Journal of Nematology 16(3):332–340. 1984.

Comparative Electrophoretic Analyses of Soluble Proteins from *Heterodera glycines* Races 1–4 and Three Other *Heterodera* Species[1]

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Abstract: Modified polyacrylamide gel and SDS-polyacrylamide gel electrophoretic systems using a low molarity tris-HCl buffer and equal pH of homogenizing buffer and stacking gel provided improved stacking for separation of soluble proteins from *Heterodera schachtii, H. trifolii, H. lespedezae,* and *H. glycines* races 1, 2, 3, and 4, compared with previous studies with cyst nematodes. The four *Heterodera* species were easily distinguished using the polyacrylamide gel system, but *H. trifolii* and *H. lespedezae* had similar protein patterns. *H. glycines* races were not separable by that system. The SDS-polyacrylamide gel system produced different protein patterns for all four *Heterodera* species although *H. trifolii* and *H. lespedezae* differed by only a single band, suggesting that these two may be subspecifically related. A protein band unique to *H. glycines* races 3 and 4 was not detected in SDS-polyacrylamide gel profiles from races 1 and 2. Molecular weight determinations were 55,000 for distinctive proteins in profiles of *H. trifolii* and 75,000 for *H. glycines* races 3 and 4.

Key words: electrophoresis, soybean cyst nematode, *Heterodera lespedezae, Heterodera schachtii, Heterodera trifolii.*

The classical approach to nematode taxonomy based on light microscopic examination of morphological features is of primary importance in the identification and classification of phytoparasitic nematodes. However, within some nematode genera, conventional taxonomic studies have failed to reveal stable features with which to separate species. Infraspecific variation and the occurrence of physiological races have necessitated the development of taxonomic techniques enabling differentiation and classification of races. Scanning electron microscopy, immunology, and electrophoresis are becoming increasingly important in supplementing data provided by classical nematode systematics.

Protein molecules are important when investigating systematic relationships, for they accurately reflect genotypic qualities of the taxon (18,25). Biochemical systematics of nematodes has relied primarily on detectable differences in protein molecules as taxonomic characters. Several researchers obtained consistently distinct soluble protein profiles when different nematode species were compared by polyacrylamide gel electrophoresis (PAGE) (8–10,16,17). The technique was considered to have great potential for characterization of interspecific variation among plant parasitic nematodes.

Proteins of species and pathotypes of *Globodera* and *Heterodera* have been compared using PAGE. Soluble proteins of several cyst-forming species and pathotypes A and E of *Heterodera* (sic *Globodera*) *rosto-*

Received for publication 27 February 1984.

[1] A portion of an M.S. Thesis, University of Illinois. The authors thank D. P. Schmitt for providing *Heterodera glycines* races 1 and 2 and A. E. Steele for providing *Heterodera schachtii* and *Heterodera trifolii.* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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chiensis were characterized and compared (31). Results from this research supported an earlier suggestion (20) that pathotypes A and E should receive specific ranking, but failed to distinguish pathotypes within the subsequently defined species, *G. rostochiensis* (Wollenweber) Behrens and *G. pallida* (Stone) Behrens. Other researchers (32) compared several populations of potato cyst nematodes having white or yellow females and found differences in protein banding between, but not within, groups of similarly colored females. They suggested that *H.* (sic *Globodera*) *rostochiensis* be split into two species even though pathotypes could not be distinguished by electrophoresis. Two pathotypes of *H. avenae* (Woll.) Filipjev, which were distinguished by the ability to reproduce on barley (*Hordeum vulgare* L. cv. Pajberg Drost), were electrophoretically identical and were considered subspecifically related (28). In a more recent study, protein patterns from different *G. rostochiensis* populations were identical but differences were observed in patterns from British and Peruvian populations of *G. pallida* (11).

