Interaction between root-knot nematodes and *Solanum* spp.

Variation in pathogenicity, cytology, proteins and DNA

De interactie tussen wortelknobbelnematoden en *Solanum* spp.

Variatie in ziekteverwekkend vermogen, cytologie, eiwitten en DNA

Promotor:	dr. R.F. Hoekstra, hoogleraar in de genetica, in het bijzonder de populatie- en kwantitatieve genetica
Co-promotoren:	dr. ir. C.H. van Silfhout, hoofd van de afdeling Virulentie en Resistentie, DLO-Instituut voor Plantenziektenkundig Onderzoek
	ir. P.W.Th. Maas, hoofd van de afdeling Ecologie en Biologische Bestrijding van Nematoden, DLO-Instituut voor Plantenziektenkundig Onderzoek

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Interaction between root-knot nematodes and *Solanum* spp.

Variation in pathogenicity, cytology, proteins and DNA

J.G. van der Beek

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Bibliographic abstract

This thesis describes genetic variation in the root-knot nematodes *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax*, particularly with respect to their pathogenicity on *Solanum* spp. Significant differences in virulence and aggressiveness were shown to exist between and within these species. Evidence for the occurrence of pathotypes of *M. chitwoodi* on *S. bulbocastanum* was obtained. Differences in virulence corresponded to differences in overall genetic variation, revealed by 2-D protein electrophoresis. A distinct species classification for *Meloidogyne* spp. was obtained by AFLPs and 2-D electrophoresis. In mating experiments *M. chitwoodi* and *M. fallax* appeared to be true biological species as testified by infertility of their hybrids. Abnormalities during meiosis in oocytes of an isolate of *M. hapla* and in spermatocytes of isolates of *M. fallax* resulted in limited sexual recombination. The combination of post-reductional meiosis and the fusion of the second polar body with the egg pronucleus is probably responsable for maintenance of heterozygosity in meiotic parthenogenetic *Meloidogyne*. The constant production of males in these populations makes the development of homogeneous isolates impossible. A method was described to conserve nematode germplasm by long-term preservation of juveniles in liquid nitrogen.

Key-words - AFLP, cryopreservation, cytology, genetic variation, interspecific hybridization, *Meloidogyne chitwoodi*, *M. fallax*, *M. hapla*, pathogenicity, pathotype, post-reductional meiosis, root-knot nematodes, *Solanum* spp., two-dimensional protein electrophoresis, virulence

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WAGENINGEN

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1. Het voorkomen van post-reductionele meiose bij *Meloidogyne* heeft als consequentie dat in meiotisch parthenogenetische populaties geen volledig homozygote individuen voorkomen.
(Dit proefschrift)

2. Het verschil tussen knotsvormige en V-vormige primordia in het tweede juveniele stadium van mannetjes van de mitotisch parthenogenetische soort *Meloidogyne incognita* is niet het gevolg van het al of niet optreden van geslachtsomkering (Papadopoulou & Triantaphyllou, 1982), maar van het tijdstip waarop deze omkering plaatsvindt.
(Papadopoulou J, Triantaphyllou AC 1982. Sex differentiation in *Meloidogyne incognita* and anatomical evidence of sex reversal. J Nematol 14:549-566; dit proefschrift)

3. Het voorkomen van variatie in *Meloidogyne*-soorten ten aanzien van virulentiefactoren maakt in resistentieproeven het testen tegen meerdere gekarakteriseerde isolaten van één soort noodzakelijk.
(Dit proefschrift)

4. De huidige kennis van de biologie van wortelknobbelnematoden bevat leemtes.

5. Het in toenemende mate gemakkelijk toegankelijk hebben van informatie zal uiteindelijk veroorzaken dat, na de blindedarm, ook ons tekstgeheugen een rudimentair orgaan zal worden. Het zal er nog wel zitten en het functioneert nog wel een beetje, maar je hebt er eigenlijk niets meer aan.
(Groen T 1995. Lezers in last; de aanzwellende tekstmassa. Onze Taal 64:98-100)

6. In de paprikakasteelt worden virusoverbrengende luizen geparasiteerd door pluimpootspinnen, die op hun beurt bestreden worden door nog grotere hagedissen (roodkeelanolis). Het lijkt erop dat deze biologische wapenwedloop kassen zal veranderen in ware dierentuinen met steeds grotere diersoorten.
(Agrarisch Dagblad 4 februari 1997)

7. De biologische classificatie van de mens als zoogdier is ontoereikend.

8. Door het ontbreken van uniforme afspraken tussen internationale tijdschriften over de "lay out" van literatuurreferenties, zijn promovendi onevenredig veel tijd kwijt aan het opstellen van de literatuurlijst bij het schrijven van hun dissertatie. Het is daarom gewenst hiervoor eenduidige afspraken te maken.

9. De generalisatie dat alle Arabieren terroristen zouden zijn, is even absurd als dat alle Nederlanders klompen zouden dragen.

Stellingen behorende bij het proefschrift getiteld "**Interaction beteen root-knot nematodes and *Solanum* spp.; variation in pathogenicity, cytology, proteins and DNA**", door J.G. van der Beek.

Wageningen, 10 oktober 1997.

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CHAPTER 1

General introduction

1.1. Background

Nematodes or eelworms (Greek *nema*, *nematos* = thread; *eidos* = likeness, resembling) belong to the phylum Nematoda. This phylum includes 12,500 species, of which 1,700 are known to be present and endemic in the Netherlands (Van Niekerken & Van Loon, 1995). It is estimated that the phylum Nematoda is amongst the 25 most unknown animal groups in the Netherlands, leaving approximately 800 species as yet unknown. This group of small inarticulated worms can be found in almost any environment in which organic matter is decomposed. On land, up to 100 species per kg soil can be detected, with on average 30,000 individuals per kg soil (Bongers, 1988). On the sea floor of the 'Waddenzee' in the Netherlands, up to 30 milllion individuals per m^2 have been found, thus forming the most numerous group after the Protozoa in the Netherlands (M.S.S. Lavaleye, mentioned by Van Niekerken & Van Loon, 1995). Nematodes comprise some economically important groups including parasites that cause serious diseases in man, animals and plants. Root-knot nematodes are amongst this last mentioned group.

Root-knot nematodes, belonging to the genus *Meloidogyne* are cosmopolitan in natural environments and in all parts of the world where agriculture is practised. Certain species are widely distributed, polyphagous and able to cause great losses in production and quality. Since 1950, crop protection has mainly been concentrated on chemical soil disinfection at which certain chemicals have proven their efficacy. However, increasing attention is paid to environmentally safe methods of crop protection, such as biological control and plant breeding for resistance and tolerance.
A breeding programme for resistance to root-knot nematodes includes searching for sources of resistance and consequently selecting for resistant genotypes. In the hypothetical absence of any pathogenic variation within a given *Meloidogyne* species, plant genotypes could be selected simply by testing against a certain number of individuals of that particular nematode species. But in reality, mutation, recombination and migration of the pathogen result in genetic variation which hampers easy screening for resistance. Moreover, parthenogenesis in many *Meloidogyne* species hinders a clear distinction of biological species, which confuses the classification of nematode populations into different species or into different races or pathotypes. A slow active migration in the soil means that a large genetic variation is expected to be found in a relatively small area. Passive migration by tillage and transport of infested root material can cause large spread of root-knot nematode populations.

1.2. Nomenclature

Root-knot nematodes belong to the genus *Meloidogyne* Goeldi, 1892 (described in 1887), subfamily Meloidogyninae Skarbilovitch, 1959, family Heteroderidae, suborder Tylenchina Chitwood, 1950, order Tylenchida Thorne, 1949, according to

the revision by Luc *et al.* (1988). At least three other systems of classification of the Tylenchida have been proposed, which reflects its taxonomic complexity: the first by Allen and Sher (1967), the second by Andrassy (1976) and the third by Sidiqi (1986). Tylenchyda are plant parasitic nematodes belonging to the subclass Tylenchia Ingliss, 1983, class Secernentea Van Linstow, 1905, phyllum Nematoda Rudolphi, 1808, the eel worms or nematodes. *Meloidogyne* spp. (Greek *melon* = apple, fruit; *eidos* = shape, resemblance; *gyne* = female) are clearly distinct from other plant parasitic species of the Tylenchida by a swollen adult stage of the female and egg deposition outside the body. Classification of root-knot nematodes was attempted since the second half of the 19-th century (Greeff, 1872; Goeldi, 1892; Müller, 1884, as mentioned by Chitwood, 1949) when they were considered to form a single species. Although the genus *Meloidogyne* was described already in 1887 by Goeldi, root-knot nematodes were generally known under names such as *Heterodera radicicola* or *H. marioni* (Chitwood, 1949; Oostenbrink, 1950; Thorne, 1961). Also other names have been designated to root-knot nematodes, including: *Anguillula marioni* Cornu, 1879, *Heterodera javanica* Treub, 1885, *Anguillula arenaria* Neal, 1889, *Tylenchus arenarius* Cobb, 1890, *Meloidogyne exigua* Goeldi, 1892, *Anguilula vialae* Lavergne, 1901, *Oxyuris incognito* Kofoid & White, 1919 and *Caconema radicicola* Cobb, 1924. Only halfway through the 20th century it was shown clearly by Christie and Albin (1944) that more than one species of root-knot nematodes exists, which could explain different host reactions found by various authors already at the end of the 19th century. Chitwood (1949) was the first who definitely identified and described different species within the genus *Meloidogyne*. He distinguished five species and one variety: *Meloidogyne exigua* Goeldi, 1887, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 (synonym *Heterodera javanica*), *Meloidogyne hapla* Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (synonym *Oxyuris incognito*), *Meloidogyne incognita* var. *acrita* Chitwood, 1949 and *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949 (synonym *Anguillula arenaria*). This classification formed the basis for many other species to be described under this genus: by 1960 four new species and subspecies had been added (Netscher, 1978); five years later, Franklin (1965) described 16 species; in 1968 the total list of *Meloidogyne* species comprised of 23 species (Whitehead, 1968). Franklin (1971) published a list of 28 species, Esser *et al.* (1976) 35 species, Sidiqi (1986) 51 species, Luc *et al.* (1988) 70 species and Eisenback and Hirschmann-Triantaphyllou (1991) 68 species. Recently, more species have been described, for example: *M. cynariensis* from artichokes in Vietnam (Fam-Thkan'Bin', 1990), *M. morocciensis* from peach rootstock in Morocco (Rammah & Hirschmann, 1990) and *M. fallax* from potato in the Netherlands (Karssen, 1996). Pais and Abrantes (1989) mention a possible new species in Portugal, based on unique esterase and malate dehydrogenase phenotypes, that was found only to infest olive trees.

As *M. chitwoodi* has long been confused with *M. hapla*, it was only recently that *M. chitwoodi* was described from potatoes by Golden *et al.* (1980). The increasing

numbers of identified *Meloidogyne* spp. since 1949 does not necessarily mean that these species came only recently into existance or that the speed by which these species are described is related with the speed of speciation in *Meloidogyne*.
The rapid increase in number of known species reflects the increasing interest in these nematode species which are difficult to identify.

1.3. Life cycle

Root-knot nematodes are endoparasitic. Their life cycle (Figure 1.1) is partly outside but mainly sedentary within the roots. The eggs are deposited in egg-masses attached to the female. The first and second-stage juveniles (J1 and J2) develop within the eggs (Taylor, 1971).
Hatched J2, slender with body length varying from 290 to 912 μm are the infective stage of the nematode (Figure 1.2). Root penetration occurs just behind the root tip, normally within two hours after contact (Wyss *et al.*, 1992). The J2 then move intracellularly towards the meristematic region in the root tip within eight hours, almost without any destruction of plant cells. They turn and move in the direction of the vascular cylinder in order to become sedentary in the root after 16 hours. No food uptake occurs untill they become stationary; the J2 then live on the lipid globules which are present in the wall of the intestine. They moult twice more, feeding on giant cells and become J4. After the fourth moult the males are slender (700 to 2,000 μm long; Figure 1.2) and leave the root. The females remain in the root. They have swollen, saccate bodies that range in median length from about 440 to 1,300 μm and in median width from about 330 to 700 μm (Eisenback & Hirschmann-Triantaphyllou, 1991; Figure 1.2), and deposit eggs in egg-masses. These egg-masses are mostly exposed outside the root, but can sometimes remain inside the root epidermis. Egg-masses of *M. chitwoodi* and *M. fallax* remain also within the epidermis of potato tubers.

1.4. Modes of reproduction

Triantaphyllou described the mode of reproduction for *Meloidogyne* spp. in a range of publications (Triantaphyllou, 1966, 1971, 1985a, 1985b, 1991, 1993; Triantaphyllou & Hirschmann, 1980). As with most species of plant parasitic nematodes, *Meloidogyne* spp. are bisexual, i.e. they are composed of male and female individuals and both sexes have easily recognizable primary and secondary sex characters. However, some cultures were described as thelytokous and produced only females (Triantaphyllou, 1993). Reproduction in *Meloidogyne* is by amphimixis (sexually) or by apomixis. Apomixis includes various types of asexual reproduction which do not result in fusion of the gametes (Rieger *et al.*, 1976; Asker & Jerling, 1992). Apomixis in *Meloidogyne* is parthenogenetic with embryos produced from female gametes.
According to the mode of reproduction, parthenogenetic species of *Meloidogyne* can

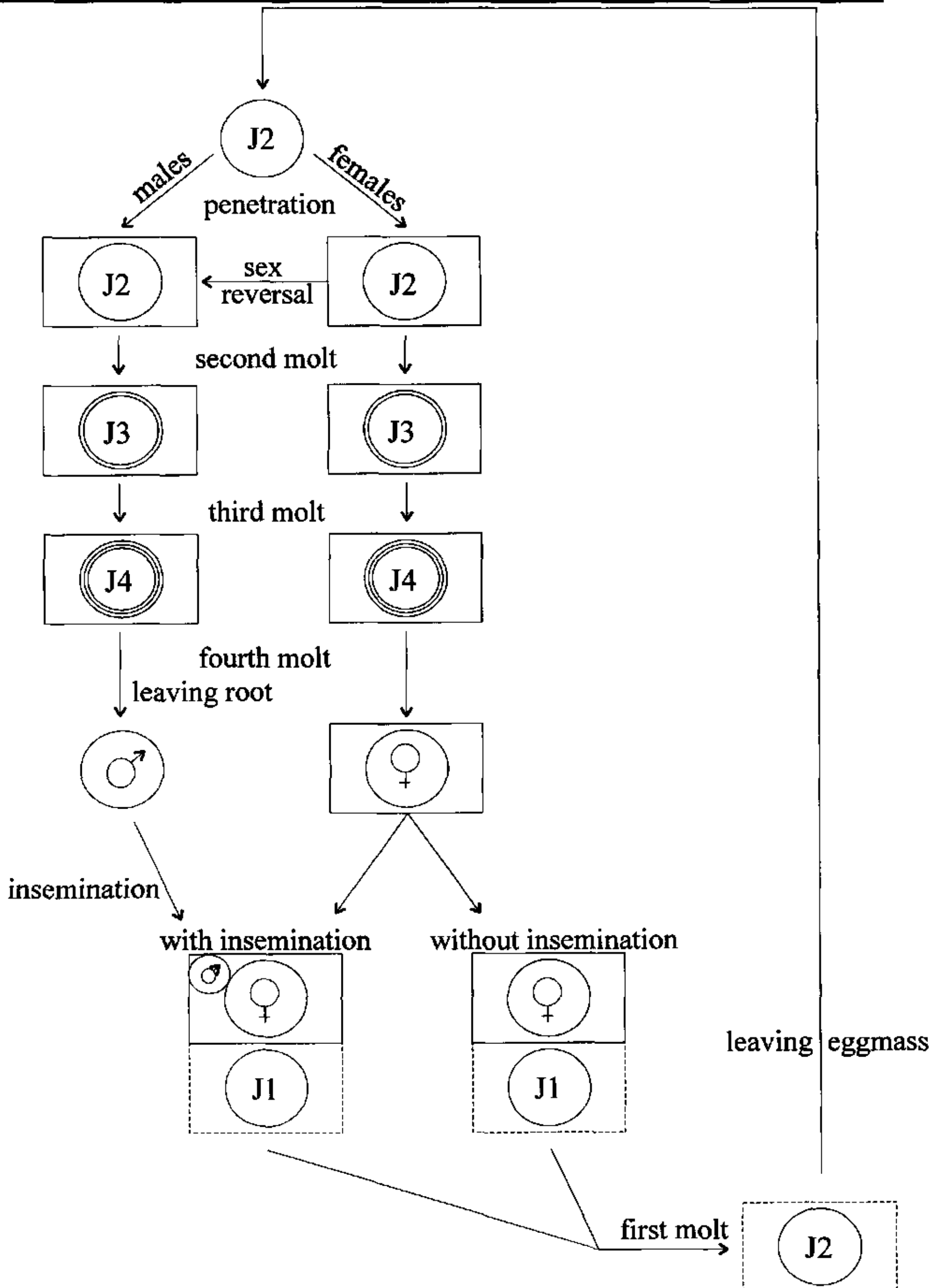


Figure 1.1. Schematic representation of the life cycle of *Meloidogyne* spp. Legenda: circle=nematode stage; double and triple circles=nematode stages within cuticula of previous stages; J1 to J4=successive juvenile stages; solid lined box=root; dotted lined box=egg-mass.

be classified as obligate or as facultative parthenogenones. Based on cytological data, parthenogenetic *Meloidogyne* spp. can be classified as meiotic somatic or as mitotic somatic. Other terms are also in use, but we shall simply refer to these types as meiotic and mitotic. For meiotic parthenogenones regular chromosome pairing and reduction of chromosome number occur, but the somatic number of chromosomes is restored by fusion of the haploid egg pronucleus and the haploid second polar nucleus (Triantaphyllou, 1985). Mitotic parthenogenones show neither reduction nor fusion of nuclei, but instead apomeiosis occurs, by which the egg nucleus develops into an embryo. Because reduction never takes place with mitotic parthenogenones, this mode of reproduction is obligate. Eisenback and Hirschmann-Triantaphyllou (1991) reported that from a total of 24 examined species six appeared to be amphimictic, seven facultative meiotic parthenogenetic and eleven obligate mitotic parthenogenetic. Three of the most common species, *M. incognita*, *M. javanica* and *M. arenaria* are mitotic parthenogenetic. *M. chitwoodi* is facultative meiotic parthenogenetic with a haploid chromosome number of n=x=14-18.

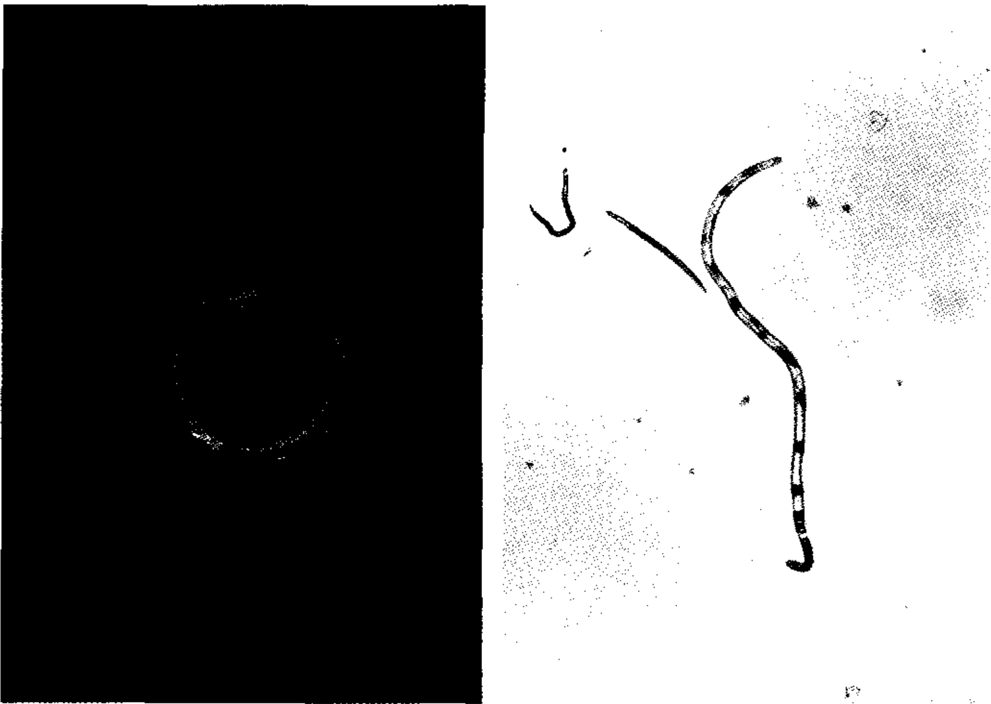


Figure 1.2. Female (left), males and second-stage juveniles (right) of *M. hapla* race B (magnification 20X)

M. hapla includes two different cytological races: race A which is also facultative meiotic parthenogenetic with n=x=14-17, and race B which is mitotic parthenogenetic with populations of 2n=3x=43-45, 2n=3x=48 and 2n=2x=30-32 (Triantaphyllou, 1985). More recently, a hermaphrodite form of *M. hapla* was found (Triantaphyllou, 1993).

Chromosomes of nematodes are holocentric, as in certain insects or plants (Triantaphyllou, 1971). This type of chromosomes is characterized by a parallel arrangement of the chromatids during maturation of the oocyte, the absence of a localized centromere and a broadside move of the chromosomes to the poles during anaphase. Unlike monocentric chromosomes, which have localized centromeres, the entire body of the chromosome forms microtubules on the poleward surface and these converge to the pole. At meiosis, the chromatids in bivalents are held together in an end-to-end association which is suggested to be caused by terminalized chiasmata (White, 1973). In *Caenorhabditis elegans* Albertson and Thomson (1993) found that either the left or the right end of the homologues could be held in association and that these holocentric chromosomes have thus two potential 'meiotic centromeres'.

Chromosomes of *Meloidogyne* spp. are small in size (1 μm) and therefore difficult to distinguish from each other. Electron microscopic observations proved that no sex chromosomes exist in *Meloidogyne* because no univalent chromosomes occurred during meiosis (Goldstein, 1981). However, there is a difference in decondensation of chromatin between males and females. During pachytene a proteinaceous structure, called the synaptonemal complex, becomes visible and forms the basis for homologous pairing. Synaptonemal complexes are inherent to the process of crossing over and consequently are not present in mitotic parthenogenones like *M. hapla* race B. The chromatin appears to be condensed along the synaptonemal complex, but specialized regions of decondensed chromatin (DCR) occur. However, Goldstein (1981) found that these DCR are not present in males. He discussed the hypothesis that DCR in *M. hapla* could be related to sex chromatin and that the male is heterogametic for DCR.

The determination of sex in *Meloidogyne* spp. is highly influenced by environmental conditions. Morphological distinction between males and females is possible from the half grown J2 (Triantaphyllou & Hirschmann, 1960). Favourable growing conditions during the J2-stage will promote the development of normal females with two ovaries, while unfavourable conditions change the sex balance toward male formation (Triantaphyllou, 1973). A change in condition during the early J2-stage, in both meiotic and mitotic parthenogenones, can even result in intersexual individuals, which are mostly non-functional and are slender as males. In *Caenorhabditis elegans*, intersexes can also be formed by disturbances during development (Nelson *et al.*, 1978).

1.5. Distribution and economic losses

Root-knot nematodes have a global distribution. The four most prevalent species are: *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*. Of these, *M. hapla* is the only one which is predominantly found in temperate zones under field conditions. The other species occur in the warmer, tropical and mediterranean, climates as well as in greenhouses where they can become major pests.
Since the description of *M. chitwoodi* in 1980, infestations with this species have been reported from different locations in the USA, including the pacific North-West (Santo *et al.*, 1980), Colorado (Pinkerton & McIntyre, 1987) and Utah (Griffin & Thomson, 1988); and in other parts of the world, including Argentine (Esbenshade & Triantaphyllou, 1985), Mexico (Cuevas et al., 1990), Portugal (Mugniery, pers. com.), South Africa (Kleynhans & Van den Berg, 1988), the Netherlands (EPPO Reporting Service, 1991) and in Belgium (Waeyenberge & Moens, 1997). *M. chitwoodi* is considered to be a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO) having A2 status (Smith *et al.*, 1997) which indicates that it is a quarantine organism for all plant species. Although *M. chitwoodi* was identified for the first time in the Netherlands in 1988 and *M. fallax* in 1992, it seems clear that these species have been present much longer (Anonymous, 1990). Three arguments support this view: 1. Infested potato tubers, collected in 1930 from the North-West of the Netherlands and conserved in formalin, appeared to be infested by *M. chitwoodi*; 2. In the South-East of the Netherlands, a build up of populations of root-knot nematodes has occurred in the past, even after cereal growing. Cereals such as wheat, barley, rye and oats are known to be good hosts of *M. chitwoodi* (O'Bannon *et al.*, 1982; Nyczepir *et al.*, 1984) and of *M. fallax* (Karssen, 1996), but not of *M. hapla*; 3. In field experiments in Belgium, infestation by *M. hapla* of barley and rye has been reported in 1955 and of oats in 1956 (Anonymous, 1990). Although these infestations were attributed to *M. hapla*, it may be concluded that either *M. chitwoodi* or *M. fallax*, or both, were most likely the causal agent. *M. fallax* is predominantly found in the South-East of the Netherlands and is sympatric with *M. chitwoodi*. Although it has been found also in Belgium (Waeyenberge & Moens, 1997) its distribution beyond that region has not yet been investigated thoroughly. Quarantine status for *M. fallax* will most likely follow soon the recently obtained status for *M. chitwoodi*.
Nematode species belonging to *Meloidogyne* are pests of major food crops, vegetables, fruits and ornamental plants. Oostenbrink (1950) reported restricted growth caused by root-knot nematodes in the Netherlands under open field conditions in potatoes, beets, peas, clovers, serradella, vetch, broad beans, dwarf beans, carrots, lettuce, salsify (scorzonera) and roses. Although not specified by Oostenbrink (1950), these crops have most likely been attacked by *M. hapla*, *M. chitwoodi* and perhaps *M. fallax*, which have all a wide range of host plants. Though many similarities do exist between these species, several characteristic host preferences do occur. One of the most important differences is that cereals are non-hosts for *M.*

hapla but hosts for *M. chitwoodi* and *M. fallax* (Santo & O'Bannon, 1981, Karssen, 1996). Also, *Lycopersicon peruvianum* is a good host for *M. chitwoodi* but a non-host for *M. hapla* (Santo & O'Bannon, 1982), and strawberry is a non-host for *M. chitwoodi* but a host for *M. hapla* (Edwards *et al.*, 1985). Only four host species were found to be specific hosts for *M. chitwoodi* on which *M. fallax* did not reproduce or only poorly, and four other host species which reacted oppositely (Karssen, 1996).

Economic losses caused by these species are due to reductions in yield and in quality. Slinger and Bird (1978) found in a greenhouse experiment with *M. hapla* on carrot, that only 58% were suitable for the market, compared to 97% when planted in nematode free soil. Affected carrots show an increased development of branched roots. Besides this reduction in quality, a pot experiment with 60 varieties of carrot resulted in a root weight reduction of about 45% due to infection with *M. hapla* (Stein, 1982). Other examples of yield reduction caused by *M. hapla* are: sprout failure, reduction in plant height and yield reduction in potato (Loue, 1977); growth reduction in alfalfa cultivars (Noel, 1977; Inserra *et al.*, 1980) and in peppermint (Eshtiaghi, 1975); growth delay up to 60% in onion (Mac Guidwin *et al.*, 1987), losses of 22, 43 and 21 % in weight of marketable produce in beet, lettuce and spinach respectively at a high infection pressure of 18,000 nematodes kg^{-1} soil (Potter & Olthof, 1974), commercial crop losses in potatoes and onions of 46 and 64 % respectively at a high infection pressure of 18,000 nematodes kg^{-1} soil (Olthof & Potter, 1972), yield reduction of 50% in pyrethrum (Parlevliet, 1971), green yield reduction of 30% in clover (Erenfelde, 1979), yield losses of 8.6% for each 10-fold increase in initial infestation of peanut (Rickard *et al.*, 1977), dry weight reduction of 24% in *Trifolium spp.* and of 76% in *Medicago sativa* when grown in a field with 24,000 nematodes kg^{-1} soil (Townsherd & Potter, 1978) and reduction of fresh top root weight of 65% (and of 60% caused by *M. chitwoodi*) when grown in soil containing 512 nematodes cm^{-3} (Griffin *et al.*, 1982).

