Fundam. appl. Nematol., 1998, **21** (4), 401-411

Genetic variation among parthenogenetic *Meloidogyne* species revealed by AFLPs and 2D-protein electrophoresis contrasted to morphology

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

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

Accepted for publication 27 April 1998.

Summary – Isolates of the ameiotic parthenogenetic species *Meloidogyne arenaria*, *M. hapla* race B, *M. incognita*, and *M. javanica* and of the meiotic parthenogenetic species *M. chitwoodi*, *M. fallax*, *M. hapla* race A, and *M. naasi* were compared for total soluble proteins using two-dimensional gel electrophoresis (2-DGE), total genomic DNA using electrophoresis of amplified fragment length polymorphisms (AFLP), and morphological characters by morphometric measurements and from literature. Data sets were converted to similarity coefficients using the Dice coefficient, based on more than 100 protein spots, 192 AFLP fragments, and 21 morphological characters. UPGMA dendrograms based on protein and DNA data were congruent: with both types of data, *M. hapla* race A and race B clustered together, the three tropical species *M. incognita*, *M. javanica*, and *M. arenaria* formed another cluster, and the species specialized on Gramineae –*M. naasi*, *M. chitwoodi*, and *M. fallax*– were distant from the rest, with high similarity between *M. chitwoodi* and *M. fallax*. The dendrogram for morphological data was different from the dendrograms for molecular data, particularly for *M. incognita* and *M. naasi*. The reasons for this discrepancy between protein and DNA studies on the one hand and morphological studies on the other hand are discussed. © Orstom/Elsevier, Paris

Résumé – ***Variabilité génétique des espèces parthénogénétiques de* Meloidogyne *telle que révélée par l'AFLP et l'électrophorèse des protéines en deux dimensions en désaccord avec les données morphologiques*** – Des isolats appartenant aux espèces parthénogénétiques améiotiques *Meloidogyne arenaria*, *M. hapla* race B, *M. incognita*, *M. javanica* et parthénogénéiques méiotiques *M. chitwoodi*, *M. fallax*, *M. hapla* race A, *M. naasi* ont été comparés quant à leurs protéines totales solubles -par électrophorèse sur gel en deux dimensions (2-DGE)-, leur ADN génomique total -par AFLP- et leurs caractères morphologiques- par mensurations directes et données provenant de la littérature. Ces données ont été transformées en coefficients de similarité en utilisant le coefficient Dice basé sur plus de 100 spots protéiniques, 192 fragments d'AFLP et 21 caractères morphologiques. Les dendogrammes, établis suivant la méthode UPGMA basés sur les données fournies par les protéines et l'ADN sont congruents. Le groupement des races A et B de *M. hapla* montre une similarité élevée, les trois espèces tropicales *M. incognita*, *M. javanica* et *M. arenaria* forment un autre groupe et les espèces spécialisées envers les graminées, *M. naasi*, *M. chitwoodi*, *M. fallax*, sont distantes des autres, les deux dernières montrant une similarité élevée. Le dendogramme basé sur les données morphologiques diffère de ceux basés sur les données moléculaires, notamment pour *M. incognita* et *M. naasi*. Il est discuté de cette non correspondance entre données provenant des protéines et de l'ADN, d'une part, et données morphologiques, d'autre part. © Orstom/Elsevier, Paris

Keywords: AFLP, electrophoresis, *Meloidogyne*, nematodes, phenetics, proteins, similarity, systematics.

The genus *Meloidogyne* comprises many plant-parasitic root-knot nematode species: the review of Eisenback and Hirschmann-Triantaphyllou (1991) included 68 nominal species, the majority of which reproduce by either facultative meiotic or obligate ameiotic (also referred to as mitotic) parthenogenesis. The parthenogenetic *Meloidogyne* spp. are taxonomically difficult to classify, because the biological species concept is difficult to apply. This is particularly true for ameiotic parthenogenetic species, in which individuals are reproductively isolated. In facultative meiotic parthenogenetic species, reproductive isolation has been demonstrated only between *M. chitwoodi* and *M. fallax* (Van der Beek & Karssen, 1997).

Traditionally, species identification in *Meloidogyne* is based on morphological and morphometric characters and host-plant response (Eisenback *et al.*, 1981; Eisenback, 1985). Some of these diagnostic characters show considerable variation, partly due to true genetic differences and partly to environmental influences. Reliable identification is needed as some economically important species are sympatric and polyphagous. For instance, *M. hapla*, *M. chitwoodi*, and *M. fallax* show large overlap in host range, which hampers the use of host preference as a diagnostic tool.

