# Molecular evidence that *Meloidogyne hapla*, *M. chitwoodi*, and *M. fallax* are distinct biological entities

Johan G. VAN DER BEEK[*], Rolf FOLKERTSMA[**], Leo M. POLEIJ[*], Paul H. G. VAN KOERT[**], and Jaap BAKKER[**]

[*] *DLO-Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands and* [**] *Agricultural University Wageningen (LUW), Department of Nematology, P.O. Box 8123, 6700 ES Wageningen, The Netherlands.*

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**Summary** – Six isolates of *Meloidogyne hapla*, including four race A and two race B isolates, eight isolates of *M. chitwoodi*, and five isolates of *M. fallax* were submitted to two-dimensional gel electrophoresis (2-DGE) to study the similarity between the various isolates of the three species based upon total soluble proteins. For each isolate, two independent samples of 50 young egg-producing females were extracted from roots. Each sample was used to produce one mini 2-D gel. Mini 2-DGE showed an average of 400 protein spots per gel. Within each species, every gel was compared to all the others to identify two types of polymorphic spots: isoelectric point (IP) variants and present-absent (PA) variants. Thirteen PA and nine IP variants were observed among *M. hapla* isolates, eight PA and no IP variants among *M. chitwoodi* isolates, and two PA and no IP variants among *M. fallax* isolates, which represents 5.0, 2.2, and 0.6 % polymorphisms among isolates of the three respective species. These percentages imply small intraspecific variation within each of these species. They also confirm these species to be clearly delineated biological groups, as similarity between these species is significantly lower than similarity between isolates within each species (Van der Beek *et al.*, unpubl.). Using UPGMA, the nine IP variants of *M. hapla* resulted in a similarity dendrogram, which clearly separated the race A isolates from the race B isolates.

**Résumé – *Données moléculaires démontrant que* Meloidogyne hapla, M. chitwoodi *et* M. fallax *sont des entités biologiques distinctes*** – Six isolats de *Meloidogyne hapla* - quatre de la race A et deux de la race B -, huit de *M. chitwoodi* et cinq de *M. fallax* ont été soumis à une électrophorèse bidimensionnelle sur gel (2-DGE) afin d'étudier la similarité intraspécifique des isolats appartenant à chacune des trois espèces, ce basé sur les protéines solubles totales. Pour chaque isolat, deux échantillons distincts de 50 jeunes femelles gravides ont été extraits des racines. Chaque échantillon a été utilisé pour réaliser un mini-gel bidimensionnnel. Les mini-DGE révèlent une moyenne de 400 spots protéiniques par gel. A l'intérieur de chaque espèce, tous les gels ont été comparés entre eux pour identifier deux types de spots polymorphiques : les variants liés au point isoélectrique (IP), et les variants présents-absents (PA). Ont été observés parmi les isolats de *M. hapla*, treize variants PA et neuf variants IP, pour *M. chitwoodi* huit PA et aucun IP et pour *M. fallax* deux PA et aucun IP, ceci correspondant à un polymorphisme de 5,0, 2,2 et 0,6% pour, respectivement, chacune des trois espèces. Ces valeurs impliquent une très faible variabilité intra-spécifique. Elles confirment également que ces espèces constituent des entités biologiques bien délimitées car la similarité entre ces espèces est significativement plus faible qu'entre isolats de la même espèce (Van der Beek *et al.*, non publié). L'utilisation de l'UPGMA pour les neuf variants IP de *M. hapla* conduit à un dendogramme de similarité séparant nettement les isolats de la race A de ceux de la race B.

**Key-words:** genetic variation, intraspecific variation, phenetics, root-knot nematodes, similarity, total soluble proteins, 2-D gel electrophoresis.

The root-knot nematode species *Meloidogyne hapla*, *M. chitwoodi*, and *M. fallax* are prevalent in agricultural fields. In particular, *M. chitwoodi* and *M. fallax* represent a threat to West-European agriculture because of their wide host range and absence of resistant cultivars for the major crops in the rotation scheme in arable farming. They are diploid, facultative meiotic parthenogenetic species that reproduce either sexually or, in the absence of male gametes, parthenogenetically (Triantaphyllou, 1985; Karssen, 1996), except for *M. hapla* race B which is an obligatory mitotic parthenogenetic species, and of which most isolates are polyploid. Species determination was traditionally based upon a relatively small number of morphological characters, sometimes leading to confusing results because of overlap between species (*e.g.*, Netscher, 1978). This was one of the reasons that no discrimination could be made between *M. chitwoodi* and meiotic parthenogenetic *M. hapla* or other *Meloidogyne* spp. until 1980 (Golden *et al.*, 1980). Also, *M. fallax* was described as a separate species only recently (Karssen, 1996), because of similarity in

morphological characters and host preference with *M. chitwoodi*.