Figures on losses and damage due to *M. chitwoodi* are reported less frequently than those due to *M. hapla* because of the recent description of the species and a more limited distribution worldwide. *M. chitwoodi* significantly suppressed tillering of wheat and barley cultivars, but plant height was not affected (Griffin, 1993). Farmers in the South-East of the Netherlands have been confronted during the past years with serious quality problems in potatoes, carrots and black salsify, often leading to an unmarketable produce. These appeared to be caused by *M. chitwoodi* and *M. fallax* (Molendijk & Mulder, 1996). From field trials with a natural infection of *M. fallax* it was estimated that an initial population density of 10 nematodes cm^{-3} affected already the physical yield of potato and that even a densitiy of 0.1 nematode cm^{-3} may cause infection of the new-formed tubers (Brommer & Molendijk, 1996). Data on yield losses caused by *M. chitwoodi* and *M. fallax* in the Netherlands have not yet been reported.

Several authors mention tolerance levels and threshold densities. Some values of tolerance levels are: 0.03 to 0.18 eggs cm^{-3} soil for winter wheat cv. Nugaines - *M.*

chitwoodi (Nyczepir *et al.*, 1984); 0.6 and 2.8 J2 or eggs cm^{-3} soil for sugarbeet - *M. hapla* and sugarbeet - *M. chitwoodi* respectively (Nyczepir *et al.*, 1984); and 0.16 J2 cm^{-3} soil for cucumber - *M. hapla* (Sagitov & Perevertin, 1988). Barker *et al.* (1976) defined a threshold density for tomato - *M. hapla* at a yield reduction of 10 to 25%. He calculated a density of 1 J2 or egg cm^{-3} soil. Other values of economic threshold densities are: 0.5 J2 cm^{-3} soil for fibre flax cv. L-1120 - *M. hapla* (Antonova, 1979) and 1.8 J2 cm^{-3} soil for cucumber - *M. hapla* (Sagitov & Perevertin, 1988).
Evidently, these high losses in yield and quality combined with low threshold densities make these root-knot nematodes a major pest in field crops in temperate regions.

1.6. Species identification

According to the biological species concept, a species is a group of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups. A species is the largest and most inclusive reproductive community of sexual and cross-fertilizing individuals that share in a common gene pool (Rieger *et al.*, 1976). Mating with production of fertile offspring is the basis of this species concept (Poinar & Hansen, 1983).
However, many definitions have been proposed to describe the concept of species. Quicke (1993) pointed out that the majority of these definitions fall into three categories: firstly the biological species concept, based on reproductive isolation, as described above. Secondly the phylogenetic and evolutionary species concept, based on basic evolutionary units as revealed by phylogenetic analysis. And thirdly *ad hoc* definitions that are based on morphological species criteria based on degrees of phenotypic or genotypic distinctiveness.
The occurrence of parthenogenetic reproduction clearly complicates species definitions in the genus *Meloidogyne*. However, biochemical and cytogenetic differences between at least the major *Meloidogyne* species support strongly the existence of relatively distinct biological groups, represented by these species.
Traditionally, in nematology the description of species is done on the morphology of different stages of juveniles and adults, and on host preference. Chitwood (1949) differentiated between species of *Meloidogyne* mainly by perineal patterns, which are the cuticular ornamentations around the anus and the vulva of the adult females formed by striae. This character was used by Taylor *et al.* (1955) to construct a key to the species of *Meloidogyne*. Eisenback (1985) pointed out why this character was used as a main descriptor for *Meloidogyne* species: 1. It is quite stable for many populations (Dropkin, 1953, Sasser, 1954); 2. Shortly after Chitwood (1949) described the first *Meloidogyne* species, the vulval cyst cones of *Heterodera* species were emphasized as useful taxonomic characters, and were thought to be analogous with perineal patterns (Mulvey, 1960). However, it appeared that a large variability existed in the configuration of the perineal pattern by which many identifications

remained inconclusive. Chitwood (1949) himself wrote: "Twice we have encountered individual females in which the perineal pattern on one side of the body was that of *Meloidogyne incognita* and on the other side it was in one instance *M. incognita acrita* and in the other case *M. javanica.*" Allen (1952) reported a large variety in perineal patterns. Thorne (1961) stated that "...diligent study of permanent slides should eventually enable a skilled worker to identify accurately upward of 90 per cent of the groups of patterns examined." Identification of individual patterns may be somewhere between 60 and 80 % reliable. Identification in mixtures of species is undoubtedly even more difficult. Netscher (1978) was not able to separate distinctively *M. javanica* from *M. incognita* in a mixture of populations, not only on the basis of perineal pattern, but also on the basis of other characters as vulval width, stylet length or interphasmidial distance. Distinction between the two species could be obtained satisfactory by the position of the excretory pore in relation to anterior end of female. However, *M. arenaria* and *M. javanica* cannot be distinguished from each other by this character (Netscher, 1978). The large variation in morphology within *Meloidogyne* causes identification to be a very laborious work, requiring much expertise.

Other morphological characters have been used to identify species (Jepson, 1984 and 1987). Fargette and Braaksma (1990), for instance, use the following biometric measurements for females: length of stylet, dorsal oesophageal gland orifice (DGO), ratio DGO/stylet length, vulval width, distance vulva - anus, ratio distance vulva - anus/vulval width; the following for males: length of stylet, length of stylet-cone, DGO, ratio cone length/stylet length, ratio DGO/stylet length; and the following for J2: body length, stylet length, DGO, diameter oesophageal valve, hemizonid, maximum body width, ratio body length/stylet length, ratio DGO/stylet length, ratio diameter valve/body length, ratio hemizonid/body length, and ratio body length/maximum body width. Nearly all characters were too variable to differentiate between populations of *Meloidogyne* spp. Only the characters ratio DGO/stylet length in female and in male were discriminative. The shapes of the male head and stylet are considered to be useful in the identification of *Meloidogyne* species (Eisenback & Hirschmann, 1982). However this character has not been widely used because of the irregular occurrence of males.

Recently, species identification by a biochemical approach has proven to be successful. Refined techniques were developed by which single adult females can be studied and their enzyme phenotypes be compared (Dalmasso & Bergé, 1978; Janati *et al.*, 1982; Esbenshade & Triantaphyllou, 1985). Esbenshade and Triantaphyllou (1990) discriminated between eight *Meloidogyne* spp., including *M. hapla* and *M. chitwoodi*, using malate-dehydrogenase and esterase to differentiate. At present, this is one of the most rapid and efficient methods to identify *Meloidogyne* spp. if an automated mini-gel electrophoresis apparatus is available. Biochemically, it was also possible to recognize occasionally intraspecific variation, e.g. between the cytological races A and B of *M. hapla* (Esbenshade & Triantaphyllou, 1987). By this technique, occurrence of undescribed biological groups could be shown (Pais *et al.*,

1989). It is interesting that nematode infection provoked novel esterase bands in root tissue (Ibrahim & Perry, 1993), which can be helpful in the understanding of plant response to nematode infection.

Currently, molecular biology offers great perspectives in species identification and in studying variation in general. Platzer (1981) already pointed out how DNA studies can be practical for nematode taxonomy and indicated the potential for studies on speciation and races. Random Fragment Length Polymorphism (RFLP) analysis revealed patterns that were useful to separate three *Meloidogyne* species, after digestion of total DNA of those species with restriction enzyme *Hin*dIII (Garate *et al.*, 1991). Although they could even separate four host races of *M. incognita* by this technique, the stability of these patterns with respect to other populations has not yet been tested. However, Carpenter *et al.* (1992) were able to separate three populations of host race 2 of *M. arenaria*, using RFLP analysis and Piotte *et al.* (1992) could also clearly demonstrate polymorphisms between populations of *M. incognita* and *M. arenaria.* A set of specific DNA fragments could be obtained to specifically separate *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla*, using probe pMiK13, and fragments could be isolated as markers for races in *M. incognita* (Piotte *et al.*, 1992). RFLP analyses require relative large quantities of nematode DNA. The Polymerase Chain Reaction (PCR) however, offers a powerful technique by which DNA of single J2 can be amplified. Harris *et al.* (1990) could separate four *Meloidogyne* spp. by amplification of mitochondrial DNA of single J2, followed by digestion with restriction enzyme *Hin*fI. PCR and Random Amplified Polymorphic DNA (RAPD) analysis are promising techniques in distinguishing populations within species. Recently, Zijlstra *et al.* (1995) developed a powerful method for species identification, based upon analysis of ITS regions of rDNA. By this reliable technique contaminations with foreign species down to 1 % could be detected.

Additionally, RFLP analyses are useful in studying relationships between and within species. Garate *et al.* (1991), Xue *et al.* (1992) and Castagnone-Sereno *et al.* (1993) described that *M. arenaria* and *M. javanica* showed closer genomic affinity to each other than to *M. incognita*, based on RFLP linkage data. *M. hapla* is remotely related to these three mitotical parthenogenetic species as the cluster analysis of these three species showed an early split off from *M. hapla* (Castagnone-Sereno *et al.*, 1993). Also, meiotic parthenogenetic populations of *M. hapla* clustered separately from mitotic parthenogenetic populations.

In conclusion, morphological taxonomy in *Meloidogyne* spp. has received some very useful additional tools like isozyme and DNA analyses, to distinguish biological groups within the genus.

1.7. Physiological variation

In nematology, nematode multiplication on a host plant is the determining factor in the assessment of resistance, in contrast to most other fields of plant pathology in

which disease or pest symptoms are observed. The ability to determine the actual numbers of the pathogen in nematology causes difficulties in applying terminology for physiological variation which is employed in other disciplines. Various expressions have been proposed, with sometimes large overlap in content, e.g. pathotype, biotype, physiologic race, host race, trophotype, virulence, pathogenicity, parasitism (Sturhan, 1971and 1985; Sidhu & Webster, 1981; Stone, 1985; Trudgill, 1986; Triantaphyllou, 1987; Müller, 1989). For the purpose of this thesis, terms related to resistance and physiological variation are defined, if applicable, on the basis of Dutch rules described by the 'Commissie voor de Terminologie van de Nederlandse Plantenziektenkundige Vereniging' (1997) and Bos and Parlevliet (1995):
reproduction: ability of a pathogen to produce live offspring;
parasite: an organism that completes its life cycle, or part of it, on a host and feeds at the expense of host tissue;
pathogen: a parasite that reproduces on a host species;
aggressiveness: the degree of reproduction of a pathogen;
virulence: ability of a pathogen to reproduce on a particular genotype of its host;
pathogenicity: ability of an organism to infect a host species and reproduce on it;
race: a group of individuals within a species sharing certain biological charactersitics, for example cytological races for *M. hapla* and host races in several *Meloidogyne* spp.;
pathotype: population within a pathogenic species that matches with a genetic factor for resistance.

In addition, the following terms are used frequently in this thesis and defined as follows:
population: a group of conspecific individuals from a common geographical site;
isolate: a (sample of a) population which is maintained in captivity;
(repeated) mono-female line: a group of genetically closely related individuals obtained after (repeated) parthenogenesis of one female, or the offspring of a female with a mitotic mode of reproduction.

Aggressiveness describes quantitatively the degree of nematode reproduction. Differences in virulence are expressed by nematode isolate-by-host genotype interaction. Aggressiveness and virulence are estimated by nematode development and reproduction. Pathogenicity comprises aspects of aggressiveness and virulence.
Parasitism as defined by Triantaphyllou (1987) is in our terminology synonymous to reproduction. Parasitism is reserved for the state of an organism in which it derives its feeding from another organism. The term biotype will be omitted. Its original definition (Johansen, 1903), referring to genetically homogenetic individuals, would only be applicable to mitotic parthenogenetic populations of *Meloidogyne* (Sidhu & Webster, 1981), which is not practical. Moreover, biotype includes a subdivision of a species by a wide range of criteria other than those of morphology. The same applies to the term race, which is used because of its wide application in *Meloidogy-*

ne. When used, it will be made clear whether the term refers to a cytological or a host race.
Studying resistance to root-knot nematodes involves research of pathogenicity, which includes aggressiveness and virulence, which are both expressed in terms of reproduction. Reproduction is mostly estimated in nematology by parameters like: number of egg-masses and number of produced eggs and J2, all related to the inoculum.

Complications in detecting physiological variation are directly connected to the lack of a conclusive way of separating *Meloidogyne* species. However, physiological variation has been observed by various authors, who could demonstrate variation in the ability to develop and reproduce on different host plants for different populations of the same species (= biological group). The International *Meloidogyne* Project (North Carolina) started in 1975 (Lawrence Apple, 1985) and had a tremendous impact on the study of variability amongst populations of *Meloidogyne* spp. from all over the world. The largest variation was found in *M. incognita*, in which four host races were described (Taylor & Sasser, 1978). For *M. chitwoodi*, a second host race was discovered in Washington State, parasitic on alfalfa (Santo & Pinkerton, 1985). A host differential set for host races 1 and 2 of *M. chitwoodi* is based on carrot cv. Red Cored Chantenay and alfalfa cv. Thor (Mojtahedi *et al.*, 1988). Additionally, host race 3 has been described, which was distinct from host race 2 by a virulent reaction on *Solanum bulbocastanum* SB22 (Mojtahedi & Santo, 1994).
Physiological variation in *M. hapla* has not been reported frequently, although variation occurs (Riggs, 1991). Di Vito and Greco (1983) mentioned a second host race to be present in Italy, unable to reproduce on watermelon cv. Charleston Grey. In Poland six 'races' within 33 populations of *M. hapla* could be distinguished using lettuce, cucumber, red beet, cabbage, onion, maize and oats as host plants (Brzeski & Baksik, 1981). Variation in host preference did also occur between nine populations of *M. hapla*, as well as between eleven of *M. arenaria* and six of *M. incognita*, from Bulgaria (Stoyanov, 1979 and 1984), but these populations have not been grouped into host races. Wofford *et al.* (1989) reported different levels of virulence of two *M. hapla* populations, isolated from alfalfa and *Onobrychis vicifolia*, but no reference to possible different host races was made. These examples illustrate the present lack of adequate understanding of the variation in pathogenicity in these *Meloidogyne* spp., which hampers efficient breeding strategies for crop resistance.

1.8. Scope of this thesis

The aim of the investigations described in this PhD-thesis is to contribute to a better understanding of the genetic variation in, and of the biology of, *M. hapla*, *M. chitwoodi* and *M. fallax*, three pathogenic nematode species of increasing importance to arable cropping in the Netherlands as well as in other parts of the world. A better understanding of genetic variation in these pathogens benefits control measurements

like developing resistant crop cultivars. For these studies in genetic variation and biology, it is important to use isolates and mono-female lines of known genetic background. In order to preserve germplasm like isolates and lines, a technique for long term storage in liquid nitrogen was optimized (Chapter 2).
Variation in virulence was investigated by studying nematode-by-plant interactions between the three *Meloidogyne* spp. and *Solanum* spp., at the level of pathogen species, isolate and within isolates, in relation to host species and cultivars.
A Petri-dish experiment was applied to investigate whether there were indications of isolate-by-potato cultivar interaction (Chapter 3). Variation in virulence in *M. hapla* (Chapter 4) and in *M. chitwoodi* and *M. fallax* (Chapter 5) was studied between species, isolates and repeated mono-female lines, on various *Solanum* spp. Isolates sharing common patterns in virulence are classified as pathotypes. Pathotype classification was discussed and a preliminary study to distinguish pathotypes in *M. chitwoodi* on *S. bulbocastanum* was performed (Chapter 6).
It was further investigated whether the observed variation in virulence was also reflected by variation in molecular polymorphisms. For this, *M. hapla*, *M. chitwoodi* and *M. fallax*, together with four other *Meloidogyne* spp., were subjected to a phenetic study by analysing large numbers of protein and DNA polymorphisms, using two-dimensional gel electrophoresis (2-DGE) and Amplified Fragment Length Polymorphism (AFLP) technique, respectively (Chapter 7). These molecular data were compared to a set of diagnostical morphological data. Subsequently, intraspecific variation in protein composition by 2-DGE was examined in *M. hapla*, *M. chitwoodi* and *M. fallax* (Chapter 8).
In *Meloidogyne* a large cytological variation occurs and *M. hapla* is one of the most variable organisms known in this respect (Triantaphyllou, 1985). A cytological method using UV microscopy was optimized for studying meiosis during oogenesis in *Meloidogyne* spp., while sperm development in the three earlier mentioned species was investigated *in situ* by confocal laser scan microscopy (Chapter 9). These techniques were applied in controlled and spontaneous crossing experiments for interspecific hybridization between *M. chitwoodi* and *M. fallax* (Chapter 10). Post-reductional meiosis was indirectly proven to occur in *Meloidogyne* spp. by comparing AFLP patterns between repeated mono-female lines and genetic consequences for conservation of heterozygosity in isolates of meiotic parthenogenetic *Meloidogyne* spp. are discussed (Chapter 11).
Finally, the consequences of the variation on the species concept, the occurrence of males, the distinction of isolates, the occurrence of new pathotypes, the testing of virulence and breeding perspectives are discussed (Chapter 12).

CHAPTER 2

Preservation of second-stage juveniles in liquid nitrogen: differences in response between isolates

2.1. Summary - A procedure for long-term preservation of germplasm of *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax* in liquid nitrogen is described, including a pretreatment with 10 % ethanediol for 2 h at room temperature and 40 % ethanediol for 45 minutes on ice. Survival rates ranged from 45 to 98 % with an average of 75 %. Comparison of three different isolates of *M. hapla*, two of *M. chitwoodi* and two of *M. fallax* revealed a significantly higher survival for one *M. hapla* isolate, while survival rates of the other six isolates were not significantly different. It was shown that higher lipid reserves in juveniles could possibly explain the high survival of this *M. hapla* isolate. Juveniles after freezing were able to reproduce on plants, but infectivity was significantly lower than for non-frozen juveniles. It is recommended to multiply juveniles, stored in liquid nitrogen, for one generation to be used as inoculum for experimentation.

2.2. Introduction

Long-term cryopreservation of obligate plant parasitic nematodes would be advantageous over preservation on plants, because the latter is laborious and demands much greenhouse space and care to avoid contamination. Additionally, certain genetic studies require maintenance of the original isolates or certain generations in order to preserve genetic variation. In general, cryopreservation seems to be a suitable method for long-term preservation of nematodes without substantial losses in survival and viability (e.g. Sayre & Hwang, 1975; Bridge & Ham, 1985).

Already in 1920, Rahm (1921, 1922) carried out cryobiological experiments with the nematode species *Plectus rhizophilus* and *P. parietinus*. In a condition of asphyxia, *Plectus* spp. could survive exposure to -272 °C for a few hours and to -192 °C for as long as 5 days. Rahm (1921) found also that, in moist condition, *Plectus* spp. could survive up to 24 h at -253 °C, but only if the nematodes were first slowly frozen in water. It was hypothesized that cold could be a stimulus to bring the nematodes in an asphyctic condition, which is favourable for successful freezing at extremely low temperatures.

Further experiments with liquid nitrogen, at -196 °C, were done by De Coninck (1951) with the free living nematode species *Anguilula silusiae*. He found 5% survival of third and fourth stage juveniles, using a two-step procedure with -30 °C and -196 °C without adding a cryoprotectant.

Gehinio and Luyet (1951) were able to induce a "cold hardening" effect by exposing vinegar eel worms (*Turbatrix aceti*) during 16 h to 95 % relative humidity causing a dehydration of the nematodes, which resulted in a survival of 90 % after freezing in -77 °C for 1 h. Freezing techniques were improved by the application of cryoprotectants of which the four most common are: methanol, ethanediol (= ethylene glycol), glycerol and dimethyl sulphoxide. Different genera and sometimes even different species within a genus react differently to these cryoprotectants. Methods

were described with various percentages survival for the following nematode species: 56 % survival of *Aphelenchoides sacchari*, 63 % survival of *Caenorhabditis briggsae*, 87 % survival of *Panagrellus redivivus* and 40 % survival of *Turbatrix aceti* (Hwang, 1970), 23 to 35 % survival of *Ditylenchus dipsaci* (Sayre & Hwang, 1975), 75 % survival of *Caenorhabditis briggsae* (Haight *et al.*, 1975), 25 % survival of *Meloidogyne graminicola* (Bridge & Ham, 1985), 50 to 90 % survival of *Meloidogyne* spp. (including *M. hapla*) and *Heterodera* spp. (Triantaphyllou & McCabe, 1989), 30 to 34 % survival of *Steinernema feltiae* (Smith *et al.*, 1990), 80 % survival of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* (Popiel & Vasquez, 1991), 17 % survival of *Bursaphelenchus* spp. (Riga & Webster, 1991) and 69 % for *Steinernema* spp. and 68 % for *Heterorhabditis* spp. (Curran *et al.*, 1992). For many *Meloidogyne* species, Bridge and Ham (1985) and Triantaphyllou and McCabe (1989) found ethanediol to be effective. However, freezing techniques for *M. chitwoodi* have not been reported at present.

Besides the choice of a competent cryoprotectant, the duration of pretreatment, temperature at pretreatment, condition (age) of the nematodes and the speed of freezing and thawing are factors determining the efficiency of the method.

The present study is aimed at verifying and optimizing the procedure described by Triantaphyllou and McCabe (1989), in order to find a quick and effective method for long-term preservation of *M. hapla, M. chitwoodi* and *M. fallax*. Special attention was paid to the effect of storage in liquid nitrogen on the infectivity of these two species and on possible differences between isolates of *M. hapla, M. chitwoodi* and *M. fallax*.

2.3. Materials and Methods

2.3.1. Nematode isolates

Three isolates of *M. hapla*: Hb, Hc and Hl, two of *M. chitwoodi*: Ce and Ci and two of *M. fallax*: Fa and Fb (Table 2.1), were used in this study. The isolates were found to be true to species by isozyme electrophoresis, as described by Esbenshade and Triantaphyllou (1990) and Karssen (1996).

Experiments for optimization of the freezing technique were mainly carried out with second-stage juveniles (J2) of *M. hapla* isolate Hl. All isolates were maintained at IPO-DLO on *Lycopersicon esculentum* cv. Moneymaker for several generations. Only J2 of 1 to 2 days after hatching were used for experimentation.

2.3.2. Two-step pretreatment with ethanediol

Because of its effectiveness in earlier research (Bridge & Ham,1985; Triantaphyllou & McCabe, 1989), ethanediol was used. Pretreatment was applied in two steps, as suggested for *Meloidogyne* spp. by Triantaphyllou and McCabe (1989). During the first step, J2 were incubated in approximately 10% ethanediol at room temperature (22 to 24 °C) and during the second step incubated in approximately 40% ethanediol on ice.

The following general procedure was pursued: 100 to 500 J2 were obtained in 100 µl suspension in an eppendorf vial. For the first step, a quantity of 900 µl 10 %

Table 2.1. Origin of the Dutch isolates of *M. hapla*, *M. chitwoodi* and *M. fallax*

Isolate	Species	Host of origin	Year of sampling	Obtained from[1]
Hb	*M. hapla*	*Astilbe*	1990	PD
Hc	*M. hapla*	*Aconicum*	1990	PD
Hl	*M. hapla*	tomato	unknown	CPRO
Ce	*M. chitwoodi*	potato	1992	CPRO
Ci	*M. chitwoodi*	tomato	1989	PD
Fa	*M. fallax*	early primrose	1992	PD
Fb	*M. fallax*	beet	1991	PD

[1] PD = Plant Protection Service, Wageningen, The Netherlands. CPRO = Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands.

ethanediol was added. Just before the second step, the vials were centrifuged at 9,000 g at a top-bench centrifuge for 0.5 min and the upper layer was removed leaving a volume of 80 µl. A quantity of 80 µl 70 % ethanediol (0 °C) was added on ice, which resulted in a final concentration of ethanediol of approximately 40 %.
This general procedure was optimized by studying the optimum periods of pretreatment in the first and second steps. The period of the first step of pretreatment was optimized in three experiments with four or five replicates. Twelve different periods were tested: 15, 30, 45, 60, 90 min, 2, 2.5, 3, 4.5, 6, 7.5 and 17 h, followed by a 45 and 90 min second step pretreatment. For optimization of the second step, five different periods were applied in an experiment with five replicates: 15, 30 45, 60 and 90 min, preceded by a first step of 2 or 17 h.

2.3.3. Three-step pretreatment

To study the effect of 50 % ethanediol on toxicity and protection, an experiment with six replications was done with a three-step pretreatment: from 10 to 30, and finally to 50 % ethanediol. Pretreatment with 30 and 50 % ethanediol was applied at 0 °C. In this experiment, the period for the first step was fixed at 2 h while the second and the third steps were applied at respectively 45 and 45 min, 60 and 30 min and 60 and 45 min.

2.3.4. Freezing, storage and thawing

For freezing, a quantity of 100 µl pretreated J2 suspension was quickly put on 0.8 x 4.0 cm pre-chilled strips of filter paper (Whatman #3) and put in 1.8 ml Nunc cryo tubes, already filled with liquid nitrogen. The tubes remained uncovered during storage and were attached to holders, containing a maximum of six tubes. Contact of the filter paper strip with the liquid nitrogen caused an immediate adhesion of the J2 to the filter paper, preventing loss of J2 and possible contamination. For experimentation purpose, juveniles were stored for 2 h in liquid nitrogen.
Juveniles which have been stored in liquid nitrogen are called hereafter in this chapter "cryo-juveniles". Thawing of cryo-juveniles was done by quickly transferring the

paper strips to Petri-dishes containing 15 to 20 ml water of approximately 35 °C. Survival was estimated 24 h after thawing as the number of motile cryo-juveniles per total cryo-juveniles.

2.3.5. Infectivity of cryo-juveniles

The infectivity of cryo-juveniles, meaning the ability of cryo-juveniles to cause infection, was estimated in terms of reproduction. This was done in two experiments by inoculating 2 to 3 week old plants of *Lycopersicon esculentum* cv. Moneymaker, a tomato cultivar susceptible to *Meloidogyne* species. The plants were grown in open-ended plastic tubes of 96 ml filled with moist silver sand to which a nutrient solution was added (Boukema *et al.*, 1984). These tubes were placed in a growth cabinet with constant temperature of 20 °C and relative humidity of 70 %.

In the first experiment, with *M. hapla* isolate Hl, 15 plants were inoculated with 5 ml of suspensions of cryo-juveniles and five plants with untreated J2 of isolate Hl with known concentrations varying from 75 to 200 nematodes ml^{-1}. In the second experiment, with *M. hapla* isolate Hc and *M. fallax* isolate Fa, ten plants were inoculated with 5 ml suspensions of cryo-juveniles and five with untreated J2 of these two isolates containing 20 nematodes ml^{-1}. Eight to 9 weeks after inoculation, infectivity was estimated by the following three reproduction parameters: number of egg-masses after one generation per initial number of J2, number of eggs per egg-mass and number of hatched J2 after one generation per initial number of J2 (Pf/Pi). Egg-masses were counted visually. Eggs and J2 were counted on a Context Vision image analyzer, using a nematode count programme for J2, as described by Been *et al.* (1996), or a modified version for egg counting. In the next generation, 400 hatched juveniles from each of the 20 plants in the first experiment, were used for the inoculation of 20 other plants, in order to study infectivity of J2 that were hatched from egg-masses originating from cryo-juveniles.

2.3.6. Effect of storage time on survival

The effect of freezing periods varying from 15 min to 2 weeks on the survival after storage was studied in two experiments with five replications.

2.3.7. Effect of short thawing intervals on survival during and after storage

The influence of a short interruption in the freezing process on the J2 was examined by exposing the paper strips for different periods ranging from 0 to 60 s to room temperature. Also, the necessity of a quick transfer of the paper strips from the liquid nitrogen to the Petri-dish with water was verified by exposing the paper strips during different periods ranging from 1 to 60 s to room temperature before this transfer. These two experiments were performed with four replicates.

2.3.8. Effect of pretreatment on survival and infectivity

In an experiment with seven replicates, the effect of solely pretreatment, without additional freezing, on the survival and on the infectivity was examined. As a control, pretreatment was followed by storage in liquid nitrogen. Plants of 'Moneymaker' were

inoculated with 5 ml of suspensions containing 20 nematodes ml^{-1}. Infectivity was estimated as described above.

2.3.9. Variation in survival between and within M. hapla, M. chitwoodi *and* M. fallax

In three experiments, the seven isolates mentioned in Table 2.1 were tested for variation between the three *Meloidogyne* species and between the different isolates. In the first experiment Hc and Fa were tested, in the second: Hb, Hl, Ce, Fb and Ci and in the third: Hb, Hc, Fa and Fb. The experimental design was a complete block with five replicates. Survival was estimated after 24 h of cryo-preservation as the percentage motile cryo-juveniles.

