# Protein variation in cyst nematodes

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STELLINGEN

1. De bewijsvoering van Brooks en Wiley voor de verenigbaarheid van de tweede wet van de thermodynamica en de evolutie van biologische systemen is wel bewonderenswaardig, maar niet overtuigend.

   Brooks, D.R. and Wiley, E.O. 1986. Evolution as entropy. University of Chicago Press.

2. De onderzoekmethoden van Braitenberg voor het bestuderen van kunstmatige intelligentie kunnen op verantwoorde wijze worden gebruikt voor de simulatie van het gedrag van nematoden in de bodem.

   Braitenberg, V. 1984. Vehicles: Experiments in synthetic psychology. MIT Press.

3. De conclusie van Ohms en Heinicke dat de door hen gevonden eiwitvarianten specifiek zijn voor pathotypen is onjuist en is mede een gevolg van voorafgaande publikaties, die gekenmerkt worden door een onzorgvuldige interpretatie van het pathotypenschema.

   Ohms, J.P. and Heinicke, D.H.K. 1985. Zeitschrift für Pflanzenkrankheit und Pflanzenschutz 92:225-232.

   Greet, D.N. and Firth, J. 1977. Nematologica 23:411-415.

   Fox, P.C. and Atkinson, H.J. 1984. Parasitology 88:131-139.

4. 'Transformation series analysis' biedt unieke mogelijkheden voor het construeren van cladogrammen op basis van 'multistate' kenmerken, maar is, in tegenstelling tot hetgeen Mickevich suggereert, in de huidige vorm slechts in een beperkt aantal situaties toepasbaar.

   Mickevich, M.F. 1982. Systematic Zoology 31 (4):461-478.

5. Bij de bestudering van interacties tussen planten en parasieten wordt te weinig onderkend dat fotodynamische reacties van zowel type I als type II vele biologische processen kunnen beïnvloeden.

6. De vraag van Gabriel en Ellingboe naar aanleiding van eiwitvariatie gemeten met elektroforese: "Is it possible that an obligate parasite such as E. graminis is so precisely adapted to its host that almost any variation is lethal?", getuigt van onvoldoende inzicht in de moleculaire aspecten van de evolutietheorie.

   Gabriel, D.W. and Ellingboe, A.H. 1982. Phytopathology 72:1496-1499.

7. Péterfy et al. gebruiken bij het voorspellen van de gevoeligheid van een ELISA een foutieve methode voor het bepalen van de affiniteitskonstante van een antilichaam.

   Péterfy, F., Kuusela, P. and Mäkelä, O. 1983. Journal of Immunology 130 (4):1809-1813.

8. 'Protein engineering' op grond van een gedetailleerde analyse van eiwitten uit aardappelcysteaaltjes zal onmisbaar zijn voor de produktie van transgene aardappelplanten met geschikte resistentie tegen aardappelmoeheid.

9. De melkklieren van transgene zoogdieren zijn ideale produktiesystemen voor het vervaardigen van humane eiwitten met therapeutische eigenschappen.

   Simons, J.P., McClenaghan, M. and Clark, A.J. 1987. Nature 328:530-532.

10. De term 'personal' computer is vooral van toepassing op de incompatibiliteit van de verschillende systemen.

Proefschrift J. Bakker
Protein variation in cyst nematodes
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J. Bakker

# Protein variation in cyst nematodes

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CONTENTS

# CHAPTER I

## INTRODUCTION

Phytonematologists are confronted with a group of organisms whose nomenclature and taxonomic groups undergo continuous change. The small size, uniformity of morphology and relatively simple basic structure of nematodes create serious difficulties in tracing discriminating characters. The limited number of conspicuous morphological features has offered ample opportunity for personal judgement in delineating species and genera. Well known examples are the cyst nematodes (45). Various species of cyst nematodes were first considered to be races of Heterodera schachtii (9). Eventually these races were described as separate species, e.g. H. schachtii, H. avenae, H. goettingiana and H. rostochiensis. The first sign that the potato cyst nematode, H. rostochiensis, might not be a single species was the discovery of populations able to overcome certain genes for resistance (23). In the early 1970s it became apparent that not all pathotypes of the potato cyst nematode H. rostochiensis interbreed freely and that two sibling species are present: G. rostochiensis and G. pallida (23). The late discovery of these species resulted from their morphological similarity. Most morphological characters are variable and overlap between the two species (43,44).

Parallel to changes in nomenclature of potato cyst nematode species, classification of their pathotypes has also been dominated by a state of instability. As with many obligate plant parasites, the number of pathotypes increased with the discovery of more sources of resistance. An additional complication was that in several European countries different systems were employed to distinguish field populations. In 1978 a number of European countries reached a consensus and proposed an international pathotype scheme (28). Currently eight pathotypes are recognized in Europe, five within G. rostochiensis and three within G. pallida (28). Resistance and virulence are thought to be mediated by genes operating on the basis of a gene for gene relationship (22,24,48). Unlike resistance against various species of fungi,

breeding programs based on major genes can be profitable, because selection towards alleles for virulence is rather slow (24,25,26,48).

In the Netherlands, the international pathotype scheme (28) forms the core on which decisions are made in controlling potato cyst nematode populations by means of resistance. However, current pathotype classification has several drawbacks in characterizing the intraspecific diversity of potato cyst nematodes. First, measuring the virulence characteristics of potato cyst nematode populations is laborious. Because no rapid assays are available, the multiplication factors of the populations on the differentials have to be estimated in a traditional way by inoculating potato plants with cysts. Second, pathotypes are delineated in an arbitrary way. Populations are classified as virulent and avirulent for a certain differential, if the multiplication factor is $> 1$ and $\leq 1$, respectively. Obviously, this way of classifying gives little information, because multiplication factors on the differentials may vary from 0 to 70. Third, the number of differentials is too limited for a proper characterization of genetic diversity of potato cyst nematode populations in Europe. Supplementary differentials have already discriminated populations classified as identical according to the current international pathotype scheme. Fourth, the expression of the nematode and host genotypes is rather variable which often makes it difficult to decide whether a population should be classified as virulent or avirulent for a given differential.

Evidently an accurate identification of potato cyst nematode populations is a necessity for an optimal control by means of resistance. Cyst nematodes are major pests of potato in Europe and an improper identification or the inability to recognize distinct populations can have drastic and costly consequences. Although traditional ways of pathotyping and species identification will always maintain their value, there is an increasing need for more efficient and accurate methods.

Diagnosis and systematics are entirely dependent on differences in characters caused by evolutionary forces. The ideal character state for delineating groups of conspecific populations, species or genera has evolved only once and has been retained by all descendants. A

powerful method to trace informative characters is the analysis of biological macromolecules. Various techniques ranging from protein electrophoresis, macromolecular sequencing and immunological assays have provided valuable information about evolutionary relationships between extant species of a variety of organisms (1,2,6,19,31,32,37) and in general there is a reasonable agreement between molecular data and classic taxonomic criteria. In the past decades it has clearly been established that proteins diverge at an approximately constant rate. The finding that a given protein accumulates amino acid substitutions at a nearly constant rate in all species forms the basis of the neutral theory of molecular evolution (27). This theory asserts that evolutionary change at the molecular level is caused mainly by random genetic drift of selectively neutral mutants rather than by positive Darwinian selection. Because mutation and random genetic drift are stochastic processes, the actual number of parallelisms and convergences are small, which makes molecular data extremely useful for inferring evolutionary relationships. Another desirable feature of molecular data is that the evolutionary rates of proteins are extremely diverse and may vary more than 100 fold, which implies that there are in theory nearly always proteins of which the evolutionary rates are adjusted to the group of populations studied. Fast evolving proteins can be explored to study conspecific populations whereas more conservative proteins are useful for studying genealogical relationships between different species , genera and families.

Compared to various other organisms, the molecular taxonomy of plant parasitic nematodes is poorly developed (16,38). Obligate plant parasites are generally of microscopic size and difficult to rear in large quantities; consequently it is difficult to use many standard biochemical techniques. For example, starch gel electrophoresis followed by specific enzyme stains, a routine technique for many organisms, has hardly (21) been applied to plant parasitic nematodes because of the relatively large amounts of protein required. French (4,5) and German investigators (36,39,40) tackled this problem by using micromethods for homogenizing and electrophoresing single specimens. Besides microelectrophoresis, nematologists have employed

various other electrophoretic techniques to study nematode proteins. For instance, the genetic variability of nematodes has been investigated with polyacrylamide gel electrophoresis at an alkaline pH (20,42,46,47), isoelectric focusing (10,11,12,13,14,17,35), immunoelectrophoresis (15,49), sodium dodecyl sulfate electrophoresis (41) and two dimensional gel electrophoresis (2-DGE)(3,7,8) according to O'Farrell (34). 2-DGE combined with a sensitive silver stain (29,30,33) seems in theory the most promising approach. A major advantage of 2-DGE over other techniques is that it is capable to resolve several hundreds of gene products on a single pattern. In the first dimension the proteins are separated according their isoelectric points and in the second dimension according their molecular weights resulting in an almost unique position of each protein. In this thesis we evaluated the merits of electrophoresis, especially 2-DGE, in exploring proteins for the systematics and diagnosis of cyst nematode populations.

Genetic distances based on qualitative data or allele frequency data from several tenths of loci are valuable measures to study divergence times of extant species. Unfortunately, current electrophoretic data on the genetic differentiation of G. rostochiensis and G. pallida allow no proper estimate of a genetic distance (3,11,12,20, 35,36,38,42,46,47,49). Similar drawbacks are associated with published data on the genetic differentiation of pathotypes. Only a small number of variants are presented in those reports and no allele frequencies are given for these variants (3,11,12,13,14,17,36,42), which hampers a refined assessment of the genetic relationships. Since many populations are not fixed for their alleles for virulence or avirulence, it is rather peculiar to conclude that such an incomplete set of data can be used to pathotype field populations (36). Pathotyping field populations with biochemical techniques is obviously far more complicated (chapter III). In this thesis we advance a more realistic approach for characterizing European potato cyst nematode populations. In chapter V we present a new concept based on the neutral theory of molecular evolution (27) to explain the relation between variations in virulence and variations revealed by electrophoretic techniques.

An accurate way to examine with 2-DGE genetic relationships between conspecific populations (chapter V and VI) or species (VII) is the comparison of homologous proteins. Although 2-DGE is a common technique in various scientific disciplines, its value for systematic studies has hardly been explored. Allele frequencies obtained by 2-DGE have never been used to construct similarity dendrograms or phylogenies and only recently qualitative variations between homologous proteins have been utilized to estimate the phylogeny of hominoid primates (18). Tracing homologues among the several hundreds of proteins resolved with 2-DGE is evidently more tedious than evaluating isozyme patterns. An additional problem with plant parasitic nematodes is that 2-DGE is not sensitive enough to detect a sufficient number of proteins from single nematodes. In the investigations described in this thesis 2-DGE patterns are made by electrophoresing a mixture of 100 or more individuals, which makes the genetic interpretation more complicated. However, as shown these difficulties can be minimized by optimalizing the experimental procedure, which is described in detail in Chapter IV.

The objectives of this study are: i) to estimate the genetic distance between G. rostochiensis and G. pallida (chapter II), ii) to isolate species specific proteins from second stage larvae of G. rostochiensis and G. pallida for developing a diagnostic test (chapter III), iii) to assess the genetic relationships between European potato cyst nematode populations (chapter V and VI) and iv) to develop an effective method to study ancient genealogical relationships with 2-DGE (chapter VII).

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## CHAPTER II

# Contrasting Rates of Protein and Morphological Evolution in Cyst Nematode Species


ABSTRACT

Bakker, J., and Bouwman-Smits, L. 1986. Contrasting rates of protein and morphological evolution in cyst nematode species. Phytopathology

The genetic differentiation of morphologically nearly indistinguishable cyst nematode species 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 time 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: potato cyst nematodes, beet cyst nematode and soybean cyst nematode

## INTRODUCTION

A variety of electrophoretic techniques have been employed to study Heterodera and Globodera spp. (3,4,10,11,12,13,25,29,30,35). Especially, the sibling species Globodera rostochiensis (Woll.) Skarbilovich and G. pallida Stone have been studied extensively. Most reports on these morphologically nearly indistinguishable cyst nematodes, which were until 1973 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) as well as two-dimensional electrophoresis followed by a Coomassie Brilliant Blue stain (35) clearly distinguished the two species.

Although protein electrophoresis has fulfilled the expectations with regard to 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 2 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, owing to 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 (population no. 1,2,3,5,9), Hilbrands laboratorium, Assen, the Netherlands (population no. 4), the Foundation for Agricultural Plant Breeding (SVP), Wageningen, the Netherlands (population no. 6) and the Department of Plant Pathology, University of Missouri, Columbia, U.S.A. (population no. 7 and 8). Potato cyst nematodes and beet cyst nematodes were reared at 18 C with 16 hr daylength on Solanum tuberosum spp. 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 daylength.

Total protein samples of mature females were made as described previously (2). Total protein samples of fourth stage females were prepared as follows. 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 10 mM tris-HCl, pH 7.4, 5% (v/v) 2-mercaptoethanol, saturated with 64 mg urea and stored at -80 C until use. 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 (clearance ca. 25 µm). Approximately 100,000 larvae were homogenized in 200 µl 10 mM tris-HCl, pH 7.4, 5% (v/v) 2-mercaptoethanol and saturated with 213 mg urea.

As a standard, 25 µg of protein was used for 2-DGE. Sample application, isoelectric focusing within the pH range 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% NaOH, 1.5% (v/v) $NH_4OH$ and 0.32% $AgNO_3$. 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 were evaluated which were consistently present in each replicate. At least three replicates per population were analyzed.

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

## RESULTS

2-DGE protein patterns of young females of G. rostochiensis, G. pallida, H. glycines and H. schachtii are shown in Fig. 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. In order to avoid arbitrary decisions with regard to quantity, these proteins were also registered as common.

The number of proteins shared and the genetic distances between the 9 populations are listed in Table 1. The average genetic distance (D) 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.

Fig. 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.

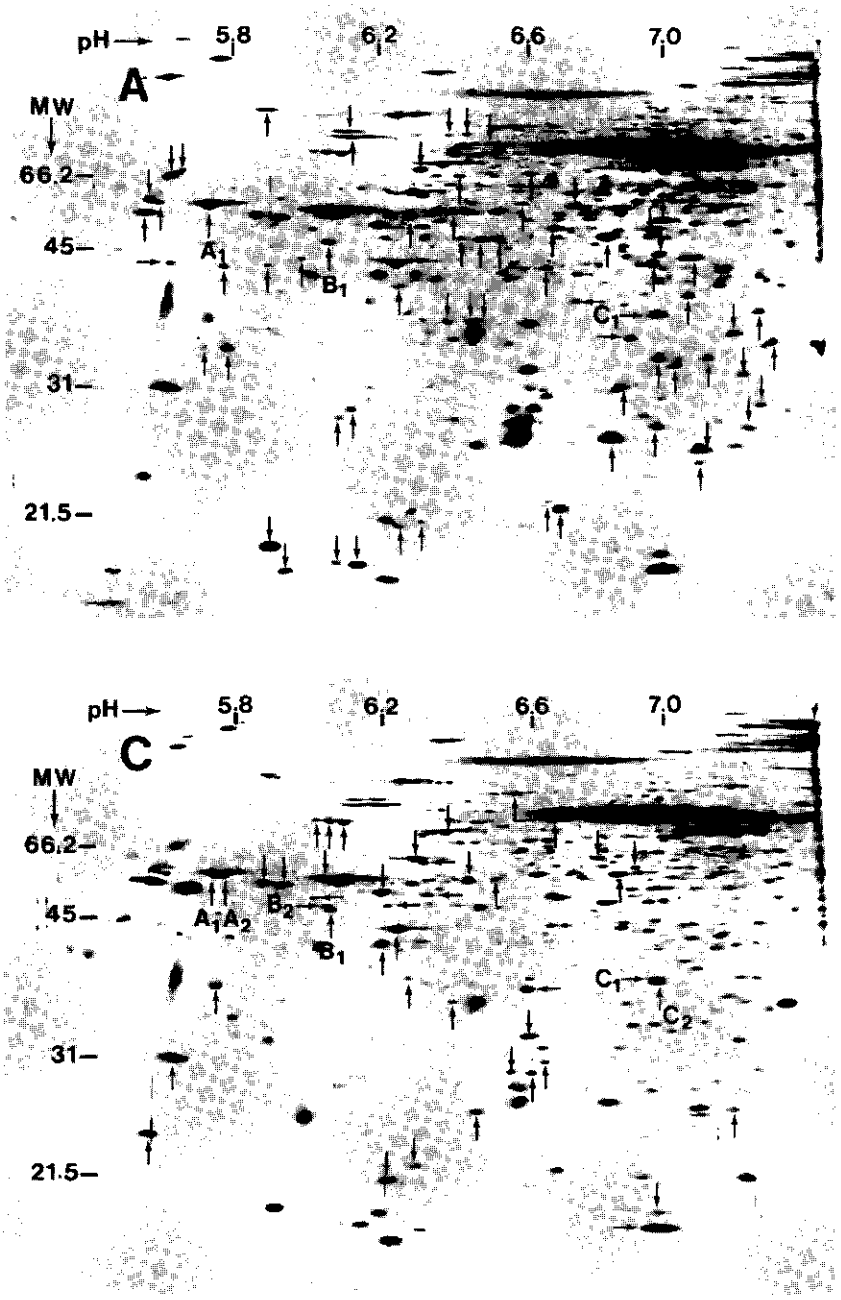
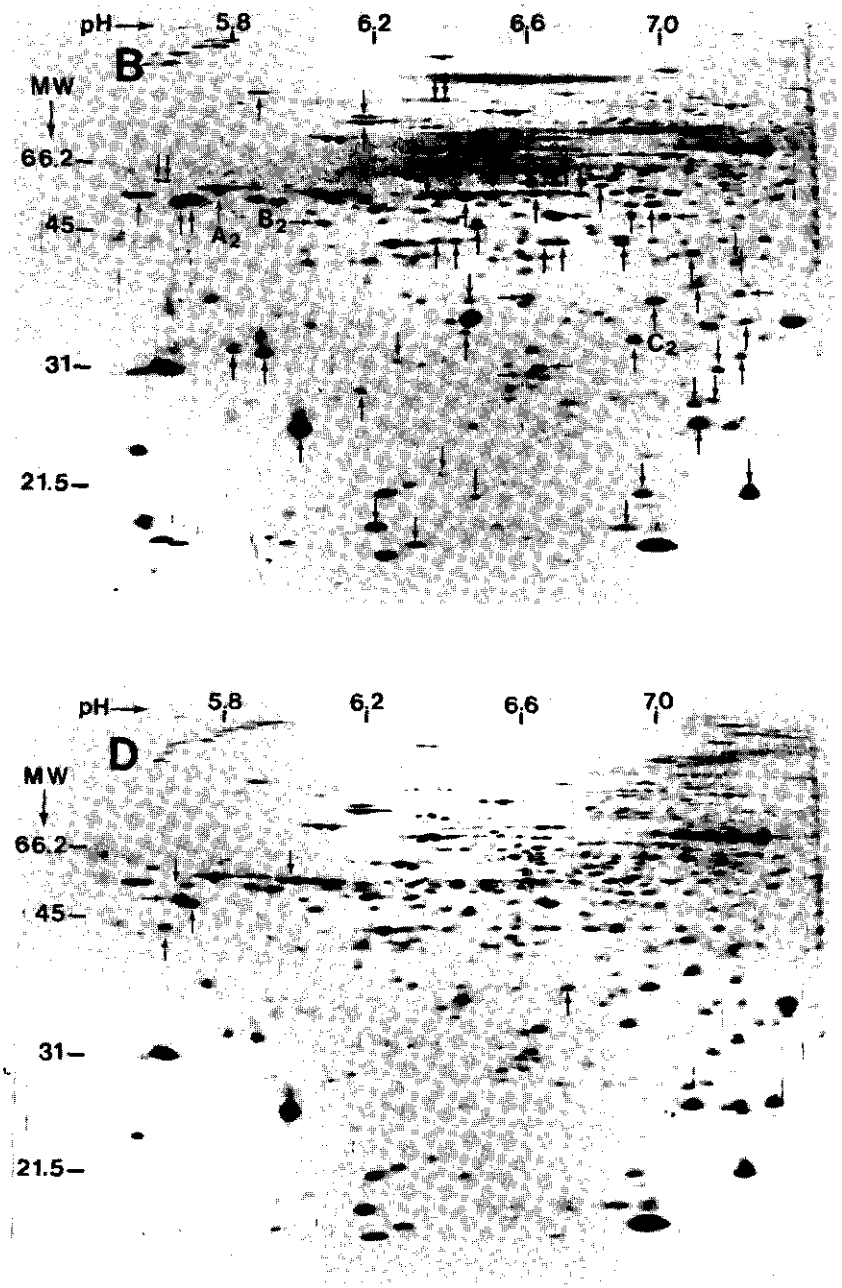



**Fig. 1** 2-DGE protein patterns (25 µg) of females of G. rostochiensis population MIER (**A**) and G. pallida population HPL-1 (**B**) in which a number of major qualitative differences are marked with arrows. Common proteins are designated in a pattern (**C**) containing an equal protein




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

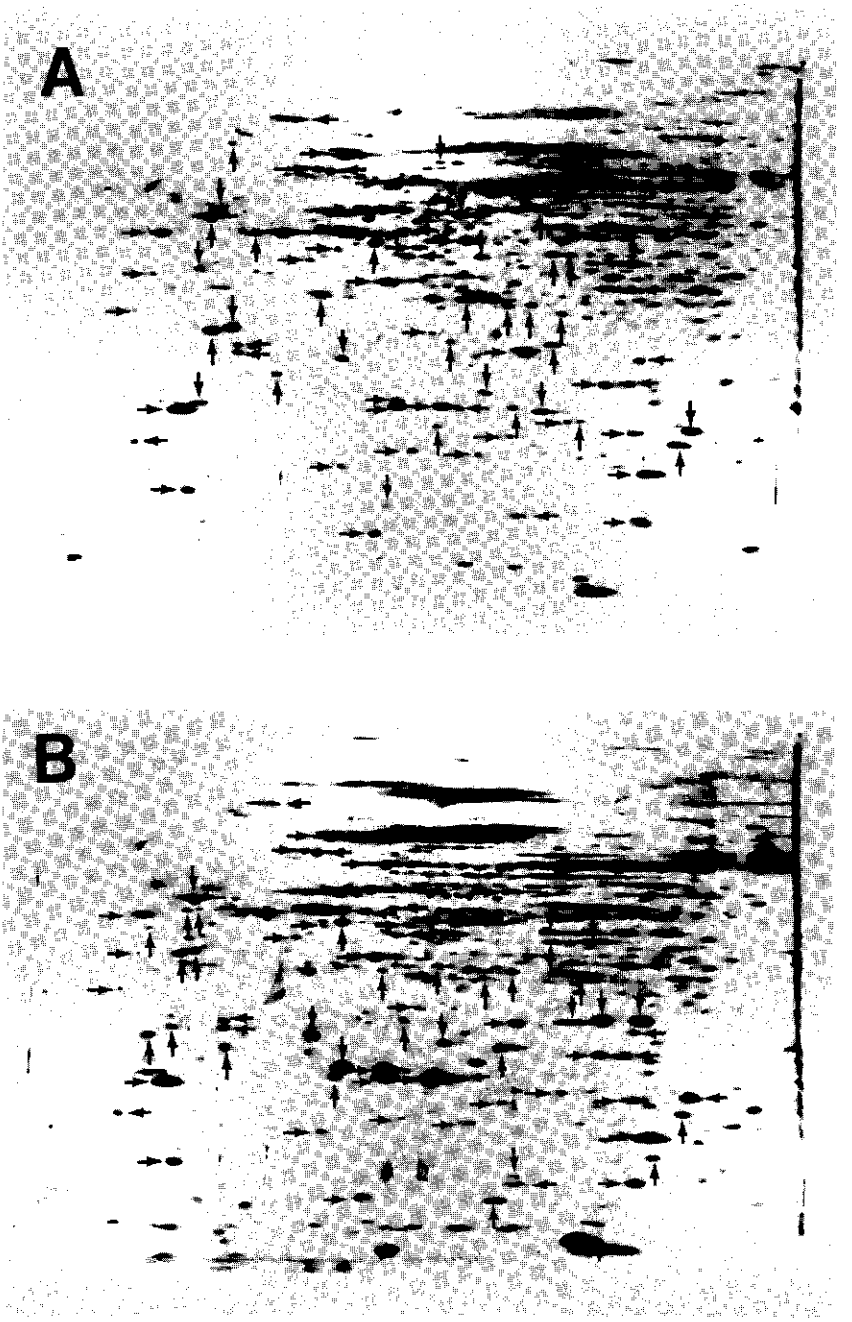



**Fig. 2.** 2-DGE protein patterns (25 µg) of females of H. glycines population R-1-92 (**A**) and H. schachtii population TINTE (**B**) in which a number of common proteins are marked with horizontal arrows. Vertical arrows refer to qualitative differences.

**Table 1** The number of proteins shared (above diagonal) and the genetic distances (D)(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

## 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 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 (D) 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 which 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 are probably to some extent also influenced by changes in the regulatory system, e.g. alterations in regulatory sequences and structural genes which influence the synthesis, processing and degradation of other proteins. However, for a 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 (D) of 0.37 (27), have diverged approximately 4 million years ago (33). Although these values cannot directly be extrapolated 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 ranging from 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 discriminates them 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 have diverged more than 10 millions of 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 authors 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 Caenorhabditis elegans and 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 on overlap. Moreover, the proteins sampled with 2-DGE seem evolutionary 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 (D) of 0.5, whereas starch gel electrophoresis of 30 enzymes revealed a distance (D) 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).

Finally it is mentioned, that 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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## CHAPTER III

# Species Specific and Thermostable Proteins from Second Stage Larvae of *Globodera rostochiensis* and *G. pallida*


ABSTRACT

Bakker, J., Schots, A., Bouwman-Smits, L. and Gommers, F.J. 1987. Species specific and thermostable proteins from second stage larvae of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. Phytopathology ..:.....

Different electrophoretic techniques were applied to extracts of second stage larvae of six *Globodera rostochiensis* and six *G. pallida* populations. Electrophoresis of native proteins clearly distinguished *G. rostochiensis* from *G. pallida*. Four major protein bands were specific for *G. rostochiensis* and five for *G. pallida*. However, repeated experiments gave large variations in intensities of most of the species specific protein bands. The species specific protein bands resolved with SDS electrophoresis were more reproducible. In contrast with other reports, no consistent intraspecific variation could be detected with one-dimensional electrophoresis. A number of the species specific proteins resolved with SDS electrophoresis appeared to be thermostable and were partially purified. Characterization of the thermostable polypeptides by two-dimensional electrophoresis (20), resolved three polypeptides specific for *G. rostochiensis* with isoelectric points (pI) and molecular masses of 20.6 kDa (pI 5.30), 20.8 kDa (pI 5.20) and 18.0 kDa (pI 6.00), which slightly differed from those specific for *G. pallida*, 21.0 kDa (pI 5.32), 20.5 kDa (pI 5.40) and 17.0 kDa (pI 5.80).



Additional keywords: potato cyst nematodes, pathotypes, silver stain.

## INTRODUCTION

Resistant potato cultivars are, together with crop rotation and the use of nematicides, essential for the control of the potato cyst nematodes Globodera rostochiensis (Woll.) Skarbilovich and G. pallida Stone. Resistance is mainly derived from Solanum tuberosum ssp. andigena Juz. and Buk. (30) and S. vernei Bitt. and Wittm. (10), and, is mediated by pathotype specific genes (3,15,23,31). However, the commercial success of resistant cultivars is limited by the widespread occurrence of virulent cyst nematode populations. At present eight pathotypes of the potato cyst nematodes (15) are recognized, five within G. rostochiensis ($Ro_1$ - $Ro_5$) and three within G. pallida ($Pa_1$ - $Pa_3$). Because large areas are infested with both species, current breeding programs are mainly focussed on resistance against most or all pathotypes of both species, which is a major constraint for the introduction of new resistant cultivars.

A reliable and quick screening test able to characterize field infestations of potato cyst nematodes according to species should improve possibilities for control by means of resistance. Diagnosis of field populations will allow growers to select cultivars with resistance against either of the two species and the introduction of new resistant cultivars will be facilitated by incorporating genes for resistance effective against either G. rostochiensis or G. pallida. The low dispersal abilities and the relative low reproduction rate of the potato cyst nematodes favor such an approach. Moreover, selection towards alleles for virulence is rather slow (11,12,13,31), and in many fields resistant cultivars can be grown without loss of yield for several years.

Identification of G. rostochiensis and G. pallida by morphological measurements is hampered by intraspecific variation and interspecific overlap (25,26,27) and, in addition, several morphological characters are influenced by environmental factors such as temperature and availability of food (4). Morphological differentiation of these sibling species is therefore time consuming and hence not suited for large scale routine purposes.

The objective of this study was to trace species specific proteins

which have suitable properties for the development of a serological assay. In addition, we speculate on the possibility for pathotyping field populations.

## MATERIALS AND METHODS

**Populations.** Twelve populations of G. rostochiensis and G. pallida were obtained from different geographical sources (Table 1). Populations no. 1,2,3,4,5,7,9 and 10 were kindly supplied by Ir. C. Miller and Ing. J. Bakker, Plant Protection Service, Wageningen, the Netherlands; population no. 12 by Ir. A. Mulder, Hilbrands Laboratorium, Assen, the Netherlands; populations no. 6 and 11 by Dr. H.J. Rumpenhorst, Department of Nematology, Muenster, Federal Republic of Germany and population no. 8 by Dr. A.R. Stone, Rothamsted Experimental Station, Harpenden, England. All populations were maintained on Solanum tuberosum ssp. tuberosum 'Eigenheimer', susceptible to all pathotypes.

**Morphological characterization.** Second stage larvae were killed in FP-4 (18) and fixed in 4% cold formalin. Further processing for measurements was as described by Seinhorst (22). On the average 20 specimens were measured per population by our taxonomist Dr. P.A.A. Loof.

**Virulence characterization.** The populations were tested for their reproductive ability on the differentials used in the international pathotype scheme of Kort et al. (15). S. tuberosum ssp. tuberosum was represented by commercial cultivars susceptible to all pathotypes. S. tuberosum ssp. andigena was replaced by the commercial cultivar Saturna having the resistance gene H1 derived from CPC 1673. In experiments in Petri dishes, carried out by our geneticist Ir. R. Janssen, S. vernei hybr. 62.33.3 was replaced by cultivar Darwina. S. kurtzianum hybr. 60.21.19 and S. multidissectum hybr. P55/7 were not included in this test.

Virulence characteristics were measured by inoculating 50 second stage larvae (two per root tip) on roots of sprouts grown in Petri dishes with water-agar (17). The number of females that appeared on the roots of the differential was expressed as percentage of the

centrifuged (105,000 g) and used immediately for isoelectric focusing.

Isoelectric focusing in the pH region 4 to 6 was carried out essentially as described by O'Farrell (20), with the following modifications. The ampholines pH range 5 to 7 were replaced by ampholines pH range 4 to 6 and gels were polymerised in glass tubes (160 mm x 2 mm inside diameter). The sample (25 - 50 µl) was loaded without prefocusing. Isoelectric focusing was carried out according the following schedule: 30 min 100 V; 30 min 200 V; 15 hr 300 V and 3 hr 400 V. In the second dimension the proteins were separated in SDS polyacrylamide gels as described above, but with a constant current of 25 mA. Between isoelectric focusing and SDS electophoresis, gels were equilibrated for 10 min in 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS in 62.5 mM tris-HCl, pH 6.8. The isoelectric points (pI) of the polypeptides were estimated by measuring the pH profile of the isoelectric focussing gel with a surface pH electrode (Bio-rad). The standard deviations of these pI values averaged 0.1 pH unit.