Despite many morphological similarities between the three species, clear differences could be demonstrated for certain stable morphological traits, isozyme phenotypes, and rDNA patterns of ITS regions (Esbenshade & Triantaphyllou, 1987; Eisenback & Hirschmann Triantaphyllou, 1991; Zijlstra *et al.*, 1995; Karssen, 1996). Interspecific variation in protein and DNA composition and morphological characters was demonstrated by Van der Beek *et al.* (unpubl.), showing relatively large genetic distances, ranging from 18 % in protein and 16 % in DNA patterns between *M. hapla* race A and *M. hapla* race B to 32 % in protein and 94 % in DNA patterns between *M. fallax* and *M. incognita*.

To interpret data from resistance and virulence testing, a better understanding of the genetic variation within these species was required. *M. hapla* has been reported as highly variable as shown by haploid chromosome numbers varying from n=13 to 17 and the occurrence of two parthenogenetic reproduction processes in this species (Triantaphyllou, 1966). *M. chitwoodi* also shows variability in chromosome number with haploid numbers ranging from n=14 to 18 (Triantaphyllou, 1985). Additionally, variation in host races was demonstrated in this species (Mojtahedi *et al.*, 1988; Mojtahedi & Santo, 1994).

The present study focussed on intraspecific variation by studying similarities within *M. hapla*, *M. chitwoodi*, and *M. fallax*, using two dimensional gel electrophoresis (2-DGE) of total soluble protein. This technique allowed the examination of approximately 400 protein encoding loci. Investigating intraspecific variation on such a large scale clarified the extent of genetic divergence within these species and allowed comparison with other well defined species. This made it possible to evaluate the validity of the differentiation between *M. hapla*, *M. chitwoodi* and *M. fallax*.

## Material and methods

### Nematode isolates and female extraction

Similarity was studied between six isolates of *Meloidogyne hapla*, eight isolates of *M. chitwoodi*, and five isolates of *M. fallax*. For *M. hapla*, all isolates originated from different sites in the Netherlands (Table 1). Two isolates were of cytological race B. Race A and race B were distinguished by observing meiosis, using Hoechst 33258 for staining the chromosomes (Van der Beek *et al.*, unpubl.). For *M. chitwoodi*, five isolates originated from different sites in the Netherlands and three from the USA (Table 1); they represented the three host races: race 1, race 2 and race 3, which are distinguished by *Daucus carota* cv. Red Cored Chantenay, *Medicago sativa* cv. Thor, and *Solanum bulbocastanum* SB22 (Mojtahedi *et al.*, 1988; Mojtahedi & Santo, 1994). All five *M. fallax* isolates originated from different sites in the Netherlands.

The isolates were maintained and propagated on *Lycopersicon esculentum* cv. Moneymaker and were tested for absence of contaminations with other species by analysing the ITS fragments of the rDNA (Zijlstra *et al.*, 1995). The plants were grown in a growth cabinet at 20 °C, 70 % relative humidity, and 16/8 hours light/dark. Seven weeks after inoculation, infected roots were cut into small pieces and treated with cellulase and a pectolytic enzyme (20 vol. % Celluclast with 20 vol. % Pectinex, both products from Novo Nordisk Ferment Ltd., Switzerland, in 60 vol. % phosphate buffer pH 4.5) for 24 to 48 h at 37 °C. This treatment caused a desintegration of cell walls and females could be collected directly from a sieve with 250 µm mesh or removed from remnants of the roots on the sieve. From each isolate, two independent samples of 50 young, milky white, egg-producing females of similar age were collected. Females were stored dry at –80 ° C until use.

### Protein sample preparation and mini two-dimensional gel electrophoresis of proteins

Two protein samples from a mixture of 50 females per isolate were prepared as described by Bakker and Bouwman-Smits (1988*a*). The protein samples were either stored at –80 °C or immediately used for electrophoresis. Mini two-dimensional gel electrophoresis of proteins (2-DGE) was performed as described by De Boer *et al.* (1992*a*) with some minor modifications (Folkertsma *et al.*, 1996). One gel was produced from each protein sample. For each *Meloidogyne* species, samples were electrophoresed simultaneously, except for *M. hapla* isolate Hbr. For *M. chitwoodi* isolate Cat, only one protein sample was prepared from 50 females, resulting in only one gel. After staining, the gels were dried with a vacuum dryer for long term preservation.