2.3.10. Neutral lipid staining of J2

Neutral lipid reserves were estimated for nematode isolates Hb, Hc, Fb and Ce by a histochemical lipid staining procedure of individual J2 using Oil-Red-O, as described by Storey (1984) for *Globodera* spp. Relative values for neutral lipid reserves were for approximately ten J2 per isolate.

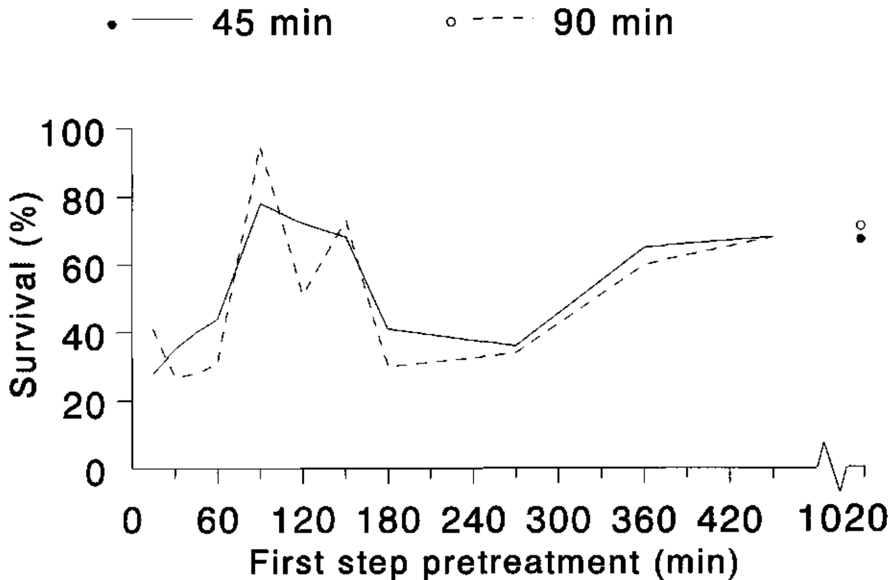


Figure 2.1. Means of survival rates after storage in liquid nitrogen of J2 of *M. hapla* isolate Hl, for different periods of the first step pretreatment with ethanediol, at 45 and at 90 min second step pretreatment

2.3.11. Statistical analysis
Means were compared by analyses of variance, using the Genstat programme (Genstat 5 Committee, 1993). Testing differences of means was done with a multiple range test at 5% confidence interval.

2.4. Results

2.4.1. Two-step pretreatment with ethanediol
The two steps in the pretreatment with the cryoprotectant ethanediol were studied in detail for *M. hapla* isolate Hl, in order to find optimum protection of the J2. In Figure 2.1 results are shown for various periods of time in the first step at two periods for the second step. Results for various periods at the first step indicated significant differences between periods. The development of the survival in the time at the second step of 90 min was largely below that at 45 min second step but in only one of the three experiments, this tendency was statistically significant.
An extremely high average survival of 94 % was obtained with a pretreatment of 90 min first step and 90 min second step. However, the 90 min second step pretreatment caused more fluctuation in survival than the 45 min second step pretreatment. A more reliable and repetitive result was obtained with the 45 min second step pretreatment. The highest average survival with the 45 min second step pretreatment was 78 % at 90 min first step. At the 45 min second step, no significant differences between survival rates were found at the periods of first step pretreatment of 90, 120, 150 min, 6, 7.5 and 17 h. Means of survival for these periods fluctuated between 65 and 78 %. In general, the confidence intervals for the means for these periods were smaller than for other periods.
A significantly lower average survival of 28 to 44 % was obtained at periods of the first step shorter than 90 min and of 3 and 4.5 h.
It was demonstrated that both steps of pretreatment were indispensable for high survival rates. A pretreatment of just the first step during 17 h resulted in only 2 % survival, and direct application of the second step during 90 min gave 27 % survival.
In Figure 2.2 survival rates are shown at various periods of second step pretreatment. No significant difference was obtained for survival between different periods when pretreated during 2 h and 17 h at the first step. Survival rates at short periods of 15 and 30 min second step treatment were significantly lower than those at periods of 45, 60 and 90 min. Optimum survival of approximately 80 % was obtained for these last three periods.

2.4.2. Three-step pretreatment
Survival at a three-step pretreatment with different periods of 30 and 50 % ethanediol were not significantly different from those at a two-step pretreatment (unpublished).

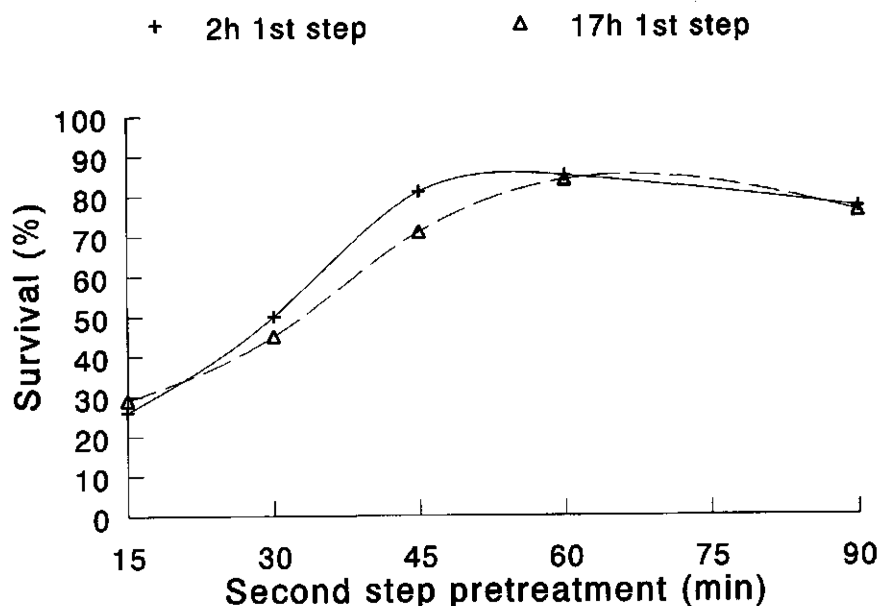


Figure 2.2. Means of survival rates after storage in liquid nitrogen of J2 of *M. hapla* isolate Hl, at different periods of the second step pretreatment with ethanediol, at 2 and at 17 h first step pretreatment

2.4.3. Infectivity of cryo-juveniles; variation in infectivity between nematode isolates
The effect of storage in liquid nitrogen on the infectivity of J2 is summarized in Figure 2.3. Clear and significant declines in Pf/Pi-values and number of egg-masses per inoculated J2 were observed. These declines varied between the three isolates Hl, Hc and Fa that were used in this experiment. However, the number of eggs per egg-mass was influenced less than the two other parameters. Only for isolate Hl the number of eggs per egg-mass of cryo-juveniles was significantly less than that of non-frozen J2. In another experiment with isolate Hl however, no significant difference (Table 2.2) was observed between eggs per egg-mass of cryo-juveniles and untreated J2.
No significant differences between infectivity of the second generation cryo-juveniles and non-frozen J2 were found (Figure 2.3B).

2.4.4. Effect of storage time on survival
No significant difference was found between means of survival for period of storage. The overall means for the two experiments were 72.2, 74.1, 72.1 and 79.1 ($lsd_{0.5}$=11.3) for 15 min, 2 h, 1 day and 2 weeks, respectively.

2.4.5. Effect of short thawing intervals on survival during and after storage
For thawing it is important to know whether short intervals of temperatures above 0 °C

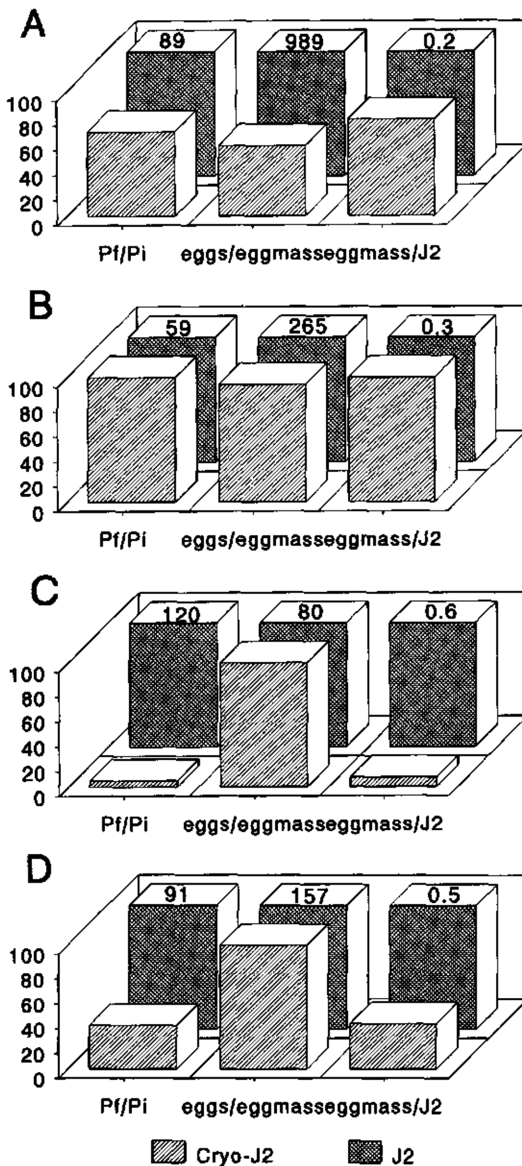


Figure 2.3. Effect of storage of J2 in liquid nitrogen on the three parameters: Pf/Pi, number of produced eggs per egg-mass and number of produced egg-masses per inoculated J2, expressed as percentages of the values for these parameters for non-frozen, untreated J2 (Y-axis: percentages). **A**. *M. hapla* isolate Hl. **B**. *M. hapla* isolate Hl, after one generation of multiplication of the frozen and non-frozen J2. **C**. *M. hapla* isolate Hc. **D**. *M. fallax* isolate Fa. (The numbers on top of the double-shaded bars correspond with the mean values for the parameters as estimated for the non-frozen, untreated J2.)

during storage and shortly before thawing have an effect on the survival (Table 2.3). During storage, cryo-juveniles on paper strips, which were exposed 15 s or longer to room temperature, resulted in a significant decrease in survival of the juveniles. One minute exposure showed a decrease in survival of 68 %. A comparable effect was obtained when paper strips were exposed to room temperature directly after storage and just before thawing: also a significantly lower survival at 15 s exposure to room temperature and an almost complete lack of survival of J2 after 1 min.

2.4.6. Effect of pretreatment on survival and infectivity

Comparison of survival of pretreated J2 with cryo-juveniles, that were pretreated and exposed to liquid nitrogen for storage, resulted in significant differences for all four parameters studied (Table 2.2). Furthermore, no significant differences occurred between the pretreated J2 and non-pretreated J2 (control), except for a higher number of eggs per egg-mass for the pretreated J2, indicating that the applied pretreatment

Table 2.2. Effect of pretreatment with and without storage in liquid nitrogen on survival and infectivity of J2 of *M. hapla* population Hl

Variable	J2[1]	Pretr.[2]	Pretr./N_2[3]
Survival (%)	88[b]	95[b]	64[a]
Egg-masses/inoculated J2	0.4[b]	0.5[b]	0.2[a]
Eggs/egg-mass	114[a]	169[b]	104[a]
Produced J2/inoculated J2	88[b]	98[b]	32[a]

[1] = J2 without pretreatment (control)
[2] = Only pretreatment, as described in text
[3] = Pretreatment followed by storage in liquid nitrogen
a,b,c means with common letters in a row do not differ significantly at $P<.05$, referring to an F-distribution

with ethanediol had no toxic effect on J2.

2.4.7. Variation in survival between and within M. hapla, M. chitwoodi *and* M. fallax
Variation in survival between isolates of *M. hapla, M. chitwoodi* and *M. fallax* was studied in three different experiments in which no interaction effects were observed between isolates and frozen or non-frozen J2, but only differences in level of survival. Statistical results of the three experiments with isolates Hb, Hc, Hl, Ce, Fa, Fb and Ci showed no significant differences in survival between the three species. However,

Table 2.3. Effect of short time periods at room temperature on the survival of frozen J2 of *M. hapla* isolate Hl during and after storage in liquid nitrogen

Time period (s)	Survival (%) during[1]	after[2]
0	94[c]	-
1	-	94[d]
5	86[c]	-
15	64[b]	74[c]
30	63[b]	18[b]
60	32[a]	4[a]

[1] =Paper strips were taken out of the liquid nitrogen during storage.
[2] =Paper strips were taken out after storage, prior to thawing.
a,b,c,d means with common letters in a column do not differ significantly at $P<.05$ referring to an F-distribution

Table 2.4. Relative values for neutral lipid reserves and values for survival after storage in liquid nitrogen of two isolates of *M. hapla*, one of *M. chitwoodi* and one of *M. fallax*

Population	Rel. lipid area (%)	Survival (%)
Hb	67[a]	58[b]
Hc	58[a]	93[a]
Ce	29[c]	49[b]
Fb	45[b]	58[b]

a,b,c means with common letters in a column do not differ significantly at $P<.05$ referring to an F-distribution

differences between isolates were demonstrated: survival was significantly higher for isolate Hc than for the other six isolates (Figure 2.4). No significant differences were found between the remaining six isolates.

2.4.8. Neutral lipid staining of J2

Analysis for lipid reserves showed that percentages area of lipid reserves of individual J2 were significantly higher for *M. hapla* isolate Hc than for *M. chitwoodi* isolate Ce and *M. fallax* isolate Fb, but not significantly different from isolate Hb (Table 2.4).

2.5. Discussion

A two-step procedure using ethanediol as cryoprotectant has been described by several authors (Ham *et al.*, 1981, James, 1981, Bridge & Ham, 1985, Triantaphyllou & McCabe, 1989). This procedure has been proven to be successful in overcoming problems in penetration and toxicity of the cryoprotectant, which are related to temperature (Ham *et al.*, 1981).

Periods for the first and the second step of the pretreatment with ethanediol were optimized in this study for cryopreservation of J2 of *M. hapla*, *M. chitwoodi* and *M. fallax*. Results from experiments with *M. hapla* isolate Hl showed for the second step a curve with a maximum starting at 45 min, indicating 45 min to be the optimum period (Figure 2.2). Periods for the first step treatment shorter than 90 min resulted in a significantly lower survival rate than at 90 - 150 min, indicating that penetration of ethanediol in the J2 during periods less than 90 min was probably insufficient to provide optimum protection for the nematodes.

Unexpectedly, however, results for the first step treatment were not unambiguously clear: at 3 and 4.5 h first step pretreatment, survival was significantly lower than at

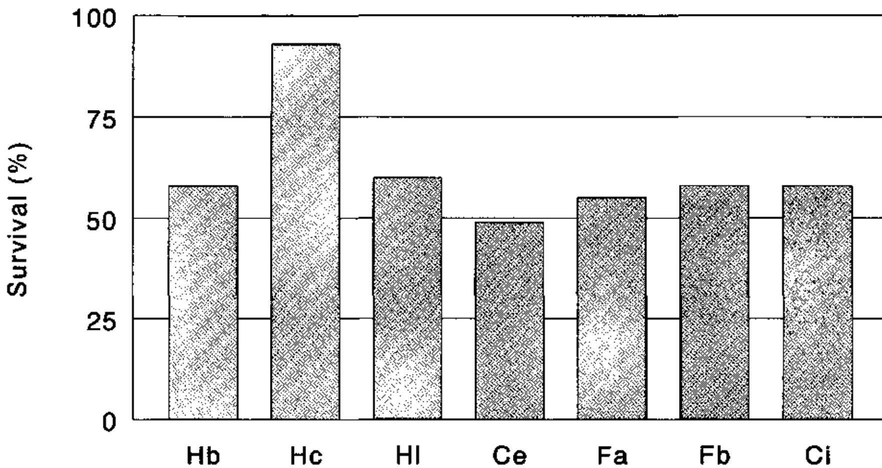


Figure 2.4. Means of survival rates after storage in liquid nitrogen of J2 of three isolates of *M. hapla* (Hb, Hc and Hl) two of *M. chitwoodi* (Ce and Ci) and two of *M. fallax* (Fa and Fb)

surrounding periods, resulting in a depression in the optimum curve (Figure 2.1). This temporal pattern of survival was observed in both experiments with the second step treatment. Ethanediol can cause toxic effects (James, 1981) and apparently this is the case at a first step pretreatment of more than 2.5 h, resulting in a low survival after freezing. Regaining a higher survival rate after pretreatment of 6 h or longer is harder to explain. To our best knowledge, this phenomenon during first step pretreatment has not been found in the literature: most authors describe experiments with pretreatments for maximum 4 h. Triantaphyllou and McCabe (1989) found that at 3 and 4 h first step pretreatment of J2 of *M. incognita*, survival was not significantly less than at 1 and 2 h. Most likely, the difference between their result and ours is due to physiological differences between nematode species and isolates. Based on the present study, it is recommended to apply a two-step pretreatment with incubation periods of 2 h (± 30 min) for the first and 45 min for the second step. For the other experiments described in this paper, these periods of pretreatment were used. Survival rates ranged at the recommended pretreatment from 45 to 98 % with an average of 75 % in ten experiments with *M. hapla* isolate Hl, which is comparable with results of Triantaphyllou and McCabe (1989) for *M. incognita*.

Short periods of exposing frozen J2 to room temperature during and immediately after the storage phase can be fatal for the survival. Based on the present study, it is recommended not to exceed a 10 s exposure of frozen J2 to room temperature. To minimize the risk of important losses, it is suggested to use units of storage that contain only a few paper strips which can be quickly taken out from and replaced in the storage container.

Survival did not change significantly with different storage periods. Although the applied periods were relatively short (from 15 s to 2 weeks), it is concluded that no

marked decline is to be expected when stored in liquid nitrogen for long periods. Curran *et al.* (1992) found no significant difference between 24 h and 3 years storage in liquid nitrogen of *Steinernema carpocapsae*.

The recommended two-step pretreatment with ethanediol had no toxic effect on J2, neither on the survival, nor on the infectivity of J2 (Table 2.2). Consequently, the decline in survival and infectivity of cryo-juveniles is completely due to the freezing, storage and thawing procedure. If required, improvement of the survival and infectivity could only be obtained by refining the freezing, storage and thawing phase. Pretreatment of J2 resulted in an unexpected, significantly higher eggs per egg-mass ratio than the non-pretreated J2 (Table 2.2). Apparently, ethanediol stimulates egg-mass filling. This conclusion needs to be verified in further experiments.

The recommended two-step pretreatment with ethanediol for long-term storage in liquid nitrogen has been applied to three isolates of *M. hapla*, two of *M. chitwoodi* and two of *M. fallax*, indicating no significant difference in survival between the three species but a significant difference between isolate Hc and the other isolates (Figure 2.4). It is unlikely that the variation in survival, resulting in the range of 45 to 98 % as described above, caused this significantly different result for Hc, because two unrelated experiments showed similar high survival rates for Hc. Also Curran *et al.* (1992) found considerable variation in survival of isolates of *Steinernema* spp. Analysis of neutral lipid reserves indicated that higher lipid reserves in individuals of isolate Hc could explain the significantly higher survival (Table 2.4). For certain nematode species it is known that higher concentrations of trehalose, lipid and/or glycogen occur when periods of desiccation have to be overcome (Womersley *et al.*, 1982). They concluded that the main purpose of lipid in free-living and plant-parasitic nematodes is the provision of food reserves which can either be used during periods of environmental stress or at the induction of, and revival from, the anhydrobiotic state. Storey (1984) obtained evidence for a correlation between neutral lipid reserves and mortality (at 20 °C), mobility and infectivity of J2 of *Globodera* spp. In order to explain the high survival after freezing of isolate Hc, as found in this study, it was hypothesized that an association could exist between the survival after freezing and the neutral lipid reserves of nematode isolates. However, satisfactory evidence for this hypothesis was not obtained because of the high value for lipid reserves of isolate Hb as well. More isolates and more individuals per isolate need to be tested for neutral lipid reserves to provide full evidence for this hypothesis.

Variation was not only demonstrated for survival, but also for infectivity, that was studied in three isolates: Hc, Hl and Fa (Figure 2.3). The decline in Pf/Pi and number of egg-masses per inoculated J2 was very high for Hc (approximately 95 %) and considerably lower for Hl and Fa (30 and 60 % respectively). In spite of the relatively high survival for isolate Hc (Figure 2.4), low values for reproduction parameters were found for this isolate. It is concluded that survival is not positively correlated with number of produced egg-masses and number of produced J2 per inoculated J2. Because of the large variation in decline of these parameters, it is recommended to store large quantities of J2 and to use J2 of a second generation of cryo-juveniles as inoculum for tests of virulence.

It is concluded that the described procedure for cryopreservation provides a suitable method for long-term preservation of *M. hapla*, *M. chitwoodi* and *M. fallax* germplasm, which can be applied to maintain large numbers of isolates for collection purposes, to preserve genetic variation for genetic studies, to provide possibilities for testing different generations simultaneously and to minimize contamination which is a considerable risk in cultures on plants.

CHAPTER 3

Isolate-by-cultivar interaction in *M. hapla*, *M. chitwoodi* and *M. fallax* on potato

3.1. Summary - To study virulence and aggressiveness in root-knot nematodes on cultivars of *Solanum tuberosum*, four isolates of *Meloidogyne hapla* race A, one of *M. hapla* race B, three of *M. chitwoodi* and two of *M. fallax* were evaluated on ten commercial potato cultivars under semi-sterile conditions in Petri-dishes. Virulence and aggressiveness were assessed in terms of nematode reproduction by penetration rate and reproduction factor, estimated by the number of produced egg-masses and produced juveniles, respectively, divided by the number of inoculated juveniles. Significant interaction for both parameters was shown between species of *Meloidogyne* and potato cultivars. Only *M. hapla* showed significant isolate-by-cultivar interaction, which was predominantly caused by the *M. hapla* race B isolate Hh. This indicates variation in virulence and suggests the occurrence of different genetic factors for virulence and resistance in *M. hapla* isolates and potato cultivars, respectively. Despite large differences, the observed levels of resistance were too low to be of practical meaning for breeding purposes, with the exception of resistance to isolate Hh. No significant differences were obtained between isolates of *M. chitwoodi* and *M. fallax* or isolate-by-cultivar interaction, indicating neither variation in aggressiveness nor in virulence in the employed isolates. A comparative greenhouse experiment gave comparable results for penetration rate but contradicting for reproduction factor, most likely due to differences in life cycle which appeared to be shorter in *M. fallax* than in *M. hapla* and *M. chitwoodi*. The Petri-dish method proved to be accurate for virulence studies of root-knot nematodes on potato cultivars. *M. fallax* was most aggressive on potato, followed by *M. chitwoodi*, *M. hapla* race A and finally by *M. hapla* race B.

3.2. Introduction

Root-knot nematodes are plant parasitic nematodes that cause economic losses in a wide range of crops (Taylor & Sasser, 1978). In the Netherlands, three species have been identified which are a potential threat for yield and quality losses in arable crops. Firstly, *Meloidogyne hapla*, which is widespread over the country and was already reported in the late fourties as a problem in many crops (Oostenbrink, 1950). Secondly, *M. chitwoodi*, which is predominantly found in the SE part of the Netherlands in an increasing number of farmers fields. Finally the recently described species *M. fallax* (Karssen, 1996), which is closely related to *M. chitwoodi* and therefore during the previous years had been regarded as a deviating type or race of *M. chitwoodi* (Van Mechelen *et al.*, 1994). *M. chitwoodi* and *M. fallax* are sympatric.

Potato (*Solanum tuberosum* L.) is economically the most important arable crop in the Netherlands and, by covering about 13% of the total agricultural area, in this respect the second crop in this country (Anonymous, 1996). Root-knot nematodes can cause

significant losses in cultivated potato (Brodie *et al.*, 1993) and an initial nematode population of 1 juvenile per 250 cc soil can cause already damage (Mojtahedi *et al.*, 1991). The losses are mainly caused by the formation of blisters on tubers caused by females which are located directly under the epidermis. These deformations may result in an unmarketable fresh product. No significant levels of resistance have been identified in roots of commercial potato cultivars in screening experiments with *M. hapla* and *M. chitwoodi* (Janssen *et al.*, 1995).

In this chapter, virulence is defined as the ability of an isolate of *Meloidogyne* spp. to reproduce on resistant host plants that prevent or suppress reproduction of non-compatible isolates of *Meloidogyne* spp. (after Roberts, 1995). The presence of virulence can be shown by a significant interaction between nematode isolates and potato cultivars. Aggressiveness is defined as the relative ability of an isolate of *Meloidogyne* spp. to reproduce on those genotypes of the host species for which it is virulent.

The present research is aimed at studying the root response of some potato cultivars to a series of isolates of *M. hapla*, *M. chitwoodi* and *M. fallax*, and at investigating the possible interaction between cultivars and isolates. The effectiveness of some experimental methods to study the variation in reproduction parameters is also evaluated.

3.3. Materials and methods

3.3.1. Plant material and nematode isolates

The following ten *S. tuberosum* cultivars were employed in this study: Anosta, Atrela, Bintje, Concurrent, Eigenheimer, Erntestoltz, Gloria, Kardal, Obelix and Saturna. These genotypes were obtained from different Dutch breeding companies and their response to one isolate of *M. hapla* or *M. fallax* was previously tested. Differences in response to these isolates of *Meloidogyne* spp. were found (pers. com. J.J. Van de Haar, RZ-Research, Metslawier, The Netherlands and G.J.W. Janssen, CPRO-DLO, Wageningen, The Netherlands). Ten Dutch nematode isolates were used (Table 3.1); four of these belonged to *M. hapla* race A, one to *M. hapla* race B, three to *M. chitwoodi* and two to *M. fallax*, all originating from geographically different sites in the Netherlands. These isolates were identified to species by isozyme electrophoresis (Esbenshade & Triantaphyllou, 1985; Karssen *et al.*, 1995) and by rDNA-analysis of ITS-regions (Zijlstra *et al.*, 1995). The distinction between isolates of *M. hapla* race A, which are meiotic parthenogenones, and race B, which are mitotic parthenogenones, was done by investigating meiosis in oocytes of adult females (Van der Beek *et al.*, G; Chapter 9). The isolates were maintained and propagated on *Lycopersicon esculentum* cv. Moneymaker.

Two different experiments were performed: the first, using a Petri-dish culture method of roots and the second by greenhouse testing of complete plants.

3.3.2. The Petri-dish experiment

Potato tubers from storage at 5 °C were exposed to light at 20 °C during two to three weeks prior to planting. Tubers were superficially sterilized for five minutes with 0.1

Table 3.1. Origin of the nematode isolates, from various locations in the Netherlands

Isolate	Race[1]	Last host	Year of sampling	Provided by[2]
M. chitwoodi				
Ca		maize	1989	PD
Ck		tomato	1989	PD
Cl		wheat	1990	PD
M. fallax				
Fa		early primrose	1992	PAGV
Fb		beet	1991	PD
M. hapla				
He	A	potato	1992	PAGV
Hh	B	fallow (weed)	1992	PAGV
Hi	A	immortelle	1992	PAGV
Hj	A	carrot	1992	PAGV
Hk	A	chicory	1992	PAGV

[1] Cytological race type of *M. hapla*: A=facultative meiotic parthenogenetic, B=obligate mitotic parthenogenetic
[2] PD = Plant Protection Service, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands.

% sodiumhypochlorite and rinsed with tap water. Sprouts were transferred to Petri-dishes containing 30 ml of 2 % water agar. The applied technique for Petri-dish culture was comparable with that described by Mugniery and Person (1976) and Mugniery (1982). Petri-dishes containing one sprout each, were kept in a plastic bag at 20 °C in an incubator without light. After three to five days, the roots were ready for inoculation.

The inoculum was prepared from second stage juveniles (J2) which were hatched from heavily infected tomato roots that were placed in a mist cabinet at 20 °C, with water of 18 °C being dispersed by nozzles, resulting in 100 % relative humidity and an aerated water layer around the roots (Seinhorst, 1988). Under these conditions immature eggs were allowed to mature and females even continued their egg production, so that during a period of several weeks inoculum could be prepared from the same roots. Within two days after hatching J2 were inoculated individually on root tips of the developed potato roots in the Petri-dishes. The experiment was set up according to a randomized complete block, with ten replicates. During ten successive weeks, one Petri-dish of each of the 100 plant genotype - isolate combinations was inoculated with ten J2, five on each of two root tips.

