Contrasting Rates of Protein and Morphological Evolution in Cyst Nematode Species

J. Bakker and L. Bouwman-Smits

Department of Nematology, Agricultural University, Binnenhaven 10, Wageningen, The Netherlands.
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ABSTRACT

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The genetic differentiation of cyst nematode species that are morphologically nearly indistinguishable was investigated by means of two-dimensional gel electrophoresis (2-DGE) followed by a sensitive silver stain. 2-DGE of total protein extracts from young females revealed an average of 245 polypeptides. The sibling species *Globodera rostochiensis* and *G. pallida* were differentiated by 70% of their polypeptides. The closely related species *Heterodera glycines* and *H. schachtii* were discriminated by 59% of their polypeptides. These large differences suggest that these nematode species have accumulated protein differences during a period of millions of years without distinct changes in morphology. These observations emphasize the importance of biochemical techniques in plant nematology for species identification and nematode systematics.

Additional keywords: beet cyst nematode, potato cyst nematode, soybean cyst nematode.

A variety of electrophoretic techniques have been employed to study *Heterodera* and *Globodera* spp. (3,4,10,11,12,13,25,29,30,35). In particular, the sibling species *Globodera rostochiensis* (Woll.) Behrens and *G. pallida* (Stone) Behrens have been studied extensively. Most reports on these morphologically nearly indistinguishable cyst nematodes, which until 1973 were regarded as pathotypes of a single species (36), deal with aspecific protein stains (3,11,12,13,25,29,35). Polyacrylamide disc electrophoresis of females revealing 14–18 major protein bands showed marked differences between the two species (13,29). However, conclusive interpretations were complicated by variations in age and conditions of the females (13). More reproducible results were obtained by electrophoresis of eggs and second-stage larvae. Polyacrylamide disc electrophoresis (13), isoelectric focusing (11,12,25), and two-dimensional electrophoresis followed by a Coomassie Brilliant Blue stain (35) clearly distinguished the two species.

Although protein electrophoresis has fulfilled the expectations for species identification, little attention has been paid to the estimation of overall genetic distances between *G. rostochiensis* and *G. pallida*. So far, the most extensive study dealt with isoelectric focusing of eggs revealing 40 protein bands and 23 enzymes (12). Only one protein band and two enzymes exhibited interspecific variation. These data probably do not represent the extent of genetic divergence of *G. rostochiensis* and *G. pallida*, because only major differences could be discerned with the methods used.

High-resolution two-dimensional gel electrophoresis (2-DGE), as originally described by O'Farrell (24), is a more refined approach for studying the genetic differentiation between nematode species. 2-DGE, combined with a sensitive silver stain (8,23), is able to detect several hundred polypeptides from crude nematode homogenates (2,3). In this study, we estimated the overall genetic distance between the potato cyst nematodes *G. rostochiensis* and *G. pallida* by means of 2-DGE of protein extracts from young females. For comparison, we also studied the sugar beet cyst nematode *Heterodera schachtii* Schmidt and the soybean cyst nematode *H. glycines* Ichinohe. These species are also morphologically nearly identical and should, according to Miller (20), be considered as subspecies of the nominate *H. schachtii*.

MATERIALS AND METHODS

The *G. rostochiensis*, *G. pallida*, *H. schachtii*, and *H. glycines* populations listed in Table 1 were supplied by: the Plant Protection Service, Wageningen, The Netherlands (populations 1, 2, 3, 5, and 9), Hilbrands laboratorium, Assen, The Netherlands (population 4), the Foundation for Agricultural Plant Breeding (SVP), Wageningen, The Netherlands (population 6), and the Department of Plant Pathology, University of Missouri, Columbia (populations 7 and 8). Potato cyst nematodes and beet cyst nematodes were reared at 18 C with 16 hr daylight on *Solanum tuberosum* ssp. *tuberosum* L. 'Eigenheimer' and *Beta vulgaris* L. 'Monohil,' respectively. Soybean cyst nematodes were grown on *Glycine max* L. 'Williams' at 25 C and 16 hr daylight.

