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Two-Dimensional Protein Patterns in *Heterodera glycines*[1]

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Abstract: Two-dimensional polyacrylamide gel electrophoretic protein patterns of *H. glycines* from southern Indiana (Posey County) and northern Indiana (Pulaski County) were largely similar, but many differences existed. The pattern of the Posey isolate was similar to patterns from isolates collected in other areas of the United States. Unique dense protein spots in the pattern of an isolate from Hokkaido, Japan, distinguished it from patterns of six U.S. isolates.

Key words: soybean cyst nematode, two-dimensional gel electrophoresis, soybean.

Although systematists generally equate the development of evolutionary novelties with morphological change, differences at macromolecular levels are receiving increased attention. Lack of synchrony between morphological change and biochemical change has been established for many taxa (9,27). Most systematic studies of protein differences have focused on population genetics (based on data comprised of selected isozyme systems), but increasingly it is recognized that proteins (and other macromolecules) may also be used as taxonomic characters in the standard sense and may be useful in discerning systematic relationships at any level (5,9,17,19). Fixation of many proteins not shared among separated isolates of taxa suggests a long absence of gene flow between isolates. Indeed, a novel protein in a single isolate may be considered comparable to a novel morphological characteristic in a single isolate.

Protein comparisons of several *Heterodera* species, including *H. glycines* Ichinohe by one-dimensional polyacrylamide gel electrophoresis (1-D PAGE), including sodium dodecyl sulfate (SDS) PAGE, were recently reported (19). Two-dimensional comparisons of proteins in other nematode genera have been reported (4,8,18,20,25). As part of a larger study of variability among isolates of soybean cyst nematode (SCN) in Indiana (all infestations discovered since 1973), we report on two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) comparisons between an isolate from Posey County in southern Indiana and one from Pulaski County in northern Indiana (the latter widely believed to have spread north from southern Indiana within the past few years). Both are classified as Race 3 populations using the standard soybean differentials (11), but they behave differently on other soybean breeding lines (unpubl. data, our laboratory) and have small morphological differences (Table 1). We also compared the protein patterns of these isolates with the pattern of an isolate from Hokkaido, Japan, type locality of *H. glycines*.

Materials and Methods

The isolates of *H. glycines* from Posey and Pulaski counties in Indiana and from Hokkaido, Japan, were increased in plant growth chambers on the susceptible soybean *Glycine max* (L.) Merr. 'Williams'. Isolates of SCN from Vanderburgh County in southern Indiana and from Virginia, Mississippi, and North Carolina, for which patterns are reproduced for comparison, were handled similarly.

Thirty young (white cyst stage) female nematodes per sample, picked from roots, were cleaned by repeated rinsing in tap water and homogenized in 0.2 M sodium borate at pH 9 (with or without the reducing agent thiodiglycol) using a ground glass homogenizer (in an ice bath) with a motor-driven pestle. The homogenate was centrifuged at 12,800 *g* for 5 minutes, and the supernatant liquid was dialyzed before radiolabeling. Unlabeled dialysates containing nematode proteins were stored in liquid nitrogen.

Nematode proteins in dialysate were labeled in vitro by reductive methylation with formaldehyde and sodium (^{3}H) borohydride (12). This labeling procedure is mild and specific for alpha-amino groups of ami-

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no terminal residues and epsilon-amino groups of lysyl residues. It is particularly well suited for labeling our small quantities of protein (25–50 μg per sample) for electrophoresis under denaturing conditions.

After radiolabeling and before electrophoresis, protein samples were stored at −80 C. Electrophoresis was carried out essentially as described by O'Farrell (16). Proteins from each isolate were run in both dimensions in the same cell with proteins from other isolates to permit the tracing of small variations in protein positions to peculiarities of individual gel runs. Molecular weight standards (Bethesda Research Laboratories, Gaithersburg, Maryland) were run in the second dimension with the nematode proteins. The standards included, with molecular weights, ^{14}C-labeled phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), alpha-chymotrypsinogen (25,700), and beta-lactoglobulin (18,400). The pH gradient was measured using 1-cm segments of the isoelectric focusing gels (16).