### Data analysis

Two types of variants were identified: isoelectric point (IP) and present-absent (PA) variants (Bakker & Bouwman-Smits, 1988*a*). IP variants are characterized by small differences in isoelectric point, similar molecular weight, and colour similarity when stained with silver (Fig. 1). The vast majority of IP variants are homologous proteins (Goldman *et al.*, 1987). PA variants are expressed by the presence or absence of a protein spot (Fig. 1). Protein profiles were evaluated visually by superimposing dried gels on a bench viewer. Only polymorphisms, observed in both protein profiles of each isolate, were registered. Concen-

***Table 1.*** *Origin of the* Meloidogyne hapla, M. chitwoodi *and* M. fallax *isolates used in this study.*

| Code | Species | Country of origin* | Last host | Sampling year | Obtained from** |
|---|---|---|---|---|---|
| Hc | *M. hapla* A | NL | *Aconicum* | 1990 | PD |
| He | *M. hapla* A | NL | potato | 1992 | PAGV |
| Hj | *M. hapla* A | NL | carrot | 1992 | PAGV |
| Hk | *M. hapla* A | NL | chichory | 1992 | PAGV |
| Hh | *M. hapla* B | NL | fallow (weed) | 1992 | PAGV |
| Hbr | *M. hapla* B | NL | *Veronica* | 1992 | PD |
| Ca | *M. chitwoodi* | NL | maize | 1989 | PD |
| Ck | *M. chitwoodi* | NL | tomato | 1989 | PD |
| Cl | *M. chitwoodi* | NL | wheat | 1990 | PD |
| Co | *M. chitwoodi* | NL | scorzonera | 1993 | PAGV |
| Caq | *M. chitwoodi* | NL | potato | 1993 | PD |
| Cat | *M. chitwoodi* | Washington (USA) | potato | | WSU |
| Cbd | *M. chitwoodi* | Washington (USA) | potato | | WSU |
| Cbh | *M. chitwoodi* | California (USA) | potato | | WSU |
| Fa | *M. fallax* | NL | early primrose | 1992 | PAGV |
| Fb | *M. fallax* | NL | beet | 1991 | PD |
| Fc | *M. fallax* | NL | potato | 1993 | PD |
| Fd | *M. fallax* | NL | potato | 1993 | PD |
| Fe | *M. fallax* | NL | unknown | 1994 | BLGG |

* Country of origin: NL = The Netherlands.

** PD = Plant Protection Service, Wageningen, The Netherlands; PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands; WSU = Washington State University, Prosser WA, USA; BLGG = Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.

trations of the protein spots of the IP variants were estimated by their optical density. For that purpose, the profiles were digitally recorded by image analysis, using MicroGop 2000 software. The density ratio of an IP variant was determined as the quotient of the optical density of that IP variant with the total density of the two or three related spots. It is noted that these density ratios are not interpreted as allele frequencies as was done by Bakker and Bouwman-Smits (1988*a*) for *Globodera* spp., because of the differences in the biology of the *Meloidogyne* spp. used.

Within each species, similarity coefficients (F) between protein patterns were calculated (Aquadro & Avise, 1981) using the formula $2n_{xy}/(n_x + n_y)$, with $n_x$ and $n_y$ being the numbers of spots observed in gel x and y respectively, and $n_{xy}$ the number of spots common to both gels.

Based upon these polymorphic protein spots, the isolates could be compared to each other for their similarities and subsequently clustered in a similarity dendrogram. The similarity among conspecific isolates, based on IP-variants, was expressed in a matrix, using the SIMINT procedure of NTSYS-pc (Rohlf, 1994) to compute euclidian distance coefficients for quantitative data. The similarity coefficients were used to construct a dendrogram with the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973), using the SAHN and TREE procedure of NTSYS-pc. The PA variants were treated as binary characters. The data were converted to similarity values using the Dice similarity coefficient (Aquadro & Avise, 1981) in the SIMQUAL procedure of NTSYS-pc. Cluster analysis was performed on the resulting similarity matrix using the SAHN procedure of NTSYS. The intraspecific relationships among the conspecific isolates were expressed in a dendrogram using the TREE procedure in NTSYS-pc. Consensus trees were constructed if the dataset allowed the formation of more than one dendrogram, using the CONSENSUS procedure in NTSYS-pc.

## Results

When the genetic differentiation of the six *M. hapla*, the eight *M. chitwoodi*, and the five *M. fallax* isolates