Heterodera glycines Ichinohe has been examined previously with electrophoresis by investigators concerned primarily with relationships among various genera (8,9,19). *H. glycines* is suited ideally for biochemical characterization by PAGE because all races can be cultured on the same susceptible cultivar under similar conditions and adult females of approximately the same physiological age are obtained readily. Investigation of *H. glycines* races by electrophoresis was undertaken because of the difficulties encountered in determining race by relative quantitative reproduction on soybean differentials. Our objectives were to assess the ability of PAGE and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to differentiate *H. glycines* races. *H. glycines, H. schachtii* Schmidt, *H. lespedezae* Golden & G. Cobb, and *H. trifolii* Goffart were compared in order to evaluate the ability of these electrophoretic systems to detect variation among closely related *Heterodera* species. All of these species occur in the north central United States and are members of the *Schachtii* group of cyst nematodes, having lemon-shaped cysts which are ambifenestrate with strongly developed bullae. These common characteristics suggest close phylogenetic relationships.

Materials and Methods

H. glycines races 1 and 2 from North Carolina and 3 and 4 from Tennessee were increased in a greenhouse on soybean (*Glycine max* (L.) Merr. cv. Essex) grown in Plainfield sand in 10.5-cm-d clay pots with four or five plants per pot. *H. lespedezae, H. schachtii,* and *H. trifolii* were reared similarly on striate lespedeza (*L. striata* (Thunb.) Hook. & Arn. cv. Kobe), cabbage (*Brassica oleracea capitata* L. cv. Early Jersey Wakefield), and white clover (*Trifolium repens* L. cv. Dutch White), respectively. *H. glycines* races and *Heterodera* species identities were verified prior to experimentation.

Females for electrophoresis were produced by inoculating appropriate host plants with approximately 8,000 freshly hatched juveniles per pot. First-generation white females were extracted from the pot cultures by Cobb's gravity sieving technique (6) using 850- over 250-μm-pore sieves. Nematodes were cultured twice, during summer 1980 and spring 1981, and divided into two groups after extraction. One group consisted of *H. glycines* races 1, 2, 3, and 4. The other comprised *H. lespedezae, H. schachtii, H. trifolii,* and *H. glycines,* the last of which was a composite of unknown proportions of each of the four races. Races or species within a group were treated similarly and tested concurrently.

Two hundred young, white, pre-egg laying females from each species or race were hand-picked with forceps and placed in a 12-ml conical centrifuge glass tube containing 6 ml tap water. Extraneous material adhering to the females was dislodged by repeatedly drawing and forcibly expelling the nematode suspension through a Pasteur pipette. The 200 washed females were transferred in 300 μl distilled water to 1 ml 0.05 M tris(hydroxymethyl)-aminomethane-HCl buffer (tris-HCl), pH 6.9, then transferred once more in 300 μl distilled water to 9 ml snap-cap plastic vials containing 1 ml 0.05 M tris-HCl, pH 6.9. The nematode suspension was then placed immediately in a freezer at −80 C for storage.

Electrophoretic methods previously used with cyst nematodes were modified for this

TABLE 1. Stock solutions used for gel preparation in electrophoresis of *Heterodera* spp. extracts.

A.	1 M hydrochloric acid	48.0 ml
	tris(hydroxymethyl)amino-methane (tris)	36.6 g
	N,N,N′,N′-tetramethyl-ethylenediamine (TEMED)	0.23 ml
	distilled water	q.s. 100.0 ml
B.	1 M HCl	46.75 ml
	tris	6.0 g
	TEMED	0.5 ml
	distilled water	q.s. 100.0 ml
C.	acrylamide	36.0 g
	N,N′-methylenebisacrylamide (BIS)	0.96 g
	distilled water	q.s. 100.0 ml
D.	acrylamide	12.0 g
	BIS	0.32 g
	distilled water	q.s. 100.0 ml
E.	ammonium persulfate	0.1 g
	distilled water	q.s. 100.0 ml
F.	sodium dodecyl sulfate	0.8 g
	distilled water	q.s. 100.0 ml