Following separation in two dimensions by combined isoelectric focusing and SDS-PAGE (16), labeled proteins were located on gels by fluorography. Six to twelve patterns were obtained for each isolate, and the transparent gel films were overlaid and compared directly. As is customary for 2-D gels, we used internal "landmark" spots to align gels for comparisons (23). Proteins and polypeptides with identical electrophoretic properties were assumed to be identical. For analysis, patterns were divided into sectors, partly for convenience but also because each sector contained constellations of spots which seemed to retain precisely the same electrophoretic relationship to each other, despite minor shifts from gel to gel between constellations (a consequence of unavoidable differences among gel runs). Each spot in each sector was numbered and its presence or absence determined for all gels of the isolates being compared.

The method of Aquadro and Avise (3) was employed to quantify overall relative similarity between isolates. The total proportion of spots shared (S) is calculated as follows: $S = 2n_{xy}/(n_x + n_y)$ in which n_x and n_y are the total number of spots scored for populations x and y, respectively, and n_{xy} is the number of spots shared by x and y.

TABLE 1. Means of selected measurements (μm) showing differences among three isolates of *Heterodera glycines*. N = 40 for all measurements.

Isolate	Total length, juvenile	Esophagus length, juvenile	Tail length, juvenile	Anterior end to excretory pore, juvenile	Stylet length, male
Posey	423.7 a	170.0 b	43.0 a	93.4 a	26.3 a
Pulaski	458.0 b	184.1 c	44.6 a	103.4 c	30.4 c
Hokkaido	473.7 c	159.8 a	47.1 b	96.6 b	29.2 b

Data analyzed by one-way analysis of variance (F ratio highly significant, $P > 0.001$, for each measurement), and Student-Newman-Keuls multiple-range test. Means within a column not followed by the same letter are significantly different ($P = 0.05$).

(For identical patterns S = 1.00.) Most of the differences scored involved simple presence or absence. In two instances (one each in sectors 2 and 4), a spot consistently small and pale in one isolate and larger and very dense in another was scored as being different in the two isolates. Only spots consistently showing good resolution were scored (average of 132 spots per isolate).

RESULTS

Protein patterns of the three SCN isolates revealed a common general pattern, with a number of evident differences (Figs. 1–3). The sketch of Figure 1 shows the protein/polypeptide spots scored for the pattern of the Posey County isolate (termed "Posey"). In the sketches for the Pulaski isolate (termed "Pulaski") and the isolate from Japan (termed "Japan") in Figures 2 and 3, the larger protein spots that are not also present in the Posey pattern are solid black for emphasis. Spots missing from the Pulaski or Japan gel patterns, but present in the Posey pattern, are omitted from the sketches accompanying the photographs of the Pulaski and Japan radiographs.

Although the patterns were highly reproducible, not every spot showed equally well in every radiograph (particularly from the standpoint of photographic reproduction). The exposure time necessary to produce good contrast in small, less dense spots results in overdevelopment for larger, denser spots. The lowest molecular weight proteins are well separated for only a brief period near the end of a plate gel run, after which they drop sufficiently to be no longer