The numbers of egg-masses were counted eight weeks after inoculation, and small root pieces containing one egg-mass each were separately placed in small vials in tap water. After three weeks of incubation at 20 °C, all hatched J2 were counted by image

analysis (Been *et al.*, 1996), using an adapted programme for *Meloidogyne* spp.

3.3.3. The greenhouse experiment

To verify results from the Petri-dish experiment, a pot experiment was designed under more natural conditions for nematodes and roots, as compared to culture in Petri-dishes. Tubers of six of the ten *S. tuberosum* cultivars were used in this pot experiment: Anosta, Bintje, Erntestoltz, Gloria, Kardal and Saturna. These cultivars showed differences in response in the Petri-dish experiment. Top sprouts of the tubers were planted in a mixture of 60 % silversand, 30 % ground earthenware and 10 % clay, enriched with 1 g NPK (12-10-18) and 200 ml Steiner's nutrient solution (Steiner, 1968) in black plastic pots of 1.5 l (modified after Been & Schomaker, 1986). The pots were placed in a greenhouse compartment at 20±2 °C and 70 % relative humidity. Perforated sheets of black plastic were placed on top of the pots to protect the soil against excessive evaporation. Water content was adjusted to 15 % of the weight of the dry soil. Irrigation was computer-controlled, by which the evapotranspiration of the leaves could be taken into account.

Two isolates of *M. hapla* race A (He and Hi), one of *M. hapla* race B (Hh), one of *M. chitwoodi* (Ca) and two of *M. fallax* (Fa and Fb) were chosen from the ten isolates used in the Petri-dish experiment (Table 3.1) and were employed in this greenhouse experiment. One- or two-days-old hatched J2 were used as inoculum. Three-weeks-old potato plants were inoculated with 1 J2 cc^{-1} soil by injection, using a 20 cm long, 2 mm wide syringe which was inserted into the soil until near the bottom of the pot and pulled up while injecting, creating an approximately uniform vertical distribution of the inoculum. A total of 25 ml inoculum was applied per plant, divided over five inoculation sites, resulting in approximately 1,500 J2 per plant. The experiment was set up according to a randomized complete block design with five replicates.

Eight weeks after inoculation, the roots were stained with Phloxine-B (Dickson & Struble, 1965) and numbers of egg-masses were estimated in 11 classes according to the following scale: class 1 = 0 egg-masses, 2 = 1-10, 3 = 11-20, 4 = 21-30, 5 = 31-40, 6 = 41-50, 7 = 51-100, 8 = 101-150, 9 = 151-200, 10 = 201-250, 11 = 251-300. Subsequently, eggs were extracted by the hypochlorite method modified after Hussey and Barker (1973), and J2 were hatched by incubating the eggs for three weeks at 20 °C without light. Finally, the numbers of obtained J2 for each root system were estimated by counting three samples of 1 ml nematode suspension with image analysing, as described by Been *et al.* (1996), using an adapted programme for *Meloidogyne* spp.

The greenhouse experiment was repeated to verify the results, using potato cultivars Bintje, Gloria and Kardal and *M. chitwoodi* isolate Ca, *M. fallax* isolates Fa and Fb, *M. hapla* race A isolate Hi and *M. hapla* race B isolate Hh.

3.3.4. Parameters of reproduction and statistical analyses

Reproduction at eight weeks after inoculation was assessed by two parameters: the penetration rate (Pr) and the reproduction factor (Rf). The penetration rate is estimated by the number of produced egg-masses divided by the number of inoculated J2. For

the greenhouse experiment Pr was calculated from the class mid-values of the numbers of egg-masses. The reproduction factor was calculated by the number of produced J2 divided by the number of inoculated J2 (Rf=Pf/Pi).
To the Pr and Rf data Generalized Linear Models (GLM's) were fitted using the method of quasi-likelihood (McCullagh & Nelder, 1989). In all experiments logistic regression models with variance proportional to binomial variance and logit-link function with replicate, cultivar, isolate and isolate by cultivar interaction as explanatory variables were fitted to the Pr data. To the Rf data log-linear regression models with variance proportional to Poisson variance and log-link function and the same explanatory variables were fitted. *F*-tests for the mean deviance ratios were used to assess treatment effects. Pair-wise differences between treatment means on the link scale were assessed using *t*-tests. Isolate-by-cultivar interaction was explored using Generalized Additive Main effects and Multiplicative Interaction effects (GAMMI) models (Van Eeuwijk, 1995, 1996) for the data in the two-way tables of observed isolate by cultivar means for Pr and Rf. For Pr data a logit-bilinear model was used and to the Rf counts a log-bilinear model. In the models interaction effects are written as a sum of multiplicative terms. The logit-bilinear model reads

$$\eta_{ij}=\nu+\alpha_i+\beta_j+\sum_{k=1}^{K}\sigma_k\gamma_{ki}\delta_{kj},\ \eta_{ij}=logit(\mu_{ij})=\log\left(\frac{\mu_{ij}}{n-\mu_{ij}}\right)$$

where μ_{ij} is the mean of Pr for isolate i and cultivar j, n number of inoculated J2, v the general mean, α_i (i=1...I) and β_j (j=1...J) the additive main effects for isolate i and cultivar j respectively. K is the number of multiplicative terms, often loosely referred to as axes. γ_{ki} and δ_{kj} denote the multiplicative isolate and cultivar scores respectively. σ_k is the singular value corresponding to axis k. It can be interpreted as a measure of association between isolate and cultivar scores, and indicates the importance of the axis. Identifiability constraints for the additive and multiplicative effects complete the model. For the log-bilinear model $\eta_{ij}=\log(\mu_{ij})$.
The first axis accounts for the largest amount of interaction (non-additivity) on the link-scale and discriminates best between isolates, followed by the second axis and so on. The number of axes K, necessary for an adequate description of the interaction should preferably be low. K attains its maximum value when it is equal to the minimum of (I-1) and (J-1). In that case the model is equivalent to the GLM model with main effects and interaction. Usually, however, one to three axes give an adequate description and a substantial gain in parsimony in modelling interaction is achieved. The criterion for inclusion of a multiplicative interaction term (axis) is an *F*-test for the ratio of the mean deviance for the particular term to the mean deviance of the rest (Gollob, 1968).
When two axes capture all or most of the relevant non-additivity biplots can be used in exploring and interpreting isolate-by-cultivar interaction (Kempton, 1984: Gower & Hand, 1996). A biplot is a graphical representation in which both isolate and cultivar scores are displayed simultaneously. The isolate and cultivar scores can be scaled as

$\gamma_{ki}^* = \gamma_{ki}\sigma_k^{1-c}$ and $\delta_{ki}^* = \delta_{ki}\sigma_k^c$ with scale factor c ($0 \leq c \leq 1$). The isolate scores $(\gamma_{1i}^*, \gamma_{2i}^*)$ are used as coordinates for a planar depiction of the isolates, and the cultivar scores $(\delta_{1i}^*, \delta_{2i}^*)$ for a similar depiction of the cultivars. In a biplot the scores determine the endpoints of the vectors with starting point at the origin (0,0). The dependence of an isolate difference on the cultivar can be expressed by the tetrad $\tau_{ijmn} = (\eta_{im}-\eta_{in})-(\eta_{jm}-\eta_{jn})$. Pairs of isolates and pairs of cultivars for which the corresponding tetrad is large show considerable interaction. Since

$$\tau_{ijmn} = \sum_{k=1}^{K} (\gamma_{ki}^* - \gamma_{kj}^*)(\delta_{km}^* - \delta_{kn}^*)$$

the tetrad equals the inproduct of the vector of difference between the corresponding isolate vectors and the vector of difference between the cultivar vectors. Hence, the value of a tetrad is obtained by multiplying the length of the projection of either difference vector onto the other and the length of the difference vector on which

Table 3.2. Mean values for penetration rate from the Petri-dish experiment in which ten juveniles of *Meloidogyne* isolates were inoculated on root tips of potato cultivars in ten replicates

	Potato cultivar										
Isolate	Co	Gl	An	At	Bi	Ei	Er	Ka	Ob	Sa	mean
Ca	3.7	2.7	2.7	1.8	4.3	4.8	4.7	3.4	3.1	4.6	3.7
Ck	3.2	6.1	4.2	3.0	5.2	5.4	4.9	1.9	3.7	5.1	4.3
Cl	5.3	4.3	4.0	3.3	3.9	4.7	3.0	3.9	3.7	4.3	4.1
mean	3.7^{abcd}	4.7^{cd}	3.6^{abc}	2.7^{a}	4.5^{cd}	5.0^{d}	4.2^{cd}	3.1^{ab}	3.5^{abc}	4.7^{cd}	4.0
Fa	6.0	5.2	5.2	4.3	6.3	6.2	6.1	3.9	4.4	4.7	5.3
Fb	4.9	6.6	5.0	4.0	6.7	7.6	6.5	3.7	5.1	5.6	5.7
mean	5.5^{abcd}	5.9^{bcd}	5.1^{abc}	4.2^{a}	6.5^{cd}	6.9^{d}	6.3^{cd}	3.8^{a}	4.8^{ab}	5.2^{abc}	5.5
He	1.6	3.0	2.6	2.2	3.3	4.0	2.1	1.0	2.5	2.7	2.6
Hi	1.8	1.8	1.4	1.0	2.6	1.6	2.0	0.8	2.2	1.8	1.7
Hj	0.9	1.6	2.0	1.9	1.5	1.1	1.2	0.4	1.2	1.3	1.3
mean	1.4^{ab}	2.1^{bc}	2.0^{bc}	1.7^{bc}	2.5^{c}	2.2^{c}	1.8^{bc}	0.7^{a}	2.0^{bc}	1.9^{bc}	1.9
Hh[1]	2.0	3.1	0.0	0.0	0.3	0.4	0.4	0.0	0.5	0.2	0.7
Hk[1]	1.3	1.0	2.0	2.3	2.0	3.1	3.4	2.0	2.7	2.7	2.4

a, b, c, d: cultivars with common letters in a row do not differ significantly on the logit-scale ($P<.05$, referring to an F-distribution for the deviance ratio statistic).

[1] Significant interaction contrast between Hh and Hk with cultivar clusters {Gloria, Concurrent} and {remaining cultivars}

projection took place. Consequently, pairs of isolates and pairs of cultivars, which show considerable interaction can be easily spotted in a biplot by looking for pairs of isolate vectors and pairs of cultivar vectors for which the length of the difference vectors is large and both difference vectors are almost parallel. A detailed treatise of bilinear analysis and its applications is given by Van Eeuwijk (1996). Significant results (P<.05) are presented.
All analyses were performed using the Genstat 5 programme (Genstat 5 Committee, 1993).

3.4. Results

3.4.1. The Petri-dish experiment

For both parameters in the Petri-dish experiment, *M. fallax* showed values 20-30 % higher than *M. chitwoodi* and *M. chitwoodi* more than 100 % higher than *M. hapla* (Tables 3.2 and 3.3). Analysis on all data showed significant isolate-by-cultivar

Table 3.3. Mean values for reproduction factor from the Petri-dish experiment in which ten juveniles of *Meloidogyne* isolates were inoculated on root tips of potato cultivars in ten replicates

	Potato cultivar										
Isolate	Co	Gl	An	At	Bi	Ei	Er	Ka	Ob	Sa	mean
Ca	86	145	49	95	165	186	252	52	99	198	137
Ck	124	154	111	103	205	175	95	39	107	154	133
Cl	168	146	107	59	161	172	155	174	101	173	144
mean	126^{abcd}	148^{bcd}	89^{ab}	86^{ab}	177^{d}	178^{d}	167^{cd}	88^{a}	102^{abc}	175^{d}	138
Fa	178	179	155	103	218	215	131	61	160	147	159
Fb	94	165	91	84	235	224	166	118	202	194	170
mean	136^{ab}	172^{b}	123^{b}	94^{ab}	227^{b}	219^{b}	148^{b}	90^{a}	181^{b}	171^{b}	164
He	93	89	116	62	143	214	80	22	92	156	116
Hi	53	78	47	24	100	94	103	17	76	96	73
Hj	24	35	43	48	52	47	69	8	87	73	48
mean	57^{bc}	67^{bc}	69^{bc}	45^{ab}	98^{bcd}	118^{d}	84^{bcd}	16^{a}	85^{bcd}	108^{cd}	79
Hh[1]	48	94	0	0	1	15	2	0	14	6	17
Hk[1]	39	51	54	18	78	73	93	80	96	108	76

a, b, c, d: cultivars with common letters in a row do not differ significantly for on the log-scale (P<.05, referring to an *F*-distribution for the deviance ratio statistic).
[1] Significant interaction contrast between Hh and Hk, with cultivar clusters {Gloria, Concurrent} and {remaining cultivars}

interaction for Pr and Rf. This interaction was further explored by analysing data for each *Meloidogyne* species separately: significant isolate-by-cultivar interaction (P<.01) was found for *M. hapla* for both parameters. In contrast, however, no significant interaction effects were found for *M. chitwoodi* and *M. fallax* isolates with cultivars. Hence, for *M. chitwoodi* and *M. fallax* the interaction was excluded from the model. Fitting the model with main effects only showed significant differences between cultivars, but differences between isolates were not significant. Ranking means of both parameters for the cultivars revealed a highly significant correlation between these two species, using Spearman's Rank Correlation Coefficient (r): r=0.84 for Pr and r=0.82 for Rf. Cultivars Kardal and Atrela were relatively resistant to *M. chitwoodi* and *M. fallax* and cv. Eigenheimer was susceptible.
Within *M. hapla*, isolate Hh (race B) showed extremely low values for Pr and Rf (Tables 3.2 and 3.3).

3.4.2. The greenhouse experiment

From the greenhouse experiment, means of Pr and Rf for the used isolates and cultivars are presented in Tables 3.4 and 3.5. Analysis of all data showed significant isolate-by-cultivar interaction for Pr and Rf, confirming the results of the Petri-dish experiment. When analysing the data for each species separately for Pr, the isolate-by-cultivar effect was significant for *M. hapla*, but not significant for *M. fallax*. *M. fallax* showed significant cultivar effects for Pr, but no significant main effects for isolates. All together, these results are in agreement with the results of the Petri-dish experiment, when analysing data of the ten isolates and ten cultivars, or restricted to the six

Table 3.4. Mean values for penetration rate from the greenhouse experiment in which 1,500 juveniles of *Meloidogyne* isolates were inoculated on potato cultivars in five replicates

	Potato cultivar						
Isolate	An	Bi	Er	Gl	Ka	Sa	mean
Ca	138[a]	125[a]	145[a]	138[a]	155[a]	135[a]	139
Fa	85	175	115	125	125	155	130
Fb	87	185	135	125	125	175	139
mean	86[a]	180[c]	125[b]	125[b]	125[b]	165[c]	135
He	163[b]	205[b]	175[b]	115[a]	195[b]	185[b]	173
Hh	25[b]	37[b]	37[b]	37[b]	7[a]	29[b]	29
Hi	125[b]	205[c]	175[bc]	63[a]	135[b]	195[c]	151
mean	104	149	129	72	112	136	116

a, b, c: cultivars with common letters in a row do not differ significantly on the logit-scale (P<.05, referring to an *F*-distribution for the deviance ratio statistic).

isolates and six cultivars used in the greenhouse experiment. However, in contrast to the results of the Petri-dish experiment for the parameter Rf, the isolate-by-cultivar interaction effect for *M. fallax* was significant (P<.01), but not significant for *M. hapla* (P=.07).
For both parameters *M. hapla* isolate Hh (race B) showed the lowest values. However, a discrepancy with the results of the Petri-dish experiment occurred in the aggressiveness of the isolates, particularly the Rf-values for the two *M. fallax* isolates were lower than expected on the basis of the Pr. In the repeated greenhouse experiment the same discrepancy occurred (data not shown).

3.4.3. Exploring the M. hapla *isolate-by-cultivar interaction*
Petri-dish experiment - To explore the isolate-by-cultivar interaction GAMMI-models with one and two multiplicative interaction terms (axes) were fitted to the Pr and Rf data. Since the difference in deviance (not shown) for the first axis largely accounts for the difference in deviance for the total interaction, a model with one axis (GAMMI-1 model) should be satisfactory. From the calculated isolate and cultivar scores for the first axis, it was concluded that isolates Hh and Hk show considerable interaction and that the cultivar scores consist of two clusters I and II: I={Gloria, Concurrent} and II={remaining cultivars} (Tables 3.2 and 3.3). Consequently, the interpretation of the interaction should be based on the contrast between the isolates Hh and Hk, and the cultivar clusters I and II. Whether the interaction contrast could give a parsimonious description of the isolate-by-cultivar interaction was investigated by fitting logistic and log-linear regression models with replicate, cultivar, the

Table 3.5. Mean values for reproduction factor from the greenhouse experiment in which 1,500 juveniles of *Meloidogyne* isolates were inoculated on potato cultivars in five replicates

	Potato cultivar						
Isolate	An	Bi	Er	Gl	Ka	Sa	mean
Ca	18.5[a]	16.3[a]	16.0[a]	14.7[a]	17.8[a]	14.0[a]	16.2
Fa	4.1[a]	4.5[a]	8.8[b]	5.5[ab]	3.1[a]	6.1[ab]	5.3
Fb	3.0[ab]	4.4[ab]	2.0[a]	6.6[bc]	6.5[bc]	6.9[c]	4.9
mean	3.6	4.5	5.4	6.1	4.8	6.5	5.1
He	10.5	11.3	3.5	4.1	7.6	13.3	8.3
Hh	1.2	1.1	2.5	0.7	0.5	3.2	1.5
Hi	8.5	9.4	11.7	2.0	9.5	12.8	9.0
mean	6.7[b]	7.3[b]	5.9[b]	2.3[a]	5.9[b]	9.8[b]	6.3

a, b, c: cultivars with common letters in a row do not differ significantly on the log-scale (P<.05, referring to an *F*-distribution for the deviance ratio statistic).

interaction contrast and the remaining isolate-by-cultivar interaction as explanatory variables to the Pr and Rf data respectively. The differences in deviance for the Pr data, corresponding to the inclusion of individual model terms, show that the interaction contrast is significant and that no remaining interaction is left (Table 3.6). The analysis of the Rf data leads to the same conclusion. Hence, GAMMI analyses have shown that the interaction is made up by a single interaction contrast, resulting in a very parsimonious description of the isolate-by-cultivar interaction.

Greenhouse experiment - To the Pr data logit-bilinear models with one and two axes were fitted. The differences in deviance for the Pr data, corresponding to the inclusion of individual model terms, are given in Table 3.7. Since the *F*-test for the deviance ratio of axis 2 was significant, a model with two axes was applied to describe the

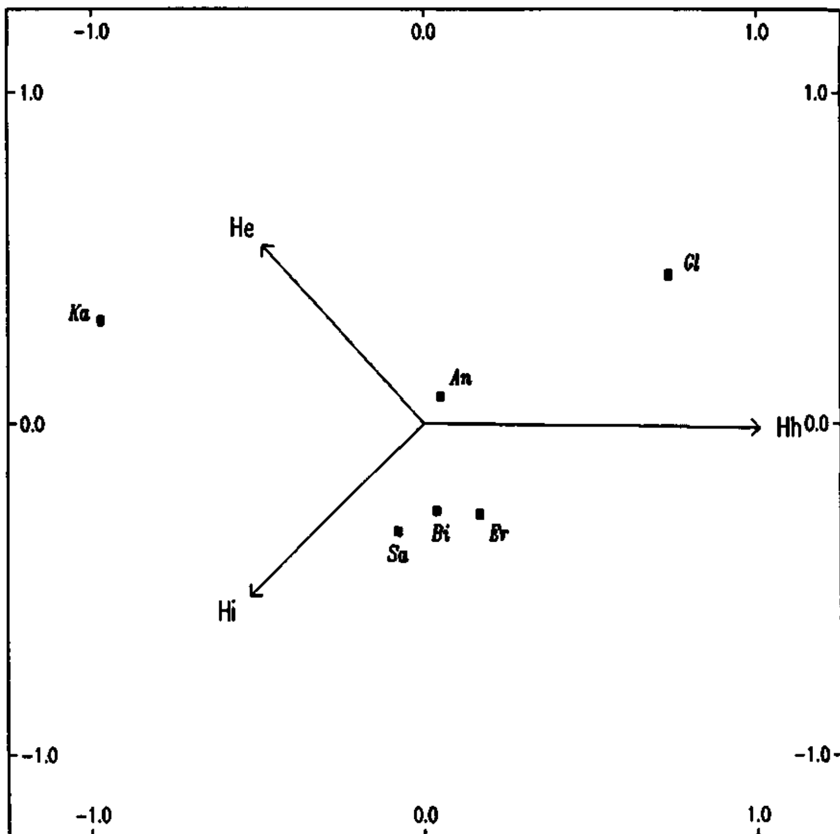


Figure 3.1. Biplot of the interaction of data for penetration rate (Pr) of *M. hapla* isolates on potato cultivars in the greenhouse experiment. The biplot was constructed from a GAMMI-2 model with logit link and variance proportional to binomial variance. Nematode isolates are represented by arrows; potato cultivars by solid squares.

isolate-by-cultivar interaction. To enhance interpretation of the interaction, a simultaneous biplot with scale factor c=0.5, was made (Figure 3.1). The association parameters for the two axes were σ_1=1.41 and σ_2=0.52, respectively. It is noticed in Figure 3.1 that especially cultivars Gloria and Kardal behaved strongly interactive as their vector endpoints are far removed from the origin. Conclusions drawn from biplots are most reliable for isolates and cultivars with high non-additivity, as they are best represented in biplots. The biplot reveals that the tetrads {He, Hh, Gl, Ka} and {Hi, Hh, Gl, Ka} show large interaction.

3.5. Discussion

With one exception, all studied isolates of *M. hapla*, *M. chitwoodi* and *M. fallax* allowed multiplication with Rf>1 on the tested *S. tuberosum* cultivars. Only *M. hapla* race B isolate Hh on Kardal showed Rf-values smaller than 1 in all experiments. These results confirm that *S. tuberosum* cultivars show varying levels of susceptibility to root-knot nematodes: significant differences for penetration rate and reproduction factor exists, not only between the three *Meloidogyne* species but also between isolates of *M. hapla*. Between *M. hapla* isolates, differences in aggressiveness and in virulence were most pronounced between the race B isolate Hh and the race A isolates (Tables 3.2, 3.3 and 3.4; Figure 3.1), but in the Petri-dish experiment significant differences existed also between race A isolates. The isolate-by-cultivar interaction suggests the existence of different genetic factors for virulence and resistance in *M. hapla* and potato cultivars, respectively. However, these genetic factors seem to express only small effects and their accumulation to attain acceptable levels of resistance will be laborious and time-consuming.

Absence of significant isolate-by-cultivar interaction terms between isolates of *M.*

Table 3.6. Deviance table for penetration rate (Pr data) for the *M. hapla* isolates on the ten potato cultivars in the Petri-dish experiment

Source	df	Deviance	Mean deviance	Deviance ratio	
Replicate	9	20.07	2.23	1.33	
Cultivar	9	40.67	4.52	2.70	
Isolate	4	142.01	35.50	21.23	
Contrast	1	91.32	91.32	54.61	***
Cultivar.Isolate	35	61.01	1.74	1.04	ns
Residual	343	573.55	1.67		
Total	401	928.62	2.32		

*** P<.001, ns= not significant (P>.05), referring to an *F*-distribution

chitwoodi and *M. fallax* indicates no variation in virulence in the used isolates of these two species. Although this conclusion is based on only three *M. chitwoodi* and two *M. fallax* isolates and therefore should be considered cautiously, it confirms similar findings in testing 14 *M. hapla*, eight *M. chitwoodi* and five *M. fallax* isolates on wild *Solanum* spp. (Van der Beek *et al.*, C and D; Chapters 4 and 5). Besides absence of virulence, the tested *M. chitwoodi* and *M. fallax* isolates showed no intraspecific variation in aggressiveness on the tested potato cultivars. In spite of the fact that significant differences in Pr and Rf existed between cultivars to *M. chitwoodi* and *M. fallax* isolates, the levels of resistance were too low to be exploited.

The results of the greenhouse experiment were comparable to those of the Petri-dish experiment in showing significant interaction between nematode species and potato cultivars for Pr and Rf, and between *M. hapla* isolates and potato cultivars for Pr. However, the results for both experiments gave dissimilar results for isolate-by-cultivar interactions for Rf: significant interaction for *M. fallax* isolates and no significant interaction for *M. hapla* isolates in the greenhouse experiment in contrast to opposite results in the Petri-dish experiment (Tables 3.5 and 3.3, respectively). Different treatments in both methods could be involved. In the greenhouse experiment Rf-values for *M. fallax* were lower than expected from the corresponding Pr-values in the Petri-dish experiment. This discrepancy is probably caused by differences between the two experimental methods, influencing the accuracy of estimating Rf. The most important difference was that egg-masses were not surrounded by soil particles but were exposed to the air in the Petri-dish experiment. This condition caused hatched J2 to remain within the gelatinous matrix of the egg-masses, while in the greenhouse experiment they would move from the egg-masses through the soil to infect new root-tips. Consequently, the Petri-dish method provides a more accurate estimation of Rf-values after one multiplication cycle of the pathogen than the greenhouse method, for which the Rf-values are underestimated. Besides accurate Rf-estimations, the Petri-

Table 3.7. Deviance table for penetration rate (Pr data) for the *M. hapla* isolates on the six potato cultivars in the greenhouse experiment

Source	df	Deviance	Mean deviance	Deviance ratio[1]	
Cultivar	5	111.18	22.24	10.6	
Isolate	2	820.22	410.11	195.3	
Axis 1	6	45.86	7.64	3.6	*
Axis 2	4	22.58	1.74	2.7	*
Residual	0	0.00			
Total	17	999.83	58.81		

[1] estimate of 2.1 for the residual deviance derived from logistic regression analysis on individual pot data. * P<.05, referring to an F-distribution with 65 degrees of freedom in the denominator

dish method entails more advantages over the greenhouse method: small quantities of inoculum are needed and the initial population (Pi) is exactly known, experiments are inexpensive in needing only limited incubator space, no care is needed during the experiments, and experimental conditions can always be maintained uniformly, particularly by absence of need for irrigation. An important consequence of the Petri-dish method is also that number of egg-masses can be considered as a direct estimation for penetration, as possible differences in attraction are eliminated by placing J2 directly on the root tips. Furthermore, the effect of competition between inoculated juveniles is probably of little importance as numbers of successful penetrations are in general agreement with those of single juvenile inoculations (data not shown). In *Globodera* for instance, the critical number of juveniles per root tip in avoiding competion effects is approximately five (Janssen *et al.*, 1990). A disadvantage of the Petri-dish method could be the use of water agar as a substrate. It was noticed that roots of cv. Atrela grew less abundantly on agar than other cultivars. It is concluded that the Petri-dish method is an accurate and inexpensive way of estimating penetration rate and reproduction factor in the root-knot nematode - potato relationship. Therefore, results on aggressiveness and virulence of *Meloidoygne* spp. on potato cultivars based upon the reproduction factor are considered to be more reliably from the Petri-dish experiment than from the greenhouse experiment.

The relatively low Rf-values for *M. fallax* isolates in the greenhouse experiment, compared to those in the Petri-dish experiment, could be explained by a shorter life cycle at 20 °C for this species on potato than for *M. hapla* and *M. chitwoodi*. This result confirms comparable observations on penetration rate and reproduction factor of isolates of *M. chitwoodi* and *M. fallax* on wild *Solanum* spp. (Van der Beek *et al.*, C; Chapter 5). Independently, M.M.A. Khan (Rijksstation voor Nematologie en Entomologie, Gent, Belgium) also demonstrated evidence of a shorter life cycle of *M. fallax* than in *M. chitwoodi* (Khan, pers. com. in Van der Beek *et al.*, C; Chapter 5). Whether the relatively short life cycle has consequences for critical population density thresholds has to be examined in further research.

The pattern of significant *M. hapla* isolate-by-cultivar interaction suggests several similarity groups for isolates, of which Hh appeared to be dissimilar to Hk, while the remaining isolates showed no significant interaction (Tables 3.2 and 3.3). In conclusion, different *M. hapla* isolates can result in different interaction effects. This is especially true for isolate Hh, suggesting genetic differences between Hh and the other tested *M. hapla* isolates for virulence to potato cultivars. Consequently, testing for resistance in *Solanum* genotypes to *M. hapla* requires the incorporation of more, well characterized isolates, considering especially *M. hapla* race A and B isolates.
Furthermore, besides to resistance of roots, in future breeding in *Solanum* spp. attention should be paid to resistance of tubers. Significant variation in tuber infection between cultivars existed, when assaying 20 cultivars in a field heavily infested with *M. chitwoodi* (Van Riel, 1993). The development of an adequate screening method will be a challenge for further research.