Netscher (1978) and Taylor and Netscher (1979) suggested that, because of the overlap between species, identification should include additional criteria, and various approaches have been introduced during the past decade. These approaches not only allowed a more accurate distinction between species, but also a better understanding of the genetic variability in the genus. Amongst them are: mode of reproduction and chromosome number (Dalmasso & Bergé, 1975; Triantaphyllou, 1985), isozyme pattern (Bergé & Dalmasso, 1975; Esbenshade & Triantaphyllou, 1987, 1990), DNA polymorphisms of mitochondrial DNA (Powers *et al.*, 1986), RFLP of genomic DNA (Carpenter *et al.*, 1992; Castagnone-Sereno *et al.*, 1993; Fargette *et al.*, 1994), RAPD of genomic DNA (Castagnone-Sereno *et al.*, 1994; Fargette *et al.*, 1994), DNA amplification fingerprinting of genomic DNA (Baum *et al.*, 1994), and ITS regions of ribosomal DNA (Zijlstra *et al.*, 1995; Petersen & Vrain, 1996). The application of ITS analysis and isozyme data for systematics is restricted by their use of a limited, non-random conserved part of the available genetic information. Other techniques, such as RFLP analysis, are more suitable for systematics, leading to stable, randomly selected genomic markers, given the application of specific molecular probes. Castagnone-Sereno *et al.* (1993), for example, used 22 RFLP fragments to study the relations between isolates of *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*.

For studies on relatedness between *Meloidogyne* spp., conclusions have so far been based on the variation of one type of characters, *e.g.*, comparison of isozymes, of DNA fragments, or of morphological characters. The present research is aimed at measuring variation at three levels: DNA, protein, and morphology. For this purpose, the potentials of the 2-DGE and the AFLP techniques were studied and combined with morphological classification.

Materials and methods

NEMATODE ISOLATES

This study was based on *M. chitwoodi*, *M. fallax*, *M. naasi*, *M. hapla* race A, *M. hapla* race B, *M. incognita*, *M. javanica*, and *M. arenaria*. The first three species reproduce by facultative meiotic parthenogenesis, the last three by obligate ameiotic parthenogenesis. It is considered that *M. hapla* consists of two cytological races: race A, reproducing by meiotic parthenogenesis, and race B, reproducing by ameiotic parthenogenesis (Triantaphyllou, 1966). For the sake of simplicity, these two races were treated as separate species in this study and in the present article. For each species, one isolate was studied (Table 1), except for *M. hapla* races A and B and *M. chitwoodi*. For these three species, the morphometry was studied on isolates other than those used for 2-D protein and DNA analyses, because the nematode material of the three species was insufficient for all three analyses.

Table 1. *Origin and isozyme phenotypes of the* Meloidogyne *isolates used.*

	Code	Origin*	Original host	Source**	Isozyme phenotype***	
					EST	MDH
M. arenaria	C4393	NL	unknown	PD	N1	A2
M. chitwoodi	Ca	NL	maize	PD	N1A	S2
M. chitwoodi	C5273-C	NL	unknown	PD	N1A	S2
M. fallax	Fb	NL	beet	PD	N1B[1]	F3[1]
M. hapla race A	Hi	NL	immortelle	PAV	H1	H1
M. hapla race A	Xbr	unknown		PD	H1	H1
M hapla race B	Hbr	NL	*Veronica* sp.	PD	H1	H1
M. hapla race B	C2552	unknown	unknown	PD	H1	H1
M. incognita	568.93	unknown	unknown	PD	N1	I1
M. javanica	C3059	China	bonsai	PD	N1	J3
M. naasi	Nb	unknown	unknown	PD	N1A	S1

* NL = The Netherlands.

** PD = Plant Protection Service, Wageningen, The Netherlands; PAV = Applied Research and Field Production of Vegetables, Lelystad, The Netherlands.

*** EST = esterase phenotypes according to Esbenshade and Triantaphyllou (1990); MDH = malate dehydrogenase phenotypes according to Esbenshade and Triantaphyllou (1990).

[1] EST and MDH phenotypes according to Van der Beek and Karssen (1997).

of the diluted reaction mixture was mixed with 50 ng of primer M00 (5-GATGAGTCCTGAGTAA), 50 ng of primer E00 (5-GACTGCGTACCAATTC), 0.4 u *Taq*-polymerase (Boehringer), 2µl of 10x PCR-buffer (Boehringer), and 0.4 µl of 10 mM dNTP in a final volume of 20 µl. PCR was performed for 30 cycles with the following cycle profile: 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. After amplification, 7 µl of the PCR product was visualised on a 1µl agarose gel and, depending on the intensities of the signals observed, the remaining preamplified template was diluted ten to twenty times.