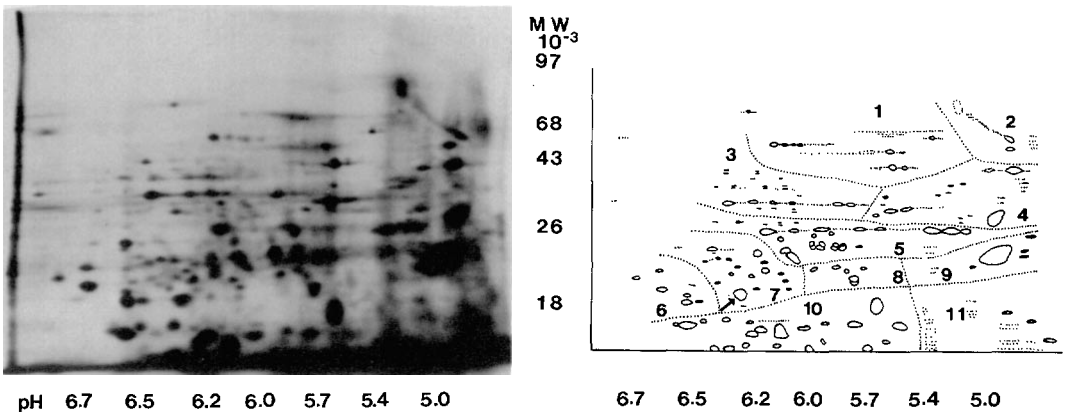

FIG. 1. 2-D PAGE protein pattern (left) for *Heterodera glycines* from Posey County, Indiana, and sketch (right) of typical pattern for this isolate. Arrow indicates large protein spot present in U.S. isolates that is missing in Japan isolate (see text).

visible on the radiograph of the gel film. Hence some radiographs of every isolate may not show them. (These comprise the "extra" row of spots along the bottom of the photograph and sketch of Figure 2. The radiographs selected for Figures 1 and 3 do not show this row of spots.) The sketches of Figures 1–3 include those proteins scored after examination and comparison of radiographs of all gels. They represent typical patterns for the isolates based on all radiographs examined and are not intended to be exact replicas of the accompanying photographs.

The Posey pattern (Fig. 1) differed from the Pulaski pattern (Fig. 2) in all sectors except 1, 2, and 9. Dense spots absent in Posey are present in sectors 4, 5, and 10 of the Pulaski pattern. Many differences occurred in the small paired spots in the upper part of sector 3. When the differences were quantified by the method of Aquadro and Avise (3), the similarity between the two isolates was 0.876.

The comparison between the Posey and Japan patterns shows more differences than between Posey and Pulaski. The large dense spots present in sector 4 of the Japan pattern (Fig. 3, arrows) distinguish this isolate immediately from all the U.S. isolates we have examined thus far (cf. patterns of four additional U.S. isolates shown in Fig. 4). A protein spot in sector 7 of the U.S. patterns, which is especially prominent in the

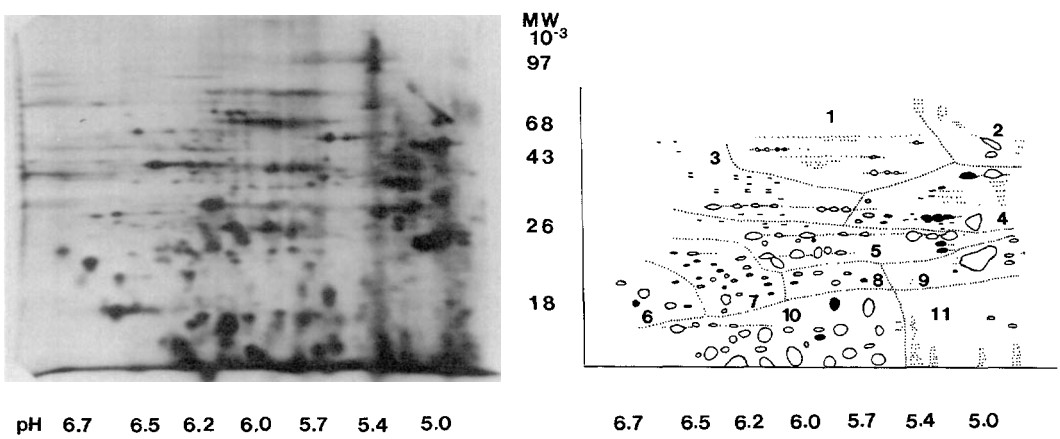

FIG. 2. 2-D PAGE protein pattern (left) for *Heterodera glycines* from Pulaski County, Indiana, and sketch (right) of typical pattern for this isolate. Protein spots identical to Posey isolate are shown in outline, new spots are solid black, and missing spots are omitted from sketch.