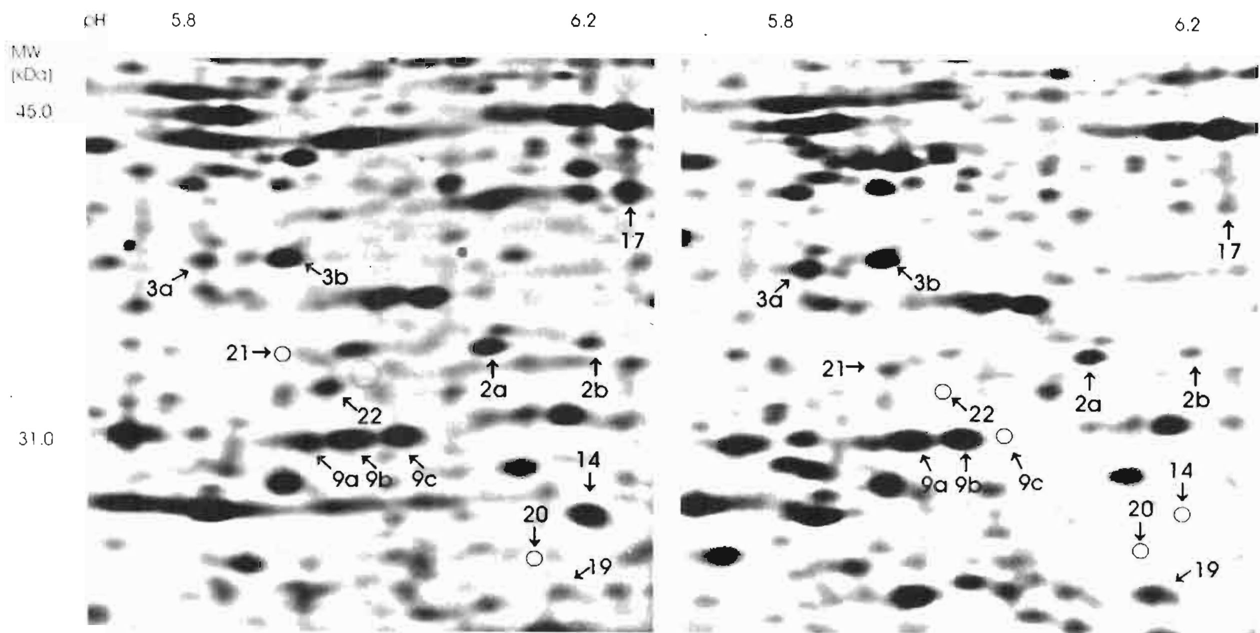


***Fig. 1.*** *Differences in two-dimensional protein extracts between* Meloidogyne hapla *isolate Hj2 (left) and Hk2 (right), showing several PA-variants (numbered 14, 17, 19, 20, 21, 22) and some of the IP-variants (IP2, 3, 9).*

was investigated with 2-DGE of total protein extracts from adult females, the total number of spots on the gels ranged from 360 to 440 with an average of about 410.

*M. HAPLA*

Comparison of the six *M. hapla* isolates revealed 418 monomorphic and 22 polymorphic protein spots, which corresponds to 5.0% polymorphisms (Fig. 2). The similarity coefficient F ranged from 0.97 to 0.99 for this species. Of these 22 polymorphisms, nine were IP-variants (Table 2) and thirteen were PA-variants (Figs 1, 2). A similarity dendrogram was constructed of the nine IP-variants, based on presence-absence data of these IP-variants. In this dendrogram, a clear separation of the *M. hapla* race B isolates from the race A isolates was noticed. When the IP-variants were treated as quantitative data, using the euclidian coefficient, UPGMA resulted in an identical clustering (Fig. 3). Density ratios for the nine IP-variants (Table 2) were used to construct this dendrogram.

Although PA-variants theoritically are less reliable than IP-variants (De Boer *et al.*, 1992*b*), the similarity dendrogram based on the thirteen PA-variants showed similar clustering as for the IP-variants in Fig. 3, except for one isolate.

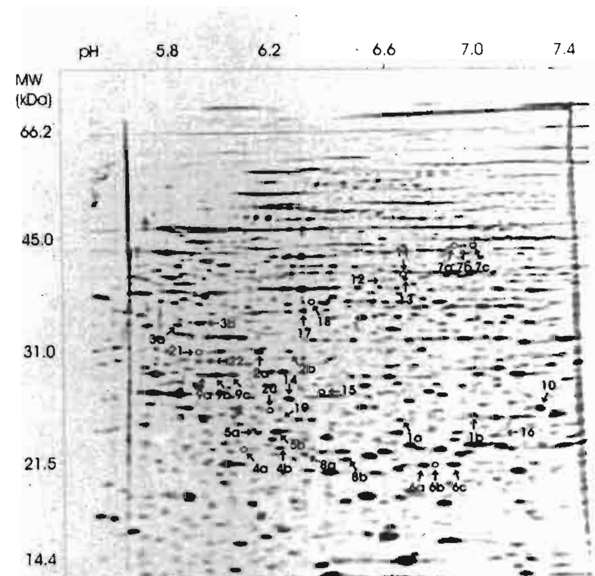


***Fig. 2.*** *Two dimensional gel of total soluble protein extracts from* Meloidogyne hapla *isolate Hj1. Isoelectric point (IP) variants are numbered 1 to 9, with each spot indicated by letters; present/absent (PA) variants are numbered 10 to 22.*