Selective amplification of the previously amplified fragments was performed with a primer corresponding to the *Eco*RI ends, consisting of the E00 sequence with two additional selective nucleotides at its 3' end, and a primer corresponding to the *Mse*I-ends, consisting of the M00 sequence with two additional selective nucleotides at its 3' end. Codes and sequences of primers used are listed in Table 2. The selective *Eco*RI-primer was radioactively end-labelled as described by Vos *et al.* (1995). PCR was performed using 5 ng of labelled selective *Eco*RI-primer, 30 ng of selective *Mse*I-primer, 5 µl of the diluted preamplified template, 0.4 u *Taq*-polymerase (Boehringer), 2 µl of 10x PCR-buffer (Boehringer), and 0.4 µl of 10 mM dNTP in a final volume of 20 µl. AFLP reactions were performed for 35 cycles with the following profile: 30 s denaturing step at 94 °C, 30 s annealing step (see below), and 1 min extension step at 72 °C. The annealing temperature in the first cycle was 65 °C, then reduced by 0.7 °C per cycle for the next twelve cycles, and remained at 56 °C for the last 22 cycles.

Table 2. *Codes and sequences of the AFLP primers used.*

Code	Sequence
E11	5-GACTGCGTACCAATTCAA-3
E19	5-GACTGCGTACCAATTC??-3
M12	5-GATGAGTCCTGAGTAAAC-3
M16	5-GATGAGTCCTGAGTAACC-3

Following amplification, reaction products were mixed with 20 µl of formamide dye, heated for 5 min at 95 °C, and quickly cooled on ice. Three µl of each sample was loaded on a CastAway Precast sequencing gel (Stratagene), then the gels were run using the CastAway Precast sequencing system (Stratagene) and processed according to the manufacture recommendations. Dried gels were exposed to X-rays films at room temperature.

Combinations of primers used were E11M12, E11M16, E19M12, and E19M16. Each experiment was repeated at least once.

DATA ANALYSIS

Protein profiles were evaluated visually by superimposing dried gels on a bench viewer. For each isolate, one of the two protein gels was chosen to be the mastergel, and 100 arbitrary protein spots, present in both gels of this isolate, were indicated. For all gels of each of the other species, the presence or absence of the 100 protein spots indicated on the master gel was evaluated. In this way, one dataset with 100 characters evaluated in fourteen gels of the other isolates was obtained for each isolate. The similarity coefficient *F* in protein patterns among isolates was estimated from these data sets, using the formula $2nxy/(nx + ny)$, where *nx* and *ny* are the number of spots observed for isolates x and y, respectively, and *2nxy* the number of identical spots observed in both patterns. *F* is an estimation of the Dice coefficient for similarity (Aquadro & Avise, 1981). All isolates were thus compared two by two, resulting in two coefficients. The means of these pairs of coefficients were calculated. The resulting similarity matrix of these mean *F* values was used in a cluster analysis by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath & Sokal, 1973). The cophenetic correlation coefficient was computed to evaluate the quality of the cluster analysis. This product-moment correlation coefficient is a measure of the agreement between the original similarity matrix and the similarity values implied by the dendrogram (Sneath & Sokal, 1973). The clusters of the dataset were expressed in a dendrogram.

AFLP autoradiograms were evaluated visually by superimposing dried gels on a bench viewer. On the AFLP autoradiograms, independent sets of equal number of inserts per isolate, present in both lanes, were marked. This resulted in a total of 192 markers. Presence of the 192 inserts in other isolates was scored affirmative only if present in both lanes, leading to a dataset of 192 characters. The resulting matrix was treated as described above, resulting in a dendrogram.

The morphometric data for juvenile characters were expressed as ratios of the body length, to obtain as much independence as possible among these measurements. Then, each of the nine characters were grouped into three categories, resulting in a binary matrix with eighteen markers. Together with the twelve morphological characters, the combined matrix consisted of 30 markers and was treated as described above, resulting in a dendrogram.

Genetic distances were estimated by the calculation of *1-F*.

Results

2-DGE OF TOTAL PROTEIN EXTRACTS

An average of 400 proteins was resolved per gel. For each isolate, 100 randomly selected spots were compared to the other isolates. 2-DGE protein patterns discriminated clearly between the species. Mean *F*-values of the two similarity coefficients of each comparison between two species are shown in Table 3. These *F*-values range from 0.99 (between *M. hapla* race A and *M. hapla* race B) to 0.54 (between *M. naasi* and *M. javanica)*. Within the cluster of the so-called tropical species: *M. javanica*, *M. incognita,* and *M. arenaria*, the *F*-values range from 0.87 to 0.81. This coefficient equals 0.86 between the supposedly related *M. fallax* and *M. chitwoodi* and 0.68 between *M. naasi* and the closest species, *M. chitwoodi.* The cophenetic correlation coefficient is 0.92, which indicates a very good fit for the cluster analysis. The dendrogram reveals a clear distinction between the eight isolates (Fig. 1A) and four clusters can be distinguished: *M. javanica*, *M. incognita* and *M. arenaria* in the first cluster, the two races of *M. hapla* in a second cluster, *M. fallax* and *M. chitwoodi* in a third cluster, and finally *M. naasi* alone and showing high dissimilarity with all other isolates.