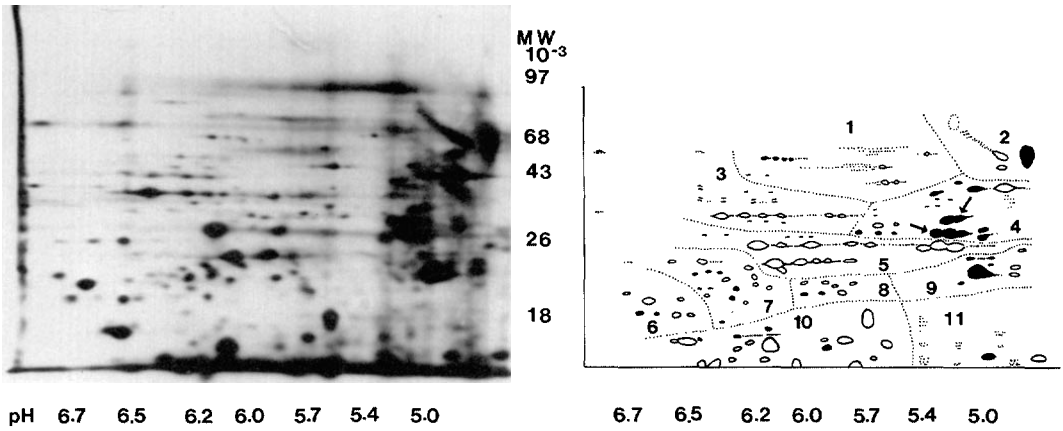

FIG. 3. 2-D PAGE protein pattern (left) for *Heterodera glycines* from Hokkaido, Japan, and sketch (right) of typical pattern for this isolate. Protein spots identical to Posey isolate are shown in outline, new spots are solid black, and missing spots are omitted from sketch. Arrows indicate large dense spots diagnostic for the Japan isolate and missing from U.S. isolates.

radiographs shown for the Indiana patterns (Fig. 1, arrow), is missing in the Japan pattern. In addition, the Japan pattern has a unique assortment of the small paired spots in the upper part of sector 3 and other differences in small spots throughout the pattern. The pattern of this isolate from Japan has an overall similarity to Posey of 0.740.

DISCUSSION

Several investigators report that O'Farrell's 2-D PAGE technique results in more conservative estimates of protein divergence than do 1-D PAGE analyses of isozymes (3,13). The 2-D PAGE protein pattern of an isolate of the fungus *Erysiphe graminis* maintained in the laboratory for 19 years was nearly identical to that of a new field isolate (10). Such data suggest that the 2-D protein pattern of a taxon is a conservative phenotypic characteristic and that changes that occur over time do so at a slow rate. Despite their apparently conserved nature, the gel patterns for SCN reveal more phenotypic differences among some geographically separated isolates than might be suspected from classical morphological data. Just as the presence or absence of a novel morphological structure enables us to discriminate quickly between taxa (even in the absence of morphometric data), the presence or absence of particularly large and distinctive protein spots enables us to discriminate quickly between protein patterns. The patterns for the U.S. isolates shown in Figure 4 look very similar to the Posey pattern (Fig. 1), but both the Pulaski pattern (Fig. 2) and the Japan pattern (Fig. 3) have distinctive dense spots unique to each isolate and immediately discernible on the gels.

Thorpe (26) recently compared published data for overall measures of electrophoretic similarity (one-dimensional enzyme electrophoresis) among taxa in a wide assortment of organisms. He concluded that the data indicate no great differences in biochemical evolution (a function of evolutionary time) among mammals, reptiles, amphibians, fishes, or invertebrates; and suggested that the amount of biochemical divergence between conspecific populations, congeneric species, etc., may be roughly similar across a wide range of taxa. Aquadro and Avise (3) used 2-D PAGE to compare six species of field mice representing levels of evolutionary divergence from different subspecies to different families. Intraspecific comparisons of mice from Georgia with those of Vermont and Oregon, revealed 95% and 92% similarity, respectively. Congener species were 81–85% similar, and species of different genera were 50–60% similar. The degree of similarity between the Posey and Pulaski isolates of SCN (0.876) is thus below the values found in intraspecific comparisons among rodents. The similarity between the Posey and Japan isolates of SCN (0.740)