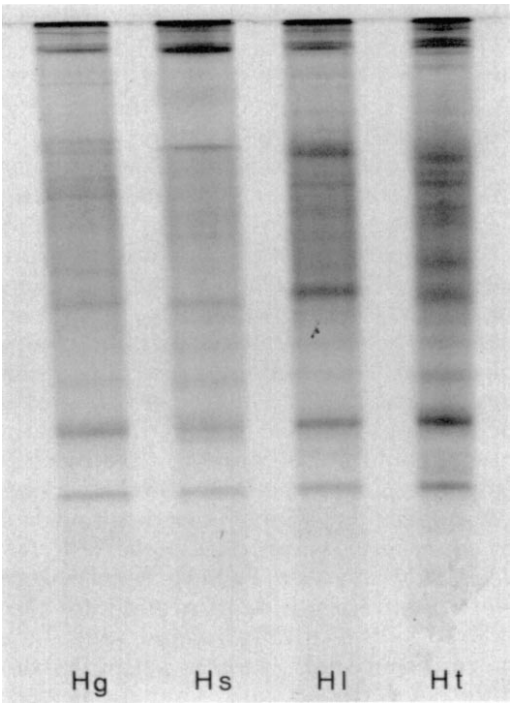

FIG. 1. Protein patterns following polyacrylamide gel electrophoresis of extracts of *Heterodera glycines* (Hg), *H. schachtii* (Hs), *H. lespedezae* (Hl), and *H. trifolii* (Ht). Lowest dark band is at Rm 0.56.

study (31). Ninety minutes before electrophoresis, a vial of nematodes was removed from the freezer and thawed but kept cold through the subsequent procedures. The nematodes were transferred in 300 μl tris-HCl to a 1.5-ml polypropylene conical centrifuge tube. After removing 270 μl buffer, 70 μl freshly prepared homogenizing solution of 0.05 M tris-HCl, pH 6.9, containing 10% sucrose, 0.1% ascorbic acid, and 0.1% cysteine hydrochloride were added to the tube of females; extracts for SDS-PAGE also contained 5% mercaptoethanol and 2% SDS in the homogenizing solution. Nematodes were macerated with a pestle in the centrifuge tube for 1 minute. The macerate was sonicated in an ultrasonic bath (Model D-100, Cole Parmer, Chicago, Illinois) for 1 minute, held in a boiling water bath for 5 minutes during the SDS-PAGE protocol, and clarified by centrifugation at 16,300 *g* for 30 minutes at 4 C. The resultant soluble protein preparation was introduced immediately into the electrophoretic cell.

The PAGE and SDS-PAGE systems used in this study were basically modifications (5,21) of the Ornstein and Davis system (7,24). Stock solutions were prepared as shown in Table 1, stored in the dark at 4 C, and used within 6 weeks of preparation. Stock solution E, however, was always prepared the day of use. Stock solutions C and D (acrylamide:BIS, 30:0.08) were filtered through Whatman Qualitative Number 1 filter paper before storage. The resolving gel solution (T = 9.24%, C = 2.67% gel, 0.378 M tris-HCl, pH 8.9) was prepared immediately before use by mixing one part solution A, two parts solution C, and one part distilled water and degassing under vacuum for 15 minutes at 25 C. Four parts solution E were then mixed with the degassed solution. One part stock solution F was substituted for the water to produce a 0.1% SDS-resolving gel.

The stacking gel (T = 3.08%, C = 2.67% gel, 0.062 M tris-HCl, pH 6.9) was prepared immediately before use by mixing one part stock solution B with two parts solution D and one part distilled water and degassing. Four parts solution E were then mixed with the degassed solution. One part stock solution F was substituted for the distilled water to produce a 0.1% SDS-stacking gel.