AFLP-ANALYSIS OF TOTAL DNA

AFLP analysis revealed a total of 192 amplified fragments. None of the fragments was shared by all species. *F*-values of all species combinations are shown in Table 3. These *F*-values range from 0.84 (between *M. hapla* race A and *M. hapla* race B) to 0.06 (between *M. incognita* and *M. fallax)*. The *F*-values range from 0.75 to 0.53 between the three tropical species and from 0.17 to 0.08 between *M. hapla* race A and these tropical species. The related species *M. fallax* and *M. chitwoodi* show an *F*-value of 0.60. The cophenetic correlation coefficient is 0.98, which indicates a very good fit for the cluster analysis. The same main clusters were obtained as with UPGMA analysis of the protein data (Fig. 1B): *M. incognita*, *M. javanica,* and *M. arenaria* in one cluster in a slightly different order, followed by *M. hapla* race A and race B in a second cluster, and finally *M. chitwoodi* and *M. fallax* in a third cluster.

ANALYSIS OF MORPHOMETRIC AND MORPHOLOGICAL DATA

Means of measurements with corresponding standard deviations of the nine morphometric characters for each isolate of the *Meloidogyne* spp. are presented in Table 4. These means are within the range presented by Jepson (1987) and Eisenback and Hirschmann-Triantaphyllou (1991). The species could be distinguished clearly by these nine characters, except for *M. fallax* and *M. hapla* race A. The similarity dendrogram based upon the combined matrix of morphometric and morphological data (Fig. 2), using the Dice coefficient for similarity, shows dissimilarity with the dendrograms based upon the molecular data: *M. incognita* is included in the cluster of *M. chitwoodi* and *M. fallax* instead of clustering with *M. arenaria* and *M. incognita*, and *M. naasi* is far from all of the other species. The cophenetic correlation coefficient is 0.88, which indicates a good fit for the cluster analysis.

Discussion

Overall genetic distances between the *Meloidogyne* species calculated from *F*-values (Table 3) based upon 2-DGE data of total soluble proteins range from 0.20 between *M. incognita* and *M. arenaria* to 0.37 between *M. chitwoodi* and *M. javanica.* These distances between species are large compared to those within species: between isolates of *M. hapla*, *M. chitwoodi*, and *M. fallax*, genetic distances ranged from 0.01 to

Table 3. *F-values based upon Dice similarity coefficients of AFLP data (above diagonal) and of 2-DGE-data (below diagonal) of eight* Meloidogyne *spp.*

	M. incognita	*M. arenaria*	*M. javanica*	*M. hapla* B	*M. hapla* A	*M. naasi*	*M. fallax*	*M. chitwoodi*
M. incognita		0.53	0.75	0.26	0.17	*	0.06	0.13
M. arenaria	0.82		0.67	0.21	0.15	*	0.18	0.16
M. javanica	0.81	0.87		0.17	0.08	*	0.12	0.10
M. hapla B	0.56	0.70	0.67		0.84	*	0.09	0.07
M. hapla A	0.63	0.66	0.62	0.99		*	0.08	0.10
M. naasi	0.58	0.64	0.54	0.61	0.58		*	*
M. fallax	0.55	0.66	0.61	0.65	0.66	0.67		0.60
M. chitwoodi	0.55	0.63	0.55	0.63	0.56	0.68	0.86	

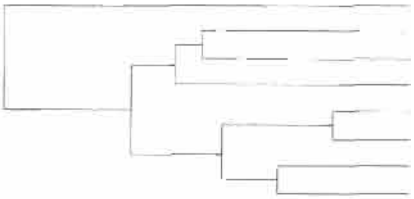
* missing value.

Table 4. *Morphometrics (mean + standard deviation) of single isolates of* Meloidogyne *spp. of two characteristics (all measurements in µm; n = 25).*