**Staining.** Staining with silver was performed according to Oakley et al. (19). In a number of experiments proteins were stained with Coomassie Brilliant Blue R250. The staining solution contained 0.2% coomassie brilliant blue, 50% (v/v) methanol and 7% (v/v) acetic acid. The gels were destained in 5% (v/v) methanol and 7% (v/v) acetic acid.

## RESULTS

In order to define our populations as well as possible, the variation in virulence and morphology was examined. In most combinations designated as compatible according to the international pathotype scheme (15), the number of females that developed on the differentials was remarkably low when compared with the general susceptible host (Table 1). For example, the relative number of females produced on S. vernei hybrid 62.33.3 by the $Pa_3$ populations no 9, 10 and 11 did not exceed 10%. This indicates that these populations are mixtures of individuals differing in their ability to overcome resistance. Furthermore, populations classified as virulent for the same differential differ in their ability to overcome resistance. For

example, populations no. 4 and 6, both classified as virulent for S. tuberosum ssp. andigena, have a value of 30.0% and 84.6%, respectively, for the relative number of females produced on S. tuberosum ssp. andigena.

Morphological characters of second stage larvae are given in Table 2. Body length and tail length differ markedly from the values given in the original descriptions of G. rostochiensis and G. pallida (25) and are here not informative in discriminating the two species. Stylet length and distance from median bulb valve to excretory pore are in concordance with the original descriptions. Although these values are variable and overlap between the two species, the average values clearly discriminate the two species. The species designation of the populations is also supported by the shape of the stylet knobs (Table 1) and the color of the females (data not shown).

The twelve populations were investigated with different types of protein electrophoresis. Electrophoresis of native proteins clearly distinguishes the two species (Fig. 1). Protein bands Rf 0.350, Rf 0.595, Rf 0.650, Rf 0.750 are specific for G. rostochiensis and Rf 0.240, Rf 0.550, Rf 0.590, Rf 0.760, Rf 0.780 for G. pallida. The intensities of several species specific proteins bands (e.g. Rf 0.350, Rf 0.650, Rf 0.760) varied between repeated experiments, probably due to a combination of small variations in the extraction procedures (e.g. time between homogenization and electrophoresis) and the instability of the native proteins. In some cases species specific protein bands were even fully absent (e.g. Rf 0.350, population 3 and 6) while present in other experiments with the same population. No consistent intraspecific differences were detectable. Storage of native protein samples at -80 C caused many of the proteins to deteriorate resulting in diffuse bands after electrophoresis (results not shown).

Fig. 2 compares the SDS denaturated protein profiles of four G. rostochiensis populations and four of G. pallida. Three major protein bands with molecular masses of 31.0 kDa, 20.7 kDa and 18.0 kDa were specific for G. rostochiensis and four major bands with molecular masses of 31.5 kDa, 21.0 kDa, 20.5 kDa and 17.0 kDa were specific for G. pallida. Subtle quantitative differences are also seen in diffuse

bands. These bands are probably composed of several polypeptides differing slightly in molecular masses. The intensities of these bands varied between repeated experiments and these are therefore not suited for species identification. In Fig. 2 only highly reproducible species specific bands are indicated. The protein bands 20.7 kDa, 18.0 kDa, 21.0 kDa, 20.5 kDa and 17.0 kDa stain grey with silver, whereas the majority of the proteins stain brown or reddish brown. Protein profiles visualized with coomassie brilliant blue (Fig. 3) again demonstrate that some of the aforementioned species specific proteins are major components of the total soluble protein fraction. With one dimensional SDS electrophoresis no intraspecific differences were found, neither qualitative nor quantitative.

Fig. 3 shows the SDS protein patterns of total soluble protein fractions and thermostable proteins from second stage larvae. The heat treatment resulted in an approximate 50-fold purification of some species specific proteins (20.7 kDa, 18.0 kDa and 21.0 kDa, 20.5 kDa, 17.0 kDa). The species specific thermostable proteins have a high affinity for the silver stain. Application of 10 ng heat treated protein is sufficient to resolve the species specific proteins.

The heat stable proteins were further characterized by combining isoelectric focusing and SDS polyacrylamide gel electrophoresis. Two-dimensional electrophoretic patterns of total protein and partially purified proteins are presented in Fig. 4. The species specific thermostable proteins are marked by their molecular masses in kDa. The 20.7 kDa protein band, specific for G. rostochiensis, is actually composed of two polypeptides of 20.6 kDa and 20.8 kDa (Fig. 4A and 4C). Two dimensional electrophoresis of an equal mixture of heat stable proteins of the two species shows the small differences in isoelectric point (Fig. 5). The specific polypeptides of G. rostochiensis, 20.6 kDa and 20.8 kDa, have an isoelectric point (pI) of 5.30 and 5.20, respectively. The specific thermostable proteins of G. pallida, 20.5 kDa and 21.0 kDa, have a slightly higher isoelectric point, 5.40 and 5.32, respectively. The polypeptide 18.0 kDa, specific for G. rostochiensis, has a pI of 6.00 and the 17.0 kDa polypeptide of G. pallida a pI of 5.80 (Fig. 5). It is noted that the differences between the pI values of the species specific proteins are constant,

whereas the estimates of the absolute pI values varied between independent measurements.

The species specific proteins showed no detectable changes in molecular mass or isoelectric point after the partial purification (Fig. 4), indicating that the proteins are not markedly affected by the heat treatment. The thermostable proteins exhibited no intraspecific variation as was assessed by two-dimensional electrophoresis of all populations (data not shown).

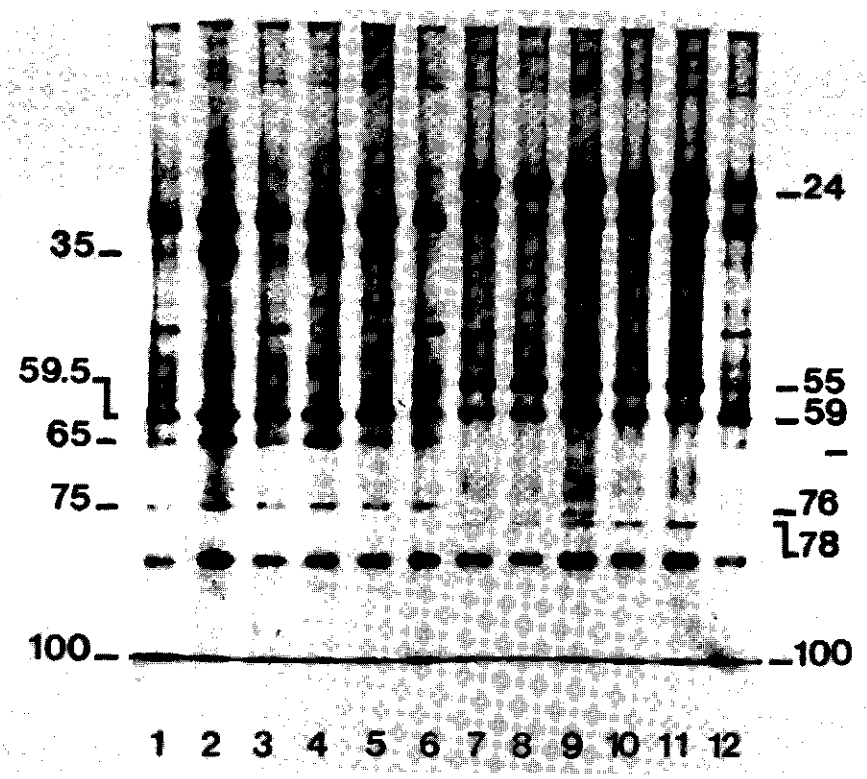



**Fig. 1.** Electrophoresis under native conditions of second stage larvae of six G. rostochiensis populations (1 - 6) and six G. pallida populations (7 - 12). The species specific protein bands are indicated with their Rf values (x $10^2$). The migration distance of bromophenol blue was given Rf 1.00. Numbers at bottom of the lanes refer to the populations in Table 1. Proteins were stained with silver. Samples contained 3 µg of protein.

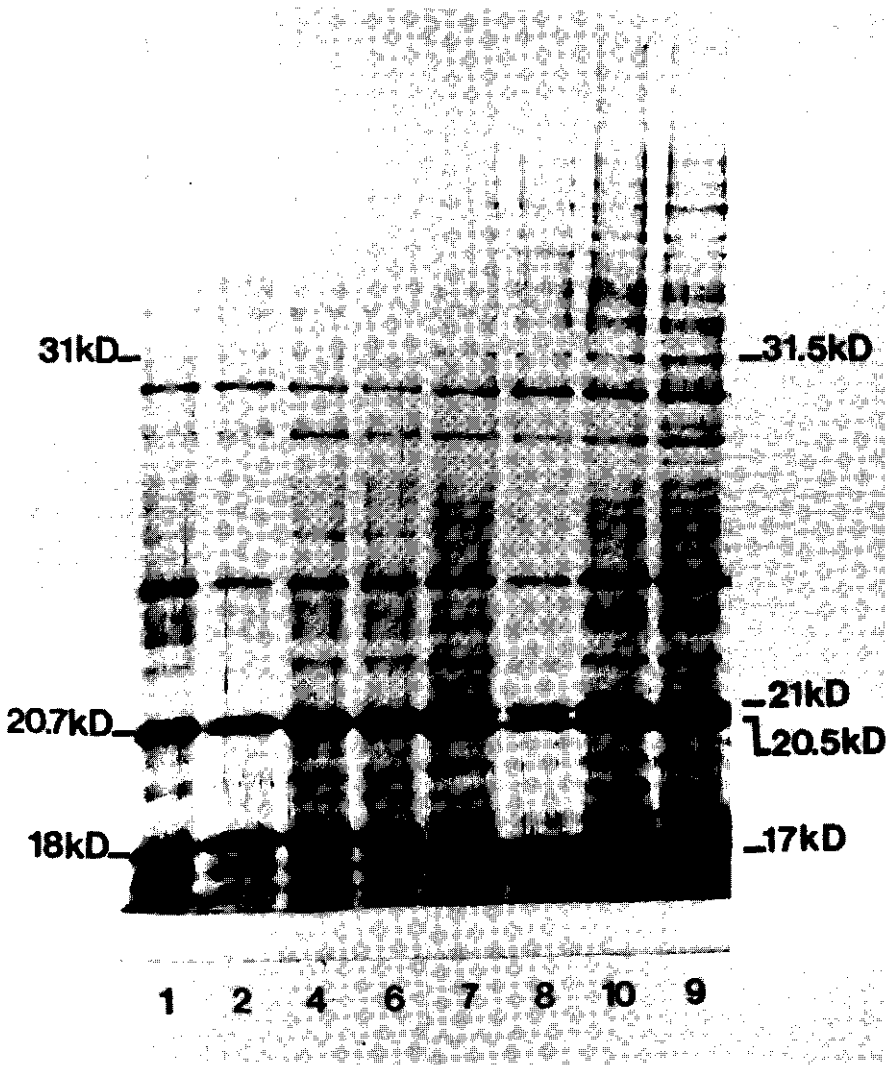



**Fig. 2.** SDS electrophoresis of second stage larvae of G. rostochiensis (1, 2, 4, 6) and G. pallida (7, 8, 10, 9). Species specific protein bands are marked. Values at left and right of the figure refer to molecular masses in kilodaltons. Numbers at bottom of the lanes refer to the populations in Table 1. Proteins were stained with silver. Samples with 3 µg of protein were used.

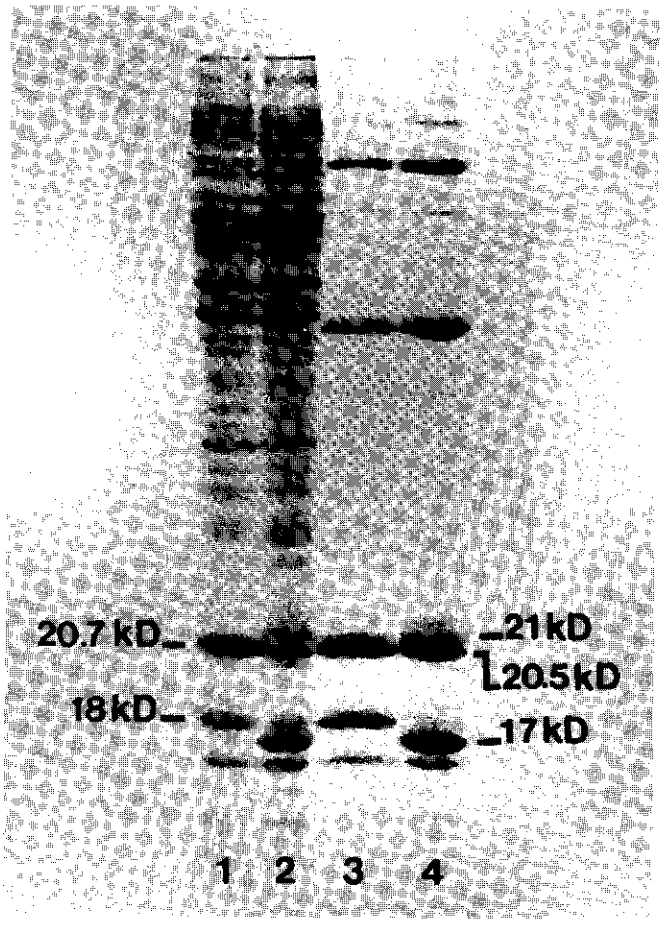



**Fig. 3.** SDS electrophoresis of crude homogenates (lane 1, 2) and thermostable proteins (lane 3, 4). The molecular masses of the thermostable proteins specific for G. rostochiensis (lane 1, 3) and G. pallida (lane 2, 4) are indicated in kilodaltons at the left and right of the figure, respectively. Samples of total soluble protein (30 µg) and thermostable protein (5 µg) were obtained from populations 1 and 8 (see Table 1). Proteins were stained with coomassie brilliant blue.

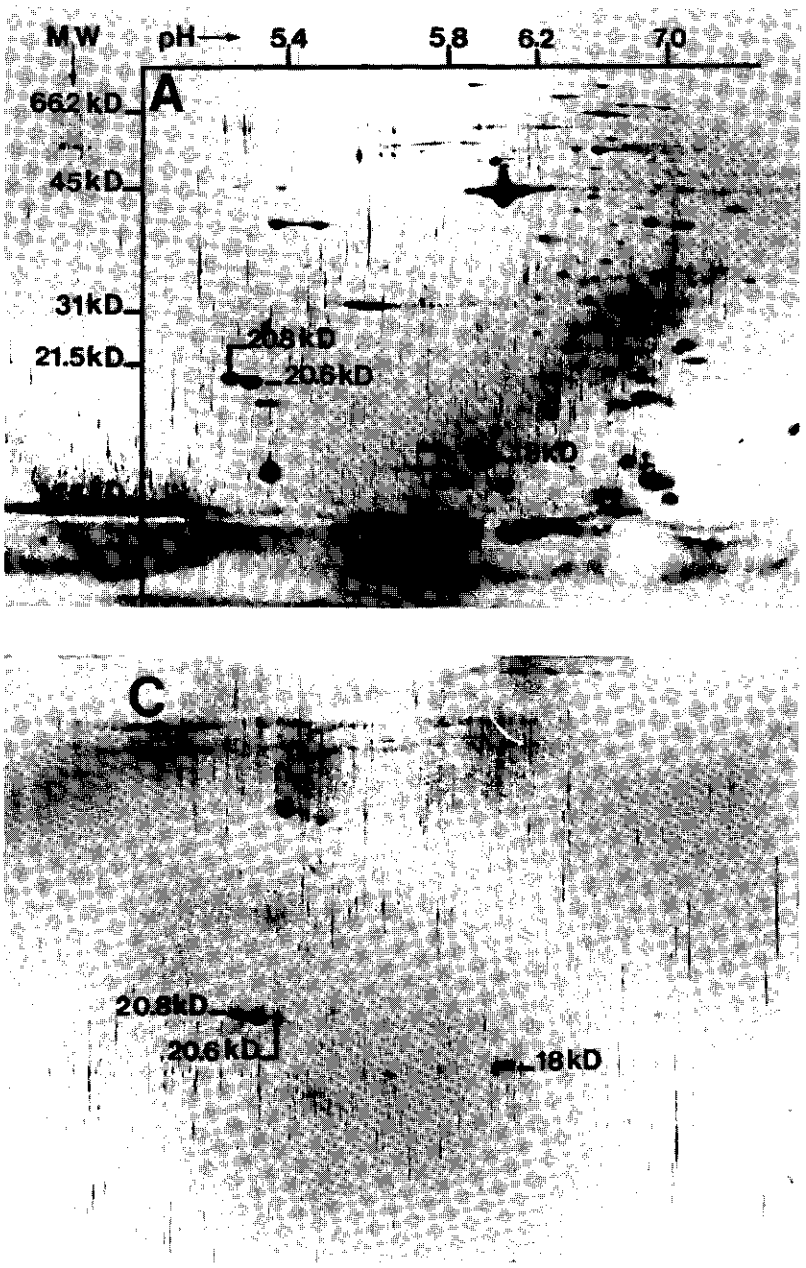
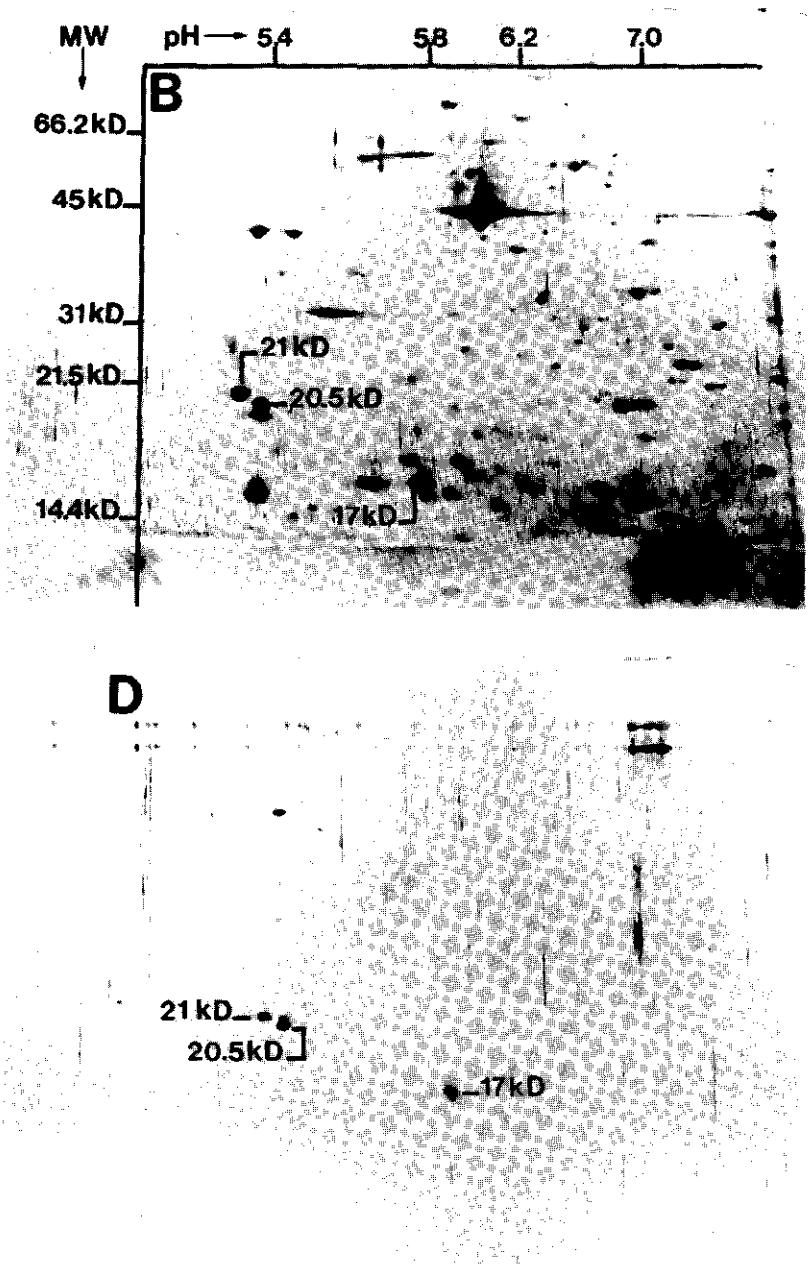



**Fig. 4.** Two dimensional electrophoretic patterns of total protein (25 µg) of G. rostochiensis (**A**) and of G. pallida (**B**) and thermostable proteins (0.5 µg) of G. rostochiensis (**C**) and of G. pallida (**D**). The molecular masses of the species specific thermostable polypeptides are given in kilodaltons. The protein band of 20.7 kilodaltons, specific




for G. rostochiensis, actually consists of two polypeptides, a peptide of 20.8 kilodaltons and of 20.6 kilodaltons. G. rostochiensis and G. pallida are represented by population 1 and 8, respectively. (see Table 1). Proteins were stained with silver.

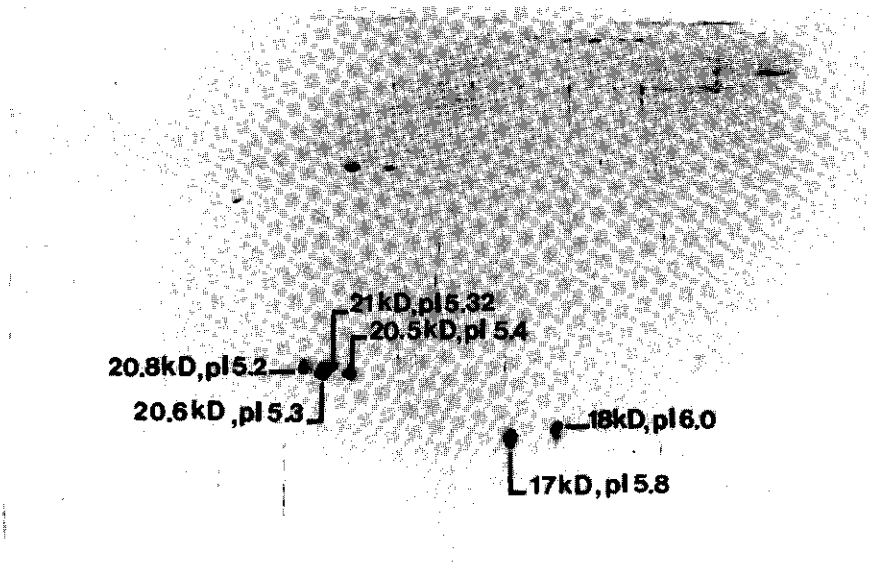



**Fig. 5.** Two dimensional electrophoretic pattern of a mixture of equal protein quantities of the thermostable proteins of G. rostochiensis (0.25 µg) and G. pallida (0.25 µg). The species specific polypeptides are indicated by their approximate isoelectric points (pI) and molecular masses in kilodaltons. Samples were prepared from populations 1 and 8 (see Table 1). Proteins were stained with silver.

**Table 1** Geographical source and virulence characteristics in the Globodera rostochiensis (Ro) and G. pallida (Pa) populations

| Pop. no. | Pathotype, collection location and code[a] | relative number of females[b] S.tuberosum ssp. andigena hybr. CPC 1673 | | S.vernei hybr. 58.1642/4 | | S. vernei hybr. 62.33.3 | |
|---|---|---|---|---|---|---|---|
| 1. | $Ro_1$, Wageningen, the Netherlands, MIER | 0 | (-) | 1 ± 0.2[c] | (-) | 0 | (-) |
| 2. | $Ro_1$, Weert, the Netherlands, A-13 | 0 | (-) | 1 ± 1.3[c] | (-) | 0 | (-) |
| 3. | $Ro_1$, Hardenberg, the Netherlands, C-133 | 1.4 ± 0.1 | (-) | 0.3 ± 0.2[c] | (-) | 0 | (-) |
| 4. | $Ro_3$, Hoogeveen, the Netherlands, C-129 | 30.0 ± 1.5 | (+) | 2.2 ± 2[c] | (-) | 0.5 ± 0.4[c] | (-) |
| 5. | $Ro_4$, Emmen, the Netherlands, F-515 | 0 | (-) | 10 ± 5[c] | (+) | 0 | (+) |
| 6. | $Ro_5$, Harmerz, Federal Republic Germany, H | 84.6 ± 3.7 | (+) | 30 ± 9[c] | (+) | 15.9 ± 1.7 | (+) |
| 7. | $Pa_1$, Glarryford, Northern Ireland, 1337 | -[d] | (+) | -[d] | (+) | 10.4 ± 2.5[c] | (-) |
| 8. | $Pa_2$, New Leake, Great Britain, ST | -[d] | (+) | -[d] | (+) | 3.3 ± 2[c] | (-) |
| 9. | $Pa_3$, Cadishead, Great Britain, E-1202 | -[d] | (+) | -[d] | (+) | 6.7 ± 0.7 | (+) |
| 10. | $Pa_3$, Far Oer, Denmark, E-1215 | -[d] | (+) | -[d] | (+) | 9.3 ± 0.7 | (+) |
| 11. | $Pa_3$, Frenswegen, Federal Republic of Germany, FR | -[d] | (+) | -[d] | (+) | 5.7 ± 0.6 | (+) |
| 12. | $Pa_2$, Veendam, the Netherlands, HPL-1 | -[d] | (+) | -[d] | (+) | 1.5 ± 0.5 | (-) |

[a] As designated in the original collections.

[b] Numbers of females produced on differentials expressed as percentages of those on susceptible plants, ± the standard deviations. (-) and (+) refer to combinations which are classified according the international pathotype scheme as incompatible and compatible, respectively.

[c] Data obtained from pot experiments.

[d] Estimates not made.

**Table 2** Morphological characters of second stage larvae of Globodera rostochiensis (Ro) and G. pallida (Pa)

| Pop. no[a] | Pathotype and code | Stylet length, µm[b] | Body length, µm[b] | Median bulb valve to excretory pore, µm[b] | Tail length, µm[b] | Stylet[c] knobs |
|---|---|---|---|---|---|---|
| 1. | $Ro_1$, MIER | 21.0 ± 0.6 | 453 ± 19 | 31.6 ± 2.0 | 50.0 ± 2.9 | 16 R, 9 R-P |
| 2. | $Ro_1$, A-13 | 20.9 ± 0.5 | 438 ± 18 | 32.5 ± 3.5 | 50.0 ± 4.4 | 14 R, 1 R-P |
| 3. | $Ro_1$, C-133 | 20.8 ± 0.6 | 436 ± 19 | 33.5 ± 2.1 | 48.8 ± 2.5 | 9 R, 5 R-P, 3 P |
| 4. | $Ro_3$, C-129 | 20.7 ± 0.6 | 420 ± 22 | 30.6 ± 2.8 | 47.5 ± 2.7 | 20 R, 5 R-P |
| 5. | $Ro_4$, F-515 | 21.4 ± 0.9 | 449 ± 18 | 32.6 ± 2.9 | 45.5 ± 2.4 | 19 R, 1 R-P |
| 6. | $Ro_5$, H | 21.0 ± 0.5 | 442 ± 20 | 29.3 ± 3.2 | 48.1 ± 3.7 | 14 R, 1 R-P |
| 7. | $Pa_1$, 1337 | 22.4 ± 1.1 | 459 ± 30 | 36.3 ± 4.0 | 49.0 ± 4.8 | 17 P, 4 R-P |
| 8. | $Pa_2$, ST | 22.4 ± 0.8 | 448 ± 19 | 38.8 ± 2.4 | 48.9 ± 3.5 | 20 P |
| 9. | $Pa_3$, E-1202 | 23.6 ± 0.9 | 449 ± 23 | 37.4 ± 2.8 | 50.2 ± 3.8 | 18 P, 2 R-P |
| 10. | $Pa_3$, E-1215 | 22.0 ± 0.5 | 418 ± 17 | 35.2 ± 2.6 | 47.3 ± 2.5 | 20 P |
| 11. | $Pa_3$, FR | 22.8 ± 0.8 | 459 ± 19 | 37.2 ± 3.0 | 49.2 ± 3.7 | 25 P |
| 12. | $Pa_2$, HPL-1 | 23.3 ± 0.8 | 458 ± 18 | 39.0 ± 4.3 | 51.1 ± 2.7 | 19 P, 1 R-P |

[a] Populations are numbered as in Table 1.

[b] ± standard deviations

[c] Numbers of specimens with basal stylet knobs rounded (R) and pointed anteriorly (P), as described for G. rostochiensis Rostoch and G. pallida Epworth, respectively (25); R - P indicates an intermediate shape.

## DISCUSSION

**Interspecific variation** A variety of electrophoretic techniques have been used with crude homogenates of G. rostochiensis and G. pallida. Using these methods several authors have reported distinct protein differences between the two species (1,5,6,7,8,9,21,24,28,29,32). So far the most promising approach as an advisory tool has been to use isoelectric focusing of native proteins of eggs and cysts (5,6). Combined with a densitometric evaluation of the protein concentrations it was possible to quantify proportions of G. rostochiensis and G. pallida in mixed samples (5,6). However, electrophoresis is probably too laborious and too expensive as a routine advisory tool.

Our approach was to search for species specific proteins having suitable properties for the development of a serological assay. Heat treatment of crude homogenates resolved several species specific proteins, (Fig. 3,4C,4D) which have desirable characteristics for the preparation of specific antibodies. These easily purified proteins are similar in molecular mass, isoelectric point and color when stained with silver. The thermostable proteins seem to be excellent targets to quantify proportions of G. rostochiensis and G. pallida in mixed samples by measuring concentrations of comparable proteins in the two species. This ability to quantify proportions may be crucial for the success of a diagnostic test, because field infestations are often mixtures of both species. Another desirable trait is that these species specific proteins are major components of the soluble protein fraction, which would facilitate the isolation of sufficient antigen. It is also noted that none of the species specific thermostable proteins manifested intraspecific variation as was ascertained with two-dimensional electrophoresis of six G. rostochiensis and six G. pallida populations. Considering the diverse provenances of the populations tested (Table 1), it seems feasible to assume that the species specific thermostable polypeptides shown in Fig. 5 are representative for many G. rostochiensis and G. pallida populations in Western Europe.

**Intraspecific variation.** Pathotypes of G. rostochiensis and G. pallida have been the focus of several electrophoretic studies (1,7,8,9,24).

Populations, representing several pathotypes of G. rostochiensis and G. pallida, have been differentiated by conventional disc-eletrophoresis of second stage larvae (9) and by two-dimensional electrophoresis of eggs (24) revealing 10 to 25 major proteins. Unlike those investigators, we were unable to detect consistent intraspecific variations with one-dimensional electrophoresis (Fig. 1,2).

It is obvious that the search for pathotype specific proteins is far more complex than implicitly suggested in the various biochemical reports dealing with pathotypes of potato cyst nematodes (1,7,9,24,32). One of the complicating factors is the way populations are classified in current pathotype schemes (3,15). Populations are designated as avirulent or virulent for a certain differential, if the reproduction factor ($P_f/P_i$) is $\leq 1$ or $>1$, respectively (3, 15). Taking a reproduction factor of 20 on the general susceptible host, which is a normal value, a reproduction factor of 1 on a differential infers that approximately 5% of the individuals are capable of development on that differential (see also Table 1). Hence, according to this definition, the number of virulent individuals for a given differential in populations classified as identical pathotypes may vary from approximately 5% to 100%. As a consequence, current pathotype classification is not accurate enough to trace correlations between intraspecific protein variations and virulent genotypes. For a proper analysis it is necessary to estimate for each differential the number of virulent genotypes (see Table 1). Until now such estimates have never been presented in biochemical studies on pathotypes (1,7,9,24,32). In view of these considerations it is evident that existing biochemical information on intraspecific variations (1,7,9,24,32) is of little use in developing tests to quantify the number of virulent genotypes in field populations.

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## CHAPTER IV

# Genetic Variation in Polypeptide Maps of two *Globodera rostochiensis* Pathotypes


ABSTRACT

Bakker, J., and Bouwman-Smits, L. 1987. Genetic variation in polypeptide maps of two *Globodera rostochiensis* pathotypes. Phytopathology ..:.....