CHAPTER 4

Variation in virulence

within *M. hapla*

on *Solanum* spp.

4.1. Summary - Virulence of eleven isolates of *Meloidogyne hapla* race A, three of *M. hapla* race B and five mono-female lines of an isolate of *M. hapla* race A, was studied in a greenhouse experiment on genotypes of *Solanum chacoense*, *S. hougasii* and *S. sparsipilum*. Virulence was expressed as total number of egg-masses counted eight weeks after inoculation with second-stage juveniles. Considerable variation in resistance and virulence was observed between plant genotypes and between nematode isolates respectively: the isolate-by-plant species interaction was highly significant. The genetic factors for (a)virulence seemed to be scattered throughout the species, regardless of the nature of the isolates, being mitotic or meiotic parthenogenetic. Several factors for (a)virulence appeared to be present in *M. hapla* corresponding with several genetic factors for resistance in the *Solanum* genotypes. This may have a major impact on breeding strategies for resistance to *M. hapla*. The interaction term for mono-female lines and *Solanum* genotypes was not significant, indicating a similar virulence factor in all lines. However, genetic differences in aggressiveness within an isolate of *M. hapla* race A appear to occur as is shown by the significant differences between the mono-female lines derived from that isolate.

4.2. Introduction

The Northern root-knot nematode (*Meloidogyne hapla*) is a well-known pest, causing serious problems in a number of dicotyledonous crops. In potato (*Solanum tuberosum*) the pathogen is able to reproduce on roots causing yield reduction. *M. hapla* causes little damage in quality by gall formation on the outside of tubers, unlike *M. chitwoodi* and *M. fallax*. Besides yield reduction in potatoes, increase of the nematode population by growing a susceptible host for *M. hapla* renders this root-knot nematode species to a potential threat in agriculture in Western Europe, as many crops in the rotation schemes are suitable hosts (Roberts, 1992). However, *M. hapla* does not reproduce on monocotyledoneous crops, which therefore have been used in rotation with dicotyledons as a control measure. Besides crop rotation with monocotyledons, at present soil fumigation is the only effective way of controlling the pathogen in potato, as well as in most other dicotyledoneous crops. New resistant cultivars would provide an environmentally more acceptable solution.

In current potato cultivars, no desirable level of resistance to *M. hapla* has been identified (Janssen *et al.*, 1995). However, for wild *Solanum* spp. various levels of resistance were reported (Brücher, 1967; Hoyman, 1974; Janssen *et al.*, 1996). Recent investigations indicated the presence of variation in host responses between different *M. hapla* isolates on genotypes of *S. bulbocastanum*, *S. hougasii*, *S. chacoense*, *S. gourlayi*, *S. sparsipilum* and *S. spegazzinii*, which were selected for resistance (Janssen *et al.*, 1997). Of these species not all genotypes appeared to be resistant.

Effective screening and breeding programmes for resistance to root-knot nematodes

requires a better understanding of the virulence in the pathogen. This study describes reasearch on the degree of variation in virulence between a relatively large number of *M. hapla* isolates, including some of cytological race B, to three *Solanum* genotypes, for which already some variation in host response was recorded. Also, variation in virulence within one isolate was studied using mono-female lines of an isolate of *M. hapla* race A, tested against the same *Solanum* genotypes. In this chapter, virulence is defined as the ability of an isolate of *M. hapla* to reproduce siginificantly on resistant host plants that prevent or suppress reproduction of non-reproducing isolates of *M. hapla* (modified after Roberts, 1995).

4.3. Materials and methods

4.3.1. Nematode isolates and plant genotypes

A total of 14 *M. hapla* isolates were used in this study (Table 4.1). All isolates, except for two of them (Haw from France and Han from South Korea) were isolated from various hosts from geographically different sites in the Netherlands. Of these isolates, 11 were of the facultative meiotic parthenogenetic race A and three (Hh, Han and Hbr) of the obligate mitotic parthenogenetic race B. Race A and B were distinguished by studying the meiosis, using Hoechst 33258 for staining of the chromosomes (Van der

Table 4.1. Isolates of *M. hapla* used in the expermiments. All isolates except Han and Haw originated from the Netherlands.

Isolate	Race[1]	Origin	Host of origin	Year of sampling	Obtained from[2]
Ha	A	Lisse	peony	1990	PD
Hb	A	Zwaanshoek	*Astilbe*	1990	PD
Hc	A	Bavel	*Aconitum*	1990	PD
He	A	Drouwenerveen	potato	1992	PAGV
Hf	A	Borgers	carrot	1992	PAGV
Hi	A	Smilde	immortelle	1992	PAGV
Hj	A	Smilde	carrot	1992	PAGV
Hk	A	Slochteren	chicory	1992	PAGV
Hak	A	Mussel	carrot	1993	HLB
Haw	A	Antibes	grape	<1977	INRA
Hbz	A	Amerzoden	rose	1990	PD
Hh	B	Wachtum	fallow (weed)	1992	PAGV
Han	B	S. Korea	*Hosta*	1990	PD
Hbr	B	Hoorn	*Veronica*	1992	PD

[1] Cytological race
[2] PD = Plant Protection Service, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands. HLB = Hilbrands Laboratory for Soil-borne Diseases and Pests, Assen, The Netherlands. INRA = Institut National de Recherche Agronomique, Montpellier, France.

Beek *et al.*, G; Chapter 9). Before the experiment the 14 isolates were characterized as *M. hapla* by isozyme electrophoresis (Esbenshade & Triantaphyllou, 1990; Karssen *et al.*, 1995) and before and after the experiment by rDNA-analysis of ITS-regions (Zijlstra *et al.*, 1995, 1997). All isolates were maintained and propagated at IPO-DLO on *Lycopersicon esculentum* cv. Moneymaker in greenhouse compartments at 20±2 °C and 70 to 80 % relative humidity.

From *M. hapla* race A isolate Ha, five mono-female lines (Ha1, Ha2, Ha4, Ha5 and Ha6) were developed by inoculating root tips of *S. tuberosum* cv. Eigenheimer growing in Petri-dishes containing 2.0 agar technical nr. 3 (Oxoid) with second stage juveniles (J2). To exclude sexual reproduction, only one J2 was inoculated per Petri-dish which resulted consequently in a parthenogenetically produced egg-mass. The offspring of one separate J2 is defined as a mono-female line. Seven consecutive times this procedure was repeated. The five resulting mono-female lines of Ha each originated from a different egg-mass of the original isolate Ha, and were propagated separately on plants of cv. Moneymaker prior to their preservation in liquid nitrogen (Van der Beek *et al.*, 1996). After thawing two more generations followed on tomato plants in order to obtain adequat numbers of J2 for the experiment.
The nematode isolates and mono-female lines were tested on genotypes of three wild tuber-bearing *Solanum* species: *S. chacoense* Bitt. 93-68-2, *S. hougasii* Corr. 93-71-6 and *S. sparsipilum* (Bitt.) Juz. et Buk. 93-107-1. These genotypes were selected from a previous experiment because of their observed variation in response to three isolates of *M. hapla* (Janssen *et al.*, 1997). The genotypes were maintained and propagated *in vitro* at CPRO-DLO, providing a rapid multiplication. Young growing shoots were cut every two to three weeks and transferred into new sterile tubes containing MS30 medium (Murashige & Skoog, 1962) in order to obtain the required number of plants per genotype. Three weeks after the last cutting plantlets were sufficiently large for *in vivo* transplantation.

4.3.2. Test for virulence

In vitro plantlets were transplanted to 350 ml clay pots, filled with moist silver sand containing 0.2 % slow release fertilizer (Osmocote; Sierra Chemical Company, Milpitas, USA) and 0.08 % NPK 12-10-18, and placed into a greenhouse compartment at 20±2 °C with additional light during 16 h per day. During the first four days after transplantation, the relative humidity was kept at 100 % and for the remaining growing period at 70 to 80 %.
In order to prepare the nematode inoculum, heavily infected tomato roots were placed into a mist cabinet at 20 °C with water temperature of 25 °C (Seinhorst, 1988). Inoculum was prepared from one- to two-days-old hatched J2, which settled on the bottom of the tray. These J2 were collected during approximately three weeks and kept at 4 °C. Prior to inoculation, the J2 were poured over a non-woven cotton sandwich disc and collected one day later, to obtain active and viable juveniles. Inoculation was done on randomly selected plants, by applying a quantity of 9 ml inoculum to a notch

of approximately 1 cm deep around the plant, while continuously stirring the inoculum. An average inoculation density of 1.6 J2 cc^{-1} pot volume was pursued, resulting in a total of approximately 560 J2 per pot. For practical reasons the experiment was split into two parts: in the first all isolates were tested and in the second the mono-female lines with isolate Ha. Both experiments were complete randomized blocks with five replicates. Due to differences in development in the first experiment, plants of the first two replicates of *S. chacoense* and *S. sparsipilum* and the first three replicates of *S. hougasii* were inoculated at day 43 after transplantation, and the rest of the plants at day 51. This was done to ensure similar developmental stages of the plants at time of inoculation. Non-inoculated control plants were included.
Eight weeks after inoculation, the roots were rinsed free from sand and egg-masses were stained red with Phloxine-B (Dickson & Struble, 1965) and counted by eye.
The number of egg-masses at eight weeks after inoculation served as criterium for nematode reproduction.

4.3.3. Statistical analyses

To the observed number of egg-masses in the experiment with the isolates, linear

Table 4.2. Mean numbers of egg-masses for the combinations of 14 *M. hapla* isolates and *S. chacoence, S. hougasii* and *S. sparsipilum*

Isolate	*S. chacoence*	*S. hougasii*	*S. sparsipilum*	Overall mean
Ha	0.5[a]	8.8[b]	0.0[a]	3.1
Hb	0.0[a]	2.0[a]	0.0[a]	0.6
Hc	0.0[a]	3.8[b]	1.2[ab]	1.6
He	1.8[a]	16.4[b]	4.5[a]	7.6
Hf	28.5[b]	0.0[a]	48.5[c]	25.6
Hi	8.3[a]	35.3[b]	41.0[b]	28.2
Hj	6.0[b]	0.0[a]	41.8[c]	15.9
Hk	0.3[a]	0.0[a]	3.0[a]	1.0
Hak	10.4[b]	0.0[a]	43.8[c]	18.1
Haw[2]	0.8	0.0	15.8	5.5
Hbz	14.5[b]	0.4[a]	2.8[a]	5.9
Hh	3.3[a]	2.2[a]	1.5[a]	2.3
Han[2]	11.4	0.0	3.8	5.1
Hbr	0.6[a]	2.2[a]	4.0[a]	2.3
mean	6.0	4.6	14.7	8.5

a,b,c: Means with common letters in a row do not differ significantly on the logit-scale (P<.05, referring to an F-distribution of the deviance ratio statistic).
[2]Isolates Haw and Han were contaminated with *M. chitwoodi* and excluded from the statistical analysis.

logistic regression models (McCullagh & Nelder, 1989) were fitted with replicates, *Solanum* genotypes, *M. hapla* isolates and the interaction between *Solanum* genotypes and isolates as explanatory variables. In the experiment with the mono-female lines, linear logistic regression models with the same variables were used with isolates being replaced by mono-female lines. The dispersion of the data was assumed to be pseudo-binomial, i.e. $var(Y)=n\sigma^2\mu(1-\mu)$, where μ is the expected proportion, n is the number of inoculated J2 and σ^2 denotes the dispersion parameter. Overall treatment effects were tested by means of *F*-statistics, which are deviance ratios based on the mean deviance of the full model.

Due to zero observations for some treatments estimates of corresponding treatment parameters would be minus infinity and *t*-tests for testing differences between treatment means on the logit-scale could not be used. The observed zeros were considered to be sampling zeros.

Pair-wise testing of differences between treatment means on the logit-scale was performed by fitting both, the model in which treatment groups to be compared were considered to be different, and the model in which they were considered to be the same. The mean deviance ratio of the reduction in residual deviance from the model (considering the groups to be different, compared to the model with one common group) and the residual deviance from the former model was used to assess pair-wise treatment differences, resulting in *F*-tests.

All statistical analyses were performed using Genstat 5 (Genstat 5 Committee, 1993).

4.4. Results

Analysis of rDNA of juveniles that were hatched from eggs collected from the roots eight weeks after inoculation revealed no contamination of the isolates with other *Meloidogyne* spp., except for Han and Haw, which both contained quantities of approximately 5 % *M. chitwoodi*. Virulence data of the two latter isolates should therefore be interpreted cautiously. Therefore, these two isolates were omitted in the statistical analysis.

Mean numbers of egg-masses for each combination of *M. hapla* isolate and *Solanum* genotype are shown in Table 4.2 and for combinations of the mono-female lines and *Solanum* genotypes in Table 4.4. None of the three *Solanum* genotypes showed absolute resistance to all isolates and none of the nematode isolates showed absolute avirulence to the three *Solanum* genotypes. However, 25% of the isolate-*Solanum* genotype combinations were incompatible by showing no reproduction. The deviance tables from fitting the regression models are in Tables 4.3 and 4.5. The plots of deviance residuals against fitted values showed no severe anomalies. Based on calculated *P*-values for the *F*-tests of the mean deviance ratios, significant effects for interaction between *Solanum* genotypes and isolates were concluded (Table 4.3). For each isolate, pair-wise differences between the means were analysed for significance and are shown in Table 4.2. Large differences in interaction were found, for example between isolates He and Hf for combinations with the three *Solanum* genotypes.

Table 4.3. Deviance table for egg-mass data of 14 *M. hapla* isolates, inoculated on genotypes of *S. chacoence*, *S. hougasii* and *S. sparsipilum*

Source	df	Deviance	Mean deviance	Deviance ratio	
Replicate	4	47.4	11.84	2.55	*
Isolates	11	1451.5	131.95	28.39	***
Solanum genotype	2	386.6	193.30	41.59	***
Isolates x *S.* genotype	22	1016.3	46.20	9.94	***
Residual	122	567.1	4.65		
Total	161	3468.9	21.55		

* $P<.05$, *** $P<.001$, referring to F-distributions

P-values in the deviance table (Table 4.5) of the five mono-female lines and isolate Ha revealed significant effects for *Solanum* genotypes and mono-female lines, but no significance for the line-by-*Solanum* genotype interaction, in contrast to the isolate-by-*Solanum* genotype interaction (Table 4.3). Mean numbers of egg-masses of the mono-female lines were higher than or equal to those of isolate Ha (Table 4.4).
The host responses of isolates Hi and Hk were compared to those of isolates HSMB and HSL, respectively. Hi and HSMB were collected in 1992 from the same soil sample from Smilde and Hk and HSL from Slochteren (both sites in the Netherlands) but maintained and propagated separately. HSMB and HSL were tested by Janssen *et al.* (1997) on the same *Solanum* genotypes as those used in this study. Large

Table 4.4. Mean number of egg-masses for the combinations of *M. hapla* isolate Ha and five mono-female lines of isolate Ha with genotypes *S. chacoence*, *S. hougasii* and *S. sparsipilum*

Line	*S. chacoence*	*S. hougasii*	*S. sparsipilum*	Overall mean
Ha	0.2	13.8	0.4	4.8^{a}
Ha1	0.8	38.7	0.6	13.4^{b}
Ha2	0.0	13.6	0.6	4.7^{a}
Ha4	0.0	3.0	0.2	1.1^{a}
Ha5	0.0	12.0	0.0	4.0^{a}
Ha6	0.8	41.0	1.2	14.3^{b}
mean	0.3^{p}	22.0^{q}	0.5^{p}	7.6

a,b and p,q,r: Marginal means with common letters for isolates and *Solanum* genotypes respectively, do not differ significantly on the logit-scale ($P<.05$, referring to an F-distribution of the deviance ratio statistic).

differences in virulence were found between the corresponding isolates (Table 4.6): isolate HSL could reproduce moderately on the genotype of *S. hougasii*, whereas isolate Hk was completely suppressed. HSMB and Hi varied on all genotypes regarding virulence.

4.5. Discussion

All isolates were found to be exclusively *M. hapla*, except for the two isolates Han and Haw which were contaminated with small amounts of *M. chitwoodi*, probably caught during maintenance of the isolates or preparation of the inoculum. This emphasizes the problems encountered in producing pure isolates and stresses the necessity of using isolates which are true to species. Verification of purity of isolates requires the application of accurate identification methods before and after virulence tests. In this study, the results for Han and Haw should be read with care, although no major differences in numbers of egg-masses are to be expected as a result of this contamination.

Considerable variability in virulence was demonstrated among the 14 isolates of *M. hapla* used, as was shown by testing against the *Solanum* genotypes used. This result is in agreement with the variability found by Janssen *et al.* (1997). The observed variability was accompanied by a highly significant interaction for mean number of egg-masses, indicating genetic differences for (a)virulence in *M. hapla* and for resistance versus susceptibility in *Solanum* genotypes. Besides this large variability in virulence, *M. hapla* is known to be a cytologically complex species, which is expressed by varying chromosome numbers, ploidy levels and reproduction systems (Triataphyllou, 1966, 1984). This genetic variability points to a relatively long evolutionary history of this species. Despite of the variability in this species, pathogenic races as have been described in *M. chitwoodi*, *M. arenaria* and *M. incognita* have not (yet) been reported for *M. hapla*. Intraspecific variation in *M. hapla* has been recorded earlier (Goplen *et al.*, 1959; Griffin & McKenry. 1989), by testing two *M. hapla*

Table 4.5. Deviance tabler for egg-mass data of the *M. hapla* isolate Ha and the five mono-female lines from isolate Ha, inoculated on genotypes of *S. chacoence*, *S. hougasii* and *S. sparsipilum*

Source	df	Deviance	Mean deviance	Deviance ratio	
Replicate	4	123.7	30.92	4.56	***
Lines	5	219.1	43.83	6.46	***
Solanum genotype	2	954.9	477.45	70.40	***
Lines x *S.* genotype	10	9.6	0.96	0.14	
Residual	61	413.9	6.79		
Total	82	1721.1	20.99		

*** $P<.001$, referring to F-distributions

isolates on two genotypes of *Medicago sativa*. In contrast, absence of a significant cultivar-by-isolate interaction in white clover was demonstrated, where eight *M. hapla* isolates from New Zealand were tested against two resistant and two susceptible cultivars (Mercer & Grant, 1993). This discrepancy may be due to the fact that *M. hapla* possibly is present with a narrower genetic basis in New Zealand than in Western Europe and the USA, or to the difference in host plant. The observed variation in virulence in *M. hapla* is also in contrast with the limited variation in *M. chitwoodi* and *M. fallax* on *Solanum* genotypes (Van der Beek *et al*., C; Chapter 5). *M. incognita*, *M. arenaria* and *M. javanica* are known to cause damage in potato in (sub)tropical regions (Greco, 1993), but nothing is known about host-by-pathogen interactions between these species and tuber-bearing *Solanum* spp.

Nematode response varied from total absence to high mean number of egg-masses on a host genotype. On an average, *S. sparsipilum* was significantly more susceptible than the other two *Solanum* genotypes, in showing more highly compatible combinations with isolates. The three *Solanum* genotypes were equally dissimilar to each other, suggesting differences in genetic control of resistance to the tested isolates. Likewise, the 14 isolates showed high dissimilarity amongst each other. No association was found between virulence and cytological race, as the race B isolates did not show similar virulence patterns to the three *Solanum* species, contrasted to those shown by the race A isolates. Under the assumption that interactions are based on genetic differences, this surprising result would signify the presence of a similar diversity of (a)virulence genes in both the meiotic parthenogenetic race A isolates, as well as in the mitotic parthenogenetic race B isolates. DNA exchange between the two races could theoretically result in such a situation. Whether the mechanisms behind such a hypothetical recombination would be of mitotic, like in certain fungi (Manners, 1993), or meiotic origin is yet unknown. However, it is generally assumed that exchange of genetic material between the two races is totally absent, because of the apomictic nature of race B. Also, it is postulated and generally accepted that mitotic populations emerged from meiotic parthenogenones or amphimicts (Triantaphyllou & Hirschmann, 1980). Additionally to a hypothetic DNA exchange, two alternative explanations concerning the formation of race B populations are possible. One interpretation for this equal diversity in virulence of race A and race B of *M. hapla* might be that race B isolates are procreated by race A isolates on a frequent basis. This would support the hypothesis that mitotic parthenogenetic populations are derived regularly from meiotic parthenogenetic populations. A second explanation would be that race B, originating at a certain moment from race A, under similar selection pressure accumulated several similar (a)virulence genes by parallel evolution. This theory presumes a common ancestral population for the two races including a diversity of factors for (a)virulence, and is supported by the fact that *M. hapla* race B isolates were clustered apart from race A isolates in experiments using isozymes (Esbenshade & Triantaphyllou, 1987), DNA (Castagnone-Sereno *et al*., 1993) and total soluble proteins (Van der Beek *et al*., A; Chapter 8).

Based on the present results and given the random choice of *M. hapla* isolates, there are strong indications that virulence factors are distributed at random over the isolates. The high number of interactions found in this study implies the presence of approximately five different virulence factors in the *M. hapla* isolates (Table 4.2), assuming that all interaction is due to genetic differences. Apparently, these genes are preserved

in the species without selection pressure from the three used *Solanum* genotypes, because these genotypes have never been introduced before in the Netherlands. It is postulated that these virulence factors are manifested in *M. hapla* during extensive periods of time and are maintained in the gene pool by a continuously changing selection pressure, assured by the polyphagous nature and the large cytological variation of the nematode species. *M. hapla* is able to reproduce on many dicotyledons, including weeds. This genetically broad supply of host genotypes might ensure preservation of many virulence factors.

The large variation in virulence renders breeding for resistance to control *M. hapla* a difficult task. Screening for resistance should include testing against a range of isolates which represent the variation in virulence in this species. Furthermore, the efficacy of resistance breeding depends on the stability of the resistance genes. Several authors indicated the ability of populations of *Meloidogyne* spp. to develop into resistance-breaking forms under laboratory conditions, e.g. Riggs and Winstead (1959), Triantaphyllou and Sasser (1960), Netscher (1977), Bost and Triantaphyllou (1982), Stephan (1982) and Jarquin-Barbarena *et al.* (1991), all of them obtained virulent isolates after several successive generations of selection for virulence, originating from avirulent isolates. This laboratory-selected virulence is sometimes confusingly referred to as 'B-race' (Riggs & Winstead, 1959). Despite of this ability of the pathogen, the *Mi*-gene in tomato was effective to *M. incognita*, *M. javanica* and *M. arenaria* from the time of its introgression from *Lycopersicon peruvianum* to the cultivated tomato, until recently (Roberts *et al.*, 1990). Selection for virulence under natural conditions is demonstrated for example in *M. incognita*, *M. arenaria* and *M. javanica* on cultivars of groundnut (*Arachis hypogea*), cotton (*Gossypium hirsutum*), strawberry (*Fragaria ananassa*) and sweet pepper (*Capsicum frutescens*), as discussed by Netscher (1978). In the present study, the striking differences between the related isolates from identical field populations (Table 4.6), indicates that large genetic variation in virulence can be expected within one field population, especially from soil samples. Divergences between two related isolates could then be explained by selection of different genotypes during multiplication, starting with a mixture of genotypes for virulence. Also contamination with other isolates of *M. hapla* can not be excluded, because at present no routine detection is available to discriminate between isolates of the same species. In contrast, no variation in virulence was found in isolate Ha by studying five mono-female lines since no mono-female line-by-*Solanum* sp, interaction was observed (Tables 4.4 and 4.5), which indicated the presence of only one common virulence factor. Apparently field populations of *M. hapla* race A may

Table 4.6. Mean numbers of egg-masses for two *M. hapla* isolates (Hi and Hk) compared to two related isolates on genotypes of three *Solanum* spp., in a separated experiment (Janssen *et al.*, 1997) under similar experimental conditions. The related isolates originated from the same soil sample in 1992, but were propagated separately from each other during four years.

Isolate	*S. chacoence*	*S. hougasii*	*S. sparsipilum*
Hi	8.3	35.3	41.0
HSMB[1]	32.8	6.0	21.3
Hk	0.3	0.0	3.0
HSL[1]	0.8	15.3	3.0

[1] *M. hapla* isolates, with numbers of egg-masses, as mentioned in Janssen *et al.* (1997)

reflect a large diversity in virulence, but once in captivity, a tendency towards homogeneity in virulence could occur due to uniformity in host and environmental conditions. Despite of the lack of variation in virulence within isolate Ha, significant differences in aggressiveness between the mono-female lines were observed (Table 4.4). The virulence pattern of Ha was comparable in the two experiments (Tables 4.2 and 4.4), indicating that the differences between the lines (Table 4.4) were caused by genetic differences in aggressiveness and were not the result of large environmental effects. As no differences in virulence were observed between the mono-female lines, aggressiveness and virulence appeared to be independently genetically controlled in *M. hapla*.

In conclusion, in resistance testing with isolates of *M. hapla* race A attention should be paid to the variation in virulence to different *Solanum* genotypes. Additionally, the possibility of more than one virulence factor in a field population should discourage the use of such heterogenous populations in resistance tests and stresses the use of more homogenous isolates such as mono-female isolates. Furthermore, the interpretation of data from resistance trials could be hindered by certain levels of heterogeneity in aggressiveness and virulence of the isolates. Finally, the presence of various genetic factors for virulence amongst *M. hapla* isolates and for resistance in the *Solanum* genotypes tested stresses the need for genetic analyses of the respective virulence and resistance.

CHAPTER 5

Variation in virulence within *M. chitwoodi* and *M. fallax* on *Solanum* spp.

5.1. Summary - Virulence of eight isolates of *Meloidogyne chitwoodi*, of five of *M. fallax* and of six mono-female lines of a *M. chitwoodi* isolate was studied by inoculating juveniles on *Solanum tuberosum* cv. Nicola and genotypes of the wild species *Solanum bulbocastanum*, *S. chacoense*, *S. hougasii* and *S. stoloniferum*, in a greenhouse assay. Virulence was expressed in terms of total nematode reproduction eight weeks after inoculation. Nematode reproduction was estimated by number of egg-masses and number of hatched second stage juveniles per inoculated juvenile. Significant differences were obtained between *Solanum* spp. To *M. chitwoodi* the response ranged from susceptible (*S. tuberosum* and *S. chacoense*), via incompletely resistant (*S. stoloniferum*) to highly resistant (*S. bulbocastanum* and *S. hougasii*). To *M. fallax*, *S. tuberosum* was susceptible whereas all four wild species were resistant. No significant isolate-by-plant genotype interaction was obtained for *M. chitwoodi* nor for *M. fallax*, indicating no or little intraspecific variation in virulence. Moreover, significant differences between isolates within species were observed, as well as between mono-female lines indicating heterogeneity of pathogenicity within meiotic parthenogenic *Meloidogyne* populations. It was shown that juveniles of *M. chitwoodi* in a species mixture with an isolate of *M. fallax* were responsible for breaking the resistance of *S. bulbocastanum* and *S. hougasii*.

5.2. Introduction

The root-knot nematode species *Meloidogyne chitwoodi* and *M. fallax* are a potential threat to the potato crop as well as to many other crops in the Netherlands. The introduction of resistant potato cultivars would improve the efficiency of crop rotation to reduce this threat. However, potato cultivars are highly susceptible to these two root-knot nematode species (Brown *et al.*, 1994; Janssen *et al.*, 1995).

Thus far, intraspecific specialization in *M. chitwoodi* is described for the occurrence of three races, which can be distinguished on alfalfa cv. Thor (non-host for race 1 and host for races 2 and 3), on carrot cv. Red Cored Chantenay (host for race 1 and non-host for races 2 and 3) and on *Solanum bulbocastanum* SB22 (non-host for races 1 and 2 and host for race 3) (Santo & Pinkerton, 1985; Mojtahedi *et al.*, 1988; Mojtahedi & Santo, 1994).

Resistances to *M. chitwoodi* and *M. fallax* have been identified in accessions of wild tuber-bearing *Solanum* species, originating mainly from North America, like *S. bulbocastanum* and *S. hougasii* (Brown *et al.*, 1989, 1991; Janssen *et al.*, 1996). Resistance to *M. chitwoodi* has been transferred from a resistant genotype of *S. bulbocastanum* to the genepool of the cultivated potato by somatic hybridization (Austin *et al.*, 1993; Brown *et al.*, 1994) and a single gene has been localized on chromosome 11 that appears to control this resistance (Brown *et al.*, 1996).