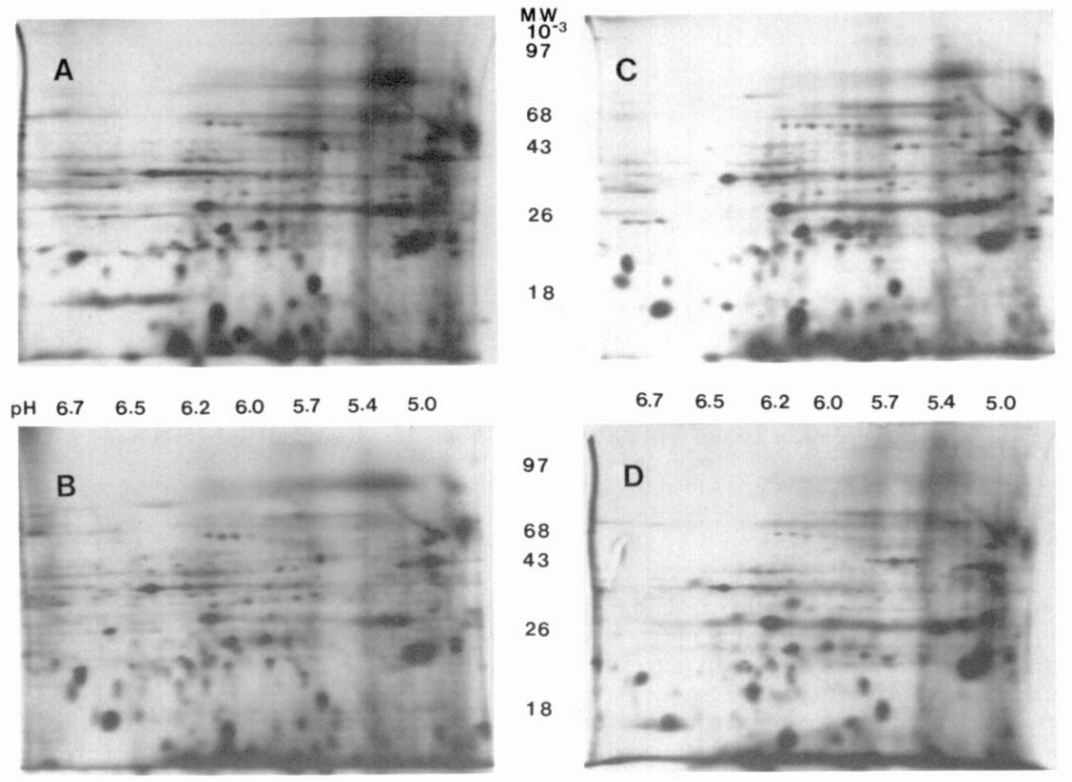

FIG. 4. 2-D PAGE protein patterns for four U.S. isolates of *Heterodera glycines*. A) Isolate from Vanderburgh County in southern Indiana. B) Mississippi isolate. C) Virginia isolate. D) North Carolina isolate. Note absence in all patterns of the large dense spots of area 4 (shown in Fig. 3) that are diagnostic for the Japan isolate.

falls below that for congeners of the rodents.

The marked protein differences between the Posey and Pulaski isolates, both classed as Race 3, reinforces suggestions (1,2,6,14,22,24) that the current race concept for SCN may obscure extensive differences among populations. Further, the pattern differences between the Pulaski isolate and the other U.S. isolates cast doubt on the view that the Pulaski infestation results from recent spread of SCN from an eastern infestation via southern Indiana (15). We do not know if the pattern of the Hokkaido isolate is typical for Japanese SCN, but if so, it is unlikely that SCN was introduced into the United States from Japan in recent times. Our data for 2-D PAGE protein patterns are consistent with the hypothesis that the U.S. populations are indigenous and derived from an ancient ancestor once widespread across Asia and North America (7,15,21). Further analyses of 2-D protein patterns of SCN isolates from Indiana and elsewhere will be reported in subsequent papers.

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