The protein composition of females of *Globodera rostochiensis* was analyzed with two dimensional gel electrophoresis (2-DGE) followed by a sensitive silver stain. Standardization of the protein extraction from young females resulted in reproducible protein profiles, which were independent of host genotypes. Protein patterns of nematodes reared on potato and tomato were indistinguishable. Isoelectric focusing within the pH range 5-7 and nonequilibrium pH gradient electrophoresis of basic proteins followed by sodium dodecyl sulfate electrophoresis resolved approximately 720 protein spots per population. Comparison of two *G. rostochiensis* populations, classified as pathotype $Ro_1$ and $Ro_5$, revealed 680 invariant and 39 variant protein spots. Twenty three variants seem to be the result of amino acid substitutions that alter net charge and involved 11 putative loci. The underlying mechanisms of the remaining 16 variants are unclear.

## INTRODUCTION

Pathotypes of the potato cyst nematodes Globodera rostochiensis (Woll.) Skarbilovich and G. pallida Stone are defined by their ability to overcome genes for resistance derived from Solanum tuberosum spp. andigena Juz. and Buk., S. kurtzianum Bitt. and Wittm. and S. vernei Bitt. and Wittm.(20). At present eight pathotypes are recognized in Europe, five within G. rostochiensis ($Ro_1$-$Ro_5$) and three within G. pallida ($Pa_1$-$Pa_3$)(20). Several authors have suggested that most genes mediating resistance and virulence might operate on the basis of a gene-for-gene relationship (15,16,43).

The fundamental processes involved with the induction and maintenance of syncytia, and the specific mechanisms underlying virulence are unknown. Second stage larvae invade the roots and, adjacent to the xylem vessels, induce multinucleate transfer cells (17,18), which serve as feeding sites. In the incompatible combinations the syncytia remain small and are often accompanied by a necrotic reaction (14) resulting in a too limited amount of food for the females to develop. It has been suggested, similar to other sedentary nematodes (2,3), that protein substances in the saliva of the potato cyst nematodes are responsible for pathogenicity (15).

A major obstacle to biochemical research on obligate organisms, such as potato cyst nematodes, is the limited amount of biological material that can be obtained. Therefore methods with high sensitivity are needed. Microelectrophoresis, for instance, has been used to study single nematodes (6,36) or even parts of them (35). However, its application is limited when dealing with complex protein mixtures.

Two-dimensional gel electrophoresis (2-DGE), as originally described by O'Farrell (29), combined with a silver stain (24,28) or autoradiography (22,29) is a powerful research tool. 2-DGE is able to resolve more than hundred gene products from minute amounts of crude nematode homogenates (1,8).

In this study we investigated the potential of 2-DGE in monitoring patterns of gene expression and genetic variability in potato cyst nematodes.

## MATERIALS AND METHODS

**Populations.** G. rostochiensis population MIER, classified as pathotype $Ro_1$ ($Ro_1$-M), was supplied by Ir. C. Miller and Ing. J. Bakker, Plant Protection Service, Wageningen, the Netherlands. G. rostochiensis population H, classified as $Ro_5$ ($Ro_5$-H), was obtained from Dr. H.J. Rumpenhorst, Department of Nematology, Muenster, Federal Republic of Germany. The former population was collected at Wageningen, the Netherlands, and the latter at Harmerz, Federal Republic of Germany. Populations were maintained on Solanum tuberosum spp. tuberosum L. 'Eigenheimer'.

The virulence characteristics of the populations were estimated by testing their reproductive ability on the differentials used in the international pathotype scheme of Kort et al. (20). S. tuberosum ssp.tuberosum, was represented by the commercial cultivar Eigenheimer, susceptible to all pathotypes. S. tuberosum ssp. andigena was replaced by the commercial cultivar Saturna having the resistance gene $H_1$, derived from CPC 1673 (20). The number of females developed on the differentials was expressed as a percentage of the number developed on the susceptible cultivar Eigenheimer. These percentages were used as an indication for the number of virulent individuals in the populations. For population $Ro_1$-M these percentages were 0% for S. tuberosum ssp. andigena hybr. CPC 1673, 0% for S. vernei hybr. 62.33.3 and 1% (0.2) for S. vernei hybr. 58.1642/4. For population $Ro_5$-H the percentages were 84.6% (3.7) for S. tuberosum ssp. andigena hybr. CPC 1673, 14.8% (1.6) for S. vernei hybr. 62.33.3 and 30% (9) for S. vernei hybr. 58.1642/4. Figures in parenthesis are the standard deviations. Values for the first two differentials were calculated from the number of females, that developed on roots of sprouts grown on water agar (26). The percentages for the latter were calculated from females developed on potatoes grown in pots.

With regard to the genetics of G. rostochiensis, it is noted that potato cyst nematodes are diploid organisms with a haploid chromosome number of nine (7). No clear evidence is available for sex determining heteromorphic chromosomes (7). Moreover sex determination seems epigenic (25).

**Preparation of protein samples.** The cultivars Eigenheimer and Mentor, susceptible to both pathotypes, were inoculated with approximately 200 cysts and grown in pots (1 L) filled with sandy loam and placed in a growth chamber at 18 C and 16 hr daylength. Populations were also reared on Lycopersicum esculentum L. 'Moneymaker'.

Females were collected in a small glass mortar and rinsed with 10 mM tris-HCL, pH 7.4 (3 x 200 µl). Total protein samples were prepared by homogenizing 100 females in 60 µl 10 mM tris-HCL, pH 7.4, and 5% (v/v) 2-mercaptoethanol and subsequently saturated with 64 mg urea. Soluble proteins were obtained by homogenizing 200 females in 90 µl 10 mM tris-HCL, pH 7.4 and 5% (v/v) 2-mercaptoethanol. The homogenate was centrifuged for 10 min at 105,000 g and the supernatant (60 µl) was saturated with 64 mg urea. All protein samples were stored at -80 C until use.

Females collected at a certain time after inoculation are always a varying mixture of e.g. white, yellow, small and large individuals. In order to exclude undesirable variations, the nematodes were handpicked under a dissecting microscope. Only healthy looking white fullgrown females were selected. As a standard the nematodes were harvested 33 to 45 days after inoculation.

Sampling errors due to genetic variations between individuals are negligible, because protein samples were prepared by homogenizing 100 or more individuals.

**Two dimensional gel electrophoresis.** Chemicals for isoelectric focusing and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis were purchased from Bio-Rad, Richmond, except for ampholines pH range 5 to 5.5 and Nonidet P-40 which were obtained from Serva, D-69 Heidelberg, West Germany and Sigma Chemical Co., St. Louis, respectively.

Thirteen µl of medium consisting of 2% ampholines pH range 3-10, 8% ampholines pH range 5-7, 10% (w/v) Nonidet P-40, 25% (v/v) 2-mercaptoethanol was added to the thawed sample. The sample was centrifuged at 105,000 g for 20 min. Protein determinations were made according to Bradford (4) by measuring the shift in extinction of Coomassie Brilliant Blue at 595 nm.

Isoelectric focusing in the pH region 5-7 was essentially as

described by O'Farrell (29) with the following modifications. Gels were polymerized in glass tubing (160 x 2 mm inside diameter). Samples (25 - 50 µl) were loaded without prefocusing. The remaining space in the tube above the sample was filled with a solution with the same buffer composition as the sample i.e. 4.75 mM tris-HCL pH 7.4, 8.9 M urea, 5% (v/v) 2-mercaptoethanol, 1% (w/v) Nonidet P-40, 0.9% ampholines pH range 5-7, 0.2% ampholines pH range 3-10. In this way variations due to application of different sample volumes are minimized. Isoelectric focusing was performed according the following schedule: 30 min 100 V; 30 min 200 V; 15 hr 300 V; 3 hr 400 V. Electrophoresis was towards the anode with basic reservoir on top and the acidic reservoir at the bottom. Equilibrium electrophoresis within the pH range 5-5.5 was carried out by replacing the ampholines pH range 5-7 by an equal amount of ampholines pH range 5-5.5.

Nonequilibrium pH gradient electrophoresis (30) was done by using the same polymerization mixture as for isoelectric focusing within the pH region 5-7. Electrophoresis was towards the cathode and terminated after 4 hr at 400 V.

After isoelectric focusing the gels were equilibrated for 10 min in a SDS buffer (29) and subjected to a discontinuous SDS gel system (21) with a 12% (w/v) acrylamide separation gel (thickness 1.5 mm, slab gel apparatus model 220 Bio-Rad). After nonequilibrium pH gradient electrophoresis a 15% (w/v) acrylamide separation gel was used. SDS electrophoresis was performed with a constant current of 25 mA.

The isoelectric points (pI) of the proteins were estimated by measuring the pH gradient with a pH contact electrode (Bio-Rad). The standard deviations of these pI values averaged 0.5 pH unit. Apparent molecular weights were estimated by using phosphorylase B, bovine serum albumine, ovalbumine, carbonic anhydrase, soybean trypsin inhibitor and lysozyme as reference proteins (Bio-Rad low molecular weight standard solution).

**Staining.** The silver staining procedure described by Oakley (28) was modified as follows. Step 1, gels were fixed for 30 min in a solution containing 50% methanol and 10% acetic acid. Step 2, stored overnight in 5% methanol with 7% acetic acid. Step 3, soaked during 1 hr in 7%

glutaraldehyde. Step 4, rinsed for 24 hr in distilled water ( 4 x 400 ml). Step 5, complexed with silver for 1 hr in an ammoniacal silver solution containing 0.075% NaOH, 1.2% (v/v) $NH_4OH$, 0.32% $AgNO_3$, 300 ml per gel. Step 6, washed in distilled water during 15 min ( 3 x 500 ml). Step 7, transferred to a clean container and immersed in a solution containing 0.001% citric acid and 0.002% formaldehyde. The proteins became visible after approximately 15 min. Protein profiles were compared visually by superimposing the original gels on a bench viewer, illuminated with fluorescent tubes. The number of replicates per object ranged from 4 to 20.

## RESULTS

Variations in hatch and development are major obstacles in obtaining highly standardized protein samples. The time at which the nematodes were harvested varied from 33 to 45 days after inoculation. These variations were mainly caused by differences in the age of the tubers and cysts. Analysis of total protein extracts with 2-DGE within the pH range 5-7 showed that our protein extraction was not influenced by these variations. White fullgrown females harvested at 33, 36, 40 and 45 days after inoculation gave identical protein profiles. The protein patterns were also not influenced by the host genotypes. Females reared on the potato cultivars Mentor and Eigenheimer and the tomato cultivar Moneymaker revealed no noticeable differences.

**Classification of variant protein spots.** Despite the high standardizaton of the 2-DGE procedure several proteins varied in intensities between repeated experiments or were even absent in some experiments. The main source of variation was found in the silver staining procedure. During the course of this study we tested several silver staining methods (23,24,28,34,46), but obtained no better results than with the procedure described here. In this study only those variants were recorded which were consistently different between the two populations.

Comparison of the 2-DGE protein patterns of population $Ro_1$-M and $Ro_5$-H revealed 680 invariant and 39 variant protein spots (Table 1). The variants were divided in three groups: i) variants which seem to

be the result of isoelectric point changing amino acid substitutions (IP-variants) (Fig. 1A and 1B), ii) variants characterized by the presence or absence of a protein (PA-variants)(Fig. 1C), iii) variants expressed by differences in concentration (C-variants)(Fig. 1D).

The corresponding IP-variants are designated as such because they have the characteristics expected from protein variations caused by mutations leading to net charge change (5,19,22,32,37,39,44). The corresponding IP-variants have a moderate difference in isoelectric point, similar molecular weight and seem to be produced in similar quantities per haploid set of chromosomes within each individual. Corresponding IP-variants are assumed to be encoded by alleles at the same locus. The IP-variants are assigned with capitals referring to the putative loci and numbers referring to the alleles. This interpretation is also supported by the color of the proteins. The color of the approximately 700 proteins studied here ranged from red (±5%), reddish brown (±70%), blackish brown (±5%), brownish grey (±5%) to grey (±15%). All corresponding IP-variants have identical colors, which is expected from proteins differing in a small number of amino acid residues (27).

In this study the IP-variant loci manifest themselves in two ways. The first group consists of IP-variant loci which are monomorphic in both populations (Table 1: locus A, C, D, G, H, and J). These IP-variants have similar spot sizes and intensities in population $Ro_1$-M and $Ro_5$-H (e.g. Fig. 1A). The second group consists of IP-variant loci which are monomorphic in population in $Ro_1$-M and polymorphic in $Ro_5$-H (Table 1: locus B, E, F, I and K). An important clue in recognizing these IP-variants as such is, that the protein quantity of each of the allele products in $Ro_5$-H is smaller than that of the corresponding allele product in $Ro_1$-M (e.g. Fig. 1B). This behaviour is expected from mutations resulting in a net charge change. The sum of the protein quantities of the allele products from a polymorphic locus should be equal or at least close to the protein quantity of the corresponding allele product from a monomorphic locus.

The remaining variants for which no putative corresponding allele products could be traced, are designated as C- or PA-variants (Fig. 1C and 1D). The C- and PA-variants are combined within one class of

variants, the nonisoelectric point variants (NIP-variants). The NIP-variants are designated with an arabic number (Fig. 1, 2 and 3, Table 1).

**2-DGE, pH range 5 - 7.** Total protein analysis resolved 393 invariant protein spots and 11 IP-, 5 PA- and 3 C-variants (Fig. 2, Table 1). 2-DGE of soluble proteins revealed 6 IP- and 2 PA-variants and 79 invariant protein spots, which were not detectable in total protein samples (Table 1). It is noted, that proteins with an isoelectric point higher than the proteins V, W, X and Y were not examined, because of the relative poor resolution and reproducibility in this area (Fig. 2).

Scrutiny of the total protein patterns of different generations revealed no disparities with the results presented here. Total protein patterns from three and two generations of $Ro_1$-M and $Ro_5$-H, respectively, were identical.

**2-DGE, pH range 5 - 5.5** Analysis of the soluble protein fraction demonstrated that C-variant no. 3 is actually composed of two variant protein spots (Table 1). This type of 2-DGE resolved in addition 1 PA- and 1C-variant not traced with 2-DGE pH range 5-7 (Table 1).

**2-DGE, basic proteins.** Nonequilibrium pH gradient electrophoresis of soluble proteins resolved 6 IP-, 3 PA-variants and approximately 170 invariant proteins not detected with 2-DGE pH range 5-7. The majority of these variant and invariant proteins have probably an isoelectric point above 6.8. This was estimated by using the proteins V, W, X and Y as references. These proteins, which are resolved both with electrophoresis within the pH range 5-7 (Fig. 2A) and with nonequilibrium pH gradient electrophoresis (Fig. 3A), have isoelectric points of 6.86, 6.92, 6.86 and 7.00. The reference proteins are easily recognized in both systems by their relative abundance and color. Protein V, W, and X are reddish brown and Y is grey. It is puzzling, that the IP-variants $\underline{I}_1$ and $\underline{I}_2$ (Fig. 3), which have a lower electrophoretic mobility towards the cathode than protein V, were not detected with 2-DGE within the pH range 5-7 (Fig. 2A and 2B).

The basic high molecular weight proteins have a low electrophoretic

mobility towards the cathode and are therefore not optimally separated by nonequilibrium pH gradient electrophoresis (Fig. 3). A better resolution of these high molecular weight proteins was obtained by extending the electrophoresis time in the first dimension to 18 hr. In this way 30 additional invariant protein spots were detected.

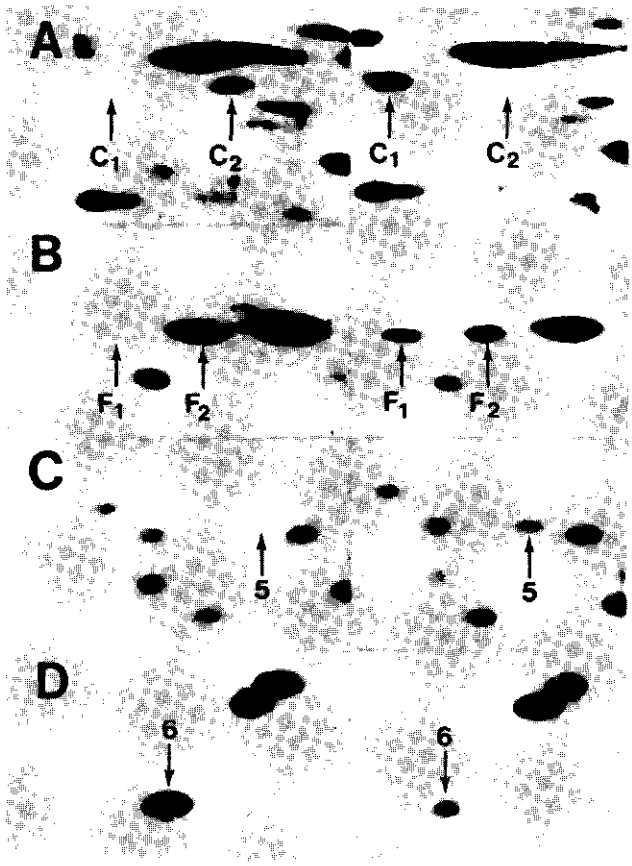



**Fig. 1** Examples of the types of variant protein spots detected by the comparison of 2-DGE protein patterns of G. rostochiensis population $Ro_1$-M (left) and $Ro_5$-H (right). **A** and **B**. Variations having the characteristics expected from amino acid substitutions that alter the isoelectric point of a protein. The isoelectric point variants (IP-variants) are assigned with capitals referring to the putative loci and numbers referring to the alleles. **C** and **D**. Variants for which no putative corresponding allele product could be traced are marked with arabic numbers. Differences expressed by presence/absence data (**C**) and differences in concentration (**D**) were combined within one class of variants, the nonisoelectric point variants (NIP-variants).


A
pH
5.4
5.8
6.2
6.6
7.0
7.4
M.W.
66.2
45
31
21.5
14.4
1
2
3
4
5
6
7
8
X
Y
Z

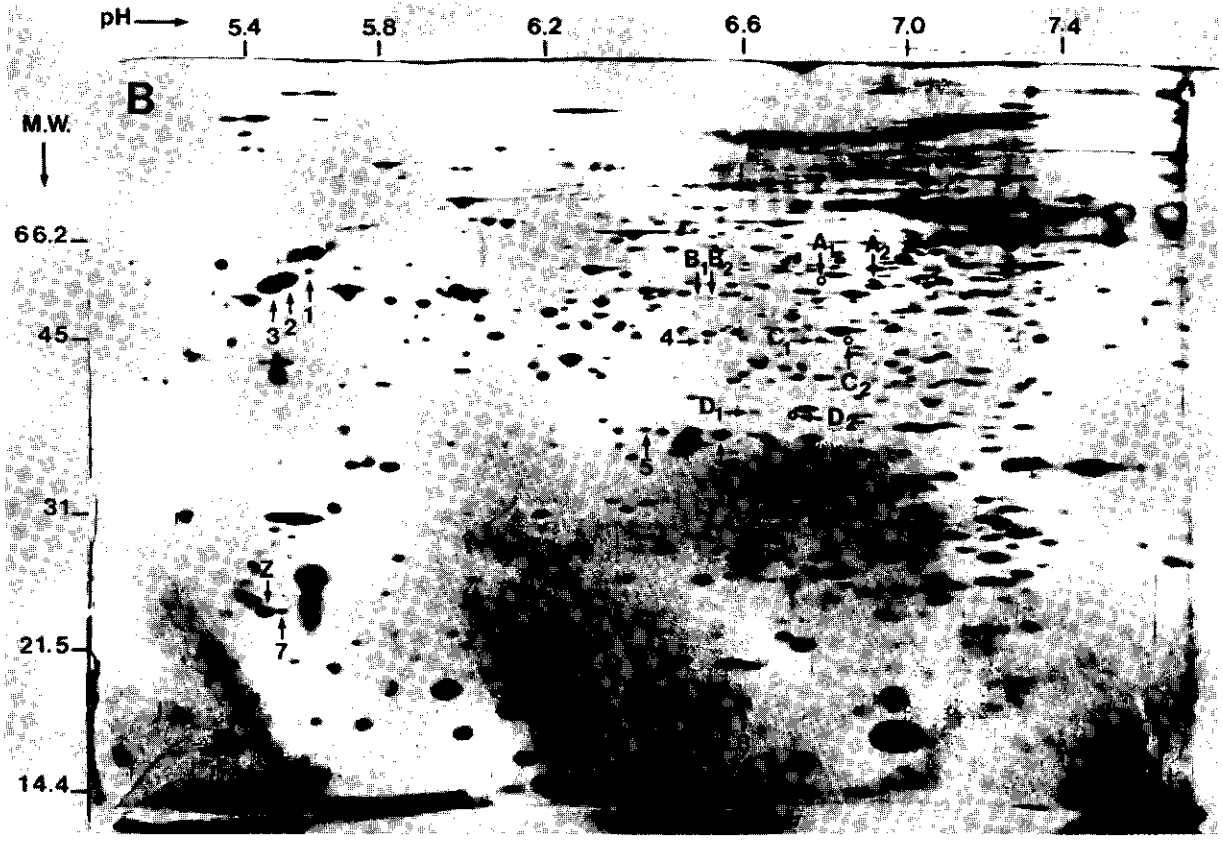
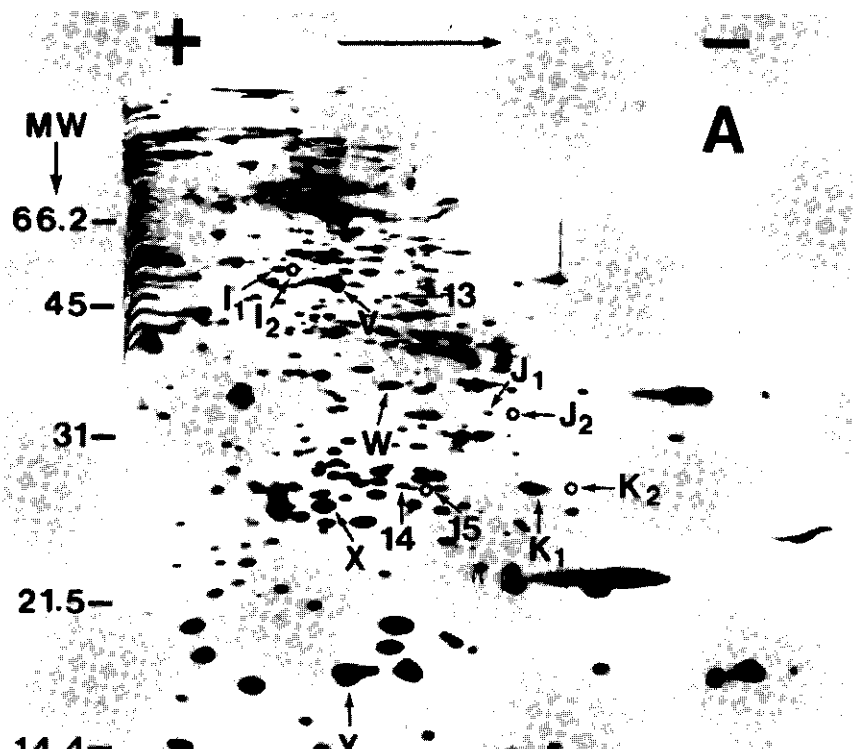



**Fig. 2** 2-DGE within the pH range 5-7 of total protein (25 µg) from G. rostochiensis population $Ro_1$-M (**A**) and $Ro_5$-H (**B**). The variant proteins that discriminate population $Ro_1$-M from $Ro_5$-H are indicated as described for Fig. 1. Open circles indicate the absence of a protein spot. A number of minor variant proteins (e.g. locus B) are only visible on the original gel patterns. Proteins V, W, X, Y and Z are referred to in the text. Molecular masses are given in kilodaltons.


+
−
MW
A
66.2
45
31
21.5
14.4
I1
I2
13
J1
J2
W
K2
14
J5
K1
X
Y

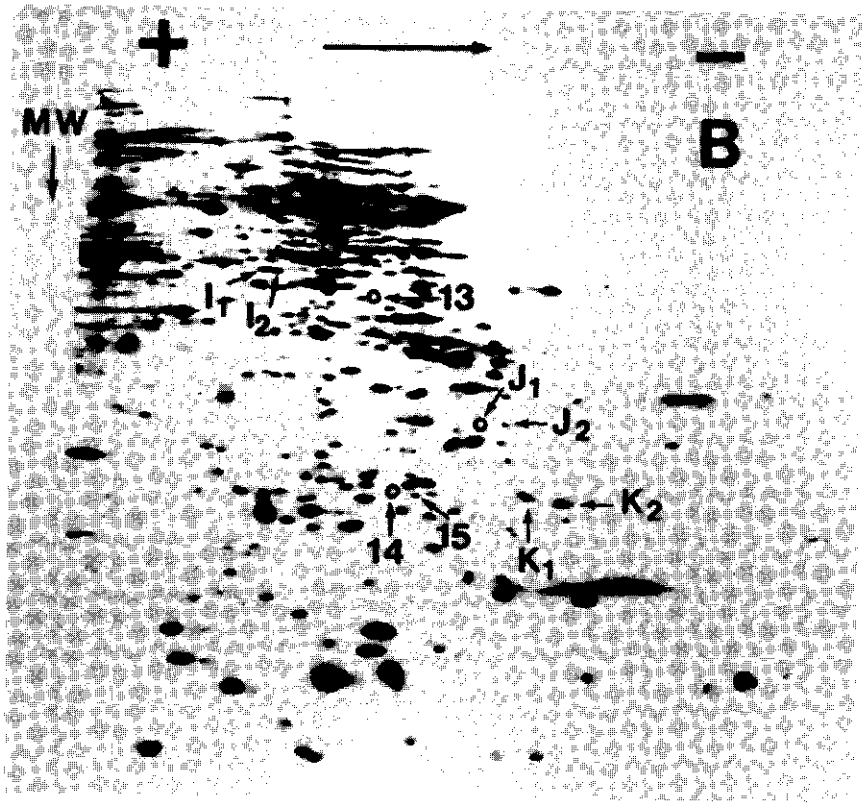



**Fig. 3** 2-DGE of basic soluble proteins (35 µg) of G. rostochiensis population $Ro_1$-M (**A**) and $Ro_5$-H (**B**). In the first dimension the proteins were separated by nonequilibrium pH gradient electrophoresis. The sample was applied at the anode. The proteins, that discriminate population $Ro_1$-M from $Ro_5$-H are marked as described under Fig. 1. Open circles indicate the absence of a protein spot. The proteins V, W, X and Y are referred to in the text. Molecular masses are given in kilodaltons.

**Table 1.** Invariant and variant proteins detected by the comparison of population $Ro_1$-M and $Ro_5$-H.

| 2-DGE system | | isoelectric point variants[a] | | | | | | | | | | | nonisoelectric point variants[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| total protein | | $A_1$ | $A_2$ | $B_1$ | $B_2$ | $C_1$ | $C_2$ | $D_1$ | $D_2$ | $E_1$ | $E_2$ | $E_3$ | 1 | 2 | 3[b] | 4 | 5 | 6 | 7 | 8 |
| pH range 5 to 7; | $Ro_1$-M | + | - | + | - | - | + | - | + | - | + | - | - | ± | ± | - | - | + | - | - |
| 393[c] | $Ro_5$-H | - | + | ± | ± | + | - | + | - | ± | - | ± | + | + | + | + | + | ± | + | + |
| | M.W.[d] | 59 | 59 | 54 | 54 | 46 | 46 | 38 | 38 | 36 | 36 | 36 | 60 | 58 | 57 | 46 | 37 | 25 | 23 | 19 |
| | pI[e] | 6.80 | 6.94 | 6.49 | 6.53 | 6.78 | 6.90 | 6.62 | 6.70 | 6.54 | 6.64 | 6.76 | 5.60 | 5.54 | 5.50 | 6.50 | 6.40 | 6.05 | 5.56 | 6.60 |
| | color[f] | r.b. | r.b. | r.b. | r.b. | r.b. | r.b. | r.b. | r.b. | b.b. | b.b. | b.b. | g. | g. | g. | g. | r.b. | r.b. | b.b. | r.b. |
| soluble protein | | $F_1$ | $F_2$ | $G_1$ | $G_2$ | $H_1$ | $H_2$ | | | | | | 9 | 10 | | | | | | |
| pH range 5 to 7; | $Ro_1$-M | - | + | - | + | - | + | | | | | | + | + | | | | | | |
| 237 (79)[c] | $Ro_5$-H | ± | ± | + | - | + | - | | | | | | - | - | | | | | | |
| | M.W.[d] | 70 | 70 | 44 | 44 | 22 | 22 | | | | | | 36 | 30 | | | | | | |
| | pI[e] | 6.24 | 6.28 | 6.46 | 6.52 | 6.20 | 6.26 | | | | | | 6.46 | 6.50 | | | | | | |
| | color[f] | r.b. | r.b. | r.b. | r.b. | r.b. | r.b. | | | | | | b.b. | r.b. | | | | | | |
| soluble protein | | | | | | | | | | | | | 3a[b] | 3b[b] | 11 | 12 | | | | |
| pH range 5 to 5.5; | $Ro_1$-M | | | | | | | | | | | | ± | ± | - | ± | | | | |
| 125 (8)[c] | $Ro_5$-H | | | | | | | | | | | | + | + | + | + | | | | |
| | M.W.[d] | | | | | | | | | | | | 57 | 57 | 28 | 24 | | | | |
| | pI[e] | | | | | | | | | | | | 5.48 | 5.51 | 5.42 | 5.41 | | | | |
| | color[f] | | | | | | | | | | | | g. | g. | g. | g. | | | | |
| soluble protein | | $I_1$ | $I_2$ | $J_1$ | $J_2$ | $K_1$ | $K_2$ | | | | | | 13 | 14 | 15 | | | | | |
| basic[g]; | $Ro_1$-M | + | - | + | - | + | - | | | | | | + | + | - | | | | | |
| 295 (170)[c] | $Ro_5$-H | ± | ± | - | + | ± | ± | | | | | | - | - | + | | | | | |
| | M.W.[d] | 52 | 52 | 34 | 34 | 27 | 27 | | | | | | 46 | 28 | 28 | | | | | |
| | color[f] | r.b. | r.b. | r.b. | r.b. | b.g. | b.g. | | | | | | r.b. | r.b. | r.b. | | | | | |

a) The presumptive loci and alleles of the isoelectric point variants (IP-variants) are designated with capitals and arabic numbers, respectively. The nonisoelectric point variants (NIP-variants) are assigned with arabic numbers. The symbols + and - indicate the presence or absence of a protein spot; ± points to a lower concentration when compared with the corresponding protein in the opposite population. For the latter three 2-DGE systems only those variants are listed which were not detected with the previous system(s).

b) NIP-variant no. 3 is composed of two proteins.

c) The total number of invariant proteins resolved. In parenthesis the number of invariant proteins not detected in the previous 2-DGE system(s).

d) Molecular masses in kilodaltons.

e) Isoelectric points.

f) Reddish brown (r.b.), blackish brown (b.b.), brownish grey (b.g.) and grey (g).

g) The majority of the variant and 170 invariant proteins resolved with nonequilibrium pH gradient electrophoresis have a pI > 6.8.

## DISCUSSION

Various electrophoretic techniques have been used to study the potato cyst nematode G. rostochiensis (9,10,11,12,31,33,40,41,42,45). The number of proteins detected in these studies ranged from 10 to 70. Conventional disc electrophoresis of females showed marked variations between protein patterns of different populations (11,12,41,42). Many of those variations appeared not to be genetically determined and were ascribed to the comparison of females differing in physiological stage (12). Because large quantities of synchronized females are difficult to obtain, several investigators switched to electrophoresis of second stage larvae and eggs, and achieved more reproducible protein profiles (9,10,12,31,40). So far the most extensive report dealt with isoelectric focusing of eggs resolving 40 major protein bands and 23 enzymes (10). Intraspecific variation within G. rostochiensis was observed at one enzyme locus (10).

In this study the sample preparation was highly standardized by a careful selection of uniform females under a dissecting microscope. In this way variations between independent experiments due to differences in age or condition of the nematodes were minimized. Unlike other investigators (12), we observed no noticeable effect of the host genotypes. Protein patterns of females reared on potato and tomato were indistinguishable.