A vertical slab electrophoresis cell (Mod-

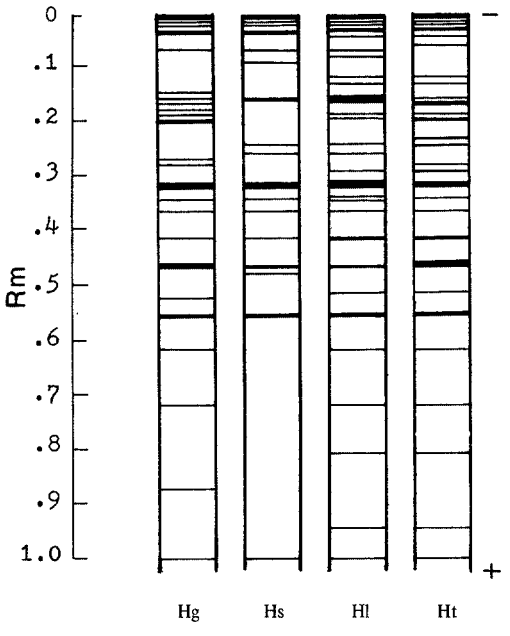

FIG. 2. Diagram of protein patterns following polyacrylamide gel electrophoresis of extracts of *Heterodera glycines* (Hg), *H. schachtii* (Hs), *H. lespedezae* (Hl), and *H. trifolii* (Ht).

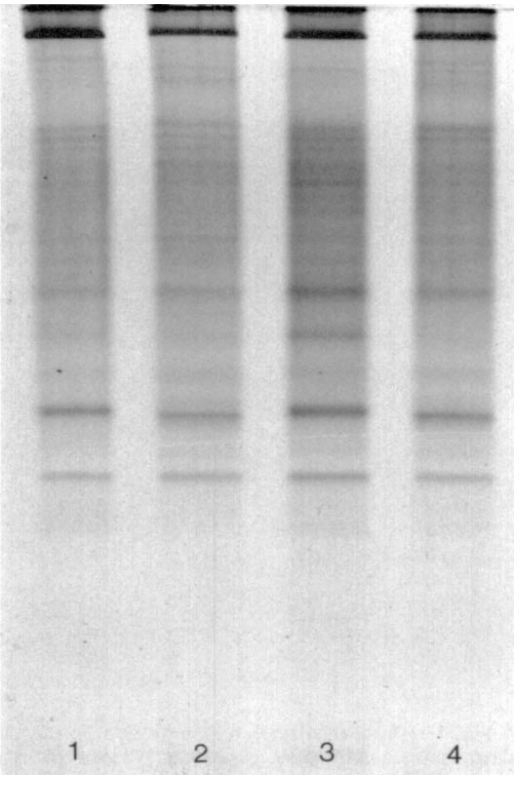

FIG. 3. Protein patterns following polyacrylamide gel electrophoresis of extracts of *Heterodera glycines* races 1, 2, 3, and 4.

el 220, Bio-Rad Laboratories, Richmond, California) was used to cast gels and separate proteins. Two 15.5- × 10.5- × 0.5-mm slabs could be cast and electrophoresis conducted simultaneously on both slabs. PAGE sample wells were 8 mm wide with eight samples applied to each gel, whereas SDS-PAGE sample wells were 12 mm wide with four samples applied per gel. Electrophoresis was conducted at 4 C.

The upper and lower electrode buffer (pH 8.3) for PAGE contained 0.005 M tris and 0.038 M glycine in chilled, distilled water. The upper and lower electrode buffer (pH 8.3) for SDS-PAGE included 0.025 M tris and 0.192 M glycine in chilled, distilled water and 0.1% (w/v) SDS. One drop of 0.1% (w/v) Bromophenol Blue was added to the upper electrode buffer as a tracking dye. The entire cell core was then lowered into the lower buffer chamber, so that the lower electrode buffer cooled the slabs during electrophoresis, and 10 μl extract containing approximately 60 μg protein (4) was underlaid into the sample well beneath the upper electrode buffer.

The following proteins were used as standards for molecular weight estimation by the SDS-PAGE system: phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). A constant current of 20 mA per slab was applied for 1 hour, then increased to 30 mA per slab for ca. 2 hours. Electrophoresis was stopped when the tracking dye had moved to within 1 cm of the bottom of the gel.