	Isolates of *Meloidogyne*							
	incognita	*arenaria*	*javanica*	*hapla* race B*	*hapla* race A*	*naasi*	*fallax*	*chitwoodi**
Female								
Stylet	16.1 ± 0.3	15.9 ± 0.4	17.1 ± 0.3	14.3 ± 0.4	14.1 ± 0.3	13.6 ± 0.4	14.5 ± 0,4	12.8 ± 0.8
DGO	3.6 ± 0.4	4.8 ± 0.4	4.8 ± 0.5	5.4+0.3	5.5 ± 0.4	3.6 ± 0.4	4.4 ± 0.6	3.1 ± 0.4
Clear lateral lines in perineal pattern	absent	present	present	present	present	absent	absent	absent
Body shape	pear-shaped	pear-shaped	pear-shaped	pear-shaped	pear-shaped	spherical	pear-shaped	pear-shaped
Male								
Stylet	23.8 ± 0.3	-	20.8 ± 0.4	19.3 ± 0.3	18.6 ± 0.5	18.0 ± 0.6	19.6 ± 0.6	17.5 ± 0.7
DGO	3.6+0.3	-	3.6 ± 0.3	4.0 ± 0.3	4.2 ± 0.4	2.9 ± 0.3	4.5+0.7	2.8 ± 0.5
Head cap	ant. flattened	ant. flattened	ant. flattened	rounded	rounded	ant. flattened	ant. flattened	ant. flattened
Lateral lips	present	absent	absent	absent	absent	present	present	present
Medial crescent shape lips	distinct	not distinct	distinct	not distinct	not distinct	distinct	distinct	distinct
Stylet form	robust	robust	robust	slender	slender	slender	slender	slender
J2								
L	387.8 ± 15.7	510.4 ± 11.8	422.7 ± 7.8	455.2 ± 9.5	399.4+10.8	424.7+8.8	408.1 ± 14.1	363.2 ± 10.8
Tail	47.6 ± 2.1	62.8 ± 0.9	52.9+1.9	58.3+2.1	51.1 ± 1.8	68.2 ± 3.5	49.5 ± 2.0	42.2+1.4
h	13.0+0.6	20.0 ± 1.3	13.8 ± 0.7	16.6 ± 1.3	12.9 ± 1.8	20.6 ± 2.2	13.7 ± 1.2	10.9 ± 1.1
Excretory pore	80.5+2.6	95.9 ± 3.1	79.4 ± 2.4	83.9 ± 1.9	75.9 ± 3.6	71.8 ± 2.6	69.2 ± 3.4	61.8 ± 3.9
DGO	3.0+0.3	4.3 ± 0.2	3.6 ± 0.3	4.2 ± 0.4	4.1+0.3	2.9+0.4	3.5 ± 0.3	3.0 ± 0.4
Stylet knobs	set off	not set off	set off	set off	set off	not set off	set off	not set off
	round	round	round	round	round	pear	round	pear
Dorsal stylet curvation	present	absent	present	absent	absent	present	absent	present
Stylet shaft broadening	present	present	present	absent	absent	absent	present	present
Body shape	round	round	round	round	round	ovoid	round	round
Tail terminus	broad	narrow	narrow	narrow	narrow	narrow	broad	broad

*: marked isolates different from those used in the molecular studies.
-: missing value.

0.03, from 0.00 to 0.02, and from 0.00 to 0.01, respectively (Van der Beek *et al.*, 1997), indicating that in the present study the data, which are based on one isolate per species, can be considered representative for their species. The distances between these eight *Meloidogyne* spp. are small when compared to genetic distances between the sibling species *Globodera rostochiensis* and *G. pallida* (0.70) and *Heterodera glycines* and *H. schachtii* (0.59) (Bakker & Bouwman-Smits, 1988*b*). The observed genetic distances are similar to that (0.19) found between the sibling species *Drosophila melanogaster* and *D. simulans* (Ohnishi *et al.*, 1983), but larger than between fifteen species of the *D. obscura*-group, with distances ranging from 0.02 to 0.11 (Acosta *et al.*, 1995), all from 2-DGE studies. The genetic distances between *Meloidogyne* spp. are larger than those between hominoid primate genera, ranging from 0.07 (between human and chimpanzee), to 0.18 (between gorilla and siamang), in studies using 2-DGE (Goldman *et al.*, 1987).