**IP- and NIP-variants.** Codominant alleles coding for proteins differing in one or more net charge changing amino acids are a prominent group among the variants detected by enzyme electrophoresis (13) and 2-DGE (5,19,22,32,37,39,44). In view of these reports and the characteristics of the isoelectric point variants (IP-variants) it seems feasible to assume that the corresponding IP-variants are the products of alleles at the same locus. Evaluating the variants detected in previous 2-DGE studies (5,19,22,32,37,39,44) shows that it is unlikely that other types of genetic variation will give rise to protein variations having the characteristics of the IP-variants. Of course, our results provide no conclusive evidence and one should always be aware of exceptions.

Eleven loci are involved with the 23 IP-variants. Population $Ro_1$-M

appeared monomorphic for all IP-variant loci, whereas $Ro_5$-H was polymorphic for 5 loci and monomorphic for 6 loci (Table 1). However, not necessarily all individuals in population $Ro_1$-M and $Ro_5$-H need to be homozygous for these monomorphic loci. The 2-DGE protein patterns represent the average protein composition of 100 individuals or more and, hence, alleles present in low frequencies may remain unnoticed. This will especially be the case for alleles producing small quantities of protein (e.g. Fig. 2A and 2B, loci B and D).

The methodology used here allows no clear cut distinction between the C-variants and the PA-variants. Proteins classified by us as C-variants may be the result of presence/absence data in individuals. The absence in one genotype (e.g. null alleles) and the presence in the other will result in a C-variant in case population $Ro_1$-M and $Ro_5$-H contain unequal proportions of these genotypes. The majority of the NIP-variants, 5 C- and 11 PA-variants, are probably genetically determined. Patterns from three subsequent generations of population $Ro_1$-M and two of $Ro_5$-H provided no evidence that they are caused by a variable expression of the genotype.

The genetic background of the NIP-variants is unclear. A number of NIP-variants may, similar to the IP-variants, be the result of net charge changing amino acid substitutions. For example, protein Z and NIP-variant no. 7 (Fig. 2A and 2B) may be encoded by alleles at the same locus, but are not recognized as such, because protein Z is recorded as invariant. It is stressed that a quantitative difference between protein Z in population $Ro_1$-M and $Ro_5$-H, which is equal to the quantity of NIP-variant no. 7, is not detectable with this system.

Some NIP-variants may also be generated by mutations in the regulatory sequences of the genome and structural genes, which influence the synthesis, processing and degradation of other proteins. In this situation one mutation may result in more than one NIP-variant.

**Virulence.** Population $Ro_1$-M and $Ro_5$-H differ widely in their capability to overcome various genes for resistance present in potato. Speculation on relationships between the IP and NIP-variants in the females and genes for (a)virulence is not yet feasible. We are investigating segregation patterns of virulence genes and the IP- and

NIP-variants.

In theory, it may be possible to trace with 2-DGE proteins encoded by genes for virulence. However, there are several points of concern. First, it is not known whether or not genes for (a)virulence are translated into proteins and, if so, whether they are in sufficient amounts to be detectable. Second, not all genetic variation is visible on a 2-DGE pattern. Only about 30% of the amino acid substitutions results in a displacement in the isoelectric focusing dimension. Furthermore, minor variations in protein concentration, e.g. those that might be caused by differences in regulatory genes influencing the synthesis of structural gene products, cannot be discerned with current 2-DGE techniques. Third, genes for virulence may not be expressed in young females, but only in the larval stages. Studying the different stages of development may therefore be necessary.

Despite aforementioned points of concern, 2-DGE remains a promising approach in elucidating the molecular processes of virulence and resistance as well as other basic mechanisms involved with the induction and maintenance of syncytia. Taking the 2000 proteins produced by the thoroughly studied nematode Caenorhabditis elegans (38) as a reference, we estimate that the 700 proteins analyzed here represent 15 to 30% of the proteins encoded by the entire G. rostochiensis genome. Probably, this figure can be increased by assaying eggs, the various larval stages and males.

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## CHAPTER V

# Monitoring Colonizing Processes of Potato Cyst Nematode Pathotypes in Europe with Two Dimensional Gel Electrophoresis of Proteins


ABSTRACT

Bakker, J., and Bouwman-Smits, L. 1987. Monitoring colonizing processes of potato cyst nematode pathotypes in Europe with two dimensional gel electrophoresis of proteins. Phytopathology ...:...

The mosaic distribution patterns of the Globodera pallida pathotypes in Europe hamper an effective control by means of resistance. These spatial variations in virulence are predominantly determined by three processes: i) the genetic structure of the initial populations introduced from South America, ii) random genetic drift and iii) gene flow. The result of these processes was studied with two dimensional gel electrophoresis (2-DGE) of total protein extracts from young females. 2-DGE of 25 European populations revealed 29 variant proteins encoded by 29 alleles at 11 loci. The majority of the populations had widely different allele frequencies at various loci. Only two populations were nearly identical. The similarity dendrogram constructed from the genetic distances of the 25 populations, which had never been exposed to resistance in Europe, showed that current pathotype classification is incapable of reflecting the genetic relationships between the G. pallida populations. Populations classified as identical pathotypes were often placed in distinct groups. Since both variation in virulence and proteins are prevalently determined by aforementioned processes, which affect the variation of the entire gene pool, the similarities and dissimilarities revealed by 2-DGE will also be reflected at virulence loci, including those loci not revealed yet by current pathotype schemes. The value of 2-DGE data for a rational breeding strategy is discussed.

## INTRODUCTION

The growth of resistant potato cultivars is in potential an effective means in the control of Globodera rostochiensis (Woll.) Skarbilovich and G. pallida Stone, because selection towards alleles for virulence is rather slow (18,19,20,43). However, in Europe a wide application of resistance, which is mediated by pathotype specific genes (24), is hampered by the presence of populations having already high numbers of virulent individuals for various sources of resistance (15). Knowledge of the virulence characteristics of the populations in an area is therefore a prerequisite for an effective introduction of new resistant cultivars. However, testing the various wild Solanum species and their progeny on suitable resistance is laborious and expensive. An additional drawback is that so far no proper monogenic resistance is available against G. pallida and breeders try to accumulate an array of genes in commercial cultivars in order to obtain resistance effective against a large part of the European populations. Because the number and nature of the genes involved is unclear (13,16,37,38), the progeny of each cross has to be tested against a large number of populations. At present the number of populations tested is arbitrary and depends mainly on the screening capacity. Evidently, knowledge of the genealogical relationships between potato cyst nematode populations, and a better understanding of the nature and extent of the processes conferring interpopulation variation in virulence will improve the efficiency of breeding for resistance.

It is hypothesized that the allele frequencies for virulence in populations, which have never been exposed to corresponding genes for resistance in Europe, are predominantly the result of three processes. First, the mosaic distribution patterns of the eight pathotypes presently known in Europe (24), five within G. rostochiensis ($Ro_1$-$Ro_5$) and three within G. pallida ($Pa_1$-$Pa_3$), may in part be explained by the genetic structure of the initial populations introduced from the Andean region, their presumed origin, or from elsewhere in South America. Native populations from different localities are often discriminated at various virulence loci (6) and

the spatial variations observed in Europe are probably in part generated by the introduction of primary founders having distinct provenances. Second, the numerous secondary founding events, e.g. by few cysts adhering to seed tubers, by which Europe has been colonized have offered ample opportunity for random genetic drift to operate. Changes in allele frequencies between generations are also likely to occur when populations are nearly eradicated, e.g. due to the infrequent growth of potatoes and the use of nematicides. Third, despite their poor dispersal abilities, gene flow can probably not be neglected. Indirect evidence that gene flow is not rare comes from the observation that G. rostochiensis and G. pallida, which have probably been introduced independently (8), often occur in mixture (23).

Although no direct evidence is available, it seems justified to assume that selection caused by adaptation to local environmental conditions is of minor importance in explaining the mosaic distribution patterns of the pathotypes. An argument against a strong selection of any type is, that it is too large a genetic load on its spread and survival. Potato cyst nematodes produce only one generation in a growing season and in a normal crop rotation the time between generations ranges from two to five years. The maximum multiplication factor is on the average 25 (31). These considerations also suggest that the contribution of mutation is irrelevant. Mutation, by itself, is a slow process and time expired between the arrival of the first founders, probably after 1850 (17), is too short for newly arisen mutants to have changed the allele frequencies noticeably.

In this study we investigated whether the diversity of the potato cyst nematodes is also reflected in variation other than virulence. A standard research tool to resolve genetic variation is electrophoresis of crude cell extracts followed by general protein or specific enzyme stains. Similar to the differentiation at virulence loci we may expect that the native populations have also diverged at other loci and that the common and distinct provenances of the initial populations are also reflected in protein variation revealed by electrophoresis.

In this report we proceed from the neutral theory of molecular evolution (21). At present sufficient molecular data are available to support the hypothesis that selection is of minor interest in

explaining intraspecific variation or at least selection is too weak to be important in this context (21). This implies that in the absence of the resistant host, both variation in virulence and proteins in the European potato cyst nematode populations are prevalently determined by the genotypes of the primary founders, random genetic drift and gene flow. Since these processes influence the whole gene pool of a population, electrophoretic data are also informative for interpopulation variation at virulence loci, including those not revealed yet by current pathotype schemes.

In previous work potato cyst nematode populations have been studied with disc electrophoresis (10,14,32,42), isoelectric focusing (9,30), immunoelectrophoresis (44) and two dimensional gel electrophoresis (2-DGE) combined with a coomassie brilliant blue stain (40). However, the number of intraspecific variants resolved was too limited for a proper assessment of the genetic relationships.

Herein we applied 2-DGE (29) followed by a sensitive silver stain (28), which allows the examination of several hundreds of gene products from crude nematode homogenates (2,3,4). Samples of 25 G. pallida populations were obtained from various locations in Europe before the relevant resistant cultivars were extensively grown and classified according the international pathotype scheme. The possibilities and perspectives for characterizing potato cyst nematode populations on a large scale are discussed.

## MATERIALS AND METHODS

Samples of the G. pallida populations listed in Table 1 were obtained from: the Plant Protection Service, Wageningen, the Netherlands (population no. 1,3,4,5,6,7,8,9,10,24,25) ; Foundation for Agricultural Plant Breeding (SVP), Wageningen, the Netherlands (population no. 11,12,15,16,17,18, 19,20,21) ; Hilbrands Laboratorium, Assen, the Netherlands (population no. 13) ; Department of Nematology, Muenster, Federal Republic of Germany (population no. 22,23) ; Department of Nematology, Rothamsted Experimental Station, Harpenden, England (population no. 2,14). The original samples ( > 100 cysts) were obtained from heavily infested spots in the field. The

populations had not or at least not significantly been exposed in Europe to potato cultivars with genes for resistance present in Solanum vernei hybrid 62.33.3. Populations were maintained in the greenhouse on cultivars susceptible to all pathotypes.

The virulence characteristics of the populations were estimated by measuring the reproduction on potatoes grown in pots. Multiplication factors of population no. 1,3,4,5,6,7,8,9 and 10 were supplied by Ir. C. Miller and Ing. J. Bakker, Plant Protection Service, and of population no. 11,12,15,16,17,18,19,20 and 21 by Ing. J.H. Vinke, Foundation for Agricultural Plant Breeding. The ability of population no. 13,22,24 and 25 to overcome resistance was determined by inoculating second stage larvae on roots of sprouts grown on water agar in Petri dishes (27). The numbers of cysts or females produced on S. vernei hybrid 62.33.3 were expressed as a percentage of those developed on a general susceptible (Table 1) and used as an indication for the number of virulent genotypes.

Total protein samples of adult females (2), second stage larvae (3) and fourth stage female larvae (3) were prepared as described. Total protein extracts from adult males were made as follows. Potato cultivar Eigenheimer was inoculated with approximately 200 cysts and after 35 days the adult males were recovered from the soil. Approximately 1200 adult males were handpicked under a dissecting microscope and homogenized in a small mortar in 60 µl 10 mM tris-HCL, pH 7.4, 5% (v/v) 2-mercaptoethanol and saturated with 64 mg urea, and stored at -80 C until use.

As a standard 35 µg protein was used for electrophoresis. Isoelectric focusing using ampholines pH range 5-7, sodium dodecyl sulfate electrophoresis in 12% acrylamide and staining (3) was done by processing eight samples simultaneously. Reference series for estimating relative protein quantities were prepared by processing 16 samples at a time. Molecular weight and isoelectric point determinations were as described (2).

Protein profiles were evaluated visually by superimposing the original gels on a bench viewer. A total of 224 protein patterns was analyzed. The minimum number of replicates per object was two. Population no. 1,3,4,13,14,22,24 and 25 (Table 1) were studied for two

or more subsequent generations. No consistent differences were observed between different generations of the same population.

The similarity dendrogram was constructed following the UPGMA method (39) with the assistance and computer facilities of Ir. T. Heyerman, Department of Entomology, Wageningen, the Netherlands.

## RESULTS

The genetic differentiation of the 25 G. pallida populations (Table 1) was investigated with two dimensional gel electrophoresis (2-DGE) of total protein extracts from adult females. 2-DGE patterns representing the protein composition of 100 individuals are shown in Fig. 1.

Comparison of the 25 populations revealed 29 variant protein spots which seem to be the result of amino acid substitutions that alter the net charge (IP-variants). The corresponding IP-variants had the typical characteristics of proteins encoded by alleles at the same locus (2). The 29 IP-variants were treated as the products of 29 alleles at 11 loci. The IP-variants are assigned with a capital referring to the locus and a number referring to the allele (2). The corresponding IP-variants had a moderate difference in isoelectric point, similar molecular weight, same color and seem to be produced in about equal quantities per haploid set of chromosomes within each individual (Fig. 1, Fig. 2, Table 1). At loci B, H and I the allele products displayed minor differences in molecular weight. Such dissimilarities in apparent molecular weight have been reported before between corresponding allele products and may be the result of deletions or additions of a stretch of amino acids, but single net charge changing substitutions may be involved as well (36). Several more IP-variants, mostly minor protein spots, were detected among the approximate 350 proteins resolved on the original patterns, but only those were included which allowed a proper quantification of their allele frequencies.

The remaining variant protein spots were designated as nonisoelectric point variants (NIP-variants) (2). Examples of NIP-variants are shown in Fig. 1. Comparison of all populations revealed

a total of 44 major NIP-variants. Variants having the characteristics of IP-variants in a number of pairwise comparisons, but of which the corresponding allele products could not be traced in all populations studied were also designated as NIP-variants. Preliminary analysis of the 44 NIP-variants showed that they support the major affinities between the 25 populations based on the IP-variants. For reasons of conciseness the NIP-variants are not included in this report.

The ratios between the protein quantities of the corresponding IP-variants were used as a measure for their allele frequencies (Fig. 2), and were estimated as follows. In case only one of the corresponding IP-variants could be detected in a population after electrophoresing 35 µg total protein, the protein quantity of this IP-variant was given a relative value of 1.0. For each locus one such a monomorphic population was chosen and used to make a reference series by electrophoresing protein quantities of 0.875 µg; 1.75 µg; 3.5 µg; 5.25 µg; 7.00 µg and so on in steps of 3.5 µg up to 45.5 µg. Hence these quantities correspond with relative values of 0.025; 0.05; 0.10; 0.15 et cetera. In populations with two or more corresponding IP-variants the protein quantities were evaluated visually by comparing the spot size and intensity with those of the reference series. If no clear cut decision could be made, e.g. whether the relative protein quantity was closer to 0.1 or 0.2 the IP-variant was given an intermediate value of 0.15. The hence obtained relative values were used to calculate the ratios, which were used as a measure for the allele frequencies. The ratios were estimated by electrophoresing a minimum of two protein samples from each population. Each replicate was evaluated twice. The average ratios and their standard deviations are listed in Table 1. The detection limits of the IP-variants were estimated from the reference series.

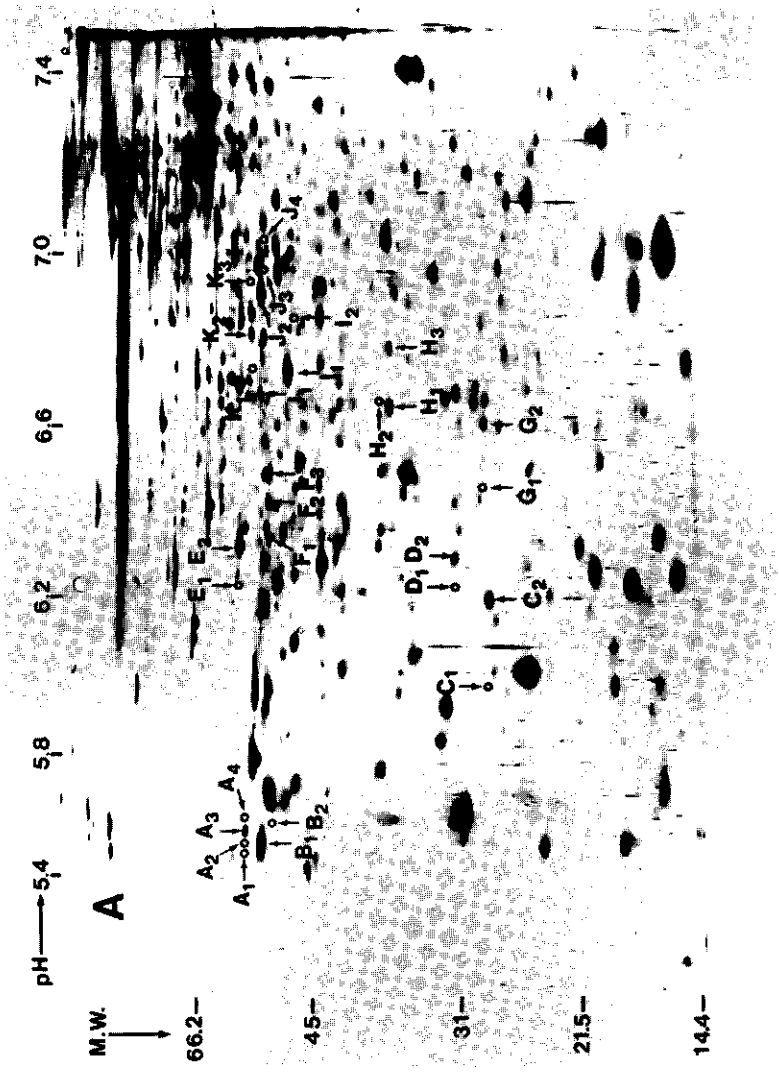
Ideally the sums of the relative values of the protein quantities of the corresponding IP-variants should equal 1.0. However, in this study the sums of the relative values usually ranged from 0.8 to 1.2 for loci B, E, F, H, I and J, and from 0.7 to 1.3 for loci A, C, D, G and K. These deviations from 1.0 were mainly caused by variations between independent experiments.

Despite these difficulties, measuring the proportion of protein

quantities of the corresponding IP-variants seems a valid approach to estimate the allele frequencies. When populations were electrophoresed and stained simultaneously in parallel experiments, deviations between the sums of the relative values for a given locus were small. Deviations between the highest and lowest values of the sums were usually less than 0.2 for loci B, E, F, H, I and J, and less than 0.3 for loci A, C, D, G, and K. Moreover, repeated electrophoresis and staining of population no. 3, 4, 13, 14, 22, 23, 24 and 25 in parallel experiments revealed no consistent differences between the sums of the relative values of any given locus, indicating that we are dealing with codominant alleles of which the products are synthesized in similar amounts.

The similarity dendrogram (Fig. 3) was constructed from a distance matrix following the UPGMA method (39). Genetic distances were obtained by interpreting the ratios between the protein quantities of the 11 IP-variant loci as allele frequencies. Distances were computed according Rogers (35). The distances between population no. 2 and the other populations were based on 10 loci, because no allele product of locus K could be traced in population no. 2 (Table 1). The distance matrix is available from the senior author upon request.

In order to define the 11 IP-variant loci as well as possible, we investigated also other developmental stages than adult females. A detailed study of population no. 13 showed, that the majority of the IP-variant encoding alleles were also expressed in adult males, fourth stage female larvae and second stage larvae (Table 1). Inspection of Table 1 demonstrates, that at locus F only one allele ($F_3$) could be traced in adult males and second stage larvae, whereas three alleles ($F_1$,$F_2$ and $F_3$) were present in adult females and fourth stage female larvae. The alleles $F_1$ and $F_2$, which both have a frequency of 0.2 in adult females, are probably also expressed in adult males and second stage larvae, but the protein quantities produced by the genes at locus F in those stages are too low to detect such low allele frequencies. In case all corresponding IP-variants were detected, the ratios between the protein quantities in those stages were similar to those in adult females (Table 1).


pH
5,4
5,8
6,2
6,6
7,0
7,4
A
M.W.
66.2
45
31
21.5
14.4
A1
A2
A3
A4
B1
B2
C1
C2
D1
D2
E1
E2
F
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J3
J4
K1
K2
K3

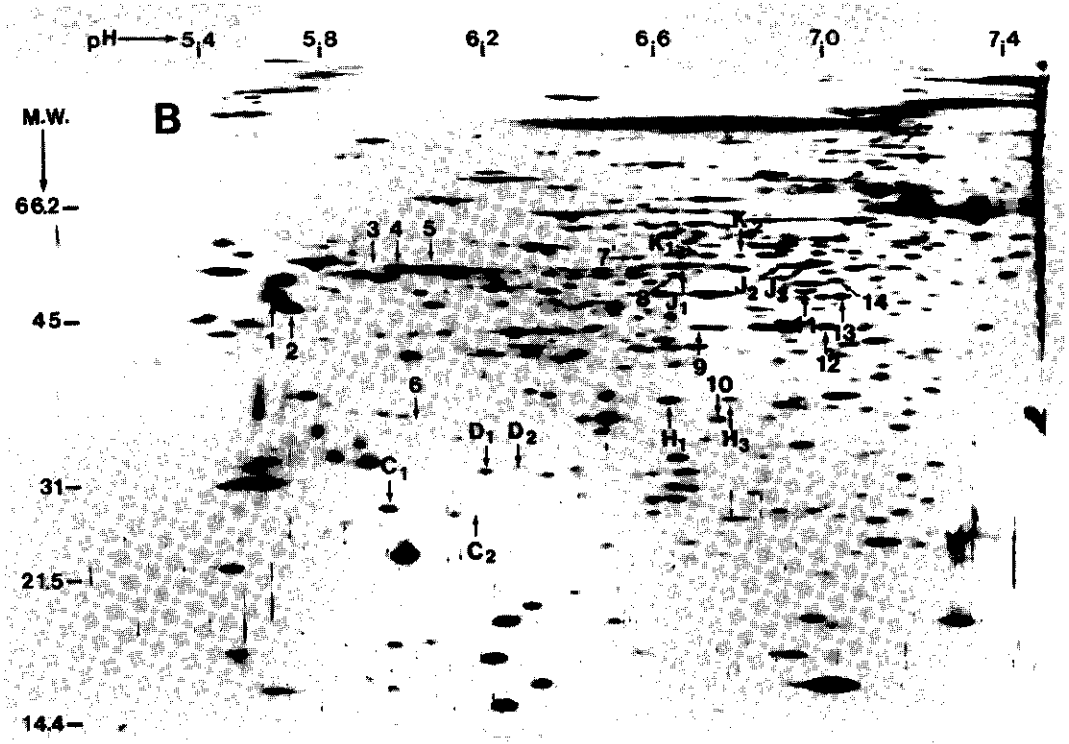


**Fig. 1** Protein patterns of a mixture of 100 adult females from G. pallida population no. 5 (**A**) and population no. 6 (**B**). The positions of the isoelectric point variants (IP-variants) detected by the comparison of 25 populations (Table 1) are marked in **A**. The capitals and numbers refer to the putative loci and alleles, respectively. Qualitative and quantitative differences between the two populations at the IP-variant loci are denoted in **B**. Dissimilarities between the two populations indicated with arabic numbers in **B** are nonisoelectric point variants (NIP-variants). Other differences visible on the photographs, e.g. variants with an isoelectric point above pH 7.05, were not consistent. Molecular masses are given in kilodaltons. Further details see text and Table 1.

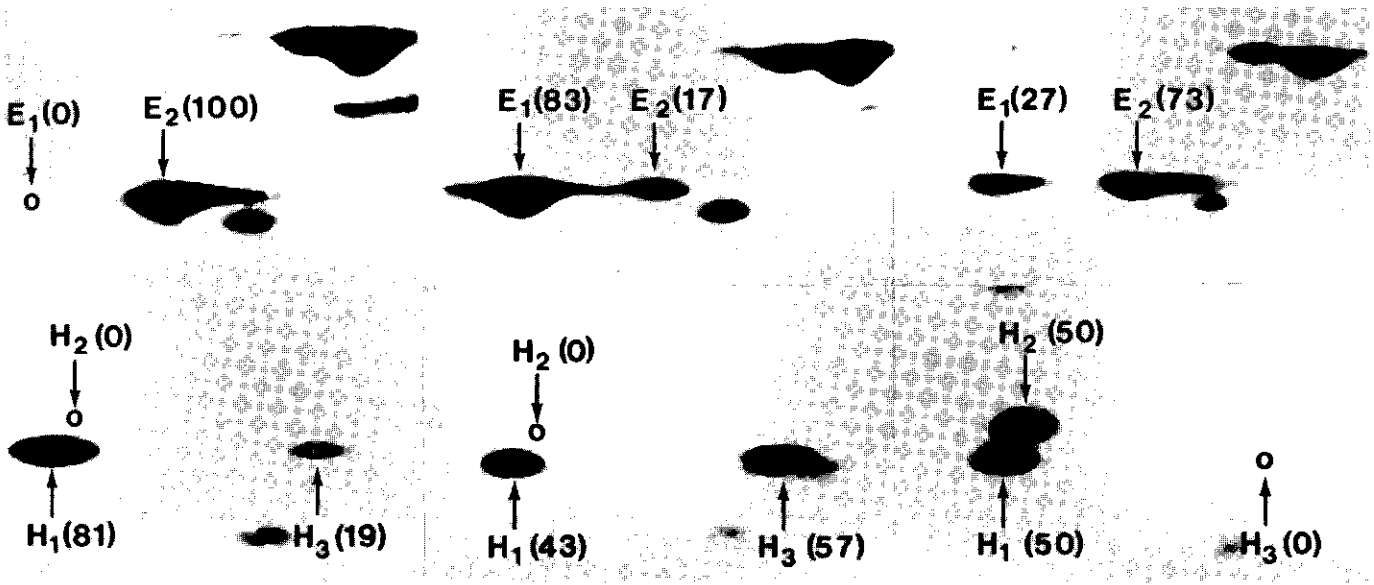


**Fig. 2** Details of protein patterns made by electrophoresing a homogenate of 100 adult females. The IP-variants are designated with capitals and numbers referring to the putative loci and alleles, respectively. In parenthesis the estimates of the ratios between the protein quantities of the corresponding IP-variants, which were used as a measure of the allele frequencies. Ratios were estimated as described in the text. **Top row.** Locus E: population no. 6 (left), no.4 (middle) and no. 15 (right). **Bottom row.** Locus H: population no. 6 (left), no. 15 (middle) and no. 13 (right).

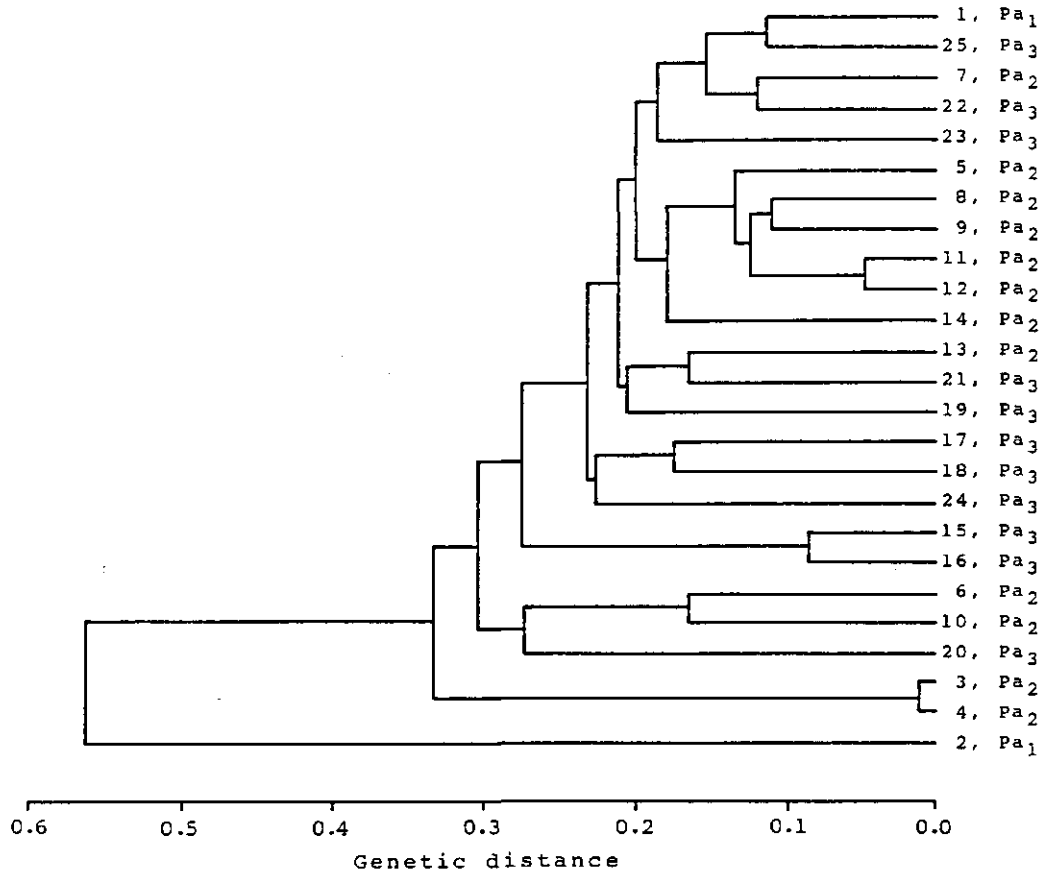


**Fig. 3** Similarity dendrogram of 25 G. pallida populations constructed from the genetic distances at 11 IP-variant loci following the UPGMA method. Populations are numbered as in Table 1.