After development of our electrophoretic systems (approximately 50 runs), the four races of *H. glycines* and four *Heterodera* spp. were studied at least four separate times in both the PAGE and SDS-PAGE systems. Since two slabs could be run simultaneously, proteins of either the four *H. glycines* races or the four *Heterodera* species could be separated on each of two gels, allowing for comparison of the summer and spring culture periods.

Proteins were detected using a procedure recommended by C. M. Wilson, USDA ARS, Urbana, Illinois (pers. comm.).

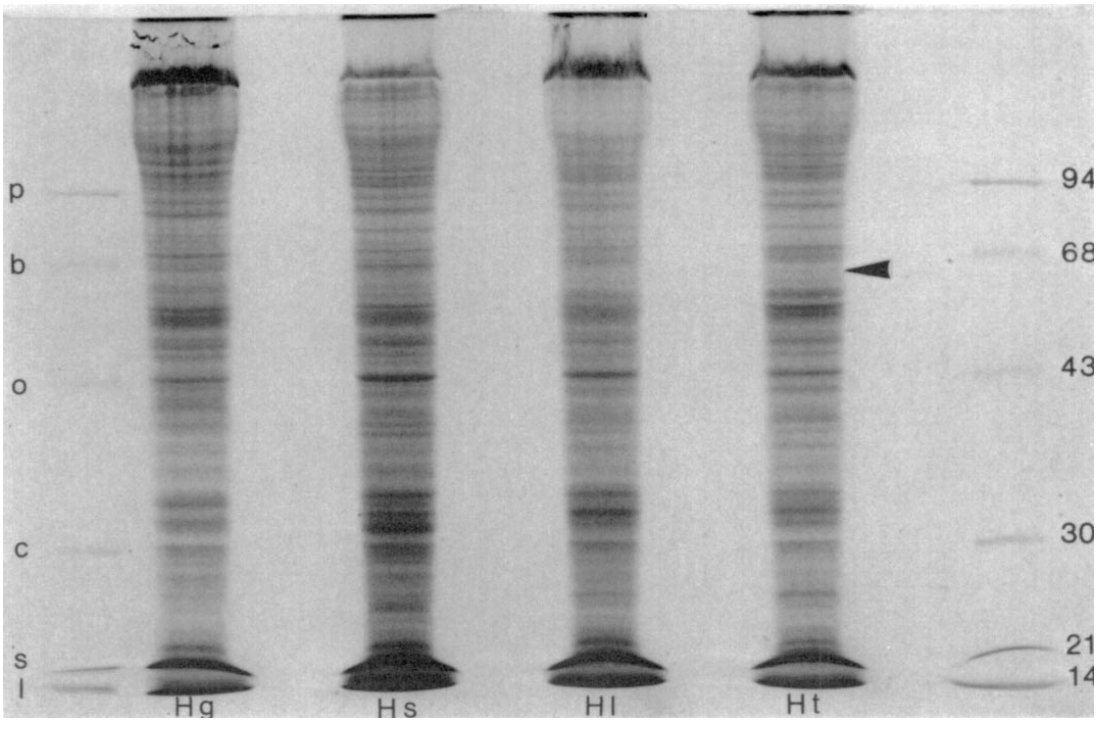

FIG. 4. Protein patterns following SDS-polyacrylamide gel electrophoresis of extracts of *Heterodera glycines* (Hg), *H. schachtii* (Hs), *H. lespedezae* (Hl), and *H. trifolii* (Ht). Arrow points to *H. trifolii* protein band which is absent from *H. lespedezae* profile. Molecular weight standards are phosphorylase (p), bovine serum albumin (b), ovalbumin (o), carbonic anhydrase (c), soybean trypsin inhibitor (s), and lysozyme (l). Numbers (right profile) are molecular weights of standards (× 1,000).