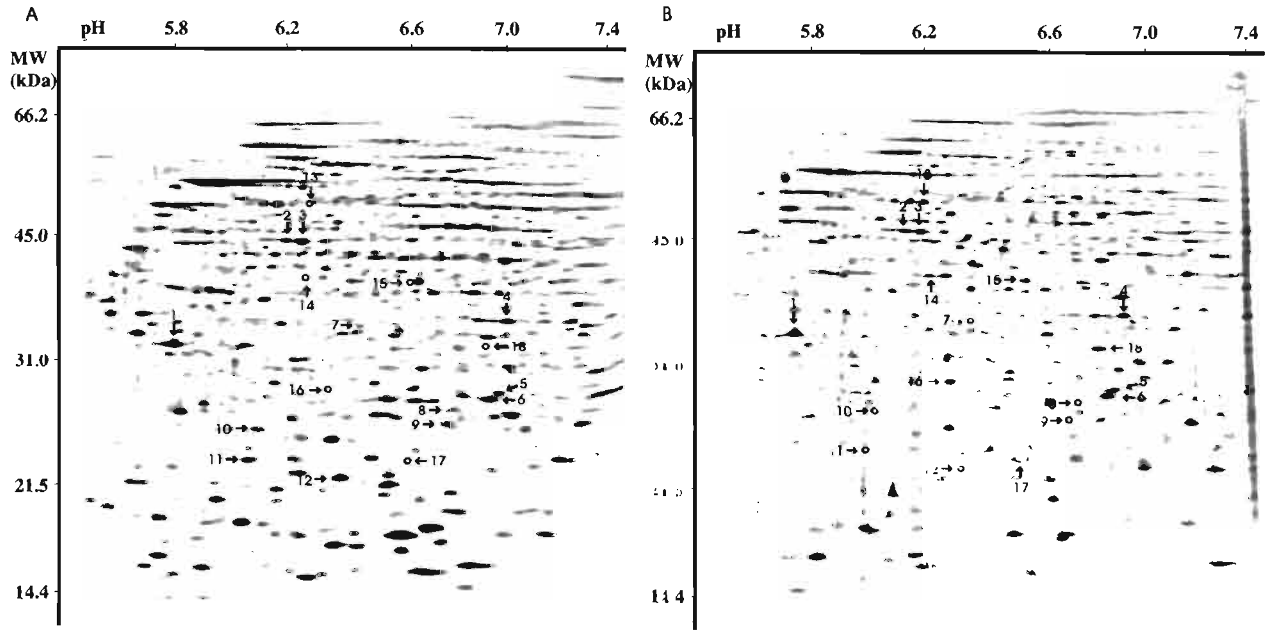


Fig. 3. 2-DGE protein pattern of Meloidogyne incognita *(A) and* M. naasi *(B), showing separation of protein spots on isoelectric point (horizontal direction) and molecular weight (vertical direction). On the patterns, six monomorphic spots (1 to 6), six spots present only in* M. incognita *(7 to 12), and six spots present only in* M. naasi *(13 to 18) are indicated.*

the major driving force of evolution, while by contrast random genetic drift is thought to have played a more important role in the evolution at the molecular level (Li & Graur, 1991). The rate in morphological evolution is thought to differ between (groups of) organisms as well as within certain organisms (Wilson *et al.*, 1974; Wilson, 1976). The present study revealed differences between molecular and morphological data (Figs 1, 2). Contrasts between molecular and morphological similarities have been demonstrated in taxonomic groups as diverse as bacteria, fungi, fish, frogs, and reptiles (*e.g.*, Wilson, 1976; Shaffer *et al.*, 1991; Lydeard *et al.*, 1995; Wägele, 1995). The large differences in clustering positions of, *e.g.*, *M. incognita* and the other two tropical species, *M. arenaria* and *M. javanica*, in the dendrograms in Figs 1 and 2, could point to different mechanisms underlying molecular and morphological evolution, with the latter resulting in a higher degree of convergent and parallel character changes. Another factor disturbing the congruency of the dendrograms may be the phenotypical variation. Unlike molecular data, morphological data are influenced by environmental factors. Also, differences in numbers of observed markers between morphological and molecular data could be involved. This emphasizes the strength of the molecular techniques, which enable the observation of large numbers of markers. Finally, it should be noted that the mutation of one gene may have dramatic effects on the morphology of an organism. It has been found that body length, growth rate, and offspring number are effected by a single gene in *Caenorhabditis elegans* (Katsura *et al.*, 1994).

Isolates of the meiotic parthenogenetic *M. hapla* race A and the ameiotic parthenogenetic *M. hapla* race B were similarly clustered in both protein and AFLP dendrograms (Fig. 1) and morphological dendrogram (Fig. 2), which is in agreement with the dendrogram based on 29 enzymes by Esbenshade and Triantaphyllou (1987). The discrimination between the two races of *M. hapla* was originally based on cytological data and their differences in reproduction mode (Triantaphyllou, 1966). Despite large cytological differences, our results confirm the similarity between the two *M. hapla* races by using molecular analyses based upon large numbers of protein and DNA markers. Presumably, a limited number of genes is involved in the difference between ameiotic and meiotic parthenogenesis.

The isolates of the ameiotic parthenogenetic species *M. incognita*, *M. javanica*, and *M. arenaria* clustered as a single group of tropical species, apart from the meiotic parthenogenetic species and *M. hapla* race B, using both protein and DNA markers. This supports