***Table 2.*** *Estimated frequencies of the nine putative isoelectric point variants (IP1 to IP9), found in the six* Meloidogyne hapla *isolates (see Fig. 1). The frequencies are presented as density ratios for each spot and for each of the two samples (1 and 2) of the isolates, as measured by image analysing of protein profiles.*

| IP-variants | Six *M. hapla* isolates (1 and 2 are replicate samples) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 to 9 | Hc1 | Hc2 | He1 | He2 | Hh1 | Hh2 | Hj1 | Hj2 | Hk1 | Hk2 | Hbr1 | Hbr2 |
| IP1a | 12 | 17 | 0 | 0 | 27 | 19 | 16 | 17 | 31 | 17 | 31 | 20 |
| IP1b | 88 | 83 | 100 | 100 | 73 | 81 | 84 | 83 | 69 | 83 | 69 | 80 |
| IP2a | 79 | 79 | 100 | 82 | 40 | 41 | 89 | 89 | 87 | 93 | 70 | 56 |
| IP2b | 21 | 21 | 0 | 18 | 60 | 59 | 11 | 11 | 13 | 7 | 30 | 44 |
| IP3a | 44 | 29 | 43 | 52 | 9 | 24 | 42 | 20 | 62 | 56 | 33 | 92 |
| IP3b | 56 | 71 | 57 | 48 | 91 | 76 | 58 | 80 | 38 | 44 | 67 | 8 |
| IP4a | 0 | 0 | 0 | 0 | 60 | 86 | 0 | 0 | 0 | 0 | 45 | 45 |
| IP4b | 100 | 100 | 100 | 100 | 40 | 14 | 100 | 100 | 100 | 100 | 55 | 55 |
| IP5a | 0 | 0 | 10 | 8 | 0 | 0 | 13 | 0 | 19 | 0 | 0 | 0 |
| IP5b | 100 | 100 | 90 | 92 | 100 | 100 | 87 | 100 | 81 | 100 | 100 | 100 |
| IP6a | 0 | 0 | 0 | 0 | 44 | 52 | 0 | 0 | 0 | 0 | 0 | 0 |
| IP6b | 32 | 38 | 39 | 41 | 0 | 0 | 48 | 46 | 0 | 0 | 41 | 39 |
| IP6c | 68 | 62 | 61 | 59 | 56 | 48 | 52 | 54 | 100 | 100 | 59 | 61 |
| IP7a | 0 | 0 | 0 | 0 | 17 | 42 | 0 | 0 | 0 | 0 | 100 | 100 |
| IP7b | 100 | 100 | 100 | 100 | 0 | 0 | 100 | 100 | 100 | 100 | 0 | 0 |
| IP7c | 0 | 0 | 0 | 0 | 83 | 58 | 0 | 0 | 0 | 0 | 0 | 0 |
| IP8a | 49 | 37 | 47 | 51 | 70 | 55 | 48 | 49 | 51 | 68 | 60 | 65 |
| IP8b | 51 | 63 | 53 | 49 | 30 | 45 | 52 | 51 | 49 | 32 | 40 | 35 |
| IP9a | 14 | 19 | 30 | 16 | 13 | 18 | 10 | 15 | 44 | 54 | 6 | 20 |
| IP9b | 44 | 43 | 45 | 46 | 38 | 42 | 47 | 45 | 56 | 46 | 41 | 36 |
| IP9c | 42 | 38 | 25 | 38 | 49 | 40 | 43 | 40 | 0 | 0 | 53 | 44 |

### *M.* CHITWOODI

Among the eight *M. chitwoodi* isolates, 368 monomorphic and eight polymorphic protein spots were observed (Fig. 4), which corresponds to 2.2 % polymorphisms. The similarity coefficient F ranged from 0.98 to 1.0 for this species, isolate Ck being least similar to the other isolates. No reliable IP-variants were recorded for *M. chitwoodi*. All eight polymorphic spots for *M. chitwoodi* were PA-variants.

### *M.* FALLAX

Among the five *M. fallax* isolates, 358 monomorphic and two polymorphic protein spots were observed (Fig. 5), which corresponds to 0.6 % polymorphisms. As for *M. chitwoodi*, no reliable IP-variants were recorded for *M. fallax*. The similarity coefficient F ranged from 0.99 to 1.0 for this species. Both polymorphic spots were PA-variants, which were only present on the profiles of isolate Fe. Consequently, no discriminative spots were found on the profiles of the remaining four *M. fallax* isolates.