After electrophoresis, gels were transferred immediately to a fixing solution of methanol:trichloroacetic acid:water (40:10:50, v:w:v) for 4 hours, stained in a solution of Coomassie Blue R-250:methanol:acetic acid:water (0.25:40:10:50, w:v:v:v) for 10–12 hours, and destained in a Model 222 Bio-Rad Slab Diffusion Destainer (Bio-Rad Laboratories, Richmond, California) against methanol:acetic acid:water (20:10:70, v:v:v) for at least 24 hours. Destained slabs were stored in 10% acetic acid, analyzed visually, and photographed.

RESULTS

Protein patterns of all species and races of *Heterodera* were highly reproducible. The PAGE system resolved 23, 16, 28, and 28 protein bands, whereas the SDS-PAGE system resolved 46, 47, 44, and 45 bands for *H. glycines, H. schachtii, H. lespedezae,* and *H. trifolii,* respectively. Slight variations in overall electrophoretic mobilities were observed among samples run at different times, but different samples run concurrently were directly comparable. No differences in protein bands were observed between culture periods of nematodes.

Distinct interspecific differences in protein banding patterns were observed among extracts of the four species of *Heterodera* run in the PAGE system (Figs. 1, 2). The lower portion of the gel was not discernible photographically (Fig. 1) because the bands were very faint. Because photographs failed to reproduce all the detail, profiles are also presented in Figure 2. The number and staining intensity of bands and electrophoretic mobilities of proteins were sufficiently different to distinguish each of the four species. However, certain common bands reflected the phylogenetic relationships of these species. Although distinct, the banding profiles of *H. trifolii* and *H. lespedezae* were quite similar. The relatively dark patterns of *H. lespedezae* and *H. trifolii* were consistently reproducible (Fig. 1). PAGE banding patterns of extracts of *H. glycines* races 1, 2, 3, and 4 showed no consistent differences; a representative gel is shown

in Figure 3. The protein profile of *H. glycines* is illustrated in diagram in Figure 2.

The more concentrated tris-glycine electrode buffer had no effect on banding quality in the PAGE system, but did improve separation and banding in the SDS-PAGE system. Gels from SDS-PAGE exhibited numerous banding differences between *H. glycines* and *H. schachtii*, distinguishing each readily from *H. trifolii* and *H. lespedezae* (Figs. 4, 5). The protein patterns of *H. lespedezae* and *H. trifolii* differed by a single faint band (Figs. 4, 5) present in the profile of *H. trifolii* but absent from that of *H. lespedezae*. The protein patterns of the four *H. glycines* races separated by SDS-PAGE were similar. However, one faint, but sharp band observed consistently in the patterns from races 3 and 4 was absent from the profiles of races 1 and 2 (Figs. 5, 6).

SDS-denatured protein standards for molecular weight determination during SDS-PAGE showed that their mobilities, when plotted against the logarithm of their molecular weights, gave a linear relationship for the higher molecular weight standards but a nonlinear relationship for the lower molecular weight standards. The estimated molecular weight for the distinctive protein band in profiles of *H. trifolii* was 55,000, whereas that for the additional band in profiles of races 3 and 4 of *H. glycines* was 75,000.

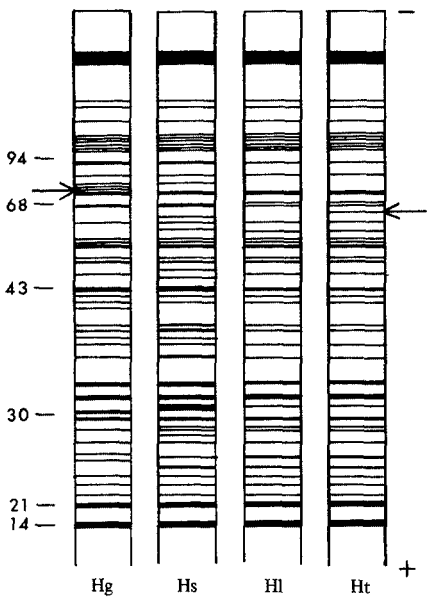

FIG. 5. Diagram of protein patterns following SDS-polyacrylamide gel electrophoresis of extracts of *Heterodera glycines* (Hg), *H. schachtii* (Hs), *H. lespedezae* (Hl), and *H. trifolii* (Ht). Arrow (right) points to *H. trifolii* protein band which is absent from *H. lespedezae* profile. The distinctive band (arrow, left) in profiles from *H. glycines* was present only in profiles of races 3 and 4 (see Fig. 6). Numbers are molecular weights of standards (× 1,000).