**Table 1.** Ratios between protein quantities produced by IP-variant encoding alleles at 11 presumptive loci in adult females of 25 *G. pallida* populations[a].

| Pathotype, collection location and code[b] | Number of females on *S. vernei* 62.33.3[c] | $A_1$ | $A_2$ | $A_3$ | $A_4$ |
|---|---|---|---|---|---|
| 1. $Pa_1$, Glarryford, Northern Ireland, 1337 | 10.4(2.5) | 0 | 23(10) | 77(10) | 0 |
| 2. $Pa_1$, Portglenone, Northern Ireland, PORT | - | 100 | 0 | 0 | 0 |
| 3. $Pa_2$, Smilde, the Netherlands, D-234 | 2.8(0.5) | 0 | 0 | 80(5) | 20(5) |
| 4. $Pa_2$, Anlo, the Netherlands, D-236 | 2.5(0.4) | 0 | 0 | 80(3) | 20(3) |
| 5. $Pa_2$, Emmen, the Netherlands, D-264 | 0.8(0.7) | 0 | 0 | 100 | 0 |
| 6. $Pa_2$, Vriezeveen, the Netherlands, D-275 | 4.3(1.6) | 0 | 0 | 100 | 0 |
| 7. $Pa_2$, Averest, the Netherlands, D-276 | 1.4(0.5) | 0 | 25(5) | 75(5) | 0 |
| 8. $Pa_2$, Hardenberg, the Netherlands, D-286 | 0.9(0.3) | 0 | 20(4) | 58(10) | 21(5) |
| 9. $Pa_2$, Oosterhesselen, the Netherlands, D-287 | 0.8(0.4) | 0 | 25(6) | 64(6) | 12(6) |
| 10. $Pa_2$, Ommen, the Netherlands, D-301 | 0.6(0.4) | 0 | 0 | 100 | 0 |
| 11. $Pa_2$, ? , the Netherlands, 1095 | 1.6(0.5) | 0 | 0 | 100 | 0 |
| 12. $Pa_2$, Coevorden, the Netherlands, P-2-22 | 0.7(0.2) | 0 | 0 | 100 | 0 |
| 13. $Pa_2$, Veendam, the Netherlands, HPL-1 | 1.5(0.5) | 0 | 50(5) | 50(5) | 0 |
| 14. $Pa_2$, New Leake, Great Brittain, ST | 3.3(2.1) | 0 | 18(4) | 68(6) | 15(7) |
| 15. $Pa_3$, Valthe, the Netherlands, ROOK | 36.7(23.8) | 0 | 16(6) | 84(6) | 0 |
| 16. $Pa_3$, Gasselte, the Netherlands, A-75-250-39 | 31.5(21.6) | 0 | 0 | 100 | 0 |
| 17. $Pa_3$, Anjum, the Netherlands, 1077 | 6.5(1.9) | 0 | 82(5) | 18(5) | 0 |
| 18. $Pa_3$, Westerbork, the Netherlands, 1112 | 11.8(5.6) | 0 | 21(6) | 79(6) | 0 |
| 19. $Pa_3$, Sleen, the Netherlands, 74-768-20 | 49.4(31.8) | 0 | 13(6) | 71(2) | 16(4) |
| 20. $Pa_3$, Vriezeveen, the Netherlands, 75-884-4 | 10.4(4.3) | 0 | 0 | 100 | 0 |
| 21. $Pa_3$, Hardenberg, the Netherlands, 1097 | 16.3(6.3) | 0 | 16(2) | 70(1) | 13(1) |
| 22. $Pa_3$, Frenswegen, Federal Republic of Germany, FR | 5.7(0.6) | 0 | 0 | 88(7) | 12(7) |
| 23. $Pa_3$, ? , Austria, RU | 47.9(10.8) | 0 | 0 | 93(10) | 7(10) |
| 24. $Pa_3$, Far Oer, Denmark, E-1215 | 9.3(0.7) | 0 | 64(5) | 36(5) | 0 |
| 25. $Pa_3$, Cadishead, Great Brittain, E-1202 | 6.7(0.7) | 0 | 12(2) | 73(4) | 15(2) |
| isoelectric point | | 5.48 | 5.52 | 5.56 | 5.60 |
| molecular mass[e] | | 59 | | | |
| color[f] | | r.b. | | | |
| detection limit[g] | | 10-15 | | | |
| 13 adult males[g] | | 0 | 33(6) | 35(7) | 0 |
| 13 fourth stage female larvae[g] | | 0 | 52(8) | 58(7) | 0 |
| 13 second stage larvae[g] | | 0 | 81(8) | 88(10) | 0 |

a) The ratios and the standard deviations (in parenthesis) were obtained as described in the text. The loci and alleles of the IP-variants are indicated with capitals and numbers, respectively.

b) As designated in the original collections.

c) Number of females expressed as a percentage of those developed on a generally susceptible cultivar.

| $B_1$ | $B_2$ | $C_1$ | $C_2$ | $D_1$ | $D_2$ | $E_1$ | $E_2$ | $F_1$ | $F_2$ | $F_3$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 100 | 0 | 24(4) | 76(4) | 25(5) | 75(5) | 0 | 100 | 21(4) | 13(4) | 66(5) |
| 60(4) | 40(4) | 100 | 0 | 100 | 0 | 0 | 100 | 14(1) | 84(2) | 2(2) |
| 100 | 0 | 0 | 100 | 0 | 100 | 83(5) | 17(5) | 15(2) | 12(2) | 73(4) |
| 100 | 0 | 0 | 100 | 0 | 100 | 82(7) | 18(7) | 20(5) | 8(2) | 72(2) |
| 100 | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 33(6) | 67(6) |
| 100 | 0 | 100 | 0 | 100 | 0 | 0 | 100 | 0 | 35(5) | 65(5) |
| 100 | 0 | 75(6) | 25(6) | 35(4) | 65(4) | 0 | 100 | 0 | 36(5) | 64(5) |
| 100 | 0 | 0 | 100 | 21(8) | 79(8) | 0 | 100 | 0 | 63(4) | 37(4) |
| 100 | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 92(5) | 8(5) |
| 100 | 0 | 100 | 0 | 92(9) | 8(9) | 0 | 100 | 0 | 13(5) | 87(5) |
| 100 | 0 | 0 | 100 | 20(5) | 80(5) | 0 | 100 | 28(4) | 67(11) | 5(7) |
| 100 | 0 | 10(12) | 90(12) | 8(9) | 92(9) | 0 | 100 | 21(5) | 71(5) | 7(3) |
| 100 | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 20(4) | 20(3) | 60(6) |
| 100 | 0 | 10(12) | 90(12) | 18(8) | 82(8) | 8(5) | 92(5) | 87(4) | 2(3) | 10(3) |
| 100 | 0 | 35(4) | 65(4) | 42(15) | 58(15) | 23(7) | 77(7) | 3(4) | 29(5) | 68(2) |
| 100 | 0 | 49(9) | 51(9) | 28(5) | 72(5) | 15(6) | 85(6) | 0 | 28(6) | 72(6) |
| 100 | 0 | 35(4) | 65(4) | 0 | 100 | 0 | 100 | 0 | 26(3) | 74(3) |
| 100 | 0 | 100 | 0 | 0 | 100 | 0 | 100 | 0 | 43(4) | 57(4) |
| 100 | 0 | 25(5) | 75(5) | 62(11) | 38(11) | 0 | 100 | 49(3) | 4(4) | 47(6) |
| 100 | 0 | 90(12) | 10(12) | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 100 | 0 | 26(5) | 74(5) | 0 | 100 | 0 | 100 | 14(5) | 53(5) | 33(4) |
| 100 | 0 | 45(4) | 55(5) | 33(6) | 67(6) | 0 | 100 | 0 | 46(4) | 54(4) |
| 100 | 0 | 10(12) | 90(12) | 35(5) | 65(5) | 17(5) | 83(5) | 0 | 9(5) | 91(5) |
| 100 | 0 | 30(9) | 70(9) | 76(5) | 24(5) | 0 | 100 | 0 | 45(4) | 55(4) |
| 100 | 0 | 42(6) | 58(6) | 35(4) | 65(4) | 0 | 100 | 33(6) | 20(6) | 47(6) |
| 5.50 | 5.58 | 5.96 | 6.20 | 6.23 | 6.28 | 6.23 | 6.29 | 6.32 | 6.42 | 6.48 |
| 56 | 54 | 28 | | 31.5 | | 60 | | 54 | | |
| r.b. | | r. | | g. | | r.b. | | r.b. | | |
| < 2.5 | | 15–20 | | 15–20 | | < 2.5 | | 2.5–5 | | |
| 43(10) | 0 | 0 | 33(7) | 0 | 0 | 0 | 34(9) | 0 | 0 | 15(6) |
| 52(10) | 0 | 0 | 74(11) | 0 | 38(9) | 0 | 35(11) | 7(4) | 10(4) | 36(8) |
| 79(11) | 0 | 0 | 78(9) | 0 | 0 | 0 | 68(10) | 0 | 0 | 9(4) |

e) Molecular masses in kilodaltons.

f) Reddish brown (r.b.), red (r.), grey (g.), blackish brown (b.b.).

g) Relative protein quantities ($x10^2$) estimated from the reference series (see text).

**Table 1.** continued

| Pathotype, collection location and code[b] | $G_1$ | $G_2$ | $H_1$ | $H_2$ | $H_3$ |
|---|---|---|---|---|---|
| 1. $Pa_1$, Glarryford, Northern Ireland, 1337 | 0 | 100 | 26(1) | 14(7) | 60(7) |
| 2. $Pa_1$, Portglenone, Northern Ireland, PORT | 100 | 0 | 0 | 0 | 100 |
| 3. $Pa_2$, Smilde, the Netherlands, D-234 | 0 | 100 | 51(2) | 0 | 49(2) |
| 4. $Pa_2$, Anlo, the Netherlands, D-236 | 0 | 100 | 52(6) | 0 | 48(6) |
| 5. $Pa_2$, Emmen, the Netherlands, D-264 | 0 | 100 | 63(5) | 0 | 37(5) |
| 6. $Pa_2$, Vriezeveen, the Netherlands, D-275 | 0 | 100 | 80(8) | 0 | 20(8) |
| 7. $Pa_2$, Averest, the Netherlands, D-276 | 0 | 100 | 31(3) | 31(3) | 38(6) |
| 8. $Pa_2$, Hardenberg, the Netherlands, D-286 | 0 | 100 | 51(2) | 0 | 49(2) |
| 9. $Pa_2$, Oosterhesselen, the Netherlands, D-287 | 0 | 100 | 60(6) | 0 | 40(6) |
| 10. $Pa_2$, Ommen, the Netherlands, D-301 | 0 | 100 | 44(8) | 0 | 56(8) |
| 11. $Pa_2$, ? , the Netherlands, 1095 | 0 | 100 | 40(5) | 0 | 60(5) |
| 12. $Pa_2$, Coevorden, the Netherlands, P-2-22 | 0 | 100 | 33(4) | 0 | 67(4) |
| 13. $Pa_2$, Veendam, the Netherlands, HPL-1 | 0 | 100 | 50(3) | 50(3) | 0 |
| 14. $Pa_2$, New Leake, Great Brittain, ST | 0 | 100 | 40(5) | 0 | 60(5) |
| 15. $Pa_3$, Valthe, the Netherlands, ROOK | 0 | 100 | 42(2) | 0 | 58(2) |
| 16. $Pa_3$, Gasselte, the Netherlands, A-75-250-39 | 0 | 100 | 49(8) | 0 | 51(8) |
| 17. $Pa_3$, Anjum, the Netherlands, 1077 | 0 | 100 | 48(4) | 52(4) | 0 |
| 18. $Pa_3$, Westerbork, the Netherlands, 1112 | 0 | 100 | 52(2) | 48(2) | 0 |
| 19. $Pa_3$, Sleen, the Netherlands, 74-768-20 | 0 | 100 | 61(12) | 0 | 39(12) |
| 20. $Pa_3$, Vriezeveen, the Netherlands, 75-884-4 | 0 | 100 | 35(7) | 0 | 65(7) |
| 21. $Pa_3$, Hardenberg, the Netherlands, 1097 | 0 | 100 | 59(2) | 0 | 41(2) |
| 22. $Pa_3$, Frenswegen, Federal Republic of Germany, FR | 0 | 100 | 59(11) | 22(5) | 19(6) |
| 23. $Pa_3$, ? , Austria, RU | 0 | 100 | 19(5) | 63(8) | 18(4) |
| 24. $Pa_3$, Far Oer, Denmark, E-1215 | 0 | 100 | 57(1) | 15(2) | 28(3) |
| 25. $Pa_3$, Cadishead, Great Brittain, E-1202 | 0 | 100 | 27(2) | 27(2) | 46(6) |
| isoelectric point | 6.44 | 6.60 | 6.62 | 6.63 | 6.78 |
| molecular mass[e] | 28.5 | | 36 | 37 | 36 |
| color[f] | r. | | b.b. | | |
| detection limit[g] | 10-15 | | 2.5-5 | | |
| 13 adult males[g] | 0 | 66(14) | 0 | 0 | 0 |
| 13 fourth stage female larvae[g] | 0 | 91(13) | 31(8) | 28(8) | 0 |
| 13 second stage larvae[g] | 0 | 68(9) | 0 | 0 | 0 |

b) As designated in the original collections.

d) No allele product of locus K could be traced in population no. 2.

e) Molecular masses in kilodaltons.

| $I_1$ | $I_2$ | $J_1$ | $J_2$ | $J_3$ | $J_4$ | $K_1$ | $K_2$ | $K_3$ |
|---|---|---|---|---|---|---|---|---|
| 100 | 0 | 35(1) | 48(3) | 16(4) | 0 | 53(5) | 47(5) | 0 |
| 100 | 0 | 0 | 100 | 0 | 0 | -d) | -d) | -d) |
| 100 | 0 | 0 | 74(4) | 0 | 26(4) | 0 | 8(9) | 92(9) |
| 100 | 0 | 0 | 77(5) | 0 | 23(5) | 0 | 8(9) | 92(9) |
| 100 | 0 | 5(3) | 95(3) | 0 | 0 | 0 | 100 | 0 |
| 100 | 0 | 56(1) | 6(2) | 38(3) | 0 | 80(5) | 20(5) | 0 |
| 100 | 0 | 71(11) | 19(8) | 10(3) | 0 | 0 | 100 | 0 |
| 100 | 0 | 25(3) | 50(6) | 25(3) | 0 | 0 | 100 | 0 |
| 100 | 0 | 64(7) | 0 | 0 | 36(7) | 0 | 100 | 0 |
| 100 | 0 | 71(10) | 29(10) | 0 | 0 | 0 | 100 | 0 |
| 100 | 0 | 11(1) | 43(2) | 0 | 46(3) | 0 | 100 | 0 |
| 100 | 0 | 11(4) | 61(6) | 0 | 28(2) | 0 | 100 | 0 |
| 100 | 0 | 78(2) | 22(2) | 0 | 0 | 48(10) | 52(10) | 0 |
| 100 | 0 | 44(5) | 26(4) | 30(1) | 0 | 20(5) | 80(5) | 0 |
| 30(4) | 70(4) | 0 | 59(2) | 41(2) | 0 | 0 | 65(5) | 35(5) |
| 18(6) | 82(6) | 0 | 72(8) | 28(8) | 0 | 0 | 76(4) | 24(4) |
| 100 | 0 | 66(6) | 34(6) | 0 | 0 | 0 | 100 | 0 |
| 100 | 0 | 23(2) | 77(2) | 0 | 0 | 0 | 100 | 0 |
| 100 | 0 | 95(3) | 0 | 5(3) | 0 | 0 | 65(4) | 35(4) |
| 100 | 0 | 45(4) | 0 | 55(4) | 0 | 74(9) | 0 | 26(9) |
| 100 | 0 | 58(7) | 8(1) | 33(7) | 0 | 65(5) | 35(5) | 0 |
| 100 | 0 | 68(4) | 0 | 32(4) | 0 | 22(9) | 88(9) | 0 |
| 100 | 0 | 26(1) | 68(2) | 0 | 6(1) | 0 | 100 | 0 |
| 100 | 0 | 0 | 37(6) | 63(6) | 0 | 0 | 100 | 0 |
| 100 | 0 | 16(4) | 58(12) | 25(12) | 0 | 17(3) | 83(3) | 0 |
| 6.71 | 6.84 | 6.68 | 6.90 | 6.97 | 7.04 | 6.72 | 6.80 | 6.94 |
| 50 | 49 | 56 | | | | 58 | | |
| r.b. | | r.b. | | | | r.b. | | |
| 2.5 | | < 2.5 | | | | 10 | | |
| 10(3) | 0 | 25(7) | 8(3) | 0 | 0 | 23(5) | 20(7) | 0 |
| 23(12) | 0 | 42(4) | 13(3) | 0 | 0 | 36(8) | 23(6) | 0 |
| 104(19) | 0 | 47(9) | 14(5) | 0 | 0 | 26(5) | 20(7) | 0 |

f) Reddish brown (r.b.), red (r.), grey (g.), blackish brown (b.b.).

g) Relative protein quantities ($x10^2$) estimated from the reference series (see text).

## DISCUSSION

The 2-DGE procedure outlined here has several desirable features in characterizing nematode populations when compared with other electrophoretic techniques. First, compared with enzyme electrophoresis, a standard research tool for many organisms, the number of loci assayed with 2-DGE is an order of a magnitude higher resulting in an increased number of variants. For instance, isoelectric focusing of 23 enzymes of the G. pallida pathotypes $Pa_1$, $Pa_2$ and $Pa_3$ revealed only two loci manifesting interpopulation variation (9). Second, the methodology applied in this report for estimating allele frequencies at polymorphic loci is efficient. 2-DGE patterns were made by electrophoresing a homogenate of 100 individuals and the ratio between the protein quantities produced by the alleles at a given locus was used as a measure for the allele frequencies. Inherent to the staining techniques, such an approach is techniqually far more difficult in case of enzyme electrophoresis. Allele frequency data of enzymes, but also in the rare cases that 2-DGE has been applied for allele frequency estimates (5,34), are in general collected by electrophoresing individuals separately. Analysing single plant parasitic nematodes is only possible by micromethods (7,33), and even than only feasible for a limited number of nematode species having adults with a large size e.g. Meloidogyne spp., Heterodera spp. and Globodera spp. Besides that, microelectrophoresis often results in loss of resolution. It is evident that electrophoresing several tens of individuals in order to estimate the allele frequencies at a single enzyme locus is too laborious for characterizing a large number of potato cyst nematode populations.

Although the methodology described here accommodates the scope of this study, it has several drawbacks when compared with electrophoresing single individuals. First, although variations due to sample sizes (100 individuals) are minimal, there is considerable variation generated by the experimental procedure. For further research it may worthwile to improve the system by a higher standardization of the silver staining procedure and by switching to a

computerized quantification of the proteins as developed in biomedical research (25). Second, alleles producing limited amounts of protein are not detected when present in low frequencies (e.g. loci A, D)(2). Third, comparison of the 2-DGE protein patterns reveals only those variants exhibiting interpopulation variation. Therefore, intrapopulation variation is not revealed in case all populations compared have about equal allele frequencies. Fourth, electrophoresing single individuals provides more insight in the genetics of the IP-variants, which diminishes the chance on incorrect genetic interpretations.

Evidently there are various ways to construct similarity dendrograms from 2-DGE patterns and for future research it will be worthwile to compare different types of data acquisition and data manipulation in estimating affinities between populations, e.g. by including NIP-variants as well. Decisive arguments for the approach outlined here are that genetic distance formulas involving allele frequencies can be applied and that estimating the ratios between the protein quantities of putative corresponding allele products diminishes the experimental variation. Replicate experiments showed that the corresponding allele products on a single pattern were always nearly proportionally affected by variations in the experimental procedure. Obviously, our approach harbors certain risks with regard to the correctness of the genetic background of all variant protein spots. However, possible misinterpretations of the genetics of a few variants will probably not significantly affect the estimates of the actual similarities between the populations.

**Pathotypes** The majority of the IP-variants recorded here, and may be all, are probably not related to virulence. Intraspecific variation in abundant proteins revealed by 2-DGE is a common feature and has been reported for various organisms such as fruit flies (5), rodents (1,22,34) and man (26,36). Plant parasitic fungi have also been studied with 2-DGE (12,41), and it was demonstrated that the majority of the variants was not associated with alleles for virulence and avirulence. In this research also none of the IP-variants could be correlated with the ability to overcome resistance in Solanum vernei hybrid 62.33.3. However, it is noted that a proper interpretation is

hampered, because, among others, the number and nature of the genes involved with resistance and virulence are unknown.

Until now intraspecific variation of G. rostochiensis and G. pallida has most extensively been studied by measuring the ability to overcome genes for resistance. These surveys have resulted in an international scheme for the identification and classification of potato cyst nematode populations (24). However, current pathotype classification is incapable of reflecting the genetic diversity among European potato cyst nematode populations. First, the number of differentials used is too limited. For example, the three G. pallida pathotypes are discriminated by only two differentials and additional test clones have already differentiated populations classified as the same pathotypes (11). Second, populations are designated as virulent or avirulent for a certain differential if the multiplication factor is $> 1$ or $\leq 1$, respectively (24). However, in compatible combinations the multiplication factors may range from 1 to 70 (24), indicating that the numbers of virulent genotypes may vary considerably among populations classified as identical pathotypes. In addition it is noted that an unambiguous classification is hampered by a variable expression of the nematode and host genotypes.

In view of this classification it is not surprising that populations of the same pathotype are often discriminated at various IP-variant loci and that genetic distances within pathotypes are not necessarily smaller than between pathotypes.

**Primary founders, random genetic drift and gene flow.** The protein variation between the 25 G. pallida populations is probably in part generated by the various unknown provenances of the primary founders, resulting in the founding of initial populations having distinct genetic structures. Evidently, it is not possible to estimate the number of different introductions involved with the 25 G. pallida populations, because the share of random genetic drift and gene flow cannot be recovered. Many descendants of the initial populations have probably diverged by random genetic drift due to reductions in population sizes caused by secondary founding events and control measures. In such cases there is also a fair chance that alleles are lost or fixed. Nevertheless, it seems that population sizes in the

past were sufficiently large to maintain genetic polymorphisms at several loci. Scrutiny of the 11 IP-variant loci listed in Table 1 shows, that the number of polymorphic loci ranges from two to nine with an average of 5.5 polymorphic loci per population. Of course, polymorphic loci can also be the result of immigration. Inherent to their passive ways of spread, gene flow will depend on factors such as, soil transported by seed tubers, machineries and wind. However, it is evident that the extent of these events is limited, because a uniform distribution of the IP-variants over a large area was not observed. The Dutch populations were collected in a relatively small area and were often discriminated from one another by various qualitative differences (Table 1 and Fig. 3).

Although there have undoubtedly been various cases in which the effect of random genetic drift and gene flow has been significant, our data provide evidence that there are on the other hand also situations in which the genetic structure can be preserved during many years. The nearly identical Dutch populations no. 3 and no. 4 have hardly diverged by gene flow and random genetic drift during the time they have been geographically separated. In theory these populations can be the descendants of parallel or sequential introductions from the same population in South America and have retained the genetic structure of the native ancestor population ever since. More likely is that those populations have separated somewhere in the Netherlands. Chance is small that those populations are not closely related by descent. Inherent to the way of spread, the number of successful primary founding events has been limited and numerous populations have common ancestors in Europe. Several others among the 25 populations may also have diverged somewhere in Europe; population no. 11 and no. 12; population no. 15 and no. 16; population no. 1 and no. 25. These populations are not identical, but share the majority of their alleles.

**Breeding for resistance** The hypothesis that in Europe the genetic differentiation at IP-variant and virulence loci is mainly caused by non-selective forces, implies that 2-DGE data are valuable indicators for interpopulation variation in virulence. In case populations have large genetic distances at IP-variant loci, there is a fair chance

that they behave differently towards various genes for resistance, e.g. because their native ancestors have differentially been exposed to genes for resistance. For instance, population no. 4 and no. 5 (Fig. 3), both classified as $Pa_2$ in the current pathotype scheme, may be discriminated in distinct pathotypes by supplementary genes for resistance. Populations that are identical or nearly so, e.g. population no. 3 and no. 4 (Fig. 3), will show resemblance at all virulence loci. Populations having relatively small genetic distances, e.g. population no. 11 and 12 (Fig. 3), have probably also small genetic distances at their virulence loci. Despite the arbitrary way populations are classified in the current pathotype scheme, the feasibility of the approach advocated here is supported by the virulence data. All populations linked at a distance less than 0.1 in the similarity dendrogram were classified as identical pathotypes and at a distance less than 0.15 nine out of the 13 populations were classified as identical.

2-DGE can be a valuable adjunctive tool for breeders, because data on the diversity present in an area are indispensible for a successful control by means of resistance. Once an area is investigated by 2-DGE of a substantial number of populations, the affinities can be used as a guidance for a representative survey in order to estimate the proportion of virulent and avirulent populations for any source of resistance to be tested. Representatives of each group should be included. How to delineate groups in such a way that they represent useful entities, i.e. a manageable number of groups with an acceptable interpopulation variation in virulence, needs further investigation.

Finally, although we proceeded from stochastic processes in explaining the spatial variation in allele frequencies, we do not exclude other mechanisms. However, in case additional genetic mechanisms are operating, e.g. balanced polymorphisms or selection due to environmental conditions, it is very unlikely that such processes are strong enough and act on sufficient loci to impair the value of 2-DGE data for a rational breeding strategy.

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## CHAPTER VI

# Spatial Variations in Proteins coinciding with the Colonization of Europe by Potato Cyst Nematode Pathotypes


ABSTRACT

Two dimensional gel electrophoresis (2-DGE) of total protein extracts from 18 Globodera rostochiensis populations demonstrated that the genetic diversity introduced into the United Kingdom has been relatively low. The maximum number of qualitative protein differences observed among populations from the United Kingdom was only 2, whereas this figure was 16 and 17 for the Netherlands and the Federal Republic of Germany, respectively. These data corroborate the observation, that $Ro_1$ is the sole G. rostochiensis pathotype found in the United Kingdom, while several Dutch ($Ro_1$, $Ro_2$, $Ro_3$ and $Ro_4$) and German pathotypes ($Ro_1$, $Ro_2$, $Ro_5$) are known. Furthermore, populations of the same pathotype were more similar to each other than to populations classified as distinct pathotypes, as was established by constructing a similarity dendrogram from a distance matrix. The coincidence between 2-DGE and virulence data can be explained by the fact that the populations studied here had not been exposed to resistance in Europe. In this situation both interpopulation variations in virulence and proteins are predominantly determined by the histories of the populations with regard to origin in South America and the occurrence of random genetic drift and gene flow in Europe. Because these three processes affect the entire gene pool of a population, similarities revealed by 2-DGE are also reflected at virulence loci.

## INTRODUCTION

The intimate association between potato cyst nematodes and their solanaceous hosts in the Andean region, their centre of origin, has resulted in an array of genes for virulence and resistance, which may in various cases operate on the basis of a gene for gene relationship (11,21). Comparison of the virulence spectra of potato cyst nematode populations from Europe and the Andean region indicated that only a limited part of the native diversity has been introduced into Europe (6). Nevertheless, the intraspecific variation of Globodera rostochiensis (Woll.) Skarbilovich and G. pallida Stone in Europe is considerable and impairs control by means of resistance. The current international pathotype scheme recognizes eight pathotypes, five within G. rostochiensis ($Ro_1$-$Ro_5$) and three within G. pallida($Pa_1$-$Pa_3$) (12).

Genetic diversity of the European populations is also manifested in variation other than virulence. Several intraspecific protein differences have been reported by various investigators (3,4,8,9,16,17,20,22). So far the most promising approach (3) dealt with two dimensional gel electrophoresis (2-DGE)(15) followed by a sensitive silver stain (14). A comparative study of 25 populations showed that the international pathotype scheme is incapable of reflecting genetic variability of G. pallida in Europe (3). The majority of the populations were discriminated from each other at various loci. Moreover, genetic distances within pathotypes were often larger than between pathotypes.

It has been argued (3) that, in the absence of the relevant genes for resistance in Europe, both interpopulation variation in virulence and proteins are predominantly determined by the same three processes. First, spatial variations in virulence and proteins can in part be explained by the genetic structures of the primary founders introduced from South America, probably after 1850 (10). Europe has probably been infested from various native localities, which has resulted in the introduction of distinct primary founders in several areas. Random genetic drift is another important process and is inherent to the numerous secondary founding events by which Europe

has been colonized. Fluctuations in allele frequencies between generations may also occur when populations are nearly eradicated by control measures. The third process is gene flow. Inherent to the passive way of spread, the extent of gene flow will depend on factors such as soil transported by wind, machineries and seed tubers.

2-DGE is a suitable tool to monitor the extent of these three processes. Because these stochastic processes affect the whole gene pool of a population, genetic relationships revealed by 2-DGE are also indicative of interpopulation variation in virulence. This report presents a study of the genetic diversity of G. rostochiensis in a number of European countries and evidence that 2-DGE offers possibilities to improve control by means of resistance.

## MATERIALS AND METHODS

Samples of populations from the United Kingdom (Table 1) were supplied Dr. A.R. Stone, Rothamsted Experimental Station, Harpenden, England. Two German populations (Table 1) were obtained from Dr. H.J. Rumpenhorst, Department of Nematology, Muenster, Federal Republic of Germany. All other populations and their reproduction values on the differentials were supplied by Ir. C. Miller and Ing. J. Bakker, Plant Protection Service, Wageningen, the Netherlands. The original samples ( >100 cysts) of the populations, which had not or not significantly been influenced by the growth of resistant cultivars, were collected from heavily infested spots in the field.

Virulence characteristics were determined by inoculating potato plants grown in pots with cysts (5). For populations no. 1, 2, 5, 17 and 19 the ability to overcome resistance in S. tuberosum ssp. andigena CPC 1673 and S. vernei hybr. 62.33.3 was estimated by inoculating second stage larvae on roots of sprouts grown in Petri dishes with water agar (5,13). The number of females produced on the differentials of the international pathotype scheme (12) or comparable resistant cultivars were expressed as a percentage of those developed on a generally susceptible host (Table 1) and used as a measure for the number of individuals able to overcome resistance (5).

Total protein extracts of adult females (1), fourth stage female

larvae (2), males (3) and second stage larvae (2) were prepared as described. As a standard, 35 µg total protein was used for electrophoresis. The minimum number of replicates per object was three. Isoelectric focusing using ampholines pH range 5 to 7, sodium dodecyl sulfate electrophoresis in 12% acrylamide and staining (2) were carried out by processing eight samples simultaneously. Reference series for estimating relative protein quantities (3) were prepared by processing 12 samples at a time. Protein patterns were judged visually by superimposing the original gels on a bench viewer. For making comparisons with a previous report (1) we refer to the molecular weights and isoelectric points of the variants. Molecular weight and isoelectric point determinations were as described (1).

Genetic distances based on isoelectric point variants (IP-variants) (1,3) were computed according Rogers (18). Similarity dendrograms were constructed from distance data following the UPGMA method (19) and kindly prepared by Ir. T. Heyerman, Department of Entomology, Wageningen, the Netherlands.

## RESULTS

Nineteen G. rostochiensis populations (Table 1) were investigated with two-dimensional gel electrophoresis (2-DGE) followed by a sensitive silver stain. Comparison of the protein profiles revealed 27 major protein spots exhibiting interpopulation variation ( Fig. 1, Table 1). Variant proteins displaying minor quantities or showing poor reproducibility with regard to quantity were excluded. The variants were divided in two classes: isoelectric point variants (IP-variants) and nonisoelectric point variants (NIP-variants) (1,3).

Corresponding IP-variants had the typical characteristics of corresponding allele products differing in one or a few amino acids (1,3). They had a moderate difference in isoelectric point and similar properties considering molecular weight, color and quantity (Fig. 1 and 2, Table 1). The 20 IP-variants were assumed to be encoded by 20 alleles at nine loci. The IP-variants are indicated with capitals referring to the putative loci and arabic numbers referring to the putative alleles ( Fig. 1 and 2, Table 1). The frequencies of the IP-

variant encoding alleles were estimated by determining the ratio between the protein quantities of corresponding IP-variants (Fig. 2). The various ratios encountered in this study were measured by making a reference series from a total protein extract of population no. 1, which is monomorphic for all loci except locus B. The spot sizes and intensities displayed by $A_2$, $B_1$, $C_2$, $D_1$, $E_1$, $F_2$, $G_2$, $H_2$ and $I_1$ after electrophoresing 35 µg total protein from population no. 1 were associated with a relative protein quantity of 1.00. The reference series was made by electrophoresing total protein quantities of 0.875 µg; 1.75 µg; 3.5 µg; 5.25 µg; 7.00 µg and so on in steps of 3.5 µg until 45.5 µg. Thus the protein quantities of these IP-variants in the reference series correlate with relative values of 0.025; 0.05; 0.10; 0.15 et cetera. The relative protein quantities of the IP-variants in the other populations and of $B_2$ in population no. 1 were estimated visually by matching their spot size and intensities with those in the reference series. These relative values were used to calculate ratios between the protein quantities of corresponding IP-variants. For further details see elsewhere (3).

With exception of locus B, the sums of the relative protein quantities of the corresponding IP-variants in a population should in theory equal 1.0. However, deviations from 1.0 are inherent to the experimental procedure (3). A significant part of the deviations were caused by the fact that a large number of samples were electrophoresed and stained independently. However, when samples of different populations were electrophoresed and stained simultaneously the differences between the highest and lowest values of the sums were usually less than 0.3. No consistent quantitative differences were observed between populations, indicating that the corresponding alleles produce similar protein quantities per haploid set of chromosomes within each individual.