## Discussion

2-DGE was applied to isolates of *M. hapla*, *M. chitwoodi*, and *M. fallax* by comparing from 360 to 440 protein spots per isolate, which are numbers large enough for estimating the overall genetic differentiation. The percentages of protein spots polymorphic between isolates of these species were low. The similarity values F ranged from 0.97 to 0.99 within *M. hapla*, from 0.98 to 1.00 within *M. chitwoodi*, and from 0.99 to 1.00 within *M. fallax*. These values are similar to those found between isolates of *Globodera rostochiensis*, *G. pallida*, and *Heterodera glycines* (Bakker & Bouwman-Smits, 1988*b*; Bakker *et al.*, 1992). The small intraspecific variation within these three *Meloidogyne* species is in contrast to the relatively large interspecific variation in protein patterns observed between these species, with a similarity of 0.64 between *M. hapla* race A and *M. chitwoodi*, 0.69 between *M. hapla* race A and *M. fallax*, and 0.74 between *M. chitwoodi* and *M. fallax* (Van der Beek *et al.*, unpubl.). These data show that there is no over-

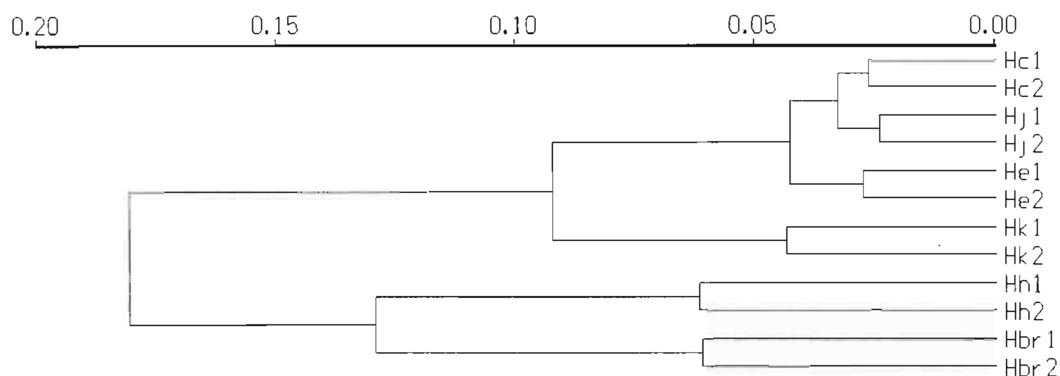


***Fig. 3.*** *Similarity dendrogram of six* Meloidogyne hapla *isolates, with each isolate represented by two independant samples. The dendrogram is constructed from the distance matrix of the nine IP-variants following the UPGMA method using the euclidian coefficient of similarity.*

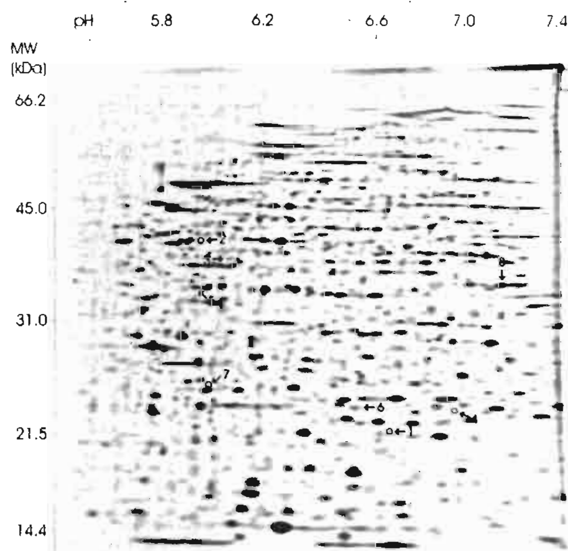


***Fig. 4.*** *Two dimensional gel of total soluble protein extracts from* Meloidogyne chitwoodi *isolate Ca1. PA-variants are numbered 1 to 8.*

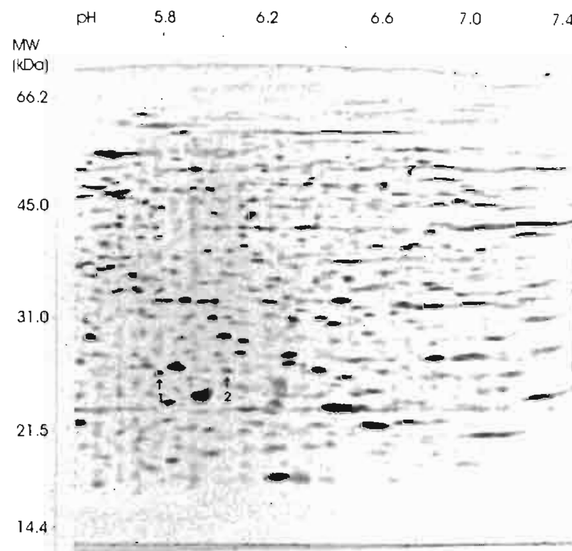


***Fig. 5.*** *Two dimensional gel of total protein extracts from* Meloidogyne fallax *isolate Fe1. The two PA-variants are numbered 1 and 2.*

lap between these species. Limited genetic variation exists within these *Meloidogyne* species when compared with the interspecific variation. It is therefore concluded that these species can be considered as distinct biological entities within the genus.