In general, the efficacy of resistance is depending largely on the genetic variation in

virulence of the pathogen and factors involved in the durability of the resistance. Often, the presence of resistance is concluded from experiments with only one or a few isolates. The present study describes the research on the variation in virulence in *M. chitwoodi* and *M. fallax* by testing various isolates and mono-female lines of one of the isolates to some of the completely resistant and incompletely resistant genotypes of *Solanum* spp. In this study, virulence is defined as the ability of an isolate of *M. chitwoodi* or *M. fallax* to reproduce significantly on resistant host plants that prevent or suppress reproduction of non-reproducing isolates of these *Meloidogyne* spp. (Roberts, 1995).

5.3. Materials and methods

5.3.1. Nematode isolates and plant genotypes

Eight isolates of *M. chitwoodi* and five of *M. fallax* were used in this study

Table 5.1. Isolates of *M. chitwoodi* and *M. fallax* used in the experiments. All isolates except for Cba originated from the Netherlands. Cba originated from Oregon (USA).

Isolate	Last host	Year of sampling	Obtained from[1]
M. chitwoodi			
Ca	maize	1989	PD
Cb	wheat	1990	PD
Ck	tomato	1989	PD
Co	black salsify	1993	PAGV
Cx	potato	1993	PD
Cy	potato	1993	PD
Caq	potato	1993	CPRO
Cba	potato		WSU
M. fallax			
Fa	early primrose	1992	PAGV
Fb	beet	1991	PD
Fc	potato	1993	PD
Fd	potato	1993	PD
Fe	unknown	1994	BLGG

[1] PD = Plant Protection Service, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands. CPRO = DLO-Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands. WSU = Washington State University, Department of Plant Pathology, Prosser, USA. BLGG = Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.

(Table 5.1). All, except for one, were obtained from geographically different sites in the Netherlands, originating from various hosts. Isolate Cba was obtained from Oregon (USA) and represents race 1. The isolates were maintained and propagated on *Lycopersicon esculentum* cv. Moneymaker at IPO-DLO. During their maintenance these isolates were regularly tested on possible contamination with other species by observing isozyme phenotypes of esterase and malate dehydrogenase (Esbenshade & Triantaphyllou, 1990; Karssen *et al.*, 1995) and by analysis of internal transcribed spacer (ITS) regions of ribosomal DNA (Zijlstra *et al.*, 1995, 1997). Additionally, during the experiment, samples of the inocula of these isolates were collected and verified for absence of mixed species, using analysis of ITS regions. The same DNA-test was performed using samples of nematode progenies of these isolates extracted from the test plants at harvest.

Originating from isolate Cb, six mono-female lines (Cb1 to Cb6) were developed by inoculating second stage juveniles (J2) on root tips of *S. tuberosum* cv. Eigenheimer growing in Petri-dishes containing 2.0 % agar technical nr. 3 (Oxoid). To ensure parthenogenetic offspring, only one J2 was inoculated per Petri-dish. Consequently, the progeny of such a juvenile is called a mono-female line. This procedure was repeated during seven consecutive cycles in Petri-dishes. Each of the mono-female lines obtained in this way originated from a different egg-mass of the original isolate Cb, with one exception: Cb1 and Cb2 were derived from two J2 from the same egg-mass after one generation of parthenogenesis. The resulting six single egg-masses were seperately propagated on plants of 'Moneymaker' prior to their preservation in liquid nitrogen (Van der Beek *et al.*, 1996; Chapter 2). After thawing, two more generations followed on 'Moneymaker' in order to obtain proper amounts of J2.

The 13 nematode isolates and the six mono-female lines were tested on susceptible *S. tuberosum* cv. Nicola and on genotypes of four wild *Solanum* spp.: *S. bulbocastanum* Dun. 93-60-2, *S. chacoense* Bitt. 93-68-2, *S. hougasii* Corr. 93-71-6 and *S. stoloniferum* Sclechtd. et Bché. 93-STOL-2. Genotypes of the wild species were selected from previous experiments because of their response to one to three isolates of *M. chitwoodi* and two or three of *M. fallax* (Janssen *et al.*, 1997). In those experiments the *S. bulbocastanum* and *S. hougasii* genotypes were completely resistant to both *Meloidogyne* spp., *S. stoloniferum* incompletely resistant to *M. chitwoodi* and completely resistant to *M. fallax*, *S. chacoense* susceptible to *M. chitwoodi* and completely resistant to *M. fallax*. Except for *S. tuberosum* cv. Nicola, these genotypes were maintained and propagated *in vitro* at CPRO-DLO ensuring a rapid multiplication. To obtain the proper number of plants per genotype, shoots were cut every two to three weeks and transferred into new tubes with MS medium containing 30 g/l sucrose (Murashige & Skoog, 1962). Three weeks after the last cutting, plantlets were sufficiently large for *in vivo* transplantation.

5.3.2. Test for virulence

In vitro plantlets and tuber pieces with top sprouts of cv. Nicola were transplanted to 350 ml clay pots, filled with moist silver sand containing 0.2 % slow release fertilizer

(Osmocote) and 0.08 % NPK 12-10-18, and placed into a greenhouse compartment at 20 ±2 °C, 70 to 80 % relative humidity and with additional light during 16 h per day. During the first four days after transplantation, the relative humidity was kept at approximately 100 % by covering the plants originating from *in vitro* culture with transparent plastic.

In order to prepare the nematode inoculum, heavily infested tomato roots were placed on trays in a mist cabinet at 20 °C with water temperature of 25 °C (Seinhorst, 1988). One- to two-days-old hatched J2, which settled on the bottom of the tray were collected during approximately three weeks and kept at 4 °C. Prior to inoculation, the J2 were poured over a non-woven cotton sandwich disc. Inoculum was collected after one day and inoculation was done on plantlets, randomly taken for genotype and replicate, by applying twice a quantity of 4.5 ml inoculum to a notch of approximately 1 cm deep around the plant, while continuously mixing the inoculum. An average inoculation density of 1.6 J2 cc^{-1} sand was pursued, resulting in a total of approximately 560 J2 per pot. Non-inoculated control plants were included in the experiment. For practical reasons the experiment was split in two parts: the first having the combinations with all isolates and the second with isolate Cb and the mono-female lines. Both experiments were carried out similarly as described above and nematode isolates and mono-female lines were tested against the same *Solanum* genotypes. Both experiments were designed as a complete randomized block with five replicates.

Eight weeks after inoculation, the roots were rinsed free from sand and stained with Phloxine-B (Dickson & Struble, 1985). Egg-masses were counted by eye.

Roots were ground in a table blender and eggs were extracted by shaking the roots for four minutes in 0.5 % NaOCl (adapted after Hussey & Barker, 1973). Eggs were separated from root particles by a series of sieves. Subsequently, they were collected and hatched on 20 µm mesh sieves and J2 were counted after three weeks incubation at 20 °C. Total numbers of hatched J2 were estimated by counting three samples of 1 ml of J2-suspension, using a Contextvision MicroGOP2000 image analysis package (Been *et al.*, 1996). The reproduction rate Pf/Pi (final population divided by initial population) was estimated by the number of hatched J2 per inoculated J2.

The root volume was estimated according to a scale with four response categories, from 'small' (=1), to 'large' root volume (=4).

5.3.3. Statistical analyses

Two parameters for reproduction were used to describe virulence: the number of egg-masses and the reproduction rate Pf/Pi. For the experiment with the mono-female lines only the number of egg-masses was used. To the observed numbers of egg-masses of each species logistic regression models were fitted with replicate, isolate, *Solanum* spp. and the isolate-by-*Solanum* spp. as explanatory variables. In the experiment with the mono-female lines logistic regression models with the same explanatory variables were used with isolate being replaced by mono-female line. The dispersion of the egg-mass data was assumed to be pseudobinomial, i.e. $var(Y)=n\sigma^2\mu(1-\mu)$, where μ denotes the expected proportion, n is the number of

inoculated J2 and σ^2 denotes the dispersion parameter. To the observed counts for Pf/Pi loglinear models were fitted with the same explanatory variables and variance proportional to Poisson variance, i.e. var(Y)= ΦE(y), where E(y) is the expected count and Φ is the dispersion parameter. The logistic and loglinear models were fitted to the data using the method of quasi-likelyhood (Welham & Thompson, 1992). Treatment effects were tested by means of *F*-statistics, which are deviance ratios based on the mean deviance of the full model. Significant results ($P<.05$) are presented. All analyses were performed using the Genstat 5 programme (Genstat 5 Committee, 1993).

5.4. Results

The means for the two parameters: number of egg-masses and Pf/Pi, are shown in Table 5.2 for the experiment with the 13 isolates and in Table 5.3 for the mono-female lines. The tested genotypes of *S. bulbocastanum* and *S. hougasii* demonstrated extremely high levels of resistance to all isolates of *M. chitwoodi* and *M. fallax* and the mono-female lines. None but three isolates could reproduce on these two *Solanum* spp. Isolate Fc was able to reproduce on the *S. bulbocastanum* and the *S. hougasii* genotypes. Isolates Fd and Ck produced only one egg-mass each on *S. bulbocastanum* and *S. hougasii*, respectively. Restriction analysis of ITS regions of rDNA, generated from J2 from inocula and from nematode progenies extracted from cv. Nicola, indicated that these three isolates contained contaminations with one other species. *M. chitwoodi* isolate Ck was contaminated for 10 % with juveniles of *M. hapla,* which might explain the single egg-mass found on *S. hougasii.* In two *M. fallax* isolates low levels of contamination with *M. chitwoodi* were found: both Fc and Fd contained approximately 2 % *M. chitwoodi* juveniles in the inocula, which resulted for both isolates in approximately 10 % contamination in their progenies on cv. Nicola eight weeks after inoculation. The resistance-breaking ability of *M. fallax* isolate Fc on *S. bulbocastanum* appeared to be totally attributed to *M. chitwoodi* which was present as a contamination in Fc, since treatment with restriction enzyme *Rsa*I of the amplified ITS regions obtained from the extracted J2 from *S. bulbocastanum,* did not reveal the *M. fallax* restriction phenotype, but the *M. chitwoodi Rsa*I ITS pattern, which is a non-digested band (Zijlstra *et al.*, 1995). Additional restriction analysis with *Dra*I confirmed the *M. chitwoodi* nature of the contamination in isolate Fc. This result was confirmed by morphological identification of juveniles of Fc reproduced on *S. bulbocastanum* (G. Karssen, Plant Protection Service, Wageningen, The Netherlands, pers. comm.). Similarly, morphological identification of the extracted progenies of Fc from *S. hougasii* and Fd from *S. bulbocastanum* after harvesting, indicated that only the *M. chitwoodi* contaminations in those *M. fallax* isolates were able to reproduce on the respective hosts. Additionally, it was confirmed that the single egg-mass produced by *M. chitwoodi* mono-female line Cb1 on *S. hougasii* (Table 5.3) was true to species. Species identification by analysis of ITS regions of rDNA revealed further absence of contamination of the isolates with *M. incognita* or other species which might occur as

Table 5.2. Mean numbers of egg-masses (Em) and Pf/Pi for the combinations of eight *M. chitwoodi* and five *M. fallax* isolates and genotypes of *S. bulbocastanum*, *S. chacoence*, *S. hougasii*, *S. stoloniferum* and *S. tuberosum*, arranged according to their increasing overall mean number of egg-masses.

	S. bulboc.		*S. chacoence*		*S. hougasii*		*S. stoloniferum*		*S. tuberosum*		overall mean	
	Em	Pf/Pi	Em	Pf/Pi	Em	Pf/Pi	Em	Pf/Pi	Em	Pf/Pi	Em	Pf/Pi
M. chitwoodi isolates:												
Co	0	0	23.8	13.6	0	0	2.4	2.2	76.4	24.3	21.4^{a}	8.6^{a}
Ck[1]	0	0	33.6	12.3	0.3	0.0	13.6	6.1	67.0	31.5	23.8	11.0
Cba	0	0	37.0	22.3	0	0	2.0	0.7	90.4	38.9	27.0^{ab}	11.8^{ab}
Ca	0	0	41.6	26.7	0	0	3.8	2.6	87.6	81.5	28.9^{ab}	24.1^{c}
Caq	0	0	53.2	39.7	0	0	5.0	7.9	84.8	57.7	29.8^{ab}	22.0^{bc}
Cb	0	0	57.6	61.2	0	0	3.2	6.8	95.0	67.1	32.5^{bc}	27.6^{c}
Cy	0	0	60.0	9.3	0	0	1.2	0.0	121.2	49.5	38.0^{bc}	12.3^{a}
Cx	0	0	58.6	43.9	0	0	1.6	6.8	144.2	79.8	42.6^{c}	27.2^{c}
mean	0	0	45.7^{b}	27.1^{b}	0.0	0.0	4.1^{a}	3.2^{a}	95.8^{c}	52.8^{c}	29.3	18.1
M. fallax isolates:												
Fe	0	0	1.6	0.0	0	0	0.2	0.6	79.2	10.8	16.9	2.5^{a}
Fa	0	0	1.8	0.2	0	0	0.4	0.0	81.8	28.2	17.5	5.9^{b}
Fd[1]	0.2[2]	0.0	0.4	0.0	0	0	0.8	0.0	92.0	42.0	19.5	8.8
Fb	0	0	0.2	0.0	0	0	0.2	0.0	107.6	26.3	22.5	5.7^{b}
Fc[1]	0.2[2]	1.3[2]	0.4	0.3	3.8[2]	0.4[2]	6.0	0.7	131.6	23.0	31.5	4.7
mean	0	0	1.0^{a}	0.1^{a}	0	0	1.5^{a}	0.2^{a}	90.2^{b}	26.8^{b}	19.1	5.7
root[3]	3.0		3.1		2.3		2.7		2.8			

[1] Contaminated isolates which were excluded from statistical analyses
[2] Means for egg-masses and Pf/Pi, entirely attributed to *M. chitwoodi* contamination in the *M. fallax* isolates. These means were omitted in the marginal means.
[3] Mean root volume according to a scale with four classes: 1='small', .., 4='large'
a,b,c: isolates or species with common letters do not differ significantly for egg-mass responses on the logit-scale and for Pf/Pi responses on the log-scale ($P<.05$ referring to an F-distribution for the deviance statistic).

contaminations under greenhouse conditions. In conclusion, absolute levels of resistance were demonstrated in *S. bulbocastanum* and *S. hougasii* genotypes to all tested *M. fallax* and *M. chitwoodi* isolates and mono-female lines, except for the contamination in *M. fallax* isolate Fc and single egg-masses by Fd and Cb1. Because the genotypes of *S. bulbocastanum* and *S. hougasii* showed no differentiation for the two parameters they did not contribute to the variation and therefore were omitted from the statistical analyses. Consequently, results of and discussion on regression analyses are solely based on the genotypes of *S. chacoence*, *S. stoloniferum* and

Table 5.3. Mean numbers of egg-masses for the combinations of *M. chitwoodi* isolate Cb and six mono-female lines of isolate Cb (Cb1 to Cb6) with genotypes of *S. bulbocastanum*, *S. chacoence*, *S. hougasii*, *S. stoloniferum* and *S. tuberosum*.

	S. bulbocast.	*S. chacoence*	*S. hougasii*	*S. stoloniferum*	*S. tuberosum*	Overall mean
Cb	0	57.8	0	2.4	92.4	31.8[bc]
Cb1	0	41.5	0.2	4.6	91.0	26.8[abc]
Cb2	0	28.8	0	3.3	97.8	25.8[ab]
Cb3	0	31.0	0	3.2	79.4	22.7[a]
Cb4	0	25.0	0	2.4	87.6	23.0[a]
Cb5	0	69.2	0	3.0	98.6	35.6[c]
Cb6	0	28.3	0	3.8	70.8	20.3[a]
mean	0	40.9[b]	0	3.2[a]	87.9[c]	26.5

a,b,c: isolates or species with common letters do not differ significantly for responses on the logit-scale ($P<.05$ referring to an F-distribution for the deviance statistic).

S. tuberosum.

For the data of the number of egg-masses and the data of the Pf/Pi, the differences in deviance corresponding to the inclusion of the individual explanatory variables are given in Tables 5.4, 5.5 and 5.6. Plots of deviance residuals against fitted values revealed no anomalies. The interaction terms for isolate-by-*Solanum* spp. (Tables 5.4 and 5.5) and lines-by-*Solanum* spp. (Table 5.6) were not significant. Differences between *Solanum* spp. were highly significant for both parameters. Differences between isolates were significant for both parameters for *M. chitwoodi* and for the mono-female lines of Cb. For *M. fallax*, differences between isolates were significant for Pf/Pi, but not for number of egg-masses. The analysis showed also a clear *Meloidogyne* spp.-by-*Solanum* spp. interaction effect (data not shown). This was mainly caused by differences in response of *S. chacoence* and *S. stoloniferum* to the two *Meloidogyne* species (Table 5.2).

M. chitwoodi isolates showed varying levels of reproduction on the genotype of *S. stoloniferum*, the latter showing a high level of resistance to all but one *M. fallax* isolates (Table 5.2). The differentiating *M. fallax* isolate was again the contaminated Fc. The *S. chacoence* genotype varied in susceptibility to *M. chitwoodi* isolates, but showed incomplete resistance to *M. fallax*. The cultivar Nicola was susceptible to both *M. fallax* and *M. chitwoodi*. On this commercial cultivar, the level of susceptibility for the parameter mean number of egg-masses was similar for *M. chitwoodi* and for *M. fallax*, but the mean Pf/Pi-value was about 50 % lower for *M. fallax* than for *M. chitwoodi*.

Table 5.4. Deviance table for egg-mass data of *M. chitwoodi* and *M. fallax* isolates, inoculated on genotypes of *S. chacoence, S. stoloniferum* and *S. tuberosum*

Source	d.f.	Deviance	Mean deviance	Deviance ratio	
For *M. chitwoodi* isolates:					
Replicate	4	157	39	2.71	*
Isolates	6	257	43	2.96	*
Solanum spp.	2	4459	2230	154.05	***
Residual	92	1331	14		
Total	104	6204	60		
For *M. fallax* isolates:					
Replicate	4	91	23	3.11	*
Isolates	2	25	12	1.70	ns
Solanum spp.	2	2919	1460	199.53	***
Residual	36	263	7		
Total	44	3298	75		

*$P<.05$, ***$P<.001$, ns=not significant, referring to an F-distribution

Analysis of variance of data for root volume revealed no significant differences between *S. tuberosum*, *S. stoloniferum* and *S. chacoense* (Table 5.2; analysis not shown), suggesting that the co-parameter root volume had no notable influence on the significant *Solanum* spp. effect for the parameters.

Using the mean deviance of the residual as an estimate of the dispersion parameter in the logistic and loglinear models results in estimates much larger than 1 (Tables 5.4, 5.5 and 5.6). This indicates overdispersion, meaning that the data are from distributions more variable than the binomial and the Poisson.

5.5. Discussion

Absence of significant plant genotype-by-nematode isolate interaction within *M. chitwoodi* and within *M. fallax*, leads to the conclusion that little variation in virulence exists among the tested isolates of *M. chitwoodi* and *M. fallax* to the three *Solanum* genotypes used in this study. This result is in striking contrast with host responses of *M. hapla* isolates, showing a highly significant interaction effect with *Solanum* genotypes (Van der Beek *et al.*, D; Chapter 4). These differences in variability in host response could conceivably be explained, at least partially, by the evolution and distribution of these species. In terms of evolution, it is likely that *M. chitwoodi* and *M. fallax* are more recent species than *M. hapla*. Furthermore, a supposed introduction of *M. chitwoodi* in the Netherlands could not only have occurred more recently than

Table 5.5. Deviance table for Pf/Pi data of *M. chitwoodi* and *M. fallax* isolates, inoculated on genotypes of *S. chacoence*, *S. stoloniferum* and *S. tuberosum*

Source	d.f.	Deviance	Mean deviance	Deviance ratio	
For *M. chitwoodi* isolates:					
Replicate	4	269	67	6.06	***
Isolates	6	497	83	7.45	***
Solanum spp.	2	1859	930	83.67	***
Residual	88	978	11		
Total	100	3603	36		
For *M. fallax* isolates:					
Replicate	4	106	26	7.04	***
Isolates	2	47	23	6.21	***
Solanum spp.	2	631	315	84.02	***
Residual	34	128	4		
Total	44	911	22		

***$P<.001$, referring to an F-distribution

that of *M. hapla*, but also could be characterized by a narrow genetic basis, resulting in high genetic similarity between isolates of the species, as could be concluded from this study. The likeness of the American *M. chitwoodi* isolate Cba with the Dutch ones would indicate the same origin or similarity in virulence patterns in *M. chitwoodi* in a wide geographical area. Evidently, further studies have to provide definite indications for this.

Despite of the low variation in virulence in relation to *Solanum* spp., the present study might indicate the presence of intraspecific specialization for virulence in

Table 5.6. Deviance table for egg-mass data of *M. chitwoodi* isolates Cb and the six mono-female lines from isolate Cb, inoculated on genotypes of *S. chacoence*, *S. stoloniferum* and *S. tuberosum*

Source	d.f.	Deviance	Mean deviance	Deviance ratio	
Replicate	4	59	15	2.66	**
Lines	6	123	20	3.73	**
Solanum spp.	2	3266	1633	296.95	***
Residual	86	473	6		
Total	98	3921	40		

**$P<.01$, ***$P<.001$, referring to an F-distribution

M. chitwoodi and its absence in *M. fallax*, because the reproduction of isolate Fc on *S. bulbocastanum* and on *S. hougasii* and of Fd on *S. bulbocastanum* was entirely caused by the presence of *M. chitwoodi*. The levels of these compatible reactions are expected to be much higher if the inoculum would consist entirely of these particular *M. chitwoodi* juveniles. They show characteristics of the previously described race 3 isolate CAMc2, which was found in California and is able to reproduce on *S. bulbocastanum* SB22 (Mojtahedi & Santo, 1994). It is remarkable that in the present study isolate Fc did not reproduce significantly on *S. chacoence*, as did the nine *M. chitwoodi* isolates (Table 5.2). Besides the possibility of intraspecific specialization, a synergistic effect of the two incompatible *Meloidogyne* spp. might explain the breaking of the resistance of *S. bulbocastanum* and *S. hougasii* to *M. chitwoodi*. Synergistic effects, inducing susceptibility, have been reported in interspecific studies in *Meloidogyne*, but involved inoculation of a compatible *Meloidogyne* species prior to the incompatible species, like *M. arenaria* or *M. hapla* inoculated prior to *M. incognita* race 1 on tobacco cv. NC95 (Eisenback, 1983) or *M. hapla* prior to *M. incognita* race 3 on *Mi*-resistant tomato cv. Celebrity (Ogallo & McClure, 1996), utilizing the split-root technique. Whether these Dutch resistance-breaking contaminants of *M. chitwoodi* belong to race 3 or possess a different, not yet described, status within the species, or reproduce on *S. bulbocastanum* and *S. hougasii* as a result of a synergistic effect between the two incompatible *Meloidogyne* spp., is being examined in supplementary research.

The significant difference in reproductive ability between *M. chitwoodi* and *M. fallax* supports the species status of *M. fallax*, which only recently has been recognized to be distinctly different from the related *M. chitwoodi* (Karssen, 1996). *M. fallax* appeared to be less virulent than *M. chitwoodi* on the tested genotypes of *S. chacoence* and *S. stoloniferum*, but both nematode species were equally (a)virulent on *S. bulbocastanum*, *S. hougasii* and *S. tuberosum* cv. Nicola (Table 5.2). In general, resistance of *Solanum* spp. is effective to both *M. chitwoodi* and *M. fallax*, as was shown by Janssen *et al.* (1996) in resistance trials testing 64 *Solanum* spp.: only genotypes of *S. chacoence* and *S. stoloniferum*, which were the same genotypes as used in this study, were differentiating between these *Meloidogyne* spp. This study specifies the consistency of this differentiation. However, for practical breeding purpose, it is important to use sources for resistance effective to both nematode species, as both species are allopatric.

Deviations from a consistency in resistance level seem to be due to contaminations, like for Ck and Fc on *S. stoloniferum* and Fc on *S. bulbocastanum*. Several reasons can be suggested to be involved in these contaminations of *M. fallax*, varying from mixed field populations to cross-infection during the maintenance programme or inoculum preparation. It is concluded that, despite of serious precautions, a constant risk for contamination is present in a programme using a range of isolates for resistance testing. Evidently, these mixed-species isolates hinder the interpretation of the results.

Only identification of reproduced juveniles, like for Fc and Fd on *S. bulbocastanum* and for Fc on *S. hougasii*, can limit these misinterpretations. These results stress the importance of employing every effort to avoid working with contaminated isolates and emphasize the importance of testing for possible contaminations. Until now, only contamination with different species can be detected. The degree of genetic homogeneity within a given isolate is not known. Differences in variation in virulence can be either due to dosage effects of avirulence genes, or to different rates of virulent and avirulent nematodes within a certain population, or to both. Results from the - experiment with the mono-female lines respond to this question. A significant main effect for mono-female lines indicated significant differences between these lines (Tables 5.3 and 5.6). These differences appeared to be nearly as pronounced as those between the *M. chitwoodi* isolates in Table 5.2. It suggests a certain genetic variation in pathogenicity present in the original isolate Cb and supports the hypothesis that a meiotic parthenogenetic *Meloidogyne* population can consist of many genotypes for pathogenicity of which all can contribute to the phenotype. The almost identical mean numbers of egg-masses for *M. chitwoodi* isolate Cb in both experiments (Tables 5.2 and 5.3) show that the significant differences between these mono-female lines most probably are genetically-based differences.

On the susceptible *S. tuberosum* cv. Nicola, mean numbers of egg-masses were similar for *M. chitwoodi* and *M. fallax*, showing high levels of virulence. However, the mean value of Pf/Pi for *M. fallax* was 50 % lower than that for *M. chitwoodi*. This phenomenon was observed in other experiments as well (Van der Beek et al., F; Chapter 3) and is presumed to be due to a shorter life cycle for *M. fallax*, by which at the time of egg extraction, many J2 had already hatched and left the egg-mass. This difference in life cycle was confirmed by M.M.A. Khan (Rijksstation voor Nematologie en Entomologie, Merelbeke, Belgium, pers. comm.), who studied the development of these two species in potato and in maize. The present results emphasize that for measuring reproduction at the interspecific level, Pf/Pi values estimated on egg or J2 countings from extraction with NaOCl, might not be representative when there are differences in length of time between life cycles. For such situations, observations of numbers of egg-masses would therefore be much more reliable than numbers of Pf/Pi.

The overdispersion for the two reproduction parameters (Tables 5.4, 5.5 and 5.6) may point towards sources of variation not taken into account in the fitted regression models. Two sources of variation can be suggested contributing to overdisperson. Firstly, differences in water dynamics between the pots can easily occur in experiments using sand as growing medium. This is especially crucial during and shortly after inoculation. Secondly, the competition between individual juveniles during infection and settlement is depending on factors like plant genotype, inoculation density and root volume. Although their effects were minimized as much as possible, these two variables remain unknown sources of variation and emphasize

the need of a better understanding of the population dynamics in *Meloidogyne* spp.

The studied genotypes of *S. bulbocastanum* and *S. hougasii* showed high levels of broad-spectrum resistance to both root-knot nematode species *M. chitwoodi* and *M. fallax*. To *M. fallax* additionally high levels of resistance were demonstrated in the genotypes of *S. chacoence* and *S. stoloniferum*. These results provide promising breeding perspectives. Intraspecific specialization in the two *Meloidogyne* spp. seems lower and less pronounced, thus much easier to handle in breeding programmes, when compared to *M. hapla* (Janssen *et al.*, 1997; Van der Beek *et al.*, D; Chapter 4). However, the virulence of *M. chitwoodi* juveniles in isolate Fc on *S. bulbocastanum* and *S. hougasii* qualifies too optimistic expectations as the spread of this and possible other virulent isolates or races in the Netherlands as well as the effect of mixtures of species is not yet known. Because ample knowledge on the durablility of the resistances in *S. bulbocastanum* and *S. hougasii* is still lacking, incomplete resistances like in the genotype of *S. stoloniferum* to *M. chitwoodi* should not be discarded.