The similarity dendrogram shown in Fig. 3 was constructed from a distance matrix (Table 2). Genetic distances were computed according Rogers (18) by using the ratios between the protein quantities of the corresponding IP-variants as a measure for the allele frequencies. The genetic distances between population no. 10 and the other populations were based on eight loci, because no allele at locus C could be traced

in population no. 10.

The NIP-variants (Fig. 1, Table 1) are a group for which no proper genetic interpretation is available and may include, among others, differences in the regulatory genes and modifying genes (1). Estimation of the protein quantities of the NIP-variants was done in a similar way as for the IP-variants. The protein quantities (Fig. 2, Table 1) were estimated relative to populations having high concentrations of a given NIP-variant. The relative protein quantities of the NIP-variants no. 4, 5, and 6 were obtained by using a reference series made from population no. 1. Each of these NIP-variants in population no. 1 was given a relative value of 1.0. The protein quantities of the other NIP-variants were obtained by making a reference series of population no. 19, in which the NIP-variants no. 1, 2, 3 and 7 were associated with a relative value of 1.0. NIP-variant no. 4 was designated as an IP-variant in a previous report (1). Due to a slightly different electrophoretic procedure the alternate allele product is probably masked here by a more abundant protein.

Inspection of Table 1 shows that many genes represented by the IP- and NIP-variants are also expressed in males, fourth stage female larvae and second stage larvae.

The IP- and NIP-variants listed in Table 1 do not seem to be associated with genes for virulence. None of the variants could unambiguously be correlated with the number of individuals able to develop on a given differential (Table 1). For instance, the relatively high number of females that developed on S. tuberosum ssp. andigena in population no. 11 (68.4%) and no. 19 (84.6%) (Table 1) could not be associated with a common IP- or NIP-variant that discriminated them either quantitatively or qualitatively from populations having no or an extreme low number of virulent genotypes for S. tuberosum ssp. andigena.

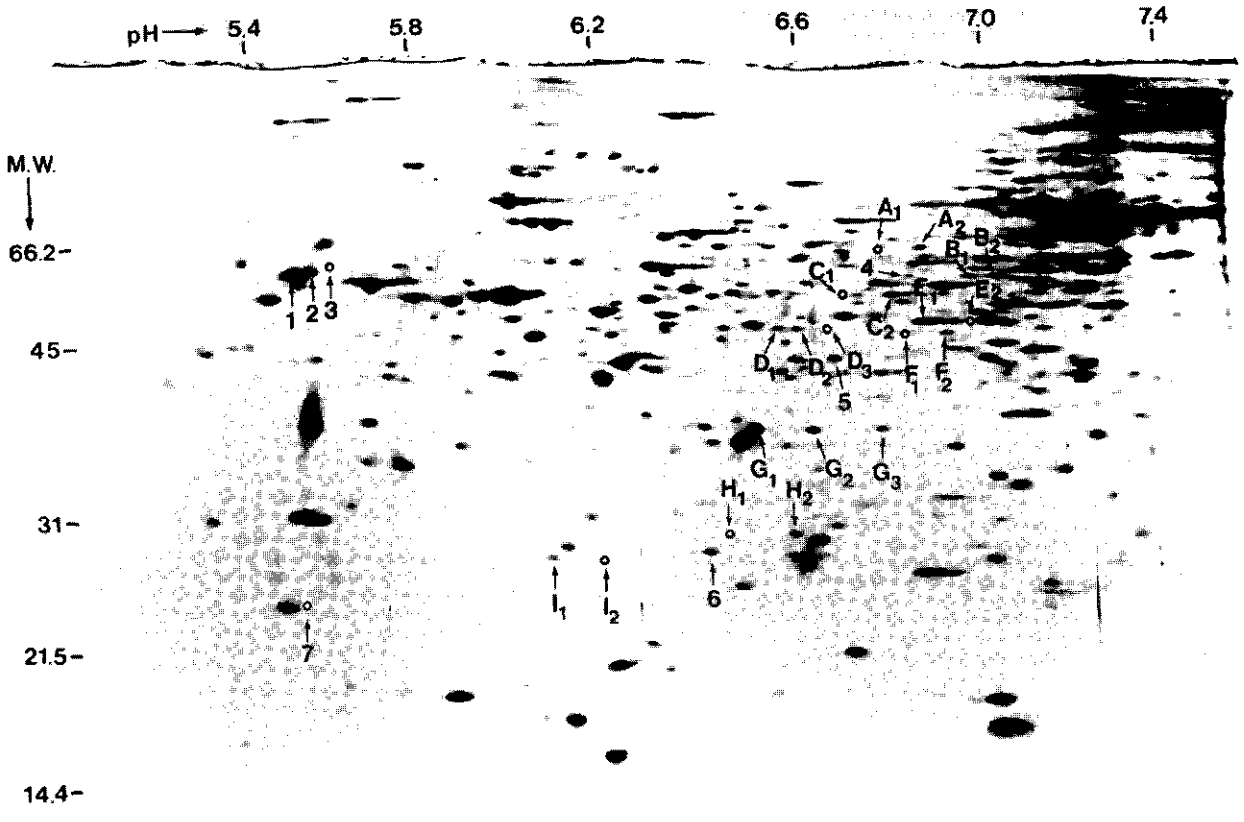


**Fig. 1** 2-DGE protein pattern of G. rostochiensis population no. 8 made by electrophoresing a mixture of 100 adult females. Variant protein spots, or their positions (open circles), revealed by the comparison of 19 G. rostochiensis populations are indicated by arrows. Isoelectric point variants (IP-variants) are designated with capitals and arabic numbers referring to the putative loci and alleles, respectively. Non-isoelectric point variants are indicated with arabic numbers. Molecular masses (at the left) are given in kilodaltons.

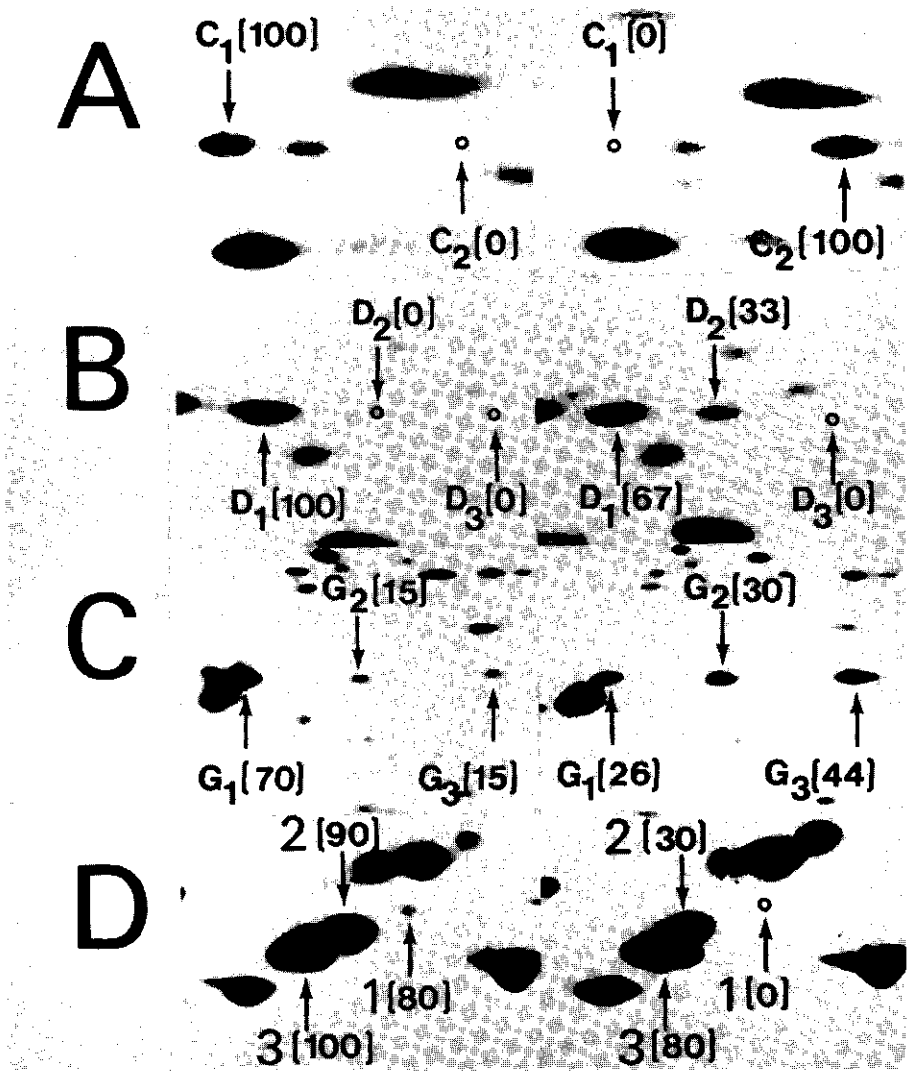


**Fig. 2** Close-ups of some IP-variants (**A, B** and **C**) and NIP-variants (**D**) in protein patterns made by electrophoresing a mixture of 100 individuals. IP-variants are indicated with capitals and arabic numbers referring to the putative loci and alleles, respectively. Figures in parenthesis are the estimates of the ratios between the protein quantities of the corresponding allele products, which were used as a measure for the allele frequencies. **A.** Locus C: population no. 13 (left) and population no. 8 (right). **B.** Locus D: population no. 2 (left) and population no. 12 (right). **C** Locus G: population no. 16 (left) and population no. 7 (right). **D.** Figures in parenthesis are the estimates of the relative protein quantities (x $10^2$) of the NIP-variants no. 1, no. 2 and no. 3 in population no. 19 (left) and population no. 1 (right). Further details see text.

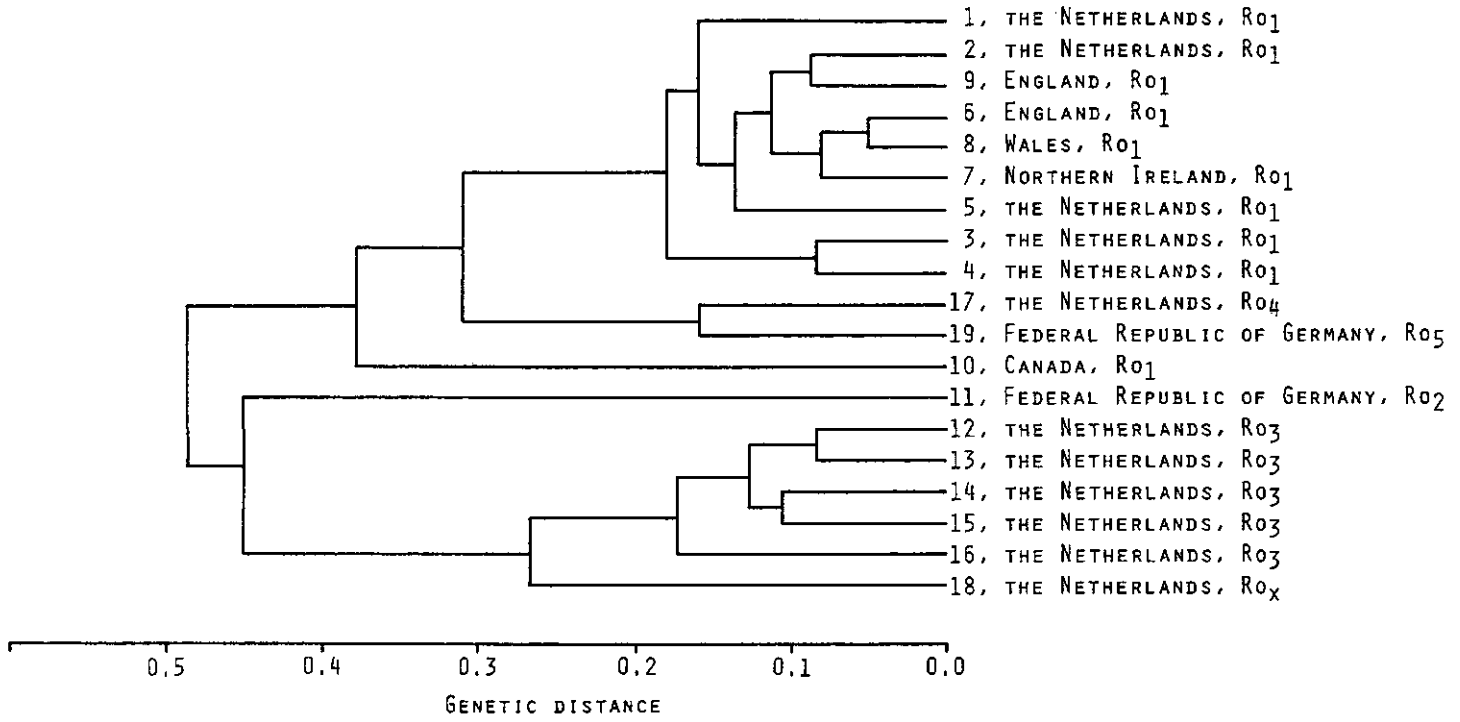


**Fig. 3** Similarity dendrogram of 19 G. rostochiensis populations constructed from the genetic distance at 9 IP-variant loci following the UPGMA method. Populations are numbered as in Table 1.

**Table 1** Protein quantities of 27 variant protein spots detected by the comparison of 2-DGE patterns of adult females from 19 populations of *G. rostochiensis*

| Pop. no. | Pathotype, collection location and code[c] | *S. tuberosum* ssp. *andigena* CPC 1673[d] | *S. vernei* hybr. 58.1642/4[d] | *S. vernei* hybr. 62.33.3[d] |
|---|---|---|---|---|
| 1. | $Ro_1$, Wageningen, the Netherlands, MIER | 0.0 | 1.1(0.2) | 0.0 |
| 2. | $Ro_1$, Bergh, the Netherlands, A-19 | 0.0 | 1.2(0.4) | 0.0 |
| 3. | $Ro_1$, Maastricht, the Netherlands, A-12 | 0.1(0.1) | 0.5(0.3) | 0.3(0.5) |
| 4. | $Ro_1$, Weert, the Netherlands, A-13 | 0.0 | 1.0(1.3) | 0.0 |
| 5. | $Ro_1$, Hardenberg, the Netherlands, C-133 | 1.4(0.1) | 0.3(0.2) | 0.0 |
| 6. | $Ro_1$, Feltwell, England, ST-1 | 0.2(0.1) | 0.3(0.2) | 0.0 |
| 7. | $Ro_1$, Ballycastle, Northern Ireland, ST-1 | 0.0 | 0.3(0.3) | 0.0 |
| 8. | $Ro_1$, Talybont, Wales, ST-1 | 1.1(0.5) | 4.2(2.1) | 0.0 |
| 9. | $Ro_1$, Johnson, England, ST-1 | 0.5(0.3) | 2.3(1.1) | 0.0 |
| 10. | $Ro_1$, Vancouver, Canada, KO[e] | 5.5(1.9) | 5.2(1.7) | 5.9(2.2) |
| 11. | $Ro_2$, Obersteinbach, Federal Republic of Germany, RU-1 | 68.4(20.3) | 0.1(0.1) | 3.1(0.5) |
| 12. | $Ro_3$, Oosterhesselen, the Netherlands, C-180 | 23.5(7.2) | 2.1(0.8) | 1.2(1.2) |
| 13. | $Ro_3$, Hoogeveen, the Netherlands, C-157 | 9.3(2.1) | 1.4(0.3) | 0.8(0.4) |
| 14. | $Ro_3$, Gramsbergen, the Netherlands, C-152 | 5.3(3.1) | 0.5(0.2) | 0.2(0.1) |
| 15. | $Ro_3$, Gramsbergen, the Netherlands, C-146 | 25.2(15.4) | 2.8(2.8) | 1.6(2.2) |
| 16. | $Ro_3$, Hoogeveen, the Netherlands, C-129 | 30.0(1.5) | 2.2(2.1) | 0.5(0.4) |
| 17. | $Ro_4$, Emmen, the Netherlands, F-515 | 0.0 | 10.2(5.2) | 0.0 |
| 18. | $Ro_x$, Anlo, the Netherlands, G-1508[f] | 19.6(7.4) | 23.3(19.3) | 1.6(0.8) |
| 19. | $Ro_5$, Harmerz, Federal Republic of Germany, RU-2 | 84.6(3.7) | 30.2(9.3) | 14.8(1.6) |
| | isoelectric point | | | |
| | molecular mass[g] | | | |
| | color[h] | | | |
| | detection limit[i] | | | |
| 1. | $Ro_1$, Wageningen, the Netherlands, MIER, adult males[i] | | | |
| 1. | $Ro_1$, Wageningen, the Netherlands, MIER, fourth stage female larvae[i] | | | |
| 1. | $Ro_1$, Wageningen, the Netherlands, MIER, second stage larvae[i] | | | |

a) Ratios between the protein quantities produced by IP-variant alleles at 9 presumptive loci. Loci and alleles of the IP-variants are indicated with capitals and numbers, respectively. Ratios and standard deviations (in parenthesis) were obtained as described in the text.

b) Relative protein quantities ($\times 10^2$) of the NIP-variants and the standard deviations were estimated from the reference series (see text).

c) As designated in the original collections.

IP-variants[a)]

| $A_1$ | $A_2$ | $B_1$ | $B_2$ | $C_1$ | $C_2$ | $D_1$ | $D_2$ | $D_3$ | $E_1$ | $E_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 100 | 81(4) | 19(4) | 0 | 100 | 100 | 0 | 0 | 100 | 0 |
| 0 | 100 | 12(4) | 88(4) | 0 | 100 | 100 | 0 | 0 | 100 | 0 |
| 0 | 100 | 57(3) | 43(3) | 0 | 100 | 95(6) | 5(6) | 0 | 100 | 0 |
| 0 | 100 | 21(7) | 79(7) | 0 | 100 | 88(8) | 12(8) | 0 | 100 | 0 |
| 0 | 100 | 19(4) | 81(4) | 0 | 100 | 55(4) | 45(4) | 0 | 100 | 0 |
| 0 | 100 | 23(4) | 77(4) | 0 | 100 | 50(5) | 50(5) | 0 | 100 | 0 |
| 0 | 100 | 36(3) | 64(3) | 0 | 100 | 75(6) | 25(6) | 0 | 100 | 0 |
| 0 | 100 | 32(3) | 68(3) | 0 | 100 | 49(5) | 51(5) | 0 | 100 | 0 |
| 0 | 100 | 32(6) | 68(6) | 0 | 100 | 100 | 0 | 0 | 100 | 0 |
| 0 | 100 | 90(3) | 10(3) | 0 | 0 | 0 | 100 | 0 | 100 | 0 |
| 0 | 100 | 100 | 0 | 100 | 0 | 0 | 100 | 0 | 6(2) | 94(2) |
| 89(8) | 11(8) | 100 | 0 | 88(11) | 12(11) | 65(4) | 35(4) | 0 | 100 | 0 |
| 57(7) | 43(7) | 100 | 0 | 100 | 0 | 76(11) | 24(11) | 0 | 100 | 0 |
| 88(11) | 12(11) | 89(4) | 11(4) | 100 | 0 | 50(9) | 50(9) | 0 | 100 | 0 |
| 80(5) | 20(5) | 100 | 0 | 100 | 0 | 93(5) | 7(5) | 0 | 100 | 0 |
| 94(8) | 6(8) | 100 | 0 | 100 | 0 | 90(9) | 10(9) | 0 | 100 | 0 |
| 0 | 100 | 89(4) | 11(4) | 0 | 100 | 0 | 0 | 100 | 100 | 0 |
| 86(9) | 14(9) | 21(4) | 79(4) | 100 | 0 | 0 | 100 | 0 | 100 | 0 |
| 0 | 100 | 82(3) | 18(3) | 0 | 100 | 100 | 0 | 0 | 100 | 0 |
| 6.75 | 6.85 | 6.95 | 7.02 | 6.69 | 6.79 | 6.55 | 6.61 | 6.67 | 6.90 | 6.98 |
| 62 | | 58 | | 54 | | 47 | | | 49 | |
| r.b. | | r.b. | | r.b. | | r.b. | | | r.b. | |
| 15–20 | | 5–10 | | 15–20 | | 10–15 | | | < 2.5 | |
| 0 | 0 | 76(13) | 13(5) | 0 | 0 | 118(11) | 0 | 0 | 86(9) | 0 |
| 0 | 87(12) | 85(10) | 18(5) | 0 | 45(10) | 106(9) | 0 | 0 | 110(8) | 0 |
| 0 | 23(6) | 98(5) | 24(5) | 0 | 0 | 105(13) | 0 | 0 | 81(9) | 0 |

d) Number of females expressed as a percentage of those developed on a generally susceptible cultivar.

e) No allele product of locus C could be detected in population no. 10.

f) Current pathotype scheme fails to classify population no. 18.

g) Molecular masses in kilodaltons

h) Reddish brown (r.b.), blackish brown (b.b.), red (r.) and grey (g).

i) Relative protein quantities (x $10^2$) estimated from the reference series (see text).

**Table 1.** continued

| Pop. no. | Pathotype, collection location and code[c] | IP-variants[a] $F_1$ | $F_2$ | $G_1$ | $G_2$ | $G_3$ | $H_1$ |
|---|---|---|---|---|---|---|---|
| 1. | $Ro_1$, the Netherlands, MIER | 0 | 100 | 0 | 100 | 0 | 0 |
| 2. | $Ro_1$, the Netherlands, A-19 | 0 | 100 | 0 | 23(2) | 77(2) | 0 |
| 3. | $Ro_1$, the Netherlands, A-12 | 30(10) | 70(10) | 0 | 36(6) | 64(6) | 0 |
| 4. | $Ro_1$, the Netherlands, A-13 | 22(15) | 78(15) | 0 | 21(3) | 79(3) | 0 |
| 5. | $Ro_1$, the Netherlands, C-133 | 30(10) | 70(10) | 0 | 100 | 0 | 8(9) |
| 6. | $Ro_1$, England, ST-1 | 16(5) | 84(15) | 32(4) | 52(2) | 16(4) | 0 |
| 7. | $Ro_1$, Northern Ireland, ST-1 | 23(16) | 77(16) | 22(2) | 31(2) | 47(2) | 0 |
| 8. | $Ro_1$, Wales, ST-1 | 0 | 100 | 40(4) | 31(3) | 29(2) | 0 |
| 9. | $Ro_1$, England, ST-1 | 0 | 100 | 25(3) | 63(3) | 12(2) | 0 |
| 10. | $Ro_1$, Canada, KO | 0 | 100 | 0 | 74(4) | 26(4) | 100 |
| 11. | $Ro_2$, Federal Republic of Germany, RU-1 | 100 | 0 | 0 | 100 | 0 | 0 |
| 12. | $Ro_3$, the Netherlands, C-180 | 100 | 0 | 36(5) | 0 | 64(5) | 0 |
| 13. | $Ro_3$, the Netherlands, C-157 | 100 | 0 | 26(4) | 0 | 74(4) | 0 |
| 14. | $Ro_3$, the Netherlands, C-152 | 100 | 0 | 30(2) | 0 | 70(2) | 0 |
| 15. | $Ro_3$, the Netherlands, C-146 | 100 | 0 | 34(3) | 25(3) | 41(2) | 0 |
| 16. | $Ro_3$, the Netherlands, C-129 | 75(5) | 25(5) | 68(2) | 15(2) | 16(2) | 0 |
| 17. | $Ro_4$, the Netherlands, F-515 | 100 | 0 | 97(4) | 0 | 3(4) | 0 |
| 18. | $Ro_x$, the Netherlands, G-1508[f] | 100 | 0 | 0 | 14(5) | 86(5) | 0 |
| 19. | $Ro_5$, Federal Republic of Germany, RU-2 | 100 | 0 | 64(4) | 0 | 36(4) | 0 |
| | isoelectric point | 6.78 | 6.90 | 6.54 | 6.64 | 6.76 | 6.49 |
| | molecular mass[g] | 46 | | 36 | | | 29 |
| | color[h] | r.b. | | b.b. | | | r. |
| | detection limit[i] | 15-20 | | 2.5-5 | | | 15-20 |
| 1. | $Ro_1$, MIER, adult males[i] | 0 | 0 | 0 | 18(5) | 0 | 0 |
| 1. | $Ro_1$, MIER, fourth stage female larvae[i] | 0 | 107(17) | 0 | 54(5) | 0 | 0 |
| 1. | $Ro_1$, MIER, second stage larvae[i] | 0 | 0 | 0 | 16(9) | 0 | 0 |

a) Ratios between the protein quantities produced by IP-variant alleles at 9 presumptive loci. Loci and alleles of the IP-variants are indicated with capitals and numbers, respectively. Ratios and standard deviations (in parenthesis) were obtained as described in the text.

b) Relative protein quantities (x $10^2$) of the NIP-variants and the standard deviations were estimated from the reference series (see text).

c) As designated in the original collections.

| | | | NIP-variants[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $H_2$ | $I_1$ | $I_2$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 100 | 100 | 0 | 80(8) | 23(5) | 0 | 88(15) | 100(14) | 93(23) | 0 |
| 100 | 100 | 0 | 0 | 0 | 0 | 103(21) | 103(23) | 98(13) | 0 |
| 100 | 45(12) | 55(12) | 38(10) | 40(8) | 0 | 83(12) | 88(29) | 40(17) | 0 |
| 100 | 55(10) | 45(10) | 22(6) | 27(6) | 0 | 73(12) | 93(23) | 46(20) | 0 |
| 92(9) | 100 | 0 | 60(18) | 15(5) | 0 | 83(15) | 80(16) | 110(34) | 0 |
| 100 | 100 | 0 | 118(13) | 33(10) | 0 | 75(17) | 68(18) | 85(12) | 0 |
| 100 | 100 | 0 | 85(13) | 23(5) | 0 | 80(14) | 96(17) | 103(25) | 0 |
| 100 | 100 | 0 | 117(15) | 66(21) | 0 | 90(14) | 96(16) | 87(15) | 0 |
| 100 | 100 | 0 | 115(14) | 45(13) | 0 | 68(10) | 63(20) | 60(26) | 0 |
| 0 | 100 | 0 | 0 | 0 | 0 | 107(11) | 0 | 110(26) | 0 |
| 100 | 0 | 100 | 0 | 0 | 0 | 113(12) | 0 | 96(23) | 0 |
| 100 | 90(12) | 10(12) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 100 | 0 | 13(6) | 23(6) | 0 | 0 | 0 | 0 | 0 |
| 100 | 45(14) | 55(14) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 50(10) | 50(10) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 25(7) | 75(7) | 3(3) | 3(3) | 0 | 38(15) | 33(15) | 11(10) | 0 |
| 100 | 100 | 0 | 102(15) | 98(18) | 85(26) | 0 | 48(8) | 76(21) | 0 |
| 100 | 35(7) | 65(7) | 2(3) | 2(3) | 0 | 93(10) | 0 | 0 | 0 |
| 100 | 100 | 0 | 107(15) | 106(11) | 95(7) | 0 | 103(21) | 112(15) | 103(9) |
| 6.58 | 6.12 | 6.22 | 5.50 | 5.54 | 5.60 | 6.80 | 6.68 | 6.43 | 5.56 |
| | 28 | | 57 | 58 | 60 | 59 | 43 | 28 | 23 |
| | g. | | g. | g. | g. | r.b. | r.b. | g. | b.b. |
| | 20-30 | | 2.5-5 | 2.5-5 | 30-40 | 25-30 | 15-20 | 15-20 | 10-15 |
| 58(11) | 50(10) | 0 | 0 | 0 | 0 | 61(9) | 0 | 28(16) | 0 |
| 122(11) | 58(9) | 0 | 0 | 0 | 0 | 96(13) | 72(10) | 37(15) | 0 |
| 85(10) | 123(8) | 0 | 0 | 0 | 0 | 124(9) | 0 | 26(7) | 0 |

f) Current pathotype scheme fails to classify population no. 18.

g) Molecular masses in kilodaltons

h) Reddish brown (r.b.), blackish brown (b.b.), red (r.) and grey (g).

i) Relative protein quantities (x $10^2$) estimated from the reference series (see text).

**Table 2** Genetic distances between 19 *G. rostochiensis* populations[a] based on 9 IP-variant loci.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10[b] | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | | | | | | | | | | | | | | | | | | |
| 2. | 0.162 | | | | | | | | | | | | | | | | | |
| 3. | 0.198 | 0.164 | | | | | | | | | | | | | | | | |
| 4. | 0.242 | 0.100 | 0.084 | | | | | | | | | | | | | | | |
| 5. | 0.161 | 0.186 | 0.228 | 0.194 | | | | | | | | | | | | | | |
| 6. | 0.185 | 0.144 | 0.211 | 0.162 | 0.076 | | | | | | | | | | | | | |
| 7. | 0.171 | 0.110 | 0.137 | 0.114 | 0.126 | 0.080 | | | | | | | | | | | | |
| 8. | 0.178 | 0.128 | 0.215 | 0.181 | 0.130 | 0.049 | 0.079 | | | | | | | | | | | |
| 9. | 0.091 | 0.085 | 0.178 | 0.165 | 0.143 | 0.094 | 0.095 | 0.087 | | | | | | | | | | |
| 10. | 0.294 | 0.411 | 0.439 | 0.471 | 0.343 | 0.327 | 0.362 | 0.311 | 0.350 | | | | | | | | | |
| 11. | 0.570 | 0.732 | 0.568 | 0.637 | 0.467 | 0.608 | 0.634 | 0.634 | 0.661 | 0.538 | | | | | | | | |
| 12. | 0.476 | 0.491 | 0.446 | 0.470 | 0.493 | 0.459 | 0.405 | 0.449 | 0.498 | 0.548 | 0.486 | | | | | | | |
| 13. | 0.544 | 0.437 | 0.418 | 0.439 | 0.474 | 0.444 | 0.365 | 0.435 | 0.457 | 0.510 | 0.463 | 0.083 | | | | | | |
| 14. | 0.544 | 0.552 | 0.409 | 0.454 | 0.539 | 0.496 | 0.473 | 0.487 | 0.567 | 0.573 | 0.419 | 0.100 | 0.141 | | | | | |
| 15. | 0.468 | 0.511 | 0.367 | 0.426 | 0.547 | 0.511 | 0.444 | 0.503 | 0.488 | 0.596 | 0.424 | 0.126 | 0.133 | 0.105 | | | | |
| 16. | 0.501 | 0.564 | 0.409 | 0.471 | 0.574 | 0.536 | 0.490 | 0.531 | 0.520 | 0.636 | 0.451 | 0.194 | 0.226 | 0.167 | 0.109 | | | |
| 17. | 0.341 | 0.406 | 0.377 | 0.416 | 0.370 | 0.329 | 0.317 | 0.326 | 0.361 | 0.486 | 0.559 | 0.385 | 0.366 | 0.441 | 0.436 | 0.472 | | |
| 18. | 0.663 | 0.521 | 0.466 | 0.421 | 0.524 | 0.497 | 0.502 | 0.589 | 0.586 | 0.600 | 0.422 | 0.273 | 0.302 | 0.173 | 0.260 | 0.313 | 0.566 | |
| 19. | 0.210 | 0.251 | 0.234 | 0.281 | 0.304 | 0.265 | 0.206 | 0.255 | 0.228 | 0.472 | 0.555 | 0.298 | 0.263 | 0.371 | 0.314 | 0.378 | 0.156 | 0.523 |

a) Populations are numbered as in Table 1.

b) Genetic distances between populations no. 10 and the other populations are based on 8 IP-variant loci.

## DISCUSSION

**Primary founders, drift and gene flow.** Degrees of similarity revealed with 2-DGE strongly suggest that the diversity of the initial populations introduced into the United Kingdom directly or indirectly from the Andean region has been relatively low. In contrast with the German and Dutch populations, populations from England, Wales and Northern Ireland were closely linked in a similarity dendrogram constructed from the distance data (Fig. 3). Similar results are obtained by a qualitative inspection of the IP- and NIP-variant data (Table 1). The maximum number of qualitative protein differences observed among populations from the United Kingdom was only two, whereas this figure was 16 and 17 for the Netherlands and the Federal Republic of Germany, respectively. We obtained no evidence that Canada has been infested from the United Kingdom as suggested by Evans and Stone (7). Population no. 10 from Vancouver is discriminated by several qualitative differences from the British populations.