Fig. 3 shows that *M. hapla* race B isolates clustered separately from race A isolates. This agrees with the results obtained by Esbenshade and Triantaphyllou (1987) using isozyme markers with four race A and two race B isolates from different geographical sites. The high similarity between race A and race B indicates genetic resemblance between the two races. The small distances between races A and B would not be a sufficient reason to exclude the occurrence of successful crosses between isolates of these two races of *M. hapla*. However, the apomictic reproduction of race B isolates makes recombination improbable. Despite these small distances, which suggests a recent divergence, the marked difference in modes of reproduction is remarkable.

Although the three species showed small intraspecific variation, considerably more genetic differentia-

tion could be demonstrated in *M. hapla* than in *M. chitwoodi* and in *M. fallax*. In the last two species, a complete lack of IP-variants among the isolates investigated suggests little genetic variation. This difference in variability between *M. hapla* on one side and *M. chitwoodi* and *M. fallax* on the other is also reflected in the number of PA-variants that were observed: thirteen among six *M. hapla* isolates and eight and two among eight *M. chitwoodi* and five *M. fallax* isolates, respectively. In comparing the intraspecific variation observed in *M. hapla* with that in *M. chitwoodi* and *M. fallax*, it is noted that a great portion of the variability within *M. hapla* was due to differences between race A and race B, as demonstrated in Fig. 3. However, after excluding the two *M. hapla* race B isolates Hh and Hbr from the data files, eight of the thirteen PA-variants and five of the nine IP-variants were still discriminating between the remaining four race A isolates. These results suggest that *M. hapla* could still be rather heterogeneous compared to other species. Before 1949, the genus *Meloidogyne* was considered to be a part of the genus *Heterodera* (Chitwood, 1949). In 1949, five *Meloidogyne* species were distinguished by Chitwood (1949) including *M. hapla*. About 30 years later, Golden *et al.* (1980) were able to distinguish *M. chitwoodi* from *M. hapla*, and recently Karssen (1996) managed to separate *M. fallax* from this species complex. Another explanation would be that these species may not be endemic in NW Europe and the gene pool that was introduced from abroad could have been larger for *M. hapla* than for *M. chitwoodi* or *M. fallax*. At present, no data are available on the origin of these species.

The limited genetic variation among isolates of *M. chitwoodi* was remarkable, given the fact that three isolates from the USA were included, representing the three host race-types, and the fact that the five Dutch isolates originated from quite different sites in the Netherlands. These results would suggest the absence of genetic differentiation between populations of this species in the North-Western part of the United States and in the Netherlands. In the Netherlands, *M. chitwoodi* was observed as early as 1930 on potato tubers and 1970 on potato cultivar Woudster (Brinkman & van Riel, 1990).

The similarity among *M. fallax* isolates was even higher than between *M. chitwoodi* isolates. The observed polymorphisms in *M. fallax* were discriminating only between one isolate and the other four. However, because these five isolates originated from a relatively small geographical area, no further inference can be drawn from this extremely high similarity.

The differences within the three *Meloidogyne* spp. revealed by 2-DGE are also reflected in variations in virulence. Previous research on variation in virulence included the same Dutch isolates of *M. hapla*, *M. chitwoodi*, and *M. fallax* (Van der Beek *et al.*, unpubl.), which were tested on genotypes of potato varieties and wild *Solanum* spp. *M. hapla* showed remarkably large variation in virulence among fourteen isolates, while no variation in virulence between eight isolates of *M. chitwoodi* and five of *M. fallax* was found. Altogether these data would support the gene pool similarity concept *i.e.*, degrees of dissimilarity between populations revealed by molecular techniques are also reflected at virulence loci, including those not yet resolved (Bakker *et al.*, 1993). This shows that the differences in intraspecific variation within these three *Meloidogyne* spp. in the Netherlands have important implications for crop protection by means of host plant resistance. Breeding for resistance against *M. chitwoodi* and *M. fallax* is more promising than against *M. hapla*.

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**References**

AQUADRO, C.F. & AVISE, J.C. (1981). Genetic divergence between rodent species assessed by using two dimensional electrophoresis. *Proc. natn. Acad. Sci. USA*, 78: 3784-3788.

BAKKER, J. & BOUWMAN-SMITS, L. (1988*a*). Genetic variation in polypeptide maps of two *Globodera rostochiensis* pathotypes. *Phytopathology*, 78: 894-900.

BAKKER, J. & BOUWMAN-SMITS, L. (1988*b*). Contrasting rates of protein and morphological evolution in cyst nematode species. *Phytopathology*, 78: 900-904.

BAKKER, J., BOUWMAN-SMITS, L. & GOMMERS, F.J. (1992). Genetic relationships between *Globodera pallida* pathotypes in Europe assessed by using two dimensional gel electrophoresis of proteins. *Fundam. appl. Nematol.*, 15: 481-490.