CHAPTER 6

Pathotypes and host races

to describe intraspecific variation

in pathogenicity:

three pathotypes identified in *M. chitwoodi*

on *S. bulbocastanum*

6.1.Summary - Testing eight Dutch *Meloidogyne chitwoodi* isolates to the differential set for host races 1 and 2 in *M. chitwoodi* provided no evidence for the existence of host race 2 in the Netherlands. The usefulness of the host race classification in *M. chitwoodi* is discussed, based upon experimental data which showed deviations from expected reactions on the differential hosts. The term pathotype is proposed for groups of isolates of one *Meloidogyne* sp. which exhibit the same level of pathogenicity on genotypes of one host species. Pathotypes can differ qualitatively or quantitatively, corresponding to virulence and aggressiveness, respectively. It is recommended to apply the pathotype classification in pathogen-host relationships when testing several genotypes of one *Meloidogyne* sp. on several genotypes of one host species or closely related species. Three qualitatively different pathotypes were identified in *M. chitwoodi* on *Solanum bulbocastanum*, suggesting at least two different genetic factors for virulence and resistance in the pathogen and the host species respectively. The occurrence of several virulence factors in *M. chitwoodi* will complicate the successful application of resistance factors from *S. bulbocastanum* for developing resistant potato cultivars.

6.2. Introduction

With respect to nematode pest control and risk avoidance, crop rotation with unsuitable or resistant host plants is one of the most effective environmentally safe strategies. Practical application, however, is complicated by intraspecific variation in pathogenicity of plant parasitic nematodes. To implement effective crop rotation, different types of variation are recognised in different denotations like: forma specialis, host race, biotype and pathotype.

To describe intraspecific pathogenic variation in *Meloidogyne* spp., two concepts are used in which groups of isolates are referred to as host races and as biotypes. The best known is the host race concept, which is based on host plant differentials to describe variation within *Meloidogyne* spp. Introduction of differential genotypes of host plant species by the International *Meloidogyne* Project (IMP) resulted in the North Carolina Differential Host Test which differentiates between *M. incognita* races 1, 2, 3 and 4, *M. javanica* (without race specification), *M. hapla* (without race specification) and *M. arenaria* races 1 and 2 by testing on specific genotypes of cotton, tobacco, pepper, watermelon, peanut and tomato (Sasser & Triantaphyllou, 1977; Taylor & Sasser, 1978; Hartman & Sasser, 1985). More recently, a differential set was proposed for *M. chitwoodi* (Table 6.1) to distinguish between host races 1, 2 and 3 by testing on specific genotypes of carrot, alfalfa and *Solanum bulbocastanum* (Mojtahedi *et al.*, 1988; Mojtahedi & Santo, 1994). Differential host tests were used to detect mixed populations consisting of more than one species and to distinguish host races (Hartman & Sasser, 1985).

The second way of classifying intraspecific pathogenic variation, as biotypes, is based

Table 6.1. Formal reaction of host races 1, 2 and 3 of *M. chitwoodi* on carrot cv. Red Cored Chantenay, alfalfa cv. Thor and *S. bulbocastanum* SB22, according to Mojtahedi *et al.* (1988) and Mojtahedi and Santo (1994).

Race	Carrot	Alfalfa	SB22
1	+	-	-
2	-	+	-
3	-	+	+

on different responses of isolates of a *Meloidogyne* species to more than one genotype of one host plant species. Roberts (1995) applied and elaborated this concept to the *M. incognita* - tomato relationship. This concept is comparable to the race concept in fungal diseases.

The aim of the present chapter is to describe the study of the intraspecific variation in *M. chitwoodi* in the Netherlands, using the race concept to distinguish between host races 1, 2 and 3. Furthermore, in this study principles are applied of the biotype concept in describing variation in virulence of *M. chitwoodi* on *S. bulbocastanum*. Based upon experimental data, terminology related to grouping of isolates of *Meloidogyne* according to pathogenicity will be discussed.

6.3. Materials and methods

6.3.1. Nematode isolates

Twelve isolates of *M. chitwoodi* were used in this study (Table 6.2). Four of the *M. chitwoodi* isolates originated from the USA; all other isolates originated from various sites in the Netherlands. The four American isolates represented the three host races: Cba (race 1), Cbd and Cbf (race 2) and Cbh (race 3). The isolates were maintained and propagated on *Lycopersicon esculentum* cv. Moneymaker. The isolates were found to be true to species by testing their isozyme phenotypes of esterase and malate dehydrogenase (Esbenshade & Triantaphyllou, 1990, Karssen *et al.*, 1995) and by analysis of internal transcribed spacer (ITS) regions of ribosomal DNA (Zijlstra *et al.*, 1995).

6.3.2. Plant genotypes

To distinguish between host races 1 and 2, seedlings of the genuine cultivars of *Daucus carota* cv. Red Cored Chantenay (susceptible to host race 1 isolates and resistant to host race 2) and *Medicago sativa* cv. Thor (resistant to host race 1 isolates and susceptible to host race 2), kindly provided by Dr. H. Mojtahedi, and of *L. esculentum* cv. Moneymaker as a susceptible control, were used. In a second experiment for further testing for host race 3 and to study nematode isolate-by-host genotype interaction, the same cultivars of carrot and alfalfa as mentioned above, and additio-

Table 6.2. Isolates of *M. chitwoodi* and *M. fallax* used in the experiment for host races 1 and 2 (Ex 1) and in the experiment for host race 3 and isolate-by-genotype interaction (Ex 2).

Isolate	Origin	Last host	Sampling year	Obtained from[1]	Ex
M. chitwoodi					
Ca	The Netherlands	maize	1989	PD	1,2
Cb	The Netherlands	wheat	1990	PD	1
Ck	The Netherlands	tomato	1989	PD	1
Co	The Netherlands	black salsify	1993	PAGV	1
Cx	The Netherlands	potato	1993	PD	1
Cy	The Netherlands	potato	1993	PD	1
Cz	The Netherlands	potato	1993	PD	1
Caq	The Netherlands	potato	1993	PD	1
Cba	Oregon (USA)	potato		WSU (ORMc12, race1)	1
Cbd	Washington	potato		WSU (WAMc30, race2)	2
Cbf	Oregon	potato		WSU (ORMc8, race2)	1
Cbh	California	potato		WSU (CAMc2, race3)	2

[1] 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, Washington, USA.

nally *S. bulbocastanum* SB22 (kindly provided by Dr. C.R. Brown), *S. bulbocastanum* 93-60-2 and *S. tuberosum* cv. Nicola as a susceptible control, were used. The host race 3 isolate of *M. chitwoodi* reproduces well on *S. bulbocastanum* SB22 and reacts like host race 2 isolates to the differential cultivars of carrot and alfalfa (Mojtahedi & Santo, 1994). *S. bulbocastanum* 93-60-2 was used in previous experiments in which this genotype appeared to posses absolute resistance to all tested isolates of *M. chitwoodi* and *M. fallax* (Janssen *et al.*, 1997) except for one *M. chitwoodi* isolate which was detected in an isolate of *M. fallax* (Van der Beek *et al.*, C; Chapter 5). To obtain the proper number of *in vitro* plants of *S. bulbocastanum* SB22 and 93-60-2 shoots were cut every two to three weeks and transferred into new tubes with MS medium containing 30 g/l sucrose (Murashige & Skoog, 1962). Three weeks after the last cut plantlets were large enough for *in vivo* transplantation.

6.3.3. Inoculation and experimental set up

Carrot, alfalfa and tomato seedlings, in vitro plantlets of the two *S. bulbocastanum* genotypes and germinating tuber buds of potato cv. Nicola were transplanted to 350 ml clay pots, filled with moist silver sand, containing 0.2 % slow release fertilizer and 0.08 % NPK 12-10-18, and placed into a greenhouse compartment at 20 ± 2 °C and 70 to 80 % relative humidity. After transplanting *in vitro* plants were kept at approximate-

ly 100 % humidity during the first three days by covering the plants with transparent plastic.

The nematode inoculum was prepared by placing heavily infested tomato roots on trays in a mist cabinet at 20 °C with water temperature of 25 °C (Seinhorst, 1988). Hatched second-stage juveniles (J2) which settled on the bottom of the tray were collected at least every other day during approximately two weeks and kept at 4 °C. Prior to inoculation, the J2 were poured over a cotton wool filter. Inoculum was collected after one day and inoculation was on plants, randomly taken for cultivar and replication, by applying 9 ml inoculum in a notch of approximately 1 cm deep around the plant, while continuously stirring the inoculum. An average density of 1.6 J2 cc^{-1} soil was pursued, resulting in approximately 560 J2 per pot. Two experiments were performed. The first experiment, to test for host races 1 and 2, involved ten *M. chitwoodi* isolates (see Table 6.1) and three host species: carrot, alfalfa and tomato in ten replicates. The second experiment, to test for host race 3 and to study nematode isolate-by-host genotype interaction on *S. bulbocastanum*, involved three *M. chitwoodi* isolates and the host species carrot, alfalfa, potato and the two *S. bulbocastanum* genotypes in six replicates. Both experiments were complete randomized block designs.

Eight weeks after inoculation, the roots were rinsed free from sand. Egg-masses were stained red with Phloxine-B (Dickson & Struble, 1965) and counted per plant.

6.3.4. Statistical analysis

The number of produced egg-masses was used to asses nematode reproduction. To the observed numbers of egg-masses in both experiments logistic regression models with replicate, isolate, host genotype and the interaction isolate-by-host genotype as explanatory variables were fitted. The dispersion of the egg-mass data was assumed to be pseudobinomial, i.e. $var(Y)=n\sigma^2\mu(1-\mu)$, where μ denotes the expected proportion,

Table 6.3. Mean numbers of egg-masses per plant of eight Dutch and two American *M. chitwoodi* isolates, inoculated with approximately 560 juveniles on 10 replicates of carrot cv. Red Cored Chantenay (C), alfalfa cv. Thor (A) and tomato cv. Moneymaker (T).
The two American isolates Cba and Cbf represent race 1 and race 2, respectively.

Isolate	C	A	T
Ca	6.9	0.0	87.0
Cb	5.0	0.1	51.3
Ck	9.4	0.0	100.3
Co	7.0	0.0	38.0
Cx	5.6	0.1	77.8
Cy	6.1	0.3	54.0
Cz	15.1	0.0	67.3
Caq	9.0	0.0	64.0
Cba	19.3	0.0	24.0
Cbf	9.2	3.0	45.5

n the number of inoculated J2 and σ^2 the dispersion parameter. Treatment effects were tested by means of F-statistics, which are deviance ratios based on the mean deviance of the full model. Significant results ($P<0.05$) are presented. The analyses were performed using the Genstat 5 programme (Genstat 5 Committee, 1993).

6.4. Results

Experiment 1, to test for host races 1 and 2, revealed estimates of mean numbers of egg-masses of the ten *M. chitwoodi* isolates, inoculated on cultivars of carrot, alfalfa and tomato, presented in Table 6.3. All isolates were pure *M. chitwoodi* isolates and no contamination with *M. hapla* or other species was noticed using the rDNA analysis. Isolate Cbf, representing host race 2, produced an average of only 3.0 egg-masses per plant on alfalfa cv. Thor. In four of the ten replicates of this isolate no egg-mass was produced on alfalfa. Some other isolates produced egg-masses on alfalfa, but only a few. These results suggest that alfalfa cv. Thor is a poor host to the *M. chitwoodi* isolates tested.
In contrast, on carrot cv. Red Cored Chantenay, all isolates reproduced moderately to well. The good reproduction of Cbf on carrot contradicts with the description of host race 2 (Mojtahedi *et al.*, 1988). Also on carrot, in some replicates, plants were observed without egg-mass development for several isolates, suggesting that carrot cv. Red Cored Chantenay is a moderate host for the *M. chitwoodi* isolates tested.
Isolates failed to reproduce in 12 % of 78 observations for host race 1 isolates on carrot cv. Red Cored Chantenay and in 50 % of 16 observations for host race 2 isolates on alfalfa cv. Thor.
A significant isolate-by-host species interaction occurred. This was mainly due to differences between the isolates on carrot and not on alfalfa. Pair-wise comparison did not reveal significant differences of Cbf and any other isolate on alfalfa.

In experiment 2 no Dutch isolate from experiment 1 was tested for host race 3, as all of them failed to reproduce on alfalfa cv. Thor. The study of isolate-by-host genotype interaction carried out in experiment 2 resulted in estimates of mean numbers of egg-masses for three *M. chitwoodi* isolates on potato cv. Nicola and two *S. bulbocastanum* genotypes, which are presented in Table 6.4. Host race 2 isolate Cbd produced a large number of egg-masses on *S. bulbocastanum* 93-60-2, but not on *S. bulbocastanum* SB 22. Host race 3 isolate Cbh produced large numbers of egg-masses on both *S. bulbocastanum* genotypes. Isolate Cbh showed no zero-reactions on its discriminating host *S. bulbocastanum* SB22.
The isolate-by-host genotype interaction appeared to be significant.

6.5. Discussion

6.5.1. Testing for host races 1 and 2

Based on the results of experiment 1 (Table 6.3), there is no indication for the

Table 6.4. Mean numbers of egg-masses per plant of three *M. chitwoodi* isolates, inoculated with approximately 560 juveniles on six replications of *S. bulbocastanum* SB22, *S. bulbocastanum* 93-60-2 and *S. tuberosum* cv. Nicola

Isolate	SB22	93-60-2	Nicola
Ca	0.0[a]	0.0[a]	101.5[c]
Cbd	0.7[a]	31.3[b]	33.8[b]
Cbh	36.7[b]	40.8[b]	77.5[c]

a,b,c isolate-*Solanum* genotype combination with common letters do not differ significantly for responses on the logit-scale ($P<0.05$ referring to an F-distribution for the deviance statistic).

presence of *M. chitwoodi* host race 2 in the Netherlands. All eight Dutch isolates tested were host race 1 according to the host differential set of Mojtahedi *et al.* (1988). The situation in the Netherlands seems to be different from the two other areas in the world which were examined for *M. chitwoodi* host race type: the Pacific North-West of the USA, with approximately 40 % of 32 isolates tested being host race 2 and 60 % host race 1 (Pinkerton *et al.*, 1987), and Tlaxcala State in Mexico where 93.5 % of 12 randomly sampled *M. chitwoodi* isolates appeared to be host race 2 and only one isolate host race 1 (Cuevas, 1995). Differences in origin and in selection pressure by different host plant genotypes may be involved in this variation in host race type in various places in the world. Possibly the generally lower pathogenicity of the Dutch isolates on carrot compared to the American would support such a difference.

Classification of *M. chitwoodi* isolates in host races 1 and 2 was not unambiguous. Variable levels of numbers of egg-masses of race 2 isolates on alfalfa have been explained by the cross-pollinating nature of this crop, resulting in heterogeneity for susceptibility to root-knot nematodes and possible gene shifts in the stock (Pinkerton *et al.*, 1987). A similar explanation may be true for carrot. Moreover, heterogeneity in meiotic parthenogenetic populations of *Meloidogyne* (Van der Beek *et al.*, H; Chapter 11) may be involved. Production of egg-masses by host race 2 isolates on carrot cv. Red Cored Chantenay, as was observed for isolate Cbf (Table 6.2), was also observed by H. Mojtahedi (pers. com.) and appeared, for as yet unknown reasons, after the isolate had been taken in culture in the greenhouse. The low numbers of egg-masses of host race 2 isolate Cbf on alfalfa and the recorded compatible reaction of this isolate on carrot hamper a clear distinction between host races 1 and 2 using carrot cv. Red Cored Chantenay and alfalfa cv. Thor as differentials. Besides these low means of numbers of egg-masses, isolates of both host races frequently failed to produce egg-

masses on the two hosts. As the differential reactions of host races 1 and 2 were not stable, conclusions on the host race status of *M. chitwoodi* isolates should be drawn cautiously.

6.5.2. Nematode isolate-by-host genotype interaction

Results from experiment 2 (Table 6.3) showed the occurrence of a significant nematode isolate-by-host genotype interaction term (data not shown) between the *M. chitwoodi* isolates on *S. bulbocastanum* genotypes, confirming similar observations by Brown *et al.* (1989). Because Ca, Cbd and Cbh show three different virulence patterns on the two *S. bulbocastanum* genotypes, this significant interaction points to at least two different (a)virulence factors in the pathogen. These factors can be mono- or polygenically controlled. This suggests that in *S. bulbocastanum* several different genes for resistance to *M. chitwoodi* are involved. These could be employed to improve the resistance of *S. tuberosum* to *M. chitwoodi*. It should be realized, however, that this resistance is pathotype-specific and its durability and usefulness has still to be assessed.

6.5.3. Host race concept versus nematode isolate-by-host genotype interaction

The classification of host races, in contrast to that of biotypes, relates pathogenic responses of isolates to single or several genotypes of different host species. Consequently, the host race concept is independent from the biotype concept and a host race can be composed of different biotypes. This is shown in a theoretical example (Table 6.5), in which host races 1 and 2 are distinguished on genotypes I of host A and host B. Isolates 'a' and 'c', both belonging to host race 1, when tested on genotype I of host A and B as differential hosts, belong to different biotypes according to their response to different genotypes of host A and B. This example further shows that the host race

Table 6.5. Theoretic example of interaction between three isolates of a hypothetic *Meloidogyne* sp. with genotypes I and II of two hypothetic host species A and B, showing in this example a host race classification based on genotypes I different from that based on genotypes II

Meloidogyne sp. isolate	Host species genotype				*Meloidogyne* sp. host race[1]	
	species A		species B			
	I	II	I	II	based on genotypes I	based on genotypes II
a	+	-	-	+	1	2
b	-	-	+	+	2	2
c	+	+	-	-	1	1

[1] host race classification based upon genotypes I and II of species A and B: host race 1 = compatible with host A and incompatible with host race B ; host race 2 = incompatible with host race A and compatible with host race B

classification is depending on the choice of genotypes. If genotypes II of host A and host B would have been chosen as differential hosts to distinguish between host races, the host race classification of isolate 'a' would have been different, due to significant isolate-by-host genotype interaction, and isolate 'a' would belong to a different host race than isolate 'c'. In conclusion, because host race designation is only applicable when using strictly the predescribed host genotypes, its validity is relative and accordingly classification is of arbitrary significance as soon as significant nematode isolate-by-host genotype interaction occurs. The nematode isolates-by-host genotype interaction, which is ignored by the host race concept, is the basis of the biotype concept. The hypothetic isolates 'a', 'b' and 'c' would belong to three different biotypes, due to significant interaction of these three isolates with the two genotypes of host A, and the same is true in relation to host B (Table 6.5).

The relative importance of the host race concept in the presence of nematode isolate-by-host genotype interaction, as described above, is illustrated by the results of experiment 2 on different *S. bulbocastanum* genotypes (Tables 6.4 and 6.6). The

Table 6.6. Pathotype and race designation of the three *M. chitwoodi* isolates (Iso) and resistance factor designation of *S. bulbocastanum* genotype 93-60-2 (Rf1) and SB22 (Rf2) from Table 6.4. For pathotypes, the index of reproduction is given as number of egg-masses produced on the *S. bulbocastanum* genotypes as a proportion of the number produced on the susceptible cv. Nicola (S). For races, a compatible reaction is denoted as + and an incompatible as -.

Isolate	S	Rf1	Rf2	Pathotype	Carrot	Alfalfa	SB22	Race
Ca	1.00	0.00	0.00	*S.bul.*0/1,2	+	-	-	1
Cbd	1.00	0.93	0.02	*S.bul.*1/2	+	+	-	2
Cbh	1.00	0.53	0.47	*S.bul.*1,2/	-	+	+	3

compatible reaction of isolate Cbh (representing host race 3) and the incompatible reaction of isolate Cbd (originally identified as host race 2) on *S. bulbocastanum* SB22 confirm the original host race status of these isolates. However, if host race 3 would have been defined using *S. bulbocastanum* 93-60-2 as a differential host, isolates Cbd and Cbh would both belong to host race 3. To avoid this confusion, we prefer to describe intraspecific pathogenic variation with respect to one host species only in terms of nematode isolate-by-host genotype interaction or aggressiveness.

6.5.4. Examples of intraspecific pathogenic variation

In various *Meloidogyne* spp. intraspecific pathogenic variation has been observed before, especially in *M. arenaria*, *M. incognita* and *M. chitwoodi*. In the soybean - *M. arenaria* relationship, it was shown that isolates of host race 1 and 2 (compatible and

incompatible with peanut cv. Florunner, respectively) could differ significantly in reproduction on soybean cultivars (Hiatt *et al.*, 1988; Carpenter & Lewis, 1991). Host race 2 isolates appeared to be more variable in aggressiveness in different environments compared to host race 1 isolates (Pedrosa *et al.*, 1994). Similar observations were done by Koenning and Barker (1992) who found differences in aggressiveness between an isolate of *M. arenaria* of host race 1 and one of host race 2 tested on four soybean cultivars. No significant interaction term was found between *M. arenaria* isolates and soybean cultivars, suggesting no variation in virulence among the employed isolates (Carpenter & Lewis, 1991; Koenning & Barker, 1992). Comparable results were obtained with host races of *M. incognita* tested on tomato (Khan & Khan, 1991a), on cauliflower (Khan & Khan, 1991b) and on *Hibiscus cannabinus* (Veech, 1992), indicating intraspecific pathogenic variation associated with host races. Also with host races of *M. chitwoodi* tested on *S. bulbocastanum* (Brown *et al.*, 1989), comparable results were obtained. It is, however, not evident that in all cases mentioned true host race specificity occurred because often only one isolate per host race was included. In different studies, the inclusion of more isolates per host race revealed significant pathogenic differences between isolates of one host race, indicating the occurrence of pathotypes. For example, significant differences among host race 1 isolates of *M. arenaria* were found by testing them on tobacco (Noe, 1992): isolate GA-7 was able to reproduce on resistant cvs. Speight G-70 and Northrup-King K326. From the results of Ibrahim *et al.* (1993), it can be concluded that interaction exists between triticale cvs. Beagle 82 and Florida 201 and *M. arenaria* host race 2 isolates Florence and Pelion, although the authors did not mention this result themselves. Different behaviour between host race 1 and 2 of *M. chitwoodi* was concluded by Brown *et al.* (1994), who reported virtual immunity to host race 1 in wild *Solanum* spp. and resistance of a quantitative nature to host race 2. This would imply that host races can be distinguished by characteristics, similar for all isolates of one host race. These host race-specific characteristics were shown on hosts (wild *Solanum* spp.) which are different from the differential hosts which separate them, namely carrot cv. Red Cored Chantenay and alfalfa cv. Thor. Our results with isolate Cbd (belonging to host race 2) on *S. bulbocastanum* do not support the quantitative nature of resistance to host race 2 isolates on wild *Solanum* spp. The differences in response between host races 1 and 2 isolates, reported by Brown *et al.* (1994), also may be explained by differences between specific isolates of *M. chitwoodi* and/or specific genotypes of *S. bulbocastanum*.

6.5.5. Terminology

In literature on *Meloidogyne* spp., the terms host race and biotype have been proposed to classify isolates according to their intraspecific variation in pathogenicity. Pathogenicity describes the ability of a pathogen to reproduce at the expense of a specific host (after Robinson, 1976), and is composed of aggressiveness and virulence. Aggressiveness corresponds to the degree in which a pathogen is able to reproduce on a genotype of a host species and is quantitative in nature. Virulence is the ability to reproduce on a

specific host genotype and corresponds to differential pathogenicity i.e. host genotype-specific pathogenicity and is consequently qualitatively characterized. For *Meloidogyne*, host race and biotype are defined as follows. The term host race, according to Hartman and Sasser (1985), reflects the ability of the isolate to reproduce on a specific genotype of a host plant species, in comparison to its inability to reproduce on a specific genotype of a different host plant species. The term biotype, according to Roberts (1995), describes the isolate's ability to reproduce on a specific genotype of a host plant species, in comparison to its inability to reproduce on a different genotype of the same host plant species. A biotype is a collective name for isolates with similar virulence patterns. Different biotypes are distinguished by different (a)virulence reactions to host plant resistance factors.
Isolates with equal patterns of intraspecific pathogenic variation have been described in terms of pathotypes in various cyst nematodes: *Globodera* spp. (Kort *et al.*, 1977), *Heterodera avenae* (Andersen & Andersen, 1982), *H. schachtii* (Trudgill, 1986; Müller, 1992), which all correspond to the biotype denotation by Roberts (1995) for *Meloidogyne* spp. Originally biotype was referring to homogenetic individuals (Johannsen, 1903) and was used afterwards to describe various types of biological variation, like in physiology, parasitic ability and resistance. In plant pathology the term pathotype would be more appropriate and is hereby proposed to describe groups of isolates of a *Meloidogyne* sp. which show equal levels of virulence on each genotype of a host species tested. Application of the term pathotype is limited to describe virulence, ignoring differences in aggressiveness. It is proposed to apply the term pathotype for groups of identically virulent genotypes, regardless of their levels of aggressiveness. Andersen and Andersen (1982) and Trudgill (1986) defined pathotypes in cyst nematodes as groups of virulent genotypes in cases of known gene-for-gene relationships concerning resistance genes in the host and virulence genes in the pathogen. Applying this definition to *Meloidogyne* spp. would require genetic studies in hybrid progenies, which is hampered by the parthenogenetic nature of many species of this genus (Roberts, 1995). For other pathogenic organisms without known sexual phase, e.g. yellow rust, a gene-for-gene relationship is assumed (Zadoks, 1961). Recently, however, Triantaphyllou (1993) and Van der Beek and Karssen (E; Chapter 10) demonstrated possibilities for hybridization in meiotic parthenogenetic *Meloidogyne* spp., which, combined with molecular characterization of isolates, indicate perspectives for genetic studies in intraspecific hybrid progenies.

The present chapter pleas for applying a pathotype scheme similar to that proposed by Roberts (1995), to describe genetic differences in virulence within a *Meloidogyne* spp. in relation to genotypes within one host species.

CHAPTER 7

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

7.1. Summary - Isolates of the mitotic 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 the 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 highly congruent. *M. hapla* race A and race B were clustered with high similarity, the tropical species *M. incognita*, *M. javanica* and *M. arenaria* were clustered together, and the species specialised on Gramineae: *M. naasi*, *M. chitwoodi* and *M. fallax* were distant from the rest showing high similarity between the latter two. The dendrogram for the morphological data deviated from those of the molecular data, particularly for *M. incognita* and *M. naasi*. The discrepancy between the protein and DNA studies on one hand and morphological studies on the other hand are discussed.

7.2. Introduction

The genus *Meloidogyne* comprises many plant parasitic root-knot nematode species: the overview of Eisenback & Hirschmann Triantaphyllou (1991) counted 68 nominal species, the majority reproducing either in a facultative meiotic or in an obligate mitotic parthenogenetic way. Particularly the parthenogenetic nature of most *Meloidogyne* spp. causes taxonomic difficulties which therefore does not always result in a distinct classification. The biological species concept is difficult to apply; in mitotic parthenogenones, reproductive isolation exists between all individuals. In facultative meiotic parthenogenones, reproductive isolation has only been demonstrated between *M. chitwoodi* and *M. fallax* (Van der Beek & Karssen, accepted; Chapter 10).

Traditionally, species identification in *Meloidogyne* is based upon morphological and morphometric characters and host plant response (Eisenback *et al.*, 1981; Eisenback, 1985). Some of these diagnostic characters show considerable variation, partly based upon true genetic differences and partly caused by the environment, such as host plant influence on morphometrics and certain morphological traits. Netscher (1978) and Taylor and Netscher (1979) among others, pointed out that large overlap between species exists and that reliable species identification should include other criteria.

Because of the mentioned limitations in applying morphological characters in the taxonomy of *Meloidogyne*, various approaches have been introduced during the past decennia. These approaches did not only allow a more accurate distinction between species, but also lead to a better understanding of the genetic variability in the genus. Amongst them are: mode of reproduction and chromosome number (Triantaphyllou, 1985; Dalmasso & Bergé, 1975), isozyme pattern (Bergé & Dalmasso, 1975; Esben-

shade & Triantaphyllou, 1987, 1990) and 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 in systematics is restricted by their use of a limited, non-random part of the available genetic information. Other techniques like RFLP analysis lead to stable, randomly selected markers, suitable for systematics. Castagnone-Sereno *et al.* (1993) for example used 22 RFLP fragments to relate isolates of *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*.

For studies on relatedness between *Meloidogyne* spp., conclusions have been based sofar on the variation at one level, like 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 potential of the 2-DGE and the AFLP techniques are studied and combined with morphological classification.

7.3. Materials and methods

7.3.1. Nematode isolates

M. chitwoodi, *M. fallax*, *M. naasi*, *M. hapla* race A, *M. hapla* race B, *M. incognita*, *M. javanica* and *M. arenaria* were used in this study. The first three of them reproduce in a facultative meiotic parthenogenetic way, the latter three are obligate mitotic parthenogenones. *M. hapla* is considered to be one species consisting of two cytological races: race A, being of meiotic parthenogenetic nature and race B, being mitotic parthenogenetic (Triantaphyllou, 1966). For reasons of simplicity, these two races were considered te be separate species throughout this chapter. From each species, one isolate was randomly chosen for this study (Table 7.1).