Evidently a full understanding of the causes generating the interpopulation variations is beyond the scope of this study. For example, the number of native ancestors associated with the 19 populations studied here cannot be estimated and it is also not possible to recover the share of drift and gene flow. Nevertheless, it is evident that random genetic drift has not been extensive. Despite the numerous bottlenecks that have accompanied the primary and secondary founding events, population sizes have been sufficiently large to maintain several polymorphic loci in most populations studied here (Table 1). Also gene flow has not been extensive. The occurrence of distinct clusters and the various qualitative differences indicate that the transport of individuals between distinct populations is restricted, which is not surprising in view of the poor dispersal abilities of the potato cyst nematodes. The contact between distinct populations is probably also impaired by the occurrence of large areas infested with rather uniform populations. Evidently for a more refined interpretation of the 2-DGE data more research is required. For instance, detailed studies of an area and data on field populations for a number of generations will provide more insight into the

occurrence of gene flow and drift. Information on the extent of random genetic drift can also be obtained by studying possible reductions in the number of polymorphic IP-variant loci by comparing European and native populations.

**Pathotypes and protein variation.** The genetic diversity of G. rostochiensis in Europe has extensively been studied by measuring the ability to overcome resistance in the differentials of the international pathotype scheme (12). However, current pathotype classification is rather arbitrary. Populations are designated as virulent or avirulent for a differential if the multiplication factor is >1 or ≤1 (12), respectively. A reproduction value of 1 indicates that approximately 5 % of the individuals can develop on a differential (5). In theory, populations classified as distinct pathotypes may have only a slightly different number of virulent genotypes, i.e. just below and above 5%. On the other hand the number of virulent genotypes between populations classified as identical pathotypes may vary in theory from 5% to 100%. In view of these considerations we prefer to give not only the pathotype classification of a population but also the numbers of individuals able to develop on the differentials (Table 1), which are used as an indication of the number of virulent genotypes in a population.

Despite the arbitrary way populations are classified as virulent or avirulent and the limited number of differentials used in the current pathotype scheme, the pathotype data strongly support the hypothesis that measuring degrees of similarity with 2-DGE is also informative for interpopulation variation at virulence loci. The uniformity of the protein patterns of the British populations (Table 1, Fig. 3) are corroborated by surveys which showed that $Ro_1$ is the solely pathotype of G. rostochiensis in the United Kingdom, whereas several pathotypes are present in the Netherlands ($Ro_1$, $Ro_2$, $Ro_3$, $Ro_4$) and the Federal Republic of Germany ($Ro_1$, $Ro_2$, $Ro_5$) (12). It is stressed that these variations in virulence are not the result of differentiating selection, because these pathotypes were already traced before resistant cultivars were grown in those countries.

Another conspicuous result is that the genetic distances between the European populations of the same pathotype were on the average smaller

than between populations classified as distinct pathotypes (Fig. 3). The finding that all nine European populations pathotyped as $Ro_1$, five from the Netherlands and four from the United Kingdom, are closely linked in the similarity dendrogram, indicates that the two countries have been infested by initial populations having a high degree of similarity and that these initial populations have contained no or a low number of alleles for virulence, which has impaired the diversification at virulence loci by random genetic drift resulting in an $Ro_1$ pathotype classification of all descendants. It is noted that these populations are classified as $Ro_1$, because they have no or an extremely low number of virulent genotypes for all differentials. These results also suggest that a large number of British and Dutch populations can be pathotyped with 2-DGE, of course on the condition that they have not or not significantly been influenced by the growth of resistant cultivars in Europe. Populations which are placed between the nine $Ro_1$ populations (Fig. 3) in a similarity dendrogram based on 2-DGE data will probably have the virulence characteristics of pathotype $Ro_1$. Such an approach is obviously less reliable for the identification of pathotype $Ro_3$. The agreement between the similarity dendrogram and the $Ro_3$ pathotype classification of the five Dutch populations is probably not representative for all populations in the Netherlands. The $Ro_3$ populations are classified as such because they are designated as virulent for S. tuberosum ssp. andigena and S. kurtzianum. As shown in Table 1 the number of individuals in the $Ro_3$ populations able to develop on S. tuberosum ssp. andigena ranges from 5.3 to 30 %, indicating that these populations are not fixed for their virulence alleles, which offers ample opportunity for drift to operate. Therefore we expect that there are also Dutch populations, which have protein characteristics very similar to the five $Ro_3$ populations studied here, but which will have less than 5% virulent genotypes and which will be classified as avirulent for S. tuberosum ssp. andigena.

Evidently, for a more thorough investigation of the correlation between virulence and 2-DGE data it will be necessary to increase the number of differentials and to obtain more insight into the genetics of resistance and virulence in order to estimate allele frequencies at

virulence loci. An interesting subject for future research will be the relation between the similarity dendrogram presented here and a dendrogram produced by estimating the genetic distances at a substantial number virulence loci.

Although there are several subjects which await further investigation, the data presented here show that 2-DGE can be valuable adjunctive tool for a breeding program aimed at obtaining resistant cultivars, which are effective against all or the large majority of the populations in an area (3). The clusters revealed by 2-DGE can be used as guidance in testing the effectiveness of new sources of resistance. Members of all distinct clusters should be included in the initial sreening. Most important for a reliable survey is that descendants of all distinct founding populations are included. The diversity generated by drift and gene flow are of secondary importance, because these stochastic processes only influence the allele frequencies of single populations and do not change the gene pool of a whole area. A major challenge will be to minimize the number of populations in testing the effectiveness of new sources of resistance without loss of information.

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## CHAPTER VII

# Phylogenetic Value of Evolutionary Conservative Proteins Resolved by Two Dimensional Gel Electrophoresis


ABSTRACT

The increased number of gene products sampled and the relatively low variation observed in those proteins indicate the potential of two dimensional gel electrophoresis (2-DGE) over conventional enzyme electrophoresis in studying ancient genealogical relationships. In this report we explored evolutionary conservative proteins to estimate remote relationships between nematode species. A suitable method to infer phylogenetic relationships proved to be the analysis of only those variant proteins which gave rise to distinct putative homologies in all species, which obviated a tedious and guideless comparison of the approximately 200 proteins resolved from each species.

## INTRODUCTION

The structures of biological macromolecules contain significant information with regard to genealogical relationships between extant species. A standard technique to study resemblances and dissimilarities at the molecular level is electrophoresis of crude cell extracts. Especially starch gel electrophoresis (SGE) followed by specific enzyme stains has been applied to a wide variety of organisms and the 15 to 30 enzyme loci assayed have proven to be informative characters in studying evolutionary relationships. However, the application of SGE has its limitations in estimating phylogenies, because a large proportion of the enzymes surveyed evolves rather fast and contains no information for species which have diverged more than 4-6 millions of years ago (1). Only few enzymes evolve slowly enough to reveal shared evolutionary events among more distantly related species.

Another relative simple technique to resolve genetic variation is two-dimensional gel electrophoresis (2-DGE) according to O'Farrell (2). Although no such phylogenies are available, it can be claimed that 2-DGE outperforms SGE in studying ancient genealogical relationships. This property is inherent to two features. First, the range in evolutionary rates of proteins is rather wide (3,4) and sampling 100 or more proteins increases the chance of tracing conservative proteins. Second, the variation detected in the abundant proteins revealed by 2-DGE is relatively low when compared to enzymes. 2-DGE studies on man (5,6), rodents (7) and Drosophila (8,9) spp. have shown that estimates of heterozygosity indices were substantially lower than those obtained by SGE. As expected from the neutral theory of molecular evolution (4), these data are corroborated by the magnitude of the overall genetic distances between species. Not only the absolute, but also the relative number of proteins sharing electrophoretic mobilities between rodent species was considerably higher (10). For example, two rodent families shared 50% of the approximately 180 protein spots analyzed with 2-DGE and only 6% of their alleles at 30 enzyme loci (10). These findings indicate that the proteins assayed with 2-DGE may be biased towards a more conservative

group of proteins. Another explanation, not mutually exclusive, is that the two techniques have differing sensitivities in tracing genic variation. SGE is thought to resolve also other alterations than amino acid substitutions involving residues of different charge, e.g. changes in the conformatiom (11,12). In any event the low variability observed makes 2-DGE in theory a powerful tool to study ancient processes of speciation. Possible disparities in sensitivity are not necessarily disturbing, because the validity of a biochemical technique as a phylogenetic assay comes only from its capacity to trace a sufficient number of shared and dissimilar evolutionary events, irrespective whether they have originated from net charge changing amino acid substitution or alterations in the conformation. Another advantage is that 2-DGE combined with sensitive silver stains (13) enables the examination of minute amounts of biological material. Various organisms, such as many obligate parasites, are difficult to obtain in sufficient quantities for SGE, macromolecular sequencing, restriction enzyme digests or immunological techniques.

Despite these advantages and its widespread application in various scientific disciplines, 2-DGE has rarely been applied to phylogenetic problems (14,15). Evidently, as discussed elsewhere (10,14), analyzing and evaluating 100 or more proteins on a single pattern is more difficult and less straightforward than interpreting SGE patterns.

In this report we evaluate the merits of 2-DGE by studying phylogenetic relationships between obligate plant parasitic nematode species and introduce an effective method to study ancient processes of speciation.

## MATERIALS AND METHODS

Females of the plant parasitic nematode species were reared on the following hosts: Heterodera schachtii (Tinte) on Beta vulgaris; H. glycines (R-1-92) on Glycine max; H. goettingiana (Bor) on Pisum sativum; H. cruciferae (Klo) on Brassica oleracea; H. mani (Ens) on Lolium perenne; H. fici (Aal) on Ficus elastica; H. humuli (Po) on Humulus lupulus; Globodera rostochiensis (Mier) on Solanum tuberosum spp. tuberosum. The codes of the populations given in parenthesis

refer to the original collections of the Plant Protection Service, Wageningen, the Netherlands, except for H. glycines, which was obtained from V. H. Dropkin, University of Missouri, Columbia, U.S.A.

Total protein samples were prepared by homogenizing one hundred fullgrown females in 60 µl 10 mM Tris-HCl, pH 7.4, 5% mercaptoethanol and saturated with urea (16). As a standard, 25 µg of protein was used for electrophoresis. Isoelectric focusing within the pH range 5 to 7, sodium dodecyl sulfate electrophoresis and molecular weight determinations were as described (17). Proteins were visualized with a silver staining procedure, which generated colors ranging from red, reddish brown, blackish brown to grey (17). Protein profiles were compared visually by superimposing the original gels on a bench viewer. To facilitate the analysis, a sample of each Heterodera species was also electrophoresed in mixture with a sample of G. rostochiensis.

Genetic distances were calculated according to Aquadro and Avise (10): $D = 1-F$ and $F = (2Nxy)/(Nx + Ny)$, wherein Nx and Ny are the total number of protein spots analyzed for population x and y, respectively, and Nxy the number of spots shared.

The phylogeny of the 7 Heterodera species was estimated from binary coded character matrices (18) using the Wagner-78 algorithm (19) with the CHG, HOM, CON and APO options. The trees were rooted with G. rostochiensis as the outgroup, which seems justified on grounds of morphology and host ranges (20, 21).

## RESULTS

Protein patterns are exemplified in Fig. 1 and were made by electrophoresing a mixture of 100 individuals. The number of protein spots detected on the original pattern after staining with silver ranged from 180 to 240. Preliminary comparisons showed that the genetic distances ranged from 0.90 to 0.99, except for H. cruciferea and H. goettingiana, and H. schachtii and H. glycines, which had a distance of approximate 0.70 and 0.60, respectively. These findings imply that the vast majority of the approximately 1400 protein spots revealed by electrophoresis of the 7 Heterodera spp. are

species specific and provide no useful information about cladistic relationships.

The phylogeny of the Heterodera spp. was estimated by the comparison of putative homologous proteins (H proteins) of which the evolution proved to be fairly well tuned to the required divergence dates (Fig. 3 and 4). The H proteins were traced by exploring the approximately constant rate at which a given protein evolves in each lineage (4), which was to a reasonable extent also reflected in the magnitude of the electrophoretic divergence. Scrutiny of two distantly related members of the study group, H. mani and H. glycines, and the outgroup G. rostochiensis revealed three classes of proteins: i) 2 invariant proteins, (protein Y and Z)(Fig. 1) ii) 10 variant proteins with distinct putative homologies in all three species ( H proteins) (Fig. 1 and Fig. 2) and iii) numerous variant proteins of which the putative homologies could not be traced in all three species.

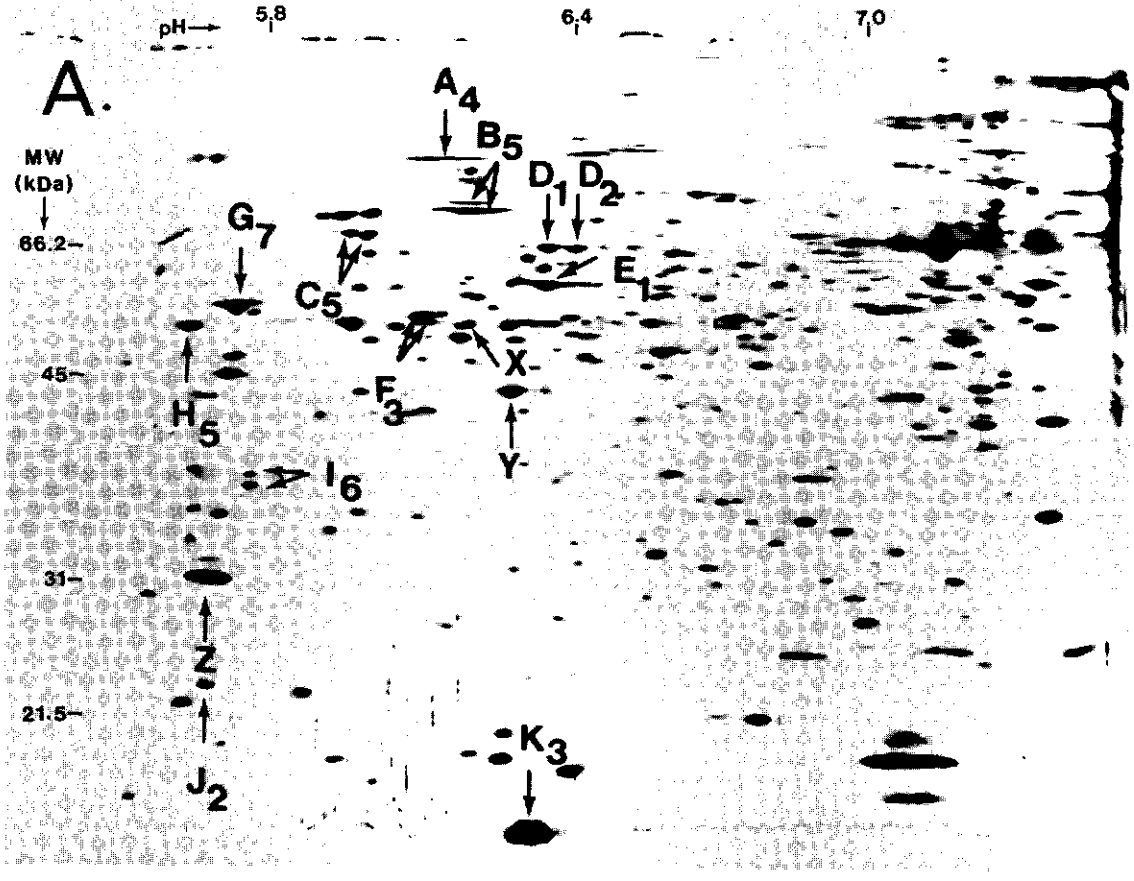
The putative homologous forms of a given H protein had marked similarities in appearance and a moderate difference in electrophoretic mobility and were clearly discriminated from neighbouring variant proteins by one or more typical characteristics with regard to quantity, shape or color (Fig. 1, Fig. 2, Table 1). Analysis of the protein patterns of all eight species showed that the three classes were representative for all other comparisons, which can be explained by the approximately constant evolutionary rates. Protein Y and Z had the same electrophoretic mobility in all species investigated and also the alternative homologous forms of the 10 H proteins could easily be detected in the other species of the study group, because they were located in the same areas of the gel (Table 1). Evidently, inherent to the fortuitous nature of molecular evolution, such a perfect resemblance between the initial and the other comparisons is not a general rule.

A number of H proteins (protein B, C, F, I) gave rise to more than one protein spot (Fig. 1 and Fig. 2). These variant protein spots behaved as expected from modifications of one and the same protein, i.e. they had fixed positions towards each other in all species studied. As shown in Fig. 1, Fig. 2 and Table 1, the detection of homologies between variant protein spots is rather facile, because the

majority of the populations appeared to be monomorphic for all H proteins as expressed by similar spot sizes and intensities. Obviously it can not be excluded that some of these populations are actually polymorphic, because alleles present in low frequencies are not registered by electrophoresing a homogenate of 100 individuals (16). Only H. humuli and H. mani were clearly polymorphic for protein B and D respectively. A typical characteristic of these polymorphisms is that the protein quantity of each of the two homologous forms is less than the quantity of the homologous forms in the monomorphic populations ( e.g. protein D, Fig. 1). Unlike spot size and intensity, the shape and the color were not affected by the lower protein quantities, which facilitates the recognition of those proteins as polymorphic H proteins.

The minimum-length Wagner tree shown in Fig. 3 was obtained by coding the homologous forms of the 10 H proteins as two-state characters, i.e., present or absent (Table 1). The Wagner tree was directly generated from the binary coded character matrix shown in Table 1. The tree had a consistency index of 0.83, which is relatively high when compared with the application of the same type of data manipulation to enzymes revealed by SGE (22). All alternative trees had a lower consistency index.

Various proteins exhibited also variations in molecular weight (Table 1, Fig. 2). Treating the molecular masses of the H proteins as discrete characters which are undergoing change during evolution, expressed by their absence or presence (Table 2), reveals several synapomorphic character states, which are not resolved when each multiple molecular form is treated as a separate character. For example, $G_6$, $G_7$ and $G_8$ yield autapomorphic character states for H. humuli, H. fici, and H. mani, respectively (Fig. 3), whereas their identical molecular mass of 57 kDa ($G_{57}$) defines a synapomorphic state for those three species (Fig.4). The tree inferred from the molecular masses had a consistency index of 0.92 and supports the three major clades resolved by the 54 homologous forms of the 10 H proteins (Fig. 3).


A.
pH
5,8
6,4
7,0
MW
(kDa)
66.2
45
31
21.5
A4
B5
D1
D2
G7
C5
E1
X
F3
H5
Y
I6
Z
K3
J2

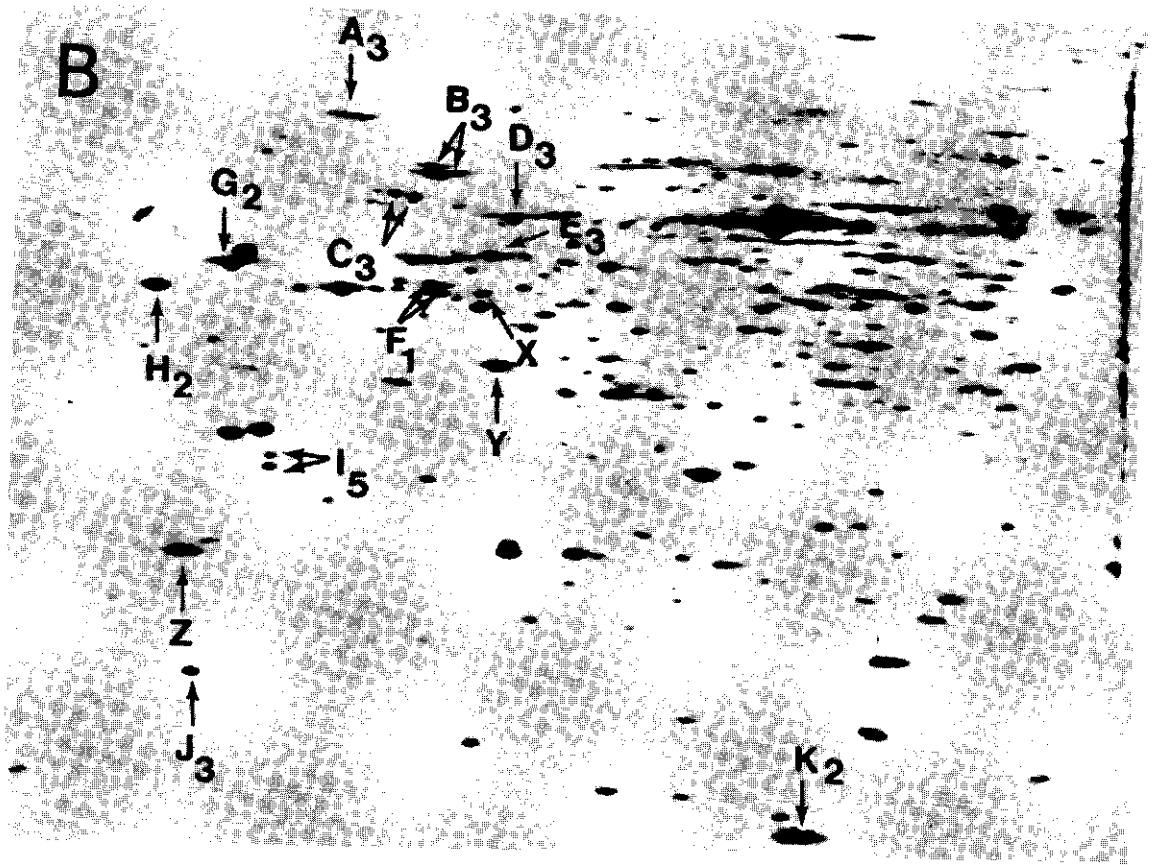



**Fig. 1** Two dimensional gels of total protein extracts form H. mani (**A**) and H. glycines (**B**). X, Y and Z are the only protein spots shared by the two species. Variant proteins of which the putative homologous forms could be traced in all species studied are marked with capitals and arabic numbers.

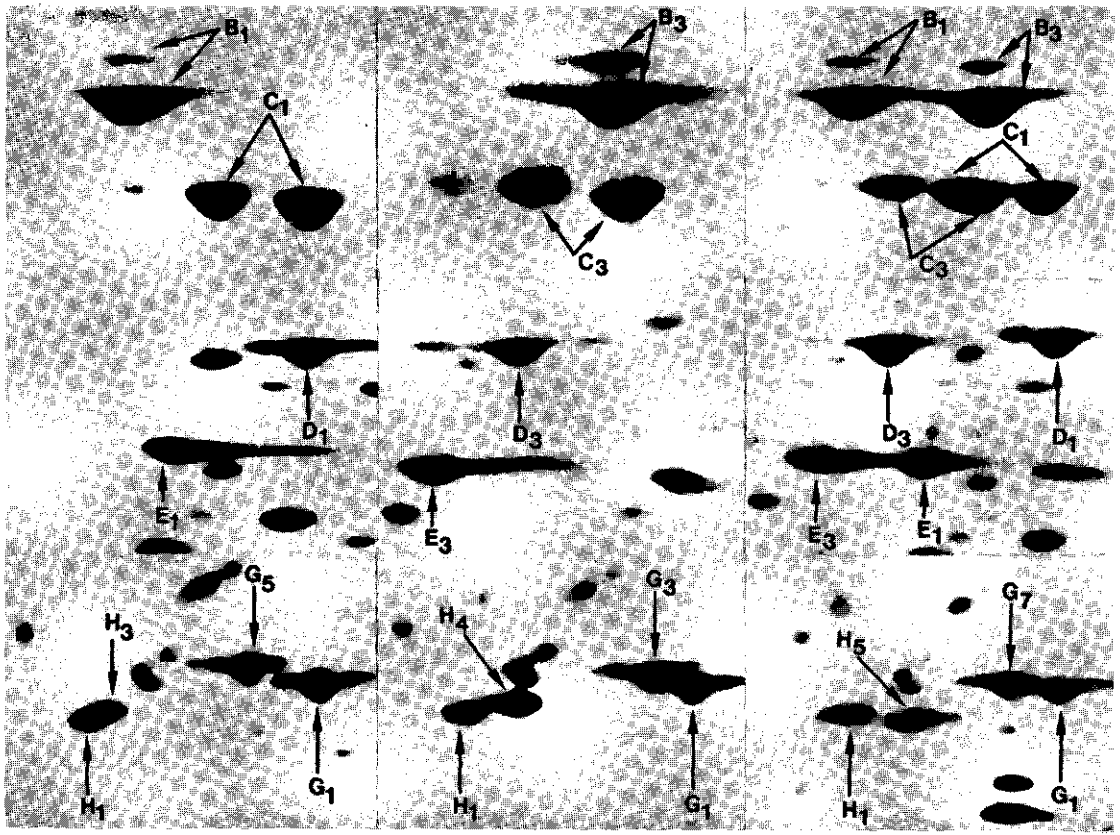



**Fig. 2** Close-ups of two dimensional gels showing variations in isoelectric point and molecular weight between the putative homologous forms of some proteins. **Top row:** protein B and C in G. rostochiensis (left), H. glycines (middle) and in a mixture of both species (right). **Middle row:** protein D and E in G. rostochiensis (left), H. glycines (middle) and in a mixture of both species. **Bottom row:** G. rostochiensis electrophoresed in mixture with H. cruciferea (left), H. goettingiana (middle) and H. mani (right). $H_1$ and $G_1$ are specific for G. rostochiensis.

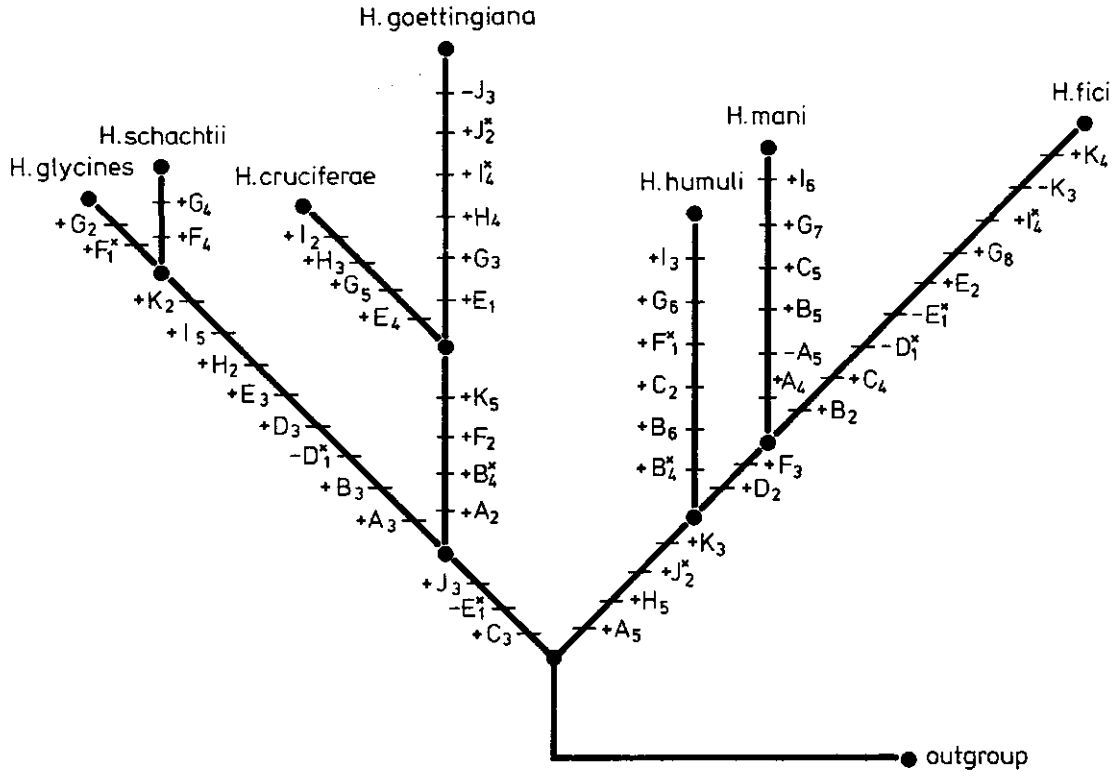



**Fig. 3** Minimum-length Wagner tree inferred from the binary coded matrix of the putative homologous forms of 10 proteins (Table 1). The synapomorphic and autapomorphic character states, expressed by the appearance (+) or loss (-) of a molecular form, are marked on the branches. Branch lengths are proportional to inferred patristic distances. Homoplasous character state changes are indicated with stars. The 9 steps associated with the branch interconnecting the outgroup and the most recent common ancestor of the study group are not shown.

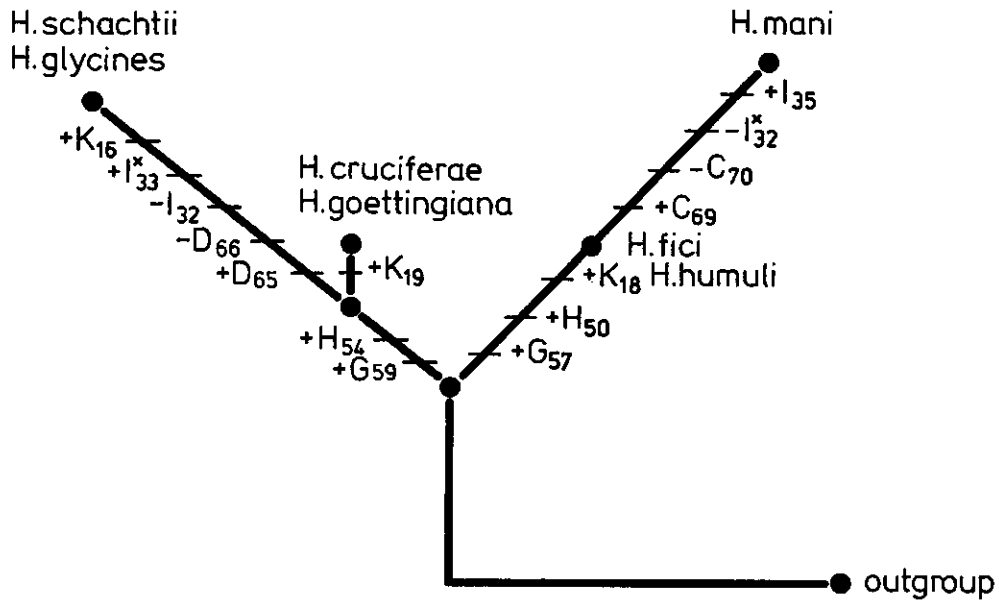



**Fig. 4** Minimum-length Wagner tree inferred from the binary coded matrix of the molecular weight values of 8 proteins (Table 2). The synapomorphic and autapomorphic character states, expressed by the appearance (+) or loss (-) of a molecular weight value, are marked on the branches. Branch lengths are proportional to inferred patristic distances. Homoplasous character state changes are indicated with stars. The 9 steps associated with the branch interconnecting the outgroup and the most recent common ancestor of the study group are not shown.

**Table 1** Presence (1) and absence (0) of the putative homologous forms of 10 proteins (A-K) in plant parasitic nematode populations.

| | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | $B_1$ | $B_2$ | $B_3$ | $B_4$ | $B_5$ | $B_6$ | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $D_1$ | $D_2$ | $D_3$ | $E_1$ | $E_2$ | $E_3$ | $E_4$ | $F_1$ | $F_2$ | $F_3$ | $F_4$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *G. rostochiensis* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *H. schachtii* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| *H. glycines* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| *H. goettingiana* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *H. cruciferea* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| *H. humuli* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *H. fici* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| *H. mani* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| molecular mass[a] | 98 | 104 | 104 | 104 | 104 | 80 | 80 | 80 | 80 | 80 | 80 | 70 | 70 | 70 | 70 | 69 | 66 | 66 | 65 | 58 | 58 | 58 | 58 | 53 | 53 | 53 | 53 |
| isoelectric point[b] | 0 | +0.5 | +2 | +5 | +9 | 0 | +2.5 | +4 | +4.5 | +5.5 | +9.5 | 0 | +1 | -2.5 | -3 | -10 | 0 | -4.5 | -7 | 0 | -4.5 | -5.5 | +6 | 0 | -2 | -4 | -4.5 |
| color[c] | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb |

a) Molecular masses in kilodaltons

b) Electrophoretic mobilities in the isoelectric focusing dimension are given in mm relative to the putative homologous form in *G. rostochiensis*; + and - refer to a higher and lower isoelectric point.

c) Reddish brown (rb), blackish brown (bb) and grey (g).