DISCUSSION

Established electrophoretic systems (5,7,21,24) were used as the starting point to develop a PAGE system in which SDS could easily and routinely be included to form SDS-denaturing gels. Thus, using the same buffer and gel stock solutions, either nondenaturing or SDS-denaturing gels could be produced readily.

Highly reproducible electrophoretic data can be obtained only when organisms are free of contamination by foreign proteins. In the case of phytoparasitic nematodes, this requirement is inherently impossible to meet because of their obligate parasitic relation with host plants. In this study care was taken to reduce foreign protein contamination. The careful and laborious white female selection and the washing and storage procedures used in this study minimized contamination.

Theoretically, in PAGE the ionic strength of the sample solution should be less than that of the stacking gel and of the same pH (14,22,33). We found that reducing the molarity of the tris-HCl homogenizing buffer from 0.10 M (31) to 0.05 M did not affect its ability to buffer the homogenate. Also, the pH of the homogenizing buffer and stacking gel were the same in this system. Stacking was improved by these two modifications. Increasing the concentration of the tris-glycine electrode buffer from 0.005 M to 0.025 M tris and from 0.038 M to 0.192 M glycine (21) improved protein separation and banding with the SDS-PAGE system but not with the PAGE system. Other improvements included submersion of the homogenate in an ultrasonic bath, use of 10% sucrose in the homogenizing buffer, and clarification of the homogenate by centrifugation at 16,000 *g* for 30 minutes rather than at 1,800 *g* for 7 minutes (31). Also, use of slabs rather than cylindrical gels gave highly reproducible protein banding patterns.