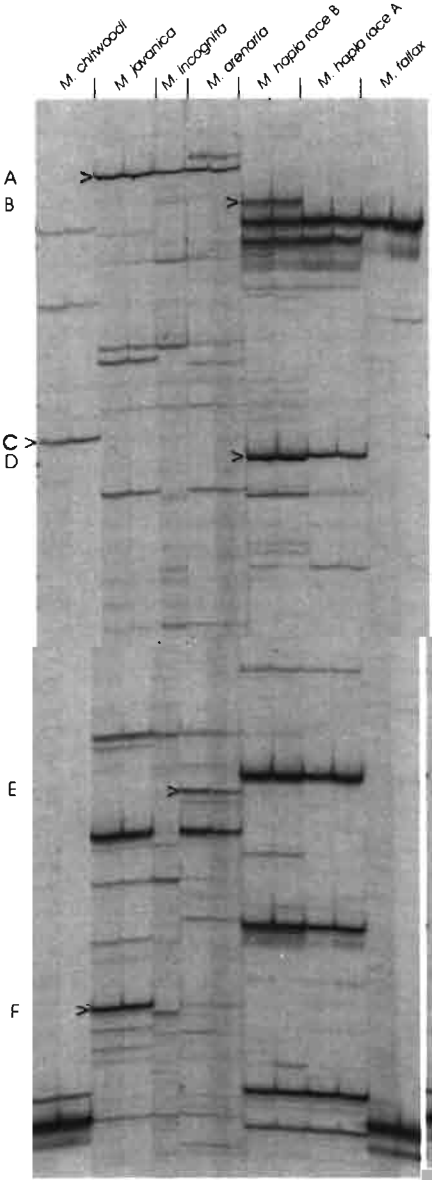


Fig. 4. *Detail of AFLP pattern, showing polymorphisms between* Meloidogyne *spp. Examples of specific inserts for: tropical* Meloidogyne *spp. (A),* M hapla *race B (B),* M. chitwoodi *(C),* M. hapla *races A and B (D),* M. arenaria *(E), and* M. javanica *(F).*

the hypothesis that the origin of the apomixis in *M. hapla* race B is different from that of the three tropical species. The separate clustering of these tropical species is in accordance with Dickson *et al.* (1971), Dalmasso and Bergé (1975), and Esbenshade and Triantaphyllou (1987), based on isozymes, Baum *et al.* (1994), based on 400 DNA fragments in amplified fingerprints, Castagnone-Sereno *et al.* (1993), based on RFLP data of genomic DNA with up to 22 DNA fragments using restriction enzyme *Bam*HI, Castagnone-Sereno *et al.* (1994), based on RAPD analysis of genomic DNA, and Fargette *et al.* (1994), based on RAPD and RFLP data of genomic DNA.

M. chitwoodi and *M. fallax* formed a third cluster of isolates. The present study is the first that links *M. fallax* to another species. The clustering of these two species was not surprising, as initially the possibility was considered that they were in fact a single species (Van Mechelen *et al.*, 1994). However, evidence for species status for *M. fallax* was recently obtained by demonstrating reproductive isolation between the two species (Van der Beek & Karssen, 1997).

The present study shows that genetic distances based on molecular data between *Meloidogyne* spp. are relatively small when compared to genetic distances between morphologically nearly indistinguishable cyst nematode species. This may indicate a more recent speciation of the *Meloidogyne* spp., which may explain in part the difficulties associated with the use of morphological characters in the taxonomy of root-knot nematodes. Secondly, the similarity dendrograms based on either molecular or morphological data reveal some pronounced discrepancies, which can be explained by parallel or convergent changes in morphological characters. Neither from an ecological point of view nor from the molecular data presented in this study, a clustering of *M. incognita* with *M. chitwoodi* and *M. fallax* would be suggested. Consequently, studies of relationships within *Meloidogyne* should not be based on morphology only but also on molecular data. Thirdly, our data indicate that apomixis is most likely not the result of a gradual accumulation of many genetic factors but seems to be caused by changes in a relatively small number of genes.

Acknowledgement

The authors thank G. Karssen for providing the morphometric and isozyme data and for verification of the perineal patterns. The critical reading of the manuscript by R.F. Hoekstra, P.W.Th. Maas, and C.H. van Silfhout is highly appreciated.

References

ACOSTA, T, PINTO, F., HERNANDES, M., GONZALES, A.M., CABRERA, V.M. & LARRUGA, J.M. (1995). Phylogeny of the *Drosophila obscura* group as inferred from one- and two-dimensional protein electrophoresis. *J. zool. Syst. Evol. Res.*, 33: 101-108.

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*). Contrasting rates of protein and morphological evolution in cyst nematode species. *Phytopathology*, 78: 900-904.

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

BAUM, T.J., GRESSHOFF, P.M., LEWIS, S.A. & DEAN, R.A. (1994). Characterization and phylogenetic analysis of four root-knot *Meloidoygne arenaria* race 2 populations. *J. Nematol.*, 24: 23-28.

BERGÉ, J.B. & DALMASSO, A. (1975). Caractéristiques biochimiques chez quelques populations de *Meloidogyne hapla* et de *M.* spp. *Cah. ORSTOM, sér. Biol.*, 10: 263-271.

BRITTEN, R.J. (1986). Rates of DNA sequence evolution differ between taxonomic groups. *Science*, 231: 1393-1398.

CARPENTER, A.S., HIATT, E.E., LEWIS, S.A. & ABBOTT, A.G. (1992). Genomic RFLP analysis of *Meloidogyne arenaria* race 2 populations. *J. Nematol.*, 24: 23-28.

CASTAGNONE-SERENO, P., PIOTTE, C., UIJTHOF, J., ABAD, P., WAJNBERG, E., VANLERGERGHE-MASUTTI, F., BONGIOVANNI, M. & DALMASSO, A. (1993). Phylogenetic relationship between amphimictic and parthenogenetic nematodes of the genus *Meloidogyne* as inferred from repetitive DNA analysis. *Heredity*, 70: 195-204.