Total protein samples of mature females were made as described previously (2). To prepare total protein samples of fourth-stage females, roots were cut in pieces of approximately 1 cm and processed for 15 sec in a blender. The root debris was removed by centrifugation in 36% (w/v) sucrose. Approximately 250 fourth-stage females were handpicked from the supernatant and homogenized in 60 µl of 10 mM Tris-HCl, pH 7.4, 5% (v/v) 2-mercaptoethanol, saturated with 64 mg of urea and stored at −80 C until used. Total protein extracts from freshly hatched second-

stage larvae were prepared by disrupting the nematodes in a glass Potter homogenizer with a tightly fitting Teflon pestle (with a clearance of about 25 μm). Approximately 100,000 larvae were homogenized in 200 μl of 10 mM Tris-HCl, pH 7.4, 5% (v/v) 2-mercaptoethanol and saturated with 213 mg of urea. As a standard, 25 μg of protein was used for 2-DGE. Sample application, isoelectric focusing within the pH range of 5–7, sodium dodecyl sulfate electrophoresis, and staining with silver were as described (2) with the following exceptions. The proteins were separated in the second dimension at a constant current of 15 mA. Complexing proteins with silver was done for 1 hr in an ammoniacal silver solution containing 0.075% (w/v) NaOH, 1.5% (v/v) NH_4OH, and 0.32% (w/v) $AgNO_3$. These modifications resulted in a reduction of the total number of spots resolved, but improved the reproducibility. Molecular weight and isoelectric point determinations were as described (2).

Protein profiles were evaluated visually by superimposing the original gels on a bench viewer. Only those proteins that were consistently present in each replicate were evaluated. At least three replicates per population were analyzed.

The genetic similarity was calculated according Aquadro and Avise (1) from the equation $F = 2N_{xy}/(N_x + N_y)$, in which N_x and N_y are the total number of proteins spots scored for populations x and y, respectively, and N_{xy} is the number of spots shared by x and y. The genetic distance is 1–F.

RESULTS

2-DGE protein patterns of young females of *G. rostochiensis*, *G. pallida*, *H. glycines*, and *H. schachtii* are shown in Figures 1 and 2. Overall genetic distances were estimated by analyzing an average of 245 protein spots per population. To facilitate comparison, equal protein quantities of two species were mixed and electrophoresed (e.g., Fig. 1C). In this way, minute differences in isoelectric point and molecular weight can be detected. For example, the proteins A_1, B_1, and C_1 in *G. rostochiensis* differ only slightly in electrophoretic mobility from the corresponding proteins A_2, B_2, and C_2 in *G. pallida* (Fig. 1A, 1B, and 1C). No mixed protein samples were made of conspecific populations, because only a few spots exhibited intraspecific variation (e.g., Fig. 1D).

The vast majority of the proteins could be scored unambiguously as shared or distinct. Approximately 95% of the proteins having shared electrophoretic mobilities also had similar spot sizes, staining intensities, and color, ranging from red (±5%), reddish brown (±70%), blackish brown (±5%), brownish grey (±5%), to grey (±15%) (2). The remaining 5% had the same color, but differed in spot sizes and/or staining intensities. To avoid arbitrary decisions about quantity, these proteins were also registered as common. The number of proteins shared and the genetic distances between the nine populations are listed in Table 1. The average genetic distance between *G. rostochiensis* and *G. pallida* was 0.70. The intraspecific distances ranged from 0.01 to 0.04 in *G. rostochiensis* and from 0.03 to 0.06 in *G. pallida*. The large genetic distances between *G. rostochiensis* and *G. pallida* were not caused by a comparison of discordant physiological or developmental stages. A detailed study of *G. rostochiensis* population MIER and *G. pallida* population HPL-1 showed that the qualitative differences between the various stages of a species were rather small. Approximately 95% of the proteins in young white females (Fig. 1) were also observed in the older yellow females. Even fourth-stage female larvae and mature females shared more than 80% of their proteins. Furthermore, comparison of the protein composition of freshly hatched second-stage larvae of *G. rostochiensis* population MIER and *G. pallida* population HPL-1 revealed a similar result as with young white females (i.e., a genetic distance of 0.65).

Figure 2 illustrates the protein differentiation between *H. schachtii* and *H. glycines*. The genetic distance between *H. glycines* and *H.schachtii* was 0.59. The genetic distance between the genus *Heterodera* and the genus *Globodera* averaged 0.98.

DISCUSSION

2-DGE and genetic distances. The protein differences between the cyst nematode species are probably not considerably influenced by the comparison of discordant physiological or developmental stages. A detailed study of *G. rostochiensis* and *G. pallida* (see Results) indicated that the qualitative protein composition of the various developmental stages of cyst nematodes is rather constant. Furthermore, the genetic distances between *H. glycines* (on soybean) and *H. schachtii* (on beet) and also between the two *Heterodera* and the two *Globodera* species are probably not significantly influenced by the host genotypes. In a previous report (2) we never found an effect of host genotypes. Potato cyst nematodes reared on tomato and potato revealed indistinguishable protein patterns, indicating that undigested host proteins that may be present in the alimentary track and gut do not interfere.