Variability in electrophoretic patterns of

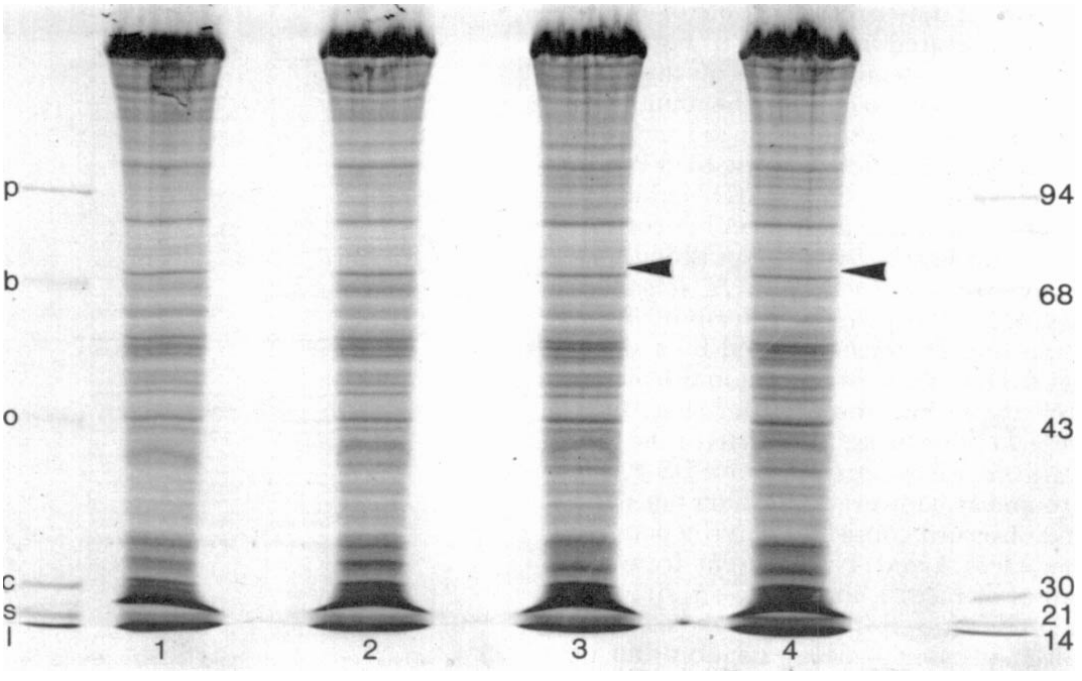

FIG. 6. Protein patterns following SDS-polyacrylamide gel electrophoresis of extracts of *Heterodera glycines* races 1, 2, 3, and 4. Arrows indicate band observed only in profiles of races 3 and 4. Molecular weight standards are explained in Figure 4.

nematode proteins can be caused by any of the following: 1) culture conditions during nematode propagation; 2) developmental stage and physiological condition of nematodes; 3) variations in sample extraction and storage conditions; 4) variability in the electrophoretic system; 5) variation in detection and analysis methods (18). These factors can influence the number, concentration, and electrophoretic mobility of proteins. We detected more proteins in our study than were reported in previous studies of the cyst nematodes using one-dimensional electrophoresis (8,11,31), but fewer than expected from animals as complex as nematodes. Although consistent and reproducible differences were observed among the species, the detected proteins represented only a small portion of the number of proteins in these species. The close phylogenetic relationship among the four *Heterodera* spp. is reflected in the similarity of their protein composition.

Identical electrophoretic patterns may be obtained from different taxa, although the proteins observed in the patterns may not be identical (3). However, similar profiles from closely related species are probably due to homologous proteins. PAGE patterns of *H. lespedezae* and *H. trifolii* were similar, although not identical. The relatively darker profiles of the two species in nondenatured gels was consistently reproducible. The differences observed on SDS-PAGE patterns between *H. lespedezae* and *H. trifolii* was no greater than the difference observed among the *H. glycines* races, indicating that *H. lespedezae* and *H. trifolii* are subspecifically related (30). Greater protein concentration may have affected banding due to protein-protein interactions, thus accounting for the discrepancy between the two systems. Further studies concerning the taxonomic relationship between *H. lespedezae* and *H. trifolii* are warranted. Protein profiles of *H. glycines* and *H. schachtii* were very distinct in both the PAGE and SDS-PAGE systems, indicating a greater phylogenetic separation between these two species than between *H. lespedezae* and *H. trifolii*. These results neither support the hypothesis that *H. glycines* is indigenous to the United States, having originated from *H. schachtii*, nor do they refute a report (23) of interbreeding between these two species.

Infraspecific differences in protein pat-

terns were not detected among the four races of *H. glycines* in the PAGE system. Preliminary investigations with *H. glycines* (27) indicating that the staining intensity of several minor bands varied between races was not supported by this work. Some modification of the electrophoretic system, such as use of two dimensional electrophoresis (1), other staining procedures (26), or determination of isozymes (2,12), might enable further distinction of *H. glycines* races. The presence or absence of one faint band with a molecular weight of 75,000 in the SDS-PAGE patterns enabled consistent separation of races 1 and 2 from races 3 and 4. This distinctive band may reflect variation related to pathogenicity or some other variable, such as physiological stage of development. The electrophoretic grouping of races 1 and 2 as distinct from races 3 and 4 does not correspond to reported morphometric, immunologic, or genetic differences among the races, as understood presently (13,15,29). The electrophoretic grouping does, however, correspond to earlier geographical distribution of these races, as races 1 and 2 were known from the North Carolina-Virginia area and races 3 and 4 were known only from the Arkansas-Tennessee region.

The SDS-PAGE system described in this study was successful in separating four *H. glycines* races into two groups. The SDS-PAGE system also demonstrated the apparent subspecific relationship between *H. lespedezae* and *H. trifolii.* Future efforts will attempt to better define *H. glycines* races and the taxonomic relationship between *H. lespedezae* and *H. trifolii.*

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