CASTAGNONE-SERENO, P., VANDERBERGHE-MASUTTI, F. & LEROY, F. (1994). Genetic polymorphism between and within *Meloidogyne* species detected wiht RAPD markers. *Genome*, 37: 904-909.

DE BOER, J.M., OVERMARS, HA., BAKKER, J. & GOMMERS, F.J. (1992). Analysis of two-dimensional protein patterns from developmental stages in the potato cyst-nematode, *Globodera rostochiensis. Parasitology*, 105: 461-474.

DICKSON, D.W., HUISINGH, D. & SASSER, J.N. (1971). Dehydrogenases, acid and alkaline phosphatases, and esterases for chemotaxonomy of selected *Meloidogyne, Heterodera* and *Aphelenchus* spp. *J. Nematol.*, 3: 1-16.

EISENBACK, J.D. (1985). Diagnostic characters useful in the identification of the four most common species of root-knot nematodes (*Meloidogyne* spp.). *In*: Sasser, J.N. & Carter, C.C. (Eds). *An advanced treatise on* Meloidogygne. *Vol. I. Biology and control.* Raleigh, NC, USA, North Carolina State University Graphics: 95-112.

EISENBACK, J.D. (1993). Morphological comparison of females, males, and second-stage juveniles of cytological races A and B of *Meloidogyne hapla*, Chitwood, 1949. *Fundam. appl. Nematol.*, 16: 259-271.

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.

EISENBACK, J.D., HIRSCHMANN, H., SASSER, J.N. & TRIANTAPHYLLOU, A.C. (1981). *A guide to the four most common species of root-knot nematodes (*Meloidogyne *species) with a pictorial key.* Raleigh, NC, USA, North Carolina State University Graphics: 48 p.

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

ESBENSHADE, P.R. & TRIANTAPHYLLOU, A.C. (1990). Isozyme phenotypes for the identification of *Meloidogyne* species. *J. Nematol.*, 22: 10-15.

FARGETTE, M., BLOK, V.C., PHILIPS, M.S. & TRUDGILL, D.L. (1994). Genetic variation in tropical *Meloidogyne* species. *In*: Lamberti, F., De Giorgi, C. & Bird, D.M. (Eds). *Advances in molecular plant nematology.* New York, USA, Plenum Press: 91-96.

FOLKERTSMA, R.T., ROUPPE VAN DER VOORT, J.N.A.M., DE GROOT, K.E., VAN ZANDVOORT, P.M., SCHOTS, A., GOMMERS, F.J., HELDER, J. & BAKKER, J. (1996). Gene pool similarities of potato cyst nematode populations assessed by AFLP analysis. *Molec. Pl. Microbe Interact.*, 1: 47-54.

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

HYMAN, B.C. & POWERS, T.O. (1991). Integration of molecular data with systematics of plant parasitic nematodes. *A. Rev. Phytopath.*, 29: 89-107.

JEPSON, S.B. (1987). *Identification of root-knot nematodes (*Meloidogyne *species).* Wallingford, UK, CAB International, 265 p.

KARSSEN, G. (1995). Morphological and biochemical differentiation in *Meloidogyne chitwoodi* populations in The Netherlands. *Nematologica*, 41: 314-315.

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

KATSURA, I., KONDO, K., AMANO, T., ISHIHARA, T. & KAWAKAMI, M. (1994). Isolation, characterization and epistasis of fluoride-resistant mutants of *Caenorhabditis elegans. Genetics*, 136: 145-154.

KIMURA, M. (1983). *The neutral theory of molecular evolution.* Cambridge, UK, Cambridge University Press, 367 p.

KING, MC. & WILSON, A.C. (1975). Evolution at two levels in humans and chimpanzees. *Science*, 188: 107-116.

KLOSE, J. (1982). Genetic variability of soluble proteins studied by two-dimensional electrophoresis on different inbred mouse strains and on different mouse organs. *J. mol. Evol.*, 18: 315-328.

KOHNE, D.E. (1970). Evolution of higher-organism DNA. *Quart. Rev. Biophys.*, 33: 327-375.

LI, W.-H. & GRAUR, D. (1991). *Fundamentals of molecular evolution.* Sunderland, MA, USA, Sinauer Associates Inc.: 284 p.

LI, W.-H., TANIMURA, M. & SHARP, P.M. (1987). An evaluation of the molecular clock hypothesis using mammalian DNA sequences. *J. mol. Evol.*, 25: 330-342.

LYDEARD, C., WOOTEN, M.C. & MEYER, A. (1995). Molecules, morphology, and area cladograms: a cladistic and biogeographic analysis of *Gambusia* (Teleostei: Poecilidae). *Syst. Biol.*, 44: 221-236.