Protein and morphological evolution. The genetic distances (D) between *G. rostochiensis* and *G. pallida* (D = 0.70), and between *H. glycines* and *H. schachtii* (D = 0.59) are remarkably large and indicate that these species diverged a long time ago. For comparison, 2-DGE studies on the sibling species *Drosophila melanogaster* and *D. simulans* revealed a genetic distance of 0.19 (27). Even two families of rodents have a smaller genetic distance (D = 0.5) (1) than these morphologically closely related cyst nematode species. Our results confirm the tendency demonstrated by Ferris et al using a comparable 2-DGE system (10). These investigators reported a genetic distance of 0.26 between conspecific populations of *H. glycines* that exhibited only minor differences in morphology.

At present it is not possible to calculate the time of divergence unambiguously from 2-DGE data. The rate at which proteins revealed by 2-DGE accumulate amino acid substitutions is unknown. Moreover, genetic distances based on 2-DGE data probably are influenced to some extent also by changes in the regulatory system (for example, alterations in regulatory sequences and structural genes that influence the synthesis, processing, and degradation of other proteins). However, for a

TABLE 1. The number of proteins shared (above diagonal) and the genetic distances (below diagonal) between cyst nematode populations as assessed by means 2-DGE of total protein extracts from females

Species and code[a]	Protein spots[b]	1.	2.	3.	4.	5.	6.	7.	8.	9.
1. *G. rostochiensis*, MIER	233		232	225	75	75	75	5	5	3
2. *G. rostochiensis*, A-12	237	0.01		228	75	75	75	5	5	3
3. *G. rostochiensis*, C-152	233	0.04	0.03		74	74	74	5	5	3
4. *G. pallida*, HPL-1	253	0.69	0.69	0.70		251	245	7	7	5
5. *G. pallida*, 1337	264	0.70	0.70	0.70	0.03		256	7	7	5
6. *G. pallida*, ROOK	269	0.70	0.70	0.69	0.06	0.04		7	7	5
7. *H. glycines*, R-1-92	234	0.98	0.98	0.98	0.97	0.97	0.97		231	99
8. *H. glycines*, R-7	238	0.98	0.98	0.98	0.97	0.97	0.97	0.02		99
9. *H.'schachtii*, TINTE	248	0.99	0.99	0.99	0.98	0.98	0.98	0.59	0.59	

[a] As designated in the original collections.
[b] Total number of proteins analyzed per population.

number of species, both 2-DGE distance data and a reasonable calculation of the divergence time have been published. For instance, *Drosophila virilis* and *D. montana*, which have a genetic distance of 0.37 (27), diverged approximately 4 million years ago (33). Although these values cannot be extrapolated directly to other organisms, it is evident that the closely related cyst nematode species diverged millions of years ago. Our data definitely exclude the possibility that *G. rostochiensis* and *G. pallida* have speciated in South America as a result of independent potato cultivation by the Amyra and Quencha Indian tribes, as was suggested by Evans et al (9).

Contrasts between genetic and organismal similarities have been revealed in a wide variety of taxonomic groups such as bacteria, snails, fish, frogs, reptiles, and birds (38). These findings support the hypothesis that organismal evolution and structural gene evolution measured by electrophoresis or other biochemical techniques go on at virtually independent rates (17,31,38,39). The evolution of proteins is thought to proceed at an approximately constant rate in all species (16), whereas the rate of organismal evolution is variable. Well-studied examples of a rapid and slow organismal evolution are placental mammals and frogs, respectively (39). Our results demonstrate that the genetic divergence measured with 2-DGE is also not correlated with morphological evolution. Similar to frogs, the cyst nematodes have accumulated protein differences during millions of years without any significant morphological changes. Another feature shared with frogs, which sets them apart from organisms such as mammals, is the capability of cyst nematodes to produce viable interspecific hybrids in spite of the large genetic distances. *H. schachtii* and *H. glycines* are able to produce fertile hybrids (20), and matings between *G. rostochiensis* and *G. pallida* result in viable second-stage larvae (22). These considerations suggest that care should be taken with mating experiments as a way to delineate nematode species and to infer taxonomic relationships.

Slow morphological divergence is probably not rare throughout the phylum Nematoda. The large genetic distances revealed here by 2-DGE are supported by the few studies in which starch gel electrophoresis has been applied to morphologically closely related nematode species (6,14,15). Starch gel electrophoresis followed by specific enzyme stains is, unlike the various electrophoretic techniques often applied in plant nematology (11,12,13,25,29,30,35), a suitable tool to estimate divergence times. For example, the morphologically nearly indistinguishable nematode species *Caenorhabditis elegans* and *C. briggsae* shared no alleles at 22 of the 24 enzyme loci assayed (6), indicating that these species diverged more than 10 million years ago.