**Table 1** continued

| | $G_1$ | $G_2$ | $G_3$ | $G_4$ | $G_5$ | $G_6$ | $G_7$ | $G_8$ | $H_1$ | $H_2$ | $H_3$ | $H_4$ | $H_5$ | $I_1$ | $I_2$ | $I_3$ | $I_4$ | $I_5$ | $I_6$ | $J_1$ | $J_2$ | $J_3$ | $K_1$ | $K_2$ | $K_3$ | $K_4$ | $K_5$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *G. rostochiensis* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *H. schachtii* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| *H. glycines* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| *H. goettingiana* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| *H. cruciferea* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| *H. humuli* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| *H. fici* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| *H. mani* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| molecular mass [a] | 56 | 59 | 59 | 59 | 59 | 57 | 57 | 57 | 52 | 54 | 54 | 54 | 50 | 36 | 32 | 32 | 32 | 33 | 35 | 24 | 23 | 23 | 17 | 16 | 18 | 18 | 19 |
| isoelectric point [b] | 0 | -1.5 | -2 | -2.5 | -3.5 | -2.5 | -3 | -3.5 | 0 | -1 | +1 | +3 | +4 | 0 | +0.5 | +1.5 | +3.5 | +4.5 | -1.5 | 0 | +3.5 | +5 | 0 | -9 | -36 | -38 | -62 |
| color [c] | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | rb | bb | bb | bb | g | g | g | g | g |

[a] Molecular masses in kilodaltons

[b] Electrophoretic mobilities in the isoelectric focusing dimension are given in mm relative to the putative homologous form in *G. rostochiensis*; + and − refer to a higher and lower isoelectric point.

[c] Reddish brown (rb), blackish brown (bb) and grey (g).

**Table 2** Matrix of character states produced by coding the molecular weight values of a given protein as either absent (0) or present (1) [a].

| | $A_{98}$ | $A_{104}$ | $C_{69}$ | $C_{70}$ | $D_{65}$ | $D_{66}$ | $G_{56}$ | $G_{57}$ | $G_{59}$ | $H_{50}$ | $H_{52}$ | $H_{54}$ | $I_{32}$ | $I_{33}$ | $I_{35}$ | $I_{36}$ | $J_{23}$ | $J_{24}$ | $K_{16}$ | $K_{17}$ | $K_{18}$ | $K_{19}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G. rostochiensis | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| H. schachtii | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| H. glycines | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| H. goettingiana | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| H. cruciferea | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| H. humuli | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| H. fici | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| H. mani | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| Homologous forms [b] | $A_1$ | $A_2$ | $C_5$ | $C_1$ | $D_3$ | $D_1$ | $G_1$ | $G_6$ | $G_2$ | $H_5$ | $H_1$ | $H_2$ | $I_2$ | $I_5$ | $I_6$ | $I_1$ | $J_2$ | $J_1$ | $K_2$ | $K_1$ | $K_3$ | $K_5$ |
| | | $A_3$ | | $C_2$ | | $D_2$ | | $G_7$ | $G_3$ | | | $H_3$ | $I_3$ | | | | $J_3$ | | | | $K_4$ | |
| | | $A_4$ | | $C_3$ | | | | $G_8$ | $G_4$ | | | $H_4$ | $I_4$ | | | | | | | | | |
| | | $A_5$ | | $C_4$ | | | | | $G_5$ | | | | | | | | | | | | | |

a) Proteins are designated with capitals and their molecular masses in kilodaltons.

b) Putative homologous forms having the same molecular mass.

## DISCUSSION

Overall genetic distances estimated from 2-DGE patterns have proven to be fair measures in studying evolutionary relationships (10, 14,15). Nonetheless, there are several points of concern with regard to phylogenetic inferences based on analyzing all proteins resolved. First, because homologies between protein spots are often obscure, experimental variation may remain unnoticed when the number of replicates is too limited and may result in incorrect phylogenetic interpretations, especially when the overall genetic distances are within a narrow range. Analyzing 100 or more proteins is always accompanied by various qualitative differences between independent experiments, e.g. due to slight differences in the electrophoretic procedure, sample preparation and physiological stage of the biological material. Second, besides structural gene products, 2-DGE monitors also the effects of genes which influence the synthesis, degradation and modification of other gene products (23). It cannot be excluded that certain mutations are given too much weight in the distance data, because, e.g., a single change in a regulatory gene may affect the presence or absence of several protein spots. Third, estimating overall genetic distances is not only a tedious, but also a devious procedure in studying ancient processes of speciation, because the majority of the proteins evolve too fast and yield protein spots unique to a single species.

In this report we introduced a method which minimizes the drawbacks associated with estimating overall genetic distances by tracing putative homologies. Considering present knowledge of protein variation, it seems feasible to assume that a considerable part of these variations, including dissimilarities in molecular weight, result from amino acid substitutions. Variations in molecular weight between corresponding allele products are not exceptional (24). Dissimilarities in apparent molecular weight can have various causes and may, e.g., be due to deletions or additions of a stretch of amino acids. But single amino acid substitutions may be involved as well. For instance, a charged and uncharged amino acid substitution in actin (25) and hypoxanthine phosphoribosyltransferase (26,27), respectively,

has been shown to result in an apparent change in molecular weight. Because such small alterations do not influence the molecular weight to a detectable extent, these single amino acid substitutions probably cause a change in the amount of SDS bound to the protein. In any event they are informative about cladistic affinities, especially between the more distantly related species, because they occur less frequently than mutations resulting in isoelectric point changes only (Table 1).

At present various types of algorithms and data manipulation are available to construct phylogenies (28); these may be suited as well to infer phylogenies from 2-DGE patterns (14,15). Here we have chosen a cladistic type of analysis for the H proteins, because it is a powerful method to study branching sequences and it readily shows its output on the branches. Another desirable feature is that the assumptions underlying the phylogenetic inferences are rather unambiguous. The type of cladistic analysis used here for the putative homologous forms of the 10 H proteins does not even require that they be homologous, since each variant is treated as a separate character. Phylogenetic inferences based on molecular weights require the hypothesis that the variants are encoded by orthologous genes, and the molecular weight values represent the condition of an ancestor gene, irrespective whether this is the condition of the structural gene itself or a modifying gene. The fact that in this qualitative approach differences in allele frequencies are not taken in account probably does not result in significant loss of information. Several reports (29, 30, 31) have shown that qualitative approaches are not inferior to a quantitative analysis when applied to the same set of data. Allele frequencies may only be relevant when studying closely related organisms (32), but are probably in most cases irrelevant when studying ancient processes of speciation, because it is unlikely that similarities in allele frequencies have been retained since the time of divergence.

An important clue in tracing homologies between variant protein spots is that the protein spots have one or more typical characteristics discriminating them from neighbouring variant protein spots. This becomes increasingly difficult when the divergence in electrophoretic mobilities becomes larger. Therefore it is not

difficult to conceive that the H proteins are biased towards a conservative group of proteins of which the evolutionary rates are fairly well adjusted to the remote divergence dates of the Heterodera spp. Other homologous proteins have diverged in electrophoretic mobilities too rapidly to be recognized as such in all species, but, as can be inferred from the large overall genetic distances, the majority of these variant protein spots yield autapomorphic character states. Invariant proteins, on the other hand, are also uninformative. Evidently, the H proteins do not include all protein spots defining synapomorphic character states, but when the phylogenetic inferences based on the homologous forms and their variations in molecular weight are stable, additional information will probably not significantly change the estimates of the genealogical relationships.

An effective method to trace homologous proteins, which are relevant in studying ancient, but also more recent processes of speciation, is to start with the comparison of the most distantly related species, in this report two members of the study group and the outgroup. Inherent to the approximately constant rates at which proteins evolve in each lineage, there is a fair chance that variant proteins giving rise to marked homologies in these species can also be identified in all other species of the study group. The observation that the 10 H proteins were sufficiently variable to distinguish even the most closely related species, indicates that the homologous forms detected in the initial comparisons differed in quite a few mutation steps. Here it is worthwhile mentioning that if the range in divergence dates within the study group is too large, the H proteins will be too conservative to resolve the affinities between the more closely related species. In those situations it will be necessary to choose a more closely related outgroup in order to trace faster evolving proteins. For example, if one wants to study closely relatives of H. schachtii one can use H. glycines as an outgroup for the initial comparisons to trace faster evolving H proteins. It is noted that the number of variant protein spots with distinct homologies increases when the genetic distances become smaller. For instance, the number of H proteins detected by comparison of the closely related species H.

schachtii (Fig. 3) and H. glycines was 35. This can, among others, be explained by the lower number of variant protein spots which diminishes confusion with neighbouring variant protein spots. In view of these findings it is evident that a proper choice of the outgroup is important. The outgroup should be unequivocally distinct from the clade being investigated , yet closely enough related to maximize the number of H proteins.

A problem shared with SGE and various other biochemical techniques is that 2-DGE is not sensitive enough to collect separate data from individuals having microscopic sizes and, when no inbred lines are used, a proper interpretation of biochemical data of mixtures of individuals may be impaired by extensive polymorphisms. However, it has clearly been established that the large majority of the loci assayed with 2-DGE appears monomorphic (7,8,9) which facilitates the recognition of homologies on protein patterns representing a mixture of individuals. In this study the observed low levels of intrapopulation variation of the H proteins (Table 1) are also inherent in the conservative nature of the H proteins. Furthermore, as shown here, polymorphic H proteins will often also be conspicuous in a mixed homogenate of individuals because of typical properties with regard to protein quantities, shape and color. The formation of distinctive colors after staining with silver is correlated with the amino acid composition (33) and for future research it may be worthwhile to switch to staining methods which enhance the formation of differentiating colors (33, 34). Nothwithstanding aforementioned findings, it can not be excluded that the homologous forms of a given H protein are obscure in one or a few species, but this has no serious consequences, because various types of data manipulation and algorithms can cope with a few omissions.

In sum, it can be concluded that 2-DGE is a suitable method to trace proteins of which the evolution is tuned to the required divergence dates and that analysis of these putative homologous proteins has several theoretical as well as pragmatic advantages over estimating overall genetic distances in studying genealogical relationships. The methodology outlined here preserves the advantages of 2-DGE and is worthy of further investigation in order to evaluate its general

application, because it offers unique possibilities for studying genealogical relationships between organisms, which are difficult to access by other biochemical techniques.

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SUMMARY

The magnitude of the protein divergence strongly suggests that Globodera rostochiensis and G. pallida have experienced hardly any morphological evolution during a time period of millions of years (chapter II). These morphologically nearly indistinguishable potato cyst nematode species are discriminated from one another by 70 % of their proteins revealed by two dimensional gel electrophoresis (2-DGE), which definitively excludes a recent divergence as has been suggested by a number of authors (3,5). The observation that even two families of rodents share more protein spots revealed by 2-DGE (1) than the two potato cyst nematode species implies that morphological evolution is not correlated with protein evolution. This fits the hypothesis that protein differences are accumulated at an approximate constant rate in all species (6) and that organismal evolution proceeds at a variable rate (7,10,11).

A small fraction of the proteins from the potato cyst nematodes appeared to be heat stable. In view of the large genetic distances between G. rostochiensis and G. pallida it is not surprising that also a considerable part of these thermostable proteins isolated from the two species are species specific (chapter III). The relative abundance and simple partial purification of these species specific proteins are suitable properties for developing a serological assay to differentiate the two species. In the near future it should be possible to use such a diagnostic test as an advisory tool. A reliable diagnosis of field populations offers possibilities to optimalize the control by means of resistance.

Because of the high resolution and the large number of gene products resolved by 2-DGE, the number of proteins exhibiting intraspecific variation was in most cases ten-fold higher than revealed with other biochemical techniques used to study the genetic variability of G. rostochiensis and G. pallida (5,9). Another desirable trait of 2-DGE is that differences in allele frequencies can be estimated in a convenient way. The variant protein spots detected by the comparison

of conspecific populations were divided in two classes: isoelectric point variants (IP-variants) and nonisolelectric point variants (NIP-variants) (chapter IV). The IP-variants have the appearance of protein differences caused by amino acid substitutions that change net charge. Corresponding IP-variants were assumed to be encoded by alleles at the same locus and the relative protein quantities were used as a measure for the allele frequencies (chapter V and VI). The NIP-variants are a remaining group for which no proper genetic interpretation is available and may include differences in regulatory genes and modifying genes. The IP-variants are the most reliable characters for estimating affinities between potato cyst nematode populations, because homologous proteins are compared. Relationships based on NIP-variants may also be informative, but should be interpreted with care, because the number of genes involved is unknown and NIP-variants are more difficult to distinguish from artefacts due to, e.g., experimental variations and differences in physiological or developmental stages.

In current breeding programs the number of populations tested in estimating the effectiveness of certain genes for resistance is rather arbitrary and depends mainly on the screening capacity. In chapter V and VI we advanced a strategy for a more rational introduction of new genes for resistance by using genetic relationships revealed with 2-DGE as a guidance for a representative survey. It is hypothesized that in the absence of selection by the relevant genes for resistance in Europe, variations in virulence and proteins among European populations are predominantly determined by the same processes: the genetic structures of the initial populations introduced from South America and the occurrence of random genetic drift and gene flow in Europe. Because these processes affect the whole gene pool of a population, genetic similarities revealed by 2-DGE are also reflected at virulence loci, including those not yet resolved by the current pathotype scheme. The feasibility of this approach is demonstrated by the observation that populations which are closely linked after constructing a similarity dendrogram often have the same pathotype designation. It is also apparent that the international pathotype

scheme is incapable of reflecting the genetic diversity introduced into Europe.

Several authors have emphasized the necessity of inferring phylogenies of extant nematode species in order to arrive at a more stable classification (2,4,8). A major constraint in reconstructing phylogenies of congeneric nematode species is the limited number of morphological features. The number of characters can in theory greatly be expanded by using biochemical techniques. In this thesis (Chapter VII) we introduced an effective method to infer phylogenies from 2-DGE protein patterns. Proteins of which the evolutionary rates are tuned to the required divergence dates were traced by taking advantage of the approximate constant rates at which proteins evolve in each lineage. This selection procedure resulted in 10 evolutionary conservative proteins which yielded valuable phylogenetic interpretations for the 7 Heterodera species studied. Starch gel electrophoresis, a standard technique for many organisms, is probably of little value in studying these ancient processes of speciation. Many of the enzymes sampled with starch gel electrophoresis evolve too fast and will reveal no shared evolutionary events between the more distantly related Heterodera species. Although 2-DGE has rarely been applied to phylogenetic problems, its general application should be evaluated. The wide application range with regard to divergence dates and the minute amounts of biological material required are desirable features in studying organisms which are difficult to investigate by other biochemical techniques.

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## SAMENVATTING

In deze samenvatting is getracht de inhoud van het proefschrift en de achtergronden zo weer te geven dat de hoofdlijnen van het onderzoek ook begrijpelijk zijn voor niet-ingewijden. Voor een vaktechnische weergave wordt verwezen naar de Engelse samenvatting.

In Nederland wordt jaarlijks ongeveer 150.000 hectare beteeld met aardappelen. Voor de bestrijding van aardappelcysteaaltjes, de veroorzakers van aardappelmoeheid, zijn een aantal wettelijke maatregelen van kracht. Bij een intensieve teelt dient de grond chemisch ontsmet te worden en is de teelt van resistente aardappelrassen verplicht. Ieder jaar wordt ongeveer 40.000 hectare behandeld met dichloorpropeen of methylisothiocyanaat. Het aandeel van deze bodemfumigantia bedraagt, uitgedrukt in volume, ongeveer 60% van alle in Nederland toegepaste bestrijdingsmiddelen. In de afgelopen jaren is ook veel geïnvesteerd in de resistentieveredeling. Vanaf de zestiger jaren zijn er rassen met verschillende vormen van resistentie op de markt verschenen. Ondanks deze inspanningen weet het aardappelcysteaaltje zich te handhaven en in veel gevallen neemt de besmettingsgraad zelfs toe. De teelt van resistente rassen levert helaas niet altijd het gewenste resultaat. Op veel percelen in Nederland komen populaties van het aardappelcysteaaltje voor waartegen de in de aardappelrassen aanwezige resistentie onvoldoende werkzaam is. Een van de problemen voor een optimaal gebruik van resistentie is dat de huidige methoden ontoereikend zijn om de verschillende typen van het aardappelcysteaaltje in een vroeg stadium te herkennen. Het onderzoek weergegeven in dit proefschrift heeft zich voornamelijk geconcentreerd op de mogelijkheden om de karakterisering van populaties van het aardappelcysteaaltje te optimaliseren.

Aardappelcysteaaltjes zijn afkomstig uit Zuid-Amerika en zijn vermoedelijk na 1850 in Europa geïntroduceerd. Er zijn twee nauw verwante soorten van het aardappelcysteaaltje bekend, Globodera rostochiensis en G. pallida. Deze soorten zijn met de lichtmicroscoop op basis van morfologische kenmerken niet of nauwelijks van elkaar te

onderscheiden en werden tot 1973 beschouwd als één soort. Populaties van beide soorten zijn onder te verdelen in een aantal groepen (pathotypen) op grond van hun vermogen (virulentie) en onvermogen (avirulentie) zich te vermeerderen op een serie aardappelplanten met verschillende vormen van resistentie ('differentials'). In het huidige pathotypenschema, gebaseerd op vijf 'differentials', worden in Europa acht pathotypen onderscheiden: vijf binnen G. rostochiensis ($Ro_1$, $Ro_2$, $Ro_3$, $Ro_4$ en $Ro_5$) en drie binnen G. pallida ($Pa_1$, $Pa_2$ en $Pa_3$). Deze pathotypen waren reeds aanwezig voordat de relevante resistente aardappelrassen in Europa werden verbouwd en zijn dus niet het gevolg van aanpassing aan in Europa geteelde resistente rassen. Deze variaties in virulentie reflecteren de genetische samenstelling van de initiële populaties. Het vermoeden bestaat dat deze introducties een aantal malen onafhankelijk van elkaar hebben plaats gevonden. In Engeland wordt van G. rostochiensis alleen het pathotype $Ro_1$ aangetroffen, terwijl meerdere pathotypen voorkomen in Nederland ($Ro_1$, $Ro_2$, $Ro_3$, $Ro_4$) en West Duitsland ( $Ro_1$, $Ro_2$, $Ro_5$). Dergelijke gegevens geven echter geen uitsluitsel over het aantal verschillende introducties. Het pathotypenschema is te onnauwkeurig om de genetische diversiteit van het aardappelcysteaaltje in Europa te weerspiegelen.

Biochemische methoden bieden perspectief voor een nauwkeurige karakterisering van aardappelcysteaaltjes. Met behulp van een gevoelige tweedimensionale eiwitscheidingstechniek (elektroforese) is het mogelijk G. rostochiensis en G. pallida te onderscheiden alsmede populaties binnen een soort. In principe is het mogelijk meer dan 700 verschillende eiwitten (polypeptiden) van het aardappelcysteaaltje zichtbaar te maken (hoofdstuk IV). Voor een eiwitpatroon is ongeveer $35 \times 10^{-6}$ gram eiwit nodig, wat overeenkomt met ongeveer 35 vrouwtjes van het aardappelcysteaaltje. Eiwitextracten voor elektroforese worden gemaakt door aardappelcysteaaltjes fijn te malen. Een dergelijk extract bevat een mengsel van verschillende polypeptiden, zoals enzymen uit de citroenzuurcyclus en eiwitten uit het spierweefsel. Onder invloed van een elektrisch veld worden de polypeptiden in de eerste dimensie gescheiden op lading (iso-elektrisch punt) en in de tweede dimensie op grootte (molecuulgewicht). Een eiwitvlekje bestaat

uit één polypeptide en is het product van één gen. De positie van een polypeptide wordt bepaald door de som van de molecuulgewichten en de ladingen van de 20 verschillende aminozuren waaruit het is opgebouwd. Het totale aantal aminozuren in polypeptiden varieert ongeveer van 60 tot 600. Indien een mutatie optreedt, waardoor een van de aminozuren vervangen wordt door een ander aminozuur, kan dit een verandering in lading tot gevolg hebben. Niet alle veranderingen in de aminozuurvolgorde geven een verschuiving in iso-elektrisch punt te zien, want slechts 5 van de 20 aminozuren hebben een lading. Deze mutaties hebben nauwelijks invloed op het molecuulgewicht van het eiwit, omdat de molecuulgewichten van de 20 aminozuren onderling weinig verschillen.

Fundamenteel onderzoek naar de evolutie van eiwitten heeft aangetoond dat de functie van eiwitten meestal niet beïnvloed wordt door veranderingen in de aminozuurvolgorde. Zo komen in het dierenrijk vele vormen van hemoglobine voor die afstammen van één voorouder-hemoglobine. Ondanks de grote verschillen in de aminozuurvolgorde die in loop van miljoenen jaren zijn ontstaan, hebben deze vormen dezelfde functie in alle dieren. De snelheid waarmee de aminozuurvolgorde van een eiwit verandert, is constant per tijdseenheid. Deze constante snelheid is niet voor alle eiwitten gelijk, maar hangt nauw samen met de functie van een eiwit. In sommige eiwitten treedt bijvoorbeeld eens per 20 miljoen jaar een verandering op en in andere eens per half miljoen jaar. Eiwitten kunnen dan ook gebruikt worden als een biologische klok. Het verschil in eiwitsamenstelling tussen twee soorten is een maat voor de tijd die verstreken is sinds deze soorten hun laatste gemeenschappelijke voorouder hebben gehad. Daarom is het verschil tussen soorten altijd groter dan tussen populaties binnen een soort.

Variaties in eiwitten zijn niet alleen geschikt om soorten of populaties binnen een soort te onderscheiden, maar ook om de mate van verwantschap te bepalen. Voor het schatten van verwantschap geven alleen die eiwitten informatie waarvan een bepaalde vorm wel in de ene groep voorkomt maar niet in een andere groep. Een eiwit dat snel

verandert, is daarom niet bruikbaar voor het bepalen van verwantschap tussen soorten, omdat alle vormen van dat eiwit soortspecifiek zullen zijn. Snel veranderende eiwitten zijn meer geschikt voor het bepalen van de verwantschap tussen populaties binnen een soort en langzaam veranderende eiwitten voor het bestuderen van soorten. Het voordeel van tweedimensionale elektroforese boven andere technieken is gelegen in het feit, dat enkele honderden eiwitten met uiteenlopende functies op een relatief simpele wijze bestudeerd kunnen worden. Hierdoor is de kans groot dat met tweedimensionale elektroforese eiwitten gevonden worden met de gewenste evolutiesnelheid.

Analyse van de eiwitpatronen gaf meer dan honderd eiwitten te zien die verschillen tussen G. pallida en G. rostochiensis. Ongeveer 70 % van de eiwitten zijn soortspecifiek (hoofdstuk II). Dergelijke grote verschillen werden ook gevonden tussen Heterodera schachtii en H. glycines (60 %), terwijl deze soorten op basis van morfologische kenmerken evenmin niet of nauwelijks te onderscheiden zijn. Uit literatuurgegevens blijkt dat deze verschillen in eiwitsamenstelling zelfs groter zijn dan tussen twee families van knaagdieren (50 %). Hieruit kan men afleiden dat G. rostochiensis en G. pallida miljoenen jaren geleden hun laatste gemeenschappelijke voorouder hebben gehad en dat beide soorten gedurende die tijd morfologisch nauwelijks veranderd zijn. Een langzame evolutie van morfologische kenmerken is niet uitzonderlijk voor lagere diersoorten. Kikkers zijn ongeveer 300 miljoen jaar geleden ontstaan, terwijl de variatie in morfologische kenmerken bijzonder gering is. Een snelle evolutie van morfologische kenmerken wordt aangetroffen bij de zoogdieren. Binnen een tijdsbestek van ongeveer 100 miljoen jaar hebben zich de geit, de koe, de wolf, de vleermuis en de walvis ontwikkeld.

Uit larven van G. rostochiensis en G. pallida zijn op eenvoudige wijze een aantal soortspecifieke polypeptiden geïsoleerd, die vermoedelijk slechts in een paar aminozuren van elkaar verschillen (hoofdstuk III). Deze eiwitten zijn bruikbaar voor het ontwikkelen van een toets om G. rostochiensis en G. pallida te onderscheiden met behulp van monoclonale antilichamen. Met een dergelijke serologische

toets kunnen in korte tijd duizenden grondmonsters worden getest op de aanwezigheid van G. rostochiensis of G. pallida. Een snelle soortsidentificatie van veldpopulaties maakt het in principe mogelijk om resistentie werkzaam tegen één van beide soorten optimaal te gebruiken.

Door eiwitpatronen van populaties te vergelijken zijn binnen G. rostochiensis 27 eiwitvlekjes gevonden die variëren en binnen G. pallida 73. De kwalitatieve verschillen in eiwitsamenstelling tussen populaties binnen een soort zijn meestal kleiner dan 7% (hoofdstuk IV, V en VI). Opvallend is dat bijna alle bestudeerde populaties, 19 van G. rostochiensis en 25 van G. pallida, van elkaar verschillen. In hoofdstuk V en VI wordt aangegeven dat deze variaties tussen Europese populaties vermoedelijk het gevolg zijn van drie processen: 1) de verschillende initiële populaties die geïntroduceerd zijn vanuit Zuid-Amerika, 2) 'random genetic drift' en 3) 'gene flow'. Mutatie en selektie hebben vermoedelijk nauwelijks een rol gespeeld (hoofdstuk V en VI). Alle eiwitvormen waren reeds aanwezig in de initiële populaties en de variaties tussen Europese populaties kunnen dan ook grotendeels verklaard worden door de verschillende herkomsten van de initiële populaties. Het tweede proces, 'random genetic drift', hangt samen met de verspreiding van de nakomelingen van de initiële populaties over Europa. In een populatie komen vaak meerdere vormen (allelen) van een eiwit voor en als een klein aantal aaltjes, bijvoorbeeld 20, een nieuw perceel besmet, is de kans groot dat enkele eiwitvormen uit de ouderpopulatie niet meer voorkomen in de nieuwe populatie. Het effect van 'random genetic drift' is te verwaarlozen indien meer dan 500 aaltjes een nieuw perceel besmetten. Evenmin treden verschuivingen op in geval maar één eiwitvorm in een populatie aanwezig is. Het derde proces, 'gene flow', vindt plaats wanneer individuen van de ene naar de andere populatie getransporteerd worden, waardoor de nakomelingen van verschillende introducties gemengd worden. 'Random genetic drift' en 'gene flow' resulteren in veranderingen tussen de opeenvolgende generaties en zijn waarschijnlijk de verklaring voor het feit dat het merendeel van de onderzochte populaties van elkaar verschillen. Slechts twee

populaties blijken identiek te zijn. Daarnaast komen binnen G. rostochiensis en G. pallida veel populaties voor die slechts in een klein aantal eiwitten van elkaar verschillen. Deze populaties vormen vaak duidelijk afgebakende groepen die gekenmerkt worden door specifieke eiwitvormen.

Het aantal pathotypen dat onderscheiden wordt is een afspiegeling van het aantal verschillende vormen van resistentie dat wordt getoetst. In het verleden werd maar één vorm van resistentie gebruikt en er werden dan ook maar twee pathotypen gevonden: populaties die zich wel en niet konden vermeerderen op aardappelplanten met resistentie afkomstig van Solanum tuberosum ssp. andigena. Thans worden er vijf pathotypen binnen G. rostochiensis onderscheiden en drie binnen G. pallida. Vanuit de resistentieveredeling zijn er aanwijzingen dat een aantal van deze pathotypen gesplitst zullen worden in 'nieuwe' pathotypen indien nieuwe vormen van resistentie in het pathotypenschema worden opgenomen. Variaties in eiwitpatronen kunnen een belangrijke bijdrage leveren aan het voorspellen van deze verborgen variaties in virulentie. Evenals variaties in eiwitten, zijn deze variaties in virulentie het resultaat van de genetische samenstelling van de initiële populaties en de effecten van 'random genetic drift' en 'gene flow'. Aangezien deze processen alle eigenschappen van een populatie beïnvloeden, kan verwantschap op basis van eiwitpatronen gebruikt worden als een indicatie voor de variatie in virulentie tussen populaties. Populaties die een grote ongelijkheid vertonen in eiwitsamenstelling komen vermoedelijk uit verschillende gebieden in Zuid-Amerika en de kans is dan ook groot dat zij verschillend zullen reageren op bepaalde vormen van resistentie. Populaties met identieke of bijna identieke eiwitpatronen zullen hetzelfde reageren op alle vormen van resistentie, omdat zij nauw verwant zijn qua afstamming. Deze benaderingswijze is alleen van toepassing op die vormen van virulentie waarvan de corresponderende vormen van resistentie niet aanwezig zijn in frequent geteelde aardappelrassen. De meeste eiwitten die geanalyseerd worden met elektroforese, houden geen verband met virulentie. Daarom zal de eiwitsamenstelling van Europese populaties die zich aanpassen aan

nieuwe vormen van resistentie, nauwelijks veranderen (hoofdstuk V en VI).

Resistente rassen zijn bijzonder effectief in de bestrijding van aardappelcysteaaltjes, omdat avirulente populaties zich slechts langzaam aanpassen aan nieuwe vormen van resistentie. Een moeilijkheid is echter het opsporen van resistentie die werkzaam is tegen een groot deel en zo mogelijk alle populaties van aardappelcysteaaltjes in Europa. De laatste jaren zijn vele honderden wilde aardappelplanten verzameld in Zuid-Amerika en getoetst op de aanwezigheid van geschikte resistentie. Een probleem voor de veredeling is echter hoeveel en welke van de duizenden populaties van aardappelcysteaaltjes uit een gebied getoetst moeten worden om een representatief beeld te verkrijgen van de effectiviteit van een bepaalde vorm van resistentie. Bij een niet-representatieve steekproef is de kans groot dat 12 tot 15 jaar veredelingswerk niet het gewenste resultaat oplevert, dat wil zeggen teveel akkerbouwers hebben populaties in de grond waartegen de nieuwe resistente aardappelrassen niet werkzaam zijn. Door een groot aantal populaties uit een gebied te karakteriseren met tweedimensionale elektroforese en door gebruik te maken van geavanceerde rekenprogramma's is het in principe mogelijk om een schatting te maken van het aantal verschillende introducties. Op basis hiervan kunnen de populaties uit een gebied ingedeeld worden in groepen (hoofdstuk V en VI). Deze indeling kan dan als leidraad gebruikt worden om nieuwe vormen van resistentie te testen. Populaties binnen een groep zullen vergelijkbaar reageren op een bepaalde vorm van resistentie, omdat zij qua afstamming nauw verwant zijn. Door een klein aantal vertegenwoordigers van elke groep te testen is het mogelijk om op een efficiënte manier geschikte vormen van resistentie op te sporen.

Cysteaaltjes zijn een belangrijke groep van gespecialiseerde planteparasieten en komen onder andere voor op bieten, sojabonen, grassen, haver, gerst, erwten, hop en peen. Het verschil in eiwitsamenstelling tussen deze Heterodera soorten is bijzonder groot (hoofdstuk VII). Meer dan 95 % van de eiwitten van het erwte-

cysteaaltje en het sojabonencysteaaltje zijn verschillend. De verwantschap tussen de soorten is bepaald aan de hand van 10 langzaam evoluerende eiwitten. Zo blijkt het koolcysteaaltje nauwer verwant te zijn aan het sojabonencysteaaltje dan aan het hopcysteaaltje. Dergelijke verwantschappen zijn moeilijk vast te stellen aan de hand van morfologische kenmerken en tweedimensionale elektroforese kan dan ook een belangrijke bijdrage leveren aan de systematiek van nematoden.

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