BAKKER, J., FOLKERTSMA, R.T., ROUPPE VAN DER VOORT, J.N.A.M., DE BOER, J. & GOMMERS, F.J. (1993). Changing concepts and molecular approaches in the management of virulence genes in potato cyst nematodes. *A. Rev. Phytopath.*, 31: 169-190.

BRINKMAN, H. & VAN RIEL, H.R. (1990). *Meloidogyne chitwoodi* - maiswortelknobbelaaltje. *In*: *Jaarboek 1989/1990*. Direktie Gewasbescherming, Plantenziektenkundige Dienst, Wageningen: 146-155.

CHITWOOD, B.G. (1949). Root-knot nematodes - Part I. A revision of the genus *Meloidogyne* Goeldi, 1887. *Proc. helminth. Soc. Wash.*, 16: 90-104.

DE BOER, J.M., OVERMARS, H.A., BAKKER, J. & GOMMERS, F.J. (1992*a*). Analysis of two-dimensional protein

patterns from developmental stages in the potato cyst-nematode, *Globodera rostochiensis*. *Parasitology*, 105: 461-474.

DE BOER, J.M., OVERMARS, H.A., BOUWMAN-SMITS, L., DE BOEVERE, M., GOMMERS, F.J. & BAKKER, J. (1992*b*). Protein polymophisms within *Globodera pallida* assessed with mini two-dimensional gel electrophoresis of single females. *Fundam. appl. Nematol.*, 15: 495-501.

EISENBACK, J.D. & HIRSCHMANN TRIANTAPHYLLOU, H. (1991). Root-knot nematodes: *Meloidogyne* species and races. *In*: Nickle, W.R. (Ed.). *Manual of agricultural nematology*. New York, USA, Marcel Dekker: 191-271.

ESBENSHADE, P.R. & TRIANTAPHYLLOU, A.C. (1987). Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). *J. Nematol.*, 19: 8-18.

FOLKERTSMA, R.T., DE GROOT, K.E., VAN KOERT, P.H.G., VAN GENT-PELZER, M.P.E., ROUPPE VAN DER VOORT, J.N.A.M., SCHOTS, A., BAKKER, J., GOMMERS, F.J. & HELDER, J. (1996). Cluster analysis of 36 *Globodera pallida* field populations using two sets of molecular markers. *Europ. J. Pl. Path.*, 102: 577-584.

GOLDEN, A.M., O'BANNON, J.H., SANTO, G.S. & FINLEY, A.M. (1980). Description and SEM observations of *Meloidogyne chitwoodi* n. sp. (Meloidogynidae), a root-knot nematode on potato in the Pacific Northwest. *J. Nematol.*, 12: 319-327.

GOLDMAN, D., RATHNA GIRI, P. & O'BRIEN, S.J. (1987). A molecular phylogeny of the hominoid primates as indicated by two-dimentsional protein electrophoresis. *Proc. natn. Acad. Sci. USA*, 84: 3307-3311.

KARSSEN, G. (1996). Description of *Meloidogyne fallax* n.sp. (Nematoda: Heteroderidae), a root-knot nematode from the Netherlands. *Fundam. appl. Nematol.*, 19: 593-599.

MOJTAHEDI, H. & SANTO, G.S. (1994). A new host of *Meloidogyne chitwoodi* from California. *Pl. Dis.*, 78: 100.

MOJTAHEDI, H., SANTO, G.S. & WILSON, J.H. (1988). Host test to differentiate *Meloidogyne chitwoodi* races 1 and 2 and *M. hapla*. *J. Nematol.*, 20: 468-473.

NETSCHER, C. (1978). *Morphological and physiological variability of species of* Meloidogyne *in West Africa and implications for their control*. Wageningen, The Netherlands, Veenman: 47 p.

ROHLF, F.J. (1994). *NTSYS-pc, numerical taxonomy and multivariate analysis system*. Exeter Software, New York, 245 p.

SNEATH, P.H.A. & SOKAL, R.R. (1973). *Numerical taxonomy: The principles and practices of numerical taxonomy*. San Francisco, CA, USA, Freeman, W.H. & Co., 573 p.

TRIANTAPHYLLOU, A.C. (1966). Polyploidy and reproductive patterns in root-knot nematode *Meloidogyne hapla*. *J. Morph.*, 118: 403-414.

TRIANTAPHYLLOU, A.C. (1985). Cytogenetics, cytotaxanomy and phylogeny of root-knot nematodes. *In*: Sasser, J.N. & Carter, C.C. (Eds). *An advanced treatise on* Meloidogyne. *Volume I: Biology and control*. Raleigh, NC, USA, North Carolina State University Graphics: 113-126.

ZIJLSTRA, C., LEVER, A.E.M., UENK, B.J. & VAN SILFHOUT, C.H. (1995). Differences between ITS regions of isolates of the root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology*, 85: 1231-1237.