Systematics and diagnosis. At present nematode systematics is still in a state of instability and is, according to Coomans, mainly based on authoritarianism rather than on scientific principles (7). Several workers have emphasized the necessity of a phylogenetic approach (7,18) in order to arrive at a more stable classification system. Moreover, estimates of genealogical relationships of plant parasitic nematode species are also valuable in studying the process of coevolution by evaluating the degree of similarity of the host and nematode phylogenies. Electrophoretic techniques can be a powerful tool to construct phylogenies (19,21,28,34). However, the application of starch gel electrophoresis of enzymes, a standard technique for many organisms, has its limitations for nematode systematics. For example, the observation that morphologically nearly indistinguishable nematode species such as *C. elegans* and

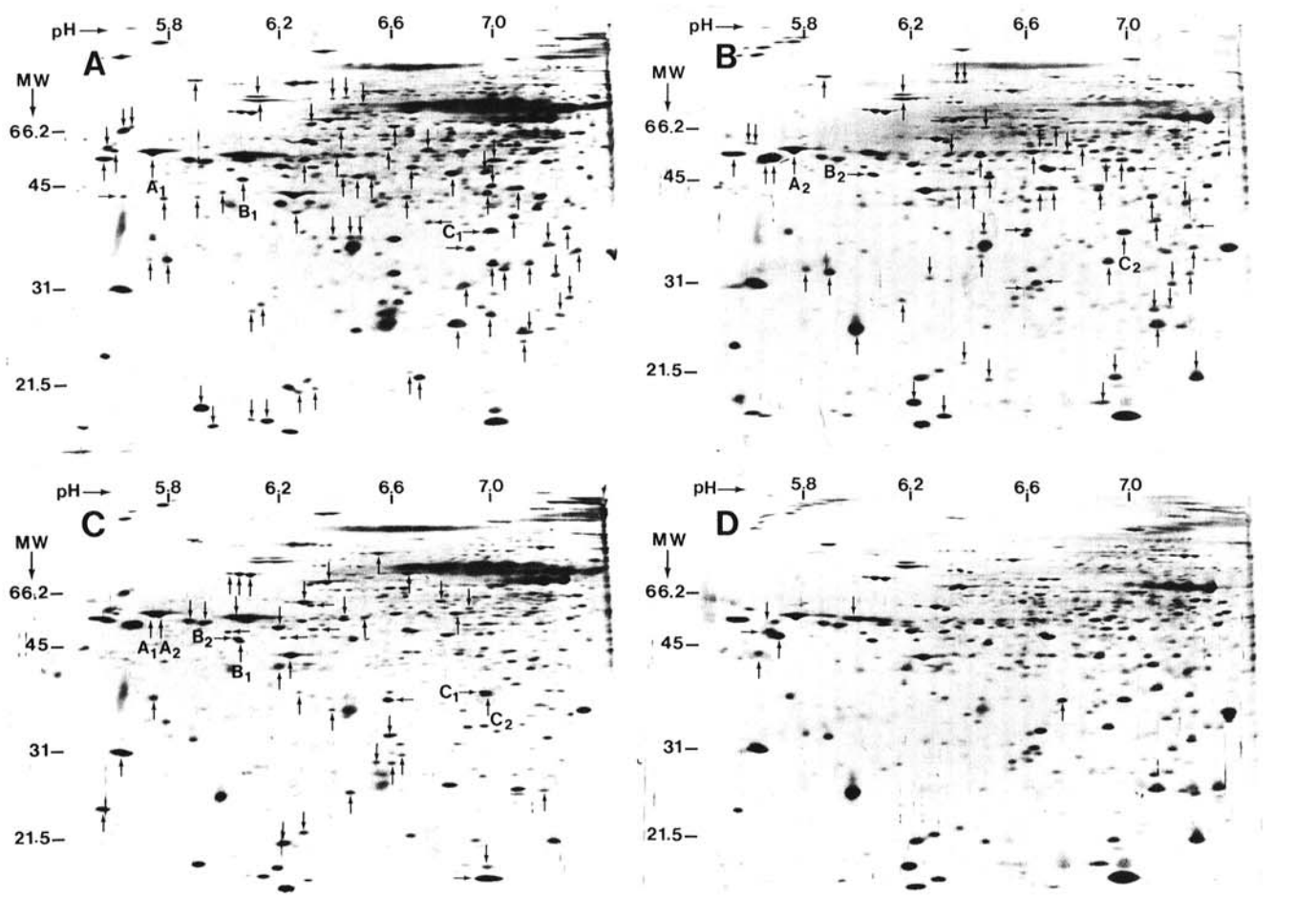

Fig. 1. Two-dimensional gel electrophoresis (2-DGE) protein patterns (25 μg) of females of *Globodera rostochiensis* population MIER (**A**) and *G. pallida* population HPL-1 (**B**) in which a number of major qualitative differences are marked with arrows. Common proteins are designated in a pattern (**C**) containing an equal protein quantity of *G. rostochiensis* MIER (12.5 μg) and *G. pallida* HPL-1 (12.5 μg). Major qualitative differences between *G. pallida* population HPL-1 (**B**) and *G. pallida* population 1337 (**D**) are indicated in **D**. Proteins marked with capitals and arabic numbers are referred to in the text. Molecular masses are given in kilodaltons.

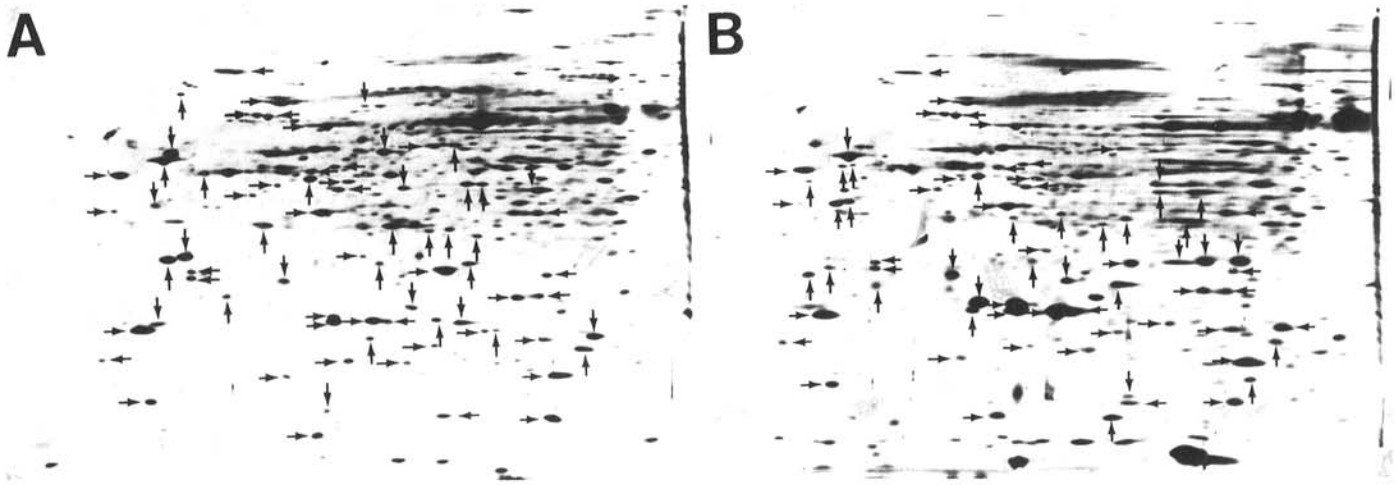

