis of the races of *Radopholus similis* indicates that they are genetically distinct. The lack of common isozyme bands at seven loci indicates that gene flow does not occur between these races even though they are sympatric and, therefore, that reproductive isolation is complete. Similar results have been reported in sibling species of *Goniobasis floridensis,* a snail, where 8 out of 18 loci are distinct (5). This is also the case in six sibling species of the marine polychaete worms, *Capitella* spp., which can be identified only on biochemical criteria (9). Even more substantial differences have been detected between the nematodes, *Caenorhabditis elegans* and *C. briggsae* (4). These two species differ in 22 out of 24 enzyme-encoding loci. Yet, the only morphological difference is in the tail bursal ray pattern of the males (8).

Those enzymes with only a single band can be assumed to be the product of a single monomorphic locus. Eight of the sixteen enzymes fall into this category. Single bands, also indicating monomorphic loci, were observed by Dickson et al. (6) in several root-knot nematode species for LDH, ADH, and ACPH. Similar results were observed in the populations of *R. similis.* In the case of ALD, there is only one band per race, but the band positions are different in each. The simplest explanation for this observation is one of complete allelic substitution via a transient polymorphism.

Five of the sixteen enzymes are less easily interpreted. In the case of G-6-PD and MDH, there is one band in the banana race and two in the citrus race. These two enzymes are known to be dimers (i.e., in heterozygous individuals, the phenotype has three bands). In a mass homogenate of a population with two alleles, three bands would be expected. As this is not the case, two interpretations are possible. First, these enzymes in the citrus race may be polymorphic, but one allele is so rare that the homodimer produced by it is present in a concentration so low that it is undetectable. This would produce a phenotype consisting only of the heterodimer and the homodimer produced by the common allele. Second, the two-banded phenotype may represent two true isozymes produced by a pair of genetic loci. In this case, the second locus may have arisen through duplication of the first, which is found in both races, and evolved a different mobility through transient polymorphism. Dickson et al. (6) found either three or four bands for MDH in *Meloidogyne* species. However, as the species used in that study were polyploids, these patterns may also have developed through divergence of duplicated genes.

In PGM and PGI, there is a third phenotype, in which two bands having the same mobility occur in each race. Phosphoglucomutase is a monomeric enzyme, and PGI is a dimer. The same interpretations apply to these isozymes as to G-6-PD and MDH. The probability of PGM being polymorphic is increased, however, because the two-banded pattern would be expected for mass homogenates of a population with a two allele polymorphism for a monomeric enzyme.

Isocitrate dehydrogenase has three bands in the banana race but only two in the citrus race. The intermediate mobility band is not midway between the fast and slow bands in the banana race, as is typical of heterodimers and is absent altogether in the citrus race. These isozymes therefore most likely represent the products of independent loci. The same situation occurs in the esterases, where the banana race has three bands and the citrus has two bands. In this last case, band three is absent from the citrus race phenotype. Esterases are well known to be both polymorphic and the products of multiple loci.

The interracial CS (0.67) of the two races of *R. similis* is representative of closely related species. For example, two species of *Drosophila,* which are similar, have a CS value of 0.65 (14).

The CS value for interracial attraction was very high, 0.95. This would be expected, as gene flow still occurs between these populations. Since individuals of *R. similis* can-

not be analyzed, other types of similarity measurements, such as Roger's coefficients of similarity or Nei's genetic distance, could not be utilized (15,17).

The proportion of clearly monomorphic enzymes in the banana race (12/16, 0.75) and citrus race (10/16, 0.62) is about as expected for diploid sexually reproducing invertebrates. However, the lack of any clear evidence for polymorphism in any of the enzymes, except perhaps PGM, was surprising. Further analysis of the nature of genetic variation in plant parasitic nematodes needs to be carried out with species that can be manipulated genetically.

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