Fig. 2. 2-DGE protein patterns (25 μg) of females of *H. glycines* population R-1-92 (**A**) and *H. schachtii* population TINTE (**B**) in which a number of common proteins are marked with horizontal arrows. Vertical arrows refer to qualitative differences.

C. briggsae share only a few enzyme encoding alleles (6) implies that enzyme electrophoresis is inadequate for constructing phylogenetic trees of more distantly related species within this genus, because no alleles will be shared. The application range of 2-DGE seems substantially larger, because the number of loci assayed is an order of a magnitude larger, which increases the chance for overlap. Moreover, the proteins sampled with 2-DGE seem evolutionarily more conservative than the 20–30 enzymes usually surveyed with starch gel electrophoresis (1,5,26,27,32). For example, analyzing approximately 190 proteins of *Peromyscus maniculatus* and *Mus musculus* with 2-DGE revealed a genetic distance of 0.5, whereas starch gel electrophoresis of 30 enzymes revealed a distance of 0.94 (1). The application range of 2-DGE has its limitations too, however. The number of proteins shared between *G. rostochiensis* and *H. schachtii* was only 3 (Table 1).

2-DGE seems a suitable tool in developing serological assays to discriminate closely related nematode species. Current technology offers possibilities to produce large quantities of specific antisera by isolating proteins directly from 2-DGE patterns (37). The contrasting rates of morphological and protein evolution among nematode species suggest that there is, in general, ample opportunity to isolate species-specific proteins from 2-DGE patterns of morphologically nearly indistinguishable species in order to develop a diagnostic test.

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