All isolates were maintained on *Lycopersicon esculentum* cv. Moneymaker, except for *M. naasi* which was maintained on *Triticum aestivum* cv. Minaret. The plants were grown in a climat chamber at 20 °C, 70 % relative humidity and 16/8 hours light/dark.

The *Meloidogyne* isolates were found to be true to species by testing them for absence of contamination with other species by analysis of ITS regions of rDNA (Zijlstra *et al.*, 1995), by isozyme electrophoresis of esterase and malate dehydrogenase (Karssen, 1995) (Table 7.1) and perineal patterns.

7.3.2. Morphometric and morphogical characterization

For practical reasons, different isolates of *M. hapla* races A and B and *M. chitwoodi* were used for measurements of morphometric characters compared to the protein and DNA evaluation.

Males and second-stage juveniles (J2) were extracted from excised root systems which were kept in a spray mist chamber (Seinhorst, 1988). Females were directly isolated

Table 7.1. Origin and isozyme phenotypes of the used *Meloidogyne* isolates

M. sp.	Code	Origin	Host of origin	Year of sampling	Source[1]	Isozyme phenotype *EST*[2]	*MDH*[3]
M. arenaria	C4393	NL			PD	N1	A2
M. chitwoodi	Ca	NL	maize	1989	PD	N1A	S2
M. chitwoodi	C5273-C	NL			PD	N1A	S2
M. fallax	Fb	NL	beet	1991	PD	N1B[4]	F3[4]
M. hapla A	Hi	NL	immortelle	1992	PAGV	H1	H1
M. hapla A	Xbr	unknown			PD	H1	H1
M hapla B	Hbr	NL	*Veronica*	1992	PD	H1	H1
M. hapla B	C2552	unknown			PD	H1	H1
M. incognita	568.93	unknown			PD	N1	I1
M. javanica	C3059	China	bonsai	?	PD	N1	J3
M. naasi	Nb	unknown			PD	N1A	S1

[1] PD = Plant Protection Service, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming, Lelystad, The Netherlands.
[2] esterase phenotypes according to Esbenshade and Triantaphyllou (1990)
[3] malate dehydrogenase phenotypes according to Esbenshade and Triantaphyllou (1990)
[4] *EST* and *MDH* phenotypes according to Van der Beek and Karssen (accepted)

from roots of infected plants. All three stages were fixed at 70 °C and mounted in TAF (Jepson, 1987), and observed with a light microscope using differential interference contrast. The following nine morphological measurements were done on 25 nematodes for each isolate, separately for females, males and J2: distance from the base of the stylet to the dorsal esophageal gland orifice (DGO), for females and males: stylet length, and only for J2: total body length, tail length, hyaline tail terminus and distance from head to excretory pore.

Besides these morphometric observations, twelve qualitative morphological characters were collected from literature for all eight species (Jepson, 1987; Eisenback & Triantaphyllou-Hirschmann, 1991; Eisenback, 1993; Karssen, 1996), which were scored as follows for females: body shape (pear or spherical), lateral lines in perineal pattern (yes or no) ; for males: head cap (rounded or anteriorly flattened), lateral lips (yes or no), medial lips (distinct or crescent shaped), form of stylet (slender or robust); and for second stage juveniles: body shape (ovoid or round), shape of stylet knob (round or pear), set off of stylet knob (yes or no), dorsal stylet carvation (yes or no), broadening of stylet shaft (yes or no), shape of tail terminus (broad or slender).

7.3.3. Female extraction for two-dimensional gel electrophoresis

Approximately seven weeks after inoculation, infected roots were cut into small pieces and treated with a liquid pectolytic enzyme and cellulase preparation (20 volume %

Pectinex with 20 volume % Celluclast in 60 volume % phosphate buffer pH 4.5) for 24 to 48 hours at 37 °C. This treatment caused a desintegration of cell walls and a breakdown of cellulose into glucose, cellobiose and higher glucose polymers, by which females could mostly be collected directly from a sieve with 250 µm mesh or removed from remnants of the roots on the sieve. For *M. naasi*, this extraction method was not practical and females had to be collected from untreated roots. From each isolate, two samples of 50 young, milk-white, egg-producing females of the same age were collected, to avoid influences of the developmental stage on the protein composition (Klose, 1982; De Boer *et al.*, 1992). Females were stored dry at -80 °C until usage.

7.3.4. 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 (1988b). The protein samples were stored at -80 °C before use for electrophoresis. Mini two-dimensional gel electrophoresis of proteins (2-DGE) was performed as described by De Boer *et al.* (1992a) with some minor modifications (Folkertsma *et al.*, 1996). One gel was run for each sample. After staining, the gels were dried on a vacuum dryer (model 543, Bio-Rad Laboratories, Richmond, California USA) in order to store the gels for a prolonged period of time.
For technical reasons, gel electrophoresis for *M. arenaria* was performed apart from the seven other species.

7.3.5. DNA extraction

DNA extraction from hatched juveniles was performed as described in Zijlstra *et al.* (1995).

7.3.6. Amplified Fragment Length Polymorfism (AFLP) procedure

AFLP was performed based on the procedures described by Vos *et al.* (1995) and Folkertsma *et al.* (1996).
To generate fragments, genomic DNA (200 ng) was incubated for 1 h at 37 °C with 10 u *Eco*RI and 5 u *Mse*I in 40 µl 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/ul BSA. Next, 10 µl of a solution containing 5 pMol *Eco*RI-adapter and 50 pMol *Mse*I adapter, 80 u T4 DNA-ligase (Biolabs), 1 mM ATP in 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA was added, and the incubation was continued for 3 h at 37 °C, followed by an over night incubation at 14 °C. After ligation the reaction mixture was diluted ten times with water.
The structures of the adapters are as follows:

```
EcoRI adapter:  5-CTCGTAGACTGCGTACC
                  CATCTGACGCATGGTTAA-5
MseI adapter:   5-GACGATGAGTCCTGAG
                      TACTCAGGACTCAT-5
```

Table 7.2. 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

Nonselective amplification of the generated fragments was performed using primers complementary to the core of the adapter sequences. Therefore 10 μl 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), 2 μl of 10x PCR-buffer (Boehringer) and 0.4 μl of 10 mM dNTP in a final volume of 20 ul. PCR was performed for 30 cycles with the following cycle profile: 30 s at 94 °C; 30 s at 56 °C; 1 min at 72 °C. After amplification 7 μl of the PCR product was visualized on a 1% 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 7.2. The selective *Eco*RI-primer was radioactively end-labeled as described by Vos *et al.* (1995). PCR was performed using 5 ng of labeled 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 ul. AFLP reactions were performed for 35 cycles with the following profile: a 30 s denaturing step at 94 °C; a 30 s annealing step (see below); a 1 min extension step at 72 °C. The annealing temperature in the first cycle was 65 °C, was subsequently reduced each cycle with 0.7 °C for the next 12 cycles, and was kept at 56 °C for the remaining 22 cycles.
Following amplification reaction products were mixed with 20 μl of formamide dye, heated for 5 min at 95 °C and then quickly cooled on ice. Of each sample 3 μl was loaded on a CastAway Precast sequencing gel (Stratagene), gels were run using the CastAway Precast sequencing system (Stratagene) and processed according to the manufactures 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.

7.3.7. Data analysis

Protein profiles were evaluated visually by superimposing dried gels on a bench viewer. One of the two protein gels was chosen for each isolate (the mastergel) and 100 arbitrary protein spots, present in both gels of each isolate, were indicated. For each species, the presence or absence of the 100 protein spots indicated on the master gel was evaluated. This resulted per mastergel in one dataset with 100 characters evaluated in 14 gels of the other isolates. The similarity coefficient F in protein patterns among isolates was estimated from these data stets, using the formula $2n_{xy}/(n_x + n_y)$, with n_x and n_y being the number of spots observed for isolate x and y respectively, and $2n_{xy}$ the number of identical spots observed in both patterns. F is an estimation of the Dice coefficient for similarity (Aquadro & Avise, 1981). This was done for all isolates by comparing them two by two, resulting in two coefficients, of which the means were calculated. The resulting similarity matrix of these mean F values was used to conduct 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 for 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 ratio's of the body length, to obtain as much independance as possible among these measurements. Then, each of the nine characters were grouped into three categories, resulting in a binary matrix with 18 markers. Together with the 12 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.

7.4. Results

7.4.1. 2-DGE of total protein extracts

An average of 400 proteins were 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 7.3. 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:

Table 7.3. F-values based upon Dice-similarity coefficients of AFLP-data (above diagonal) and of 2-DGE-data (below diagonal) of eight *Meloidogyne* spp.

	M. in.	*M. ar.*	*M. ja.*	*M. h.B*	*M. h.A*	*M. na.*	*M. fa.*	*M. ch.*
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	

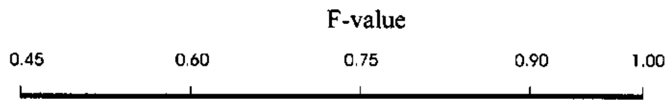
M. javanica, *M. incognita* and *M. arenaria*, the F-values range from 0.87 to 0.81. Between the supposedly related *M. fallax* and *M. chitwoodi* this coefficient 0.86 equals and between *M. naasi* and the closest other species *M. chitwoodi*, 0.68. The cophenetic correlation coefficient is 0.92 indicating a very good fit for the cluster analysis. The dendrogram reveals a clear distinction between the eight isolates (Figure 7.1A) and four main clusters can be distinguished. *M. javanica*, *M. incognita* and *M. arenaria* are clustered together and are separated from the cluster with the two races of *M. hapla*. *M. fallax* and *M. chitwoodi* were grouped together, remote from the two previously mentioned groups. Finally, *M. naasi* shows high dissimilarity with all other isolates and is branched loosely to *M. chitwoodi* and *M. fallax*.

7.4.2. 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 7.3. 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*. Between the three tropical species, these values range from 0.75 to 0.53. The F-values between *M. hapla* race A and these tropical species range from 0.17 to 0.08. The related species *M. fallax* and *M. chitwoodi* show an F-value of 0.60. The cophenetic correlation coefficient is 0.98, indicating a very good fit for the cluster analysis. The same main clusters were obtained as with UPGMA analysis of the protein data (Figure 7.1B): *M. incognita*, *M. javanica* and *M. arenaria* in one cluster in a slighly different order, followed by *M. hapla* race A and race B in a separate cluster, and finally *M. chitwoodi* and *M. fallax* in a third cluster, remote from the rest.

7.4.3. Analysis of morphometric and morphological data

Means of measurements with corresponding standard deviations of the nine


A
F-value
0.45
0.60
0.75
0.90
1.00
M. naasi
M. chitwoodi
M. fallax
M. hapla race A
M. hapla race B
M. arenaria
M. javanica
M. incognita

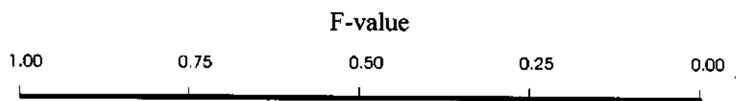

B
F-value
1.00
0.75
0.50
0.25
0.00

Figure 7.1. (p. 102) Similarity dendrograms of isolates of eight *Meloidogyne* spp., based upon **A**. protein spots from 2-DGE analysis, and **B**. DNA fragments from AFLP analysis. *M. naasi* was absent in **B**. The dendrograms were based on the Dice coefficient of similarity and constructed with UPGMA.

morphometric characters for each isolate of the *Meloidogyne* spp. are presented in Table 7.4. These means were 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 for morphometric and morphological data (Figure 7.2), using the Dice coefficient for similarity, shows dissimilarity with the dendrograms based upon the molecular data: *M. incognita* is grouped together with the cluster of *M. chitwoodi* and *M. fallax* instead of forming one cluster with *M. arenaria* and *M. incognita*, and *M. naasi* is clustered remote from all other species. The cophenetic correlation coefficient is 0.88, indicating a good fit for the clustering analysis.

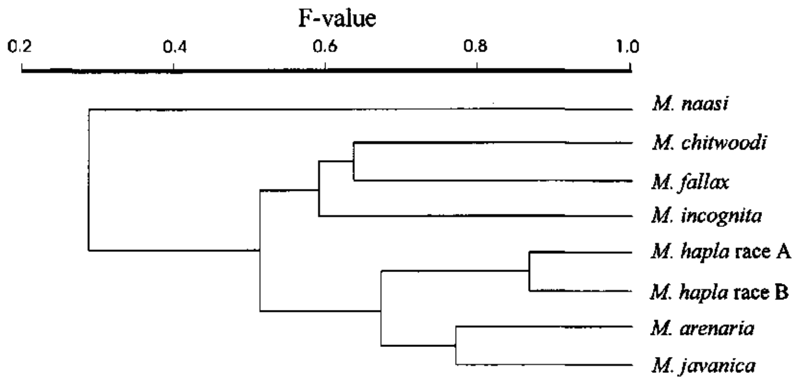


Figure 7.2. Similarity dendrogram based upon 21 morphological data of eight *Meloidogyne* spp., constructed with UPGMA using the Dice coefficient for similarity

7.5. Discussion

Overall genetic distances between the *Meloidogyne* species which were calculated from F-values (Table 7.3) and based upon 2-DGE data of total soluble proteins (Figure 7.3), range from 0.20 between *M. incognita* and *M. arenaria* to 0.37 between *M. chitwoodi* and *M. javanica*. These distances between the species are

Table 7.4. Means of morphometric data with standard deviations of single isolates of *Meloidogyne* spp. of two characteristics of 25 adult females, two of 25 adult males and five of 25 second stage juveniles per isolate

iso[1]	stl-f	dgo-f	stl-m	dgo-m	bl-j	tl-j	htt-j	hex-j	dgo-j
inc	16.1±0.3	3.6±0.4	23.8±0.3	3.6±0.3	387.8±15.7	47.6±2.1	13.0±0.6	80.5±2.6	3.0±0.3
are	15.9±0.4	4.8±0.4	mv[2]	mv	510.4±11.8	62.8±0.9	13.8±0.7	79.4±2.4	3.6±0.3
haB*	14.3±0.4	5.4±0.3	19.3±0.3	4.0±0.3	455.2± 9.5	58.3±2.1	16.6±1.3	83.9±1.9	4.2±0.4
haA*	14.1±0.3	5.5±0.4	18.6±0.5	4.2±0.4	399.4±10.8	51.1±1.8	12.9±1.8	75.9±3.6	4.1±0.3
naa	13.6±0.4	3.6±0.4	18.0±0.6	2.9±0.3	424.7± 8.8	68.2±3.5	20.6±2.2	71.8±2.6	2.9±0.4
fal	14.5±0.4	4.4±0.6	19.6±0.6	4.5±0.7	408.1±14.1	49.5±2.0	13.7±1.2	69.2±3.4	3.5±0.3
chi*	12.8±0.8	3.1±0.4	17.5±0.7	2.8±0.5	363.2±10.8	42.2±1.4	10.9±1.1	61.8±3.9	3.0±0.4

[1] Legenda:
iso = isolate of *Meloidogyne* spp. (see Table 7.1). The *-marked isolates were different in the molecular studies.
stl-f = stylet length in adult female (μm)
dgo-f = distance from the base of the stylet to the dorsal esophageal gland orifice in adult female (μm)
stl-m = stylet length in adult male (μm)
dgo-m = distance from the base of the stylet to the dorsal esophageal gland orifice in adult male (μm)
bl-j = total body lenth of second stage juvenile (μm)
tl-j = tail length from anus to tail tip in second stage juvenile (μm)
htt-j = hyaline tail terminus in second stage juvenile (μm)
hex-j = distance from head to excretory pore (μm)
dgo-j = distance from the base of the stylet to the dorsal esophageal gland orifice in second stage juvenile (μm)
[2] mv = missing value

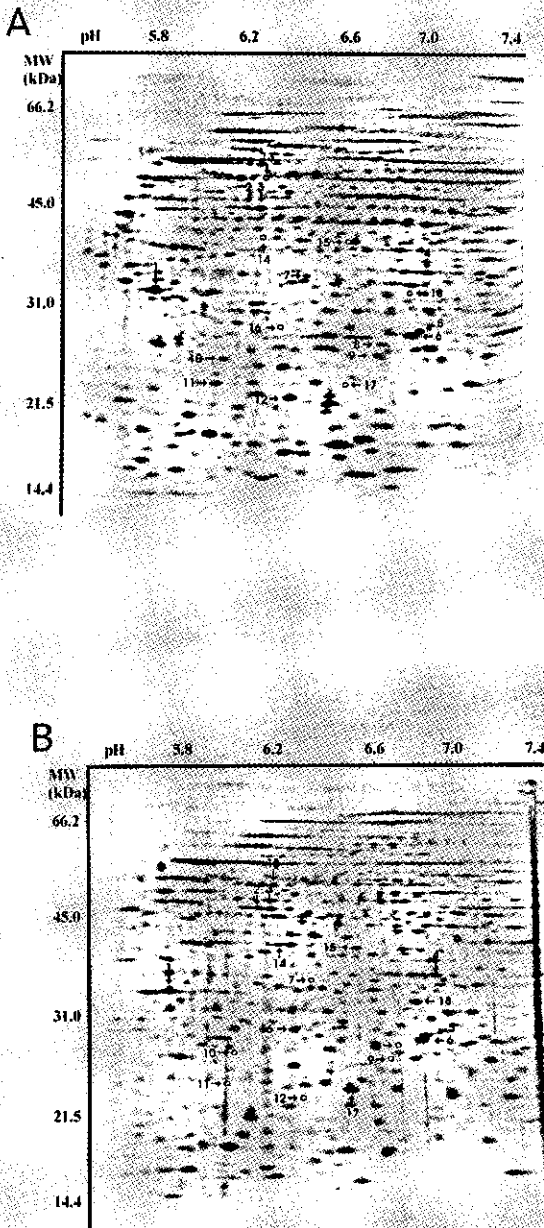
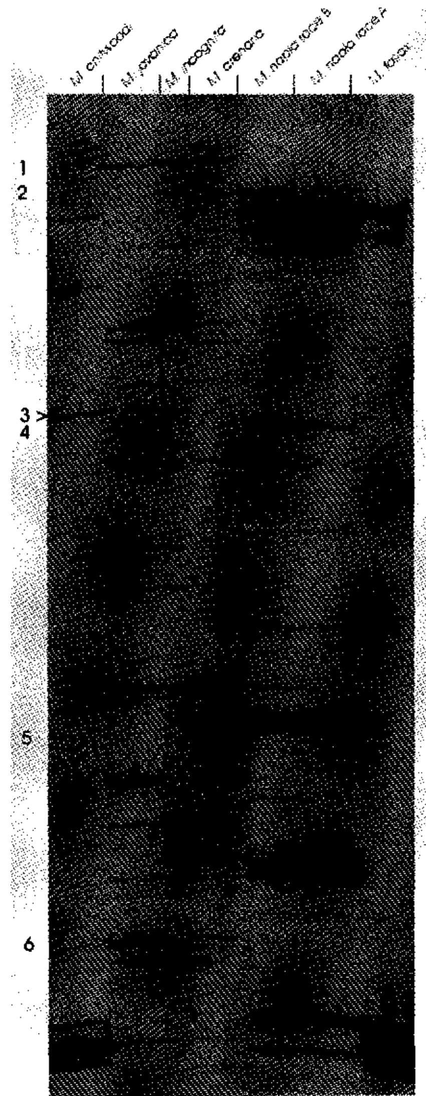


Figure 7.3. 2-DGE protein patterns of **A.** *M. incognita* and of **B.** *M. naasi*, showing separation of protein spots on isoelectric point (horizontal direction) and molecular weight (vertical direction). On the patterns, six monomorphic spots (1 to 6), six spots only present in *M. incognita* (7 to 12) and six spots only present in *M. naasi* (13 to 18) are indicated.

Figure 7.4. Detail of AFLP pattern, showing polymorphisms between *Meloidogyne* spp. Examples of specific inserts for 1. tropical *Meloidogyne* spp., 2. *M. hapla* race B, 3. *M. chitwoodi*, 4. *M. hapla* races A and B, 5. *M. arenaria*, 6. *M. javanica*.



large compared to those within species: between isolates of *M. hapla*, *M. chitwoodi* and *M. fallax* genetic distances ranged from 0.01 to 0.03, from 0.00 to 0.02 and from 0.00 to 0.01, respectively (Van der Beek *et al.*, A; Chapter 8), indicating that in the present study the data, which are based on one isolate for each 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*, being 0.70, and *Heterodera glycines* and *H. schachtii*, being 0.59 (Bakker & Bouwman-Smits, 1988b). The observed distances are in the same range as found between the sibling species *Drosophila melanogaster* and *D. simulans*, showing a genetic distance of 0.19 (Ohnishi *et al.*, 1983), but larger than between 15 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).

Estimates of genetic distance based on this methodology are considered not to be significantly influenced by differences in developmental stage of the females or physiological factors, as these differences were minimized. Although *M. naasi* was maintained on wheat, influence of the host on the protein profile is likely to be of little to no importance, as Bakker and Bouwman-Smits (1988a) found no differences between profiles obtained from *Globodera rostochiensis*, maintained on various

hosts: potato cvs Mentor and Eigenheimer and tomato cv. Moneymaker.

The genetic distances between the species, which were calculated from F-values (Table 7.3) and based upon AFLP-data (Figure 7.4), range from 0.75 between *M. incognita* and *M. javanica* to 0.96 between *M. fallax* and *M. incognita*. Castagnone-Sereno *et al.* (1993) found that genetic distances, based upon RFLP and also using the Dice coefficient, within the group of tropical species ranged from 0.27 to 0.60 and that the distances from *M. hapla* race A to this group ranged from 0.97 to 1.00. This is comparable to the results presented in this paper, ranging from 0.25 to 0.47 and 0.83 to 0.92, respectively. The genetic distance estimates from the results of the DNA techniques are larger than those from the 2-DGE data of total protein because, among others, the AFLP technique samples also non-coding regions. Moreover, also in the coding regions 2-DGE is less effective in recovering differences in DNA sequence, including those resulting in an amino acid substitution. Overall it is estimated that about one third of all amino acid substitutions results in a shift in isoelectric point (Pasteur *et al.*, 1988).

The order of clustering in the two dendrograms derived from protein and DNA data was highly similar (Figure 7.1). This is not surprising, considering protein and DNA evolution evolve both at fairly constant rates. Kimura (1983) showed that rate-constancy for a given protein exists, although this constancy is an approximate one. The rate of molecular evolution seems not to be constant per year among lineages of diverse taxonomic groups, and differences in substitution rate are among others explained by the generation-time effect (Kohne, 1970) and by differences in the efficiency of the DNA repair system (Britten, 1986). Nevertheless, Li *et al.* (1987) found nearly equal substitution rates in rats and mice. Thus, although a global evolutionary clock will not exist, a local clock may exist for groups of relatively closely related species (Li & Graur, 1991). In contrast, it has been hypothesized that morphological and molecular evolution, measured by electrophoresis and other biochemical techniques, evolve at practically independent rates (Wilson *et al.*, 1974; King & Wilson, 1975; Prager & Wilson, 1975). For morphological traits, natural selection has been considered as the major driving force of evolution, while in 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, as has been found in frogs (Wilson *et al.*, 1974; Wilson, 1976). The present study revealed differences between molecular and morphological data (Figures 7.1 and 7.2). Contrasts between molecular and morphological similarities have been revealed in a range of taxonomic groups such 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 for instance *M. incognita* and the other two tropical species, *M. arenaria* and *M. javanica*, in the dendrograms in Figures 7.1 and 7.2, could point to different mechanisms underlying

molecular and morphological evolution, with the latter resulting in a higher degree of convergent and parallel character changes. An other 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 the morphological and molecular data could be involved. It emphasizes the strength of the molecular techniques by enabling the observation of large numbers of markers. Finally, it is 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 is effected by a single gene in *Caenorhabditis elegans* (Katsura *et al.*, 1994).

Isolates of the meiotic parthenogenetic *M. hapla* race A and the mitotic parthenogenetic *M. hapla* race B were clustered similarly in the dendrograms of the protein and AFLP data (Figure 7.1) and of the morphological data (Figure 7.2), 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 mitotic and meiotic parthenogenesis.
The isolates of the mitotic parthenogenetic species *M. incognita*, *M. javanica* and *M. arenaria* clustered in one group of tropical species, apart from the meiotic parthenogenetic species and *M. hapla* race B, using both protein and DNA markers. This suggests 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 Esbenshade and Triantaphyllou (1987), Dalmasso and Bergé (1975) based on isozymes, Dickson *et al.* (1971) 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 relates *M. fallax* to other species. The clustering of these two species was not surprising, as initially the possibility was considered that both belong to the same species (Van Mechelen *et al.*, 1994). However, evidence for the species status for *M. fallax* was recently obtained by demonstrating reproductive isolation between the two species (Van der Beek & Karssen, accepted; Chapter 10).
M. chitwoodi, *M. fallax* and *M. naasi* which clustered distantly from the other species of this study, are able to reproduce on monocotyledons, contrary to the other five. This confirms the only other available reference on the relationship of *M. naasi*

and *M. chitwoodi* (Esbenshade & Triantaphyllou, 1987).

The present study shows that the genetic distances, based on molecular data between *Meloidogyne* spp. are relatively small when compared to morphologically nearly indistinguishable cyst nematode species. This may indicate a more recent speciation of the *Meloidogyne* spp. and hence may explain in part the difficulties in applying morphological characters in the taxonomy of the root-knot nematodes. Secondly, the similarity dendrograms based on molecular and 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 on relationships in *Meloidogyne* should not solely be based on morphology but also on molecular data. Thirdly, our data indicate that apomixis most likely is 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.

CHAPTER 8

Molecular evidence for *M. hapla*, *M. chitwoodi* and *M. fallax* being distinct biological entities

8.1. Summary - Six isolates of *Meloidogyne hapla*, among which four of race A and two of race B, eight of *M. chitwoodi* and five of *M. fallax* were submitted to two-dimensional gel electrophoresis (2-DGE) to study similarity between isolates within 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 revealed an average of 400 protein spots per gel. Within each species, all gels were compared mutually to identify two types of polymorphic spots: isoelectric point (IP) variants and present-absent (PA) variants. Among *M. hapla* isolates, 13 PA and nine IP variants, among *M. chitwoodi* isolates, eight PA and no IP variants and among *M. fallax* isolates, two PA and no IP variants were observed, corresponding with 5.0, 2.2 and 0.6 % polymorphisms among isolates of the three respective species. These percentages imply small intraspecific variation within these species. They also confirm these species to be clearly delineated biological groups, as similarity between these species is significantly lower than between isolates within these species (Van der Beek *et al.*, B; Chapter 8). 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.

8.2. Introduction

Root-knot nematode species *Meloidogyne hapla* race A, *M. chitwoodi* and *M. fallax* are diploid, facultative meiotic parthenogenetic species, that either reproduce sexually, or by absence of male gametes reproduce parthenogenetically (Triantaphyllou, 1985; Karssen, 1996). *M. hapla* race B is obligate mitotic parthenogenetic of which most isolates are polyploid. Species determination has traditionally been 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* as well as 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.* (B; Chapter 8), 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*.
Especially *M. chitwoodi* and *M. fallax* form a threat to West-European agriculture by their wide host range and absence of resistant cultivars of the major crops in the rotation scheme in arable farming. To interpret data from resistance and virulence testing, a better understanding of the genetic variation within these species is required. *M. hapla* has been reported as highly variable which is expressed by varying haploid chromosome numbers from n=13 to 17 and the occurrence of two parthenogenetic ways of reproduction in this species (Triantaphyllou, 1966). In *M. chitwoodi* also a variability in chromosome number was shown with haploid numbers ranging from n=14 to18 (Triantaphyllou, 1985). Additionally, variation in host races was demonstrated in this species (Mojtahedi *et al.*, 1988; Mojtahedi & Santo, 1994).
The present study is 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 allows the examination of approximately 400 protein encoding loci. Investigating intraspecific variation on such a large scale provides insight in the extent of the genetic divergence within these species and allows comparison with other well established species. In this way, the validity of the distinction between *M. hapla*, *M. chitwoodi* and *M. fallax* can be evaluated.

8.3. Material and methods

8.3.1. Nematode isolates and female extraction

Similarity was studied between six isolates of *Meloidogyne hapla*, eight of *M. chitwoodi* and five of *M. fallax*. For *M. hapla*, all isolates originated from different sites in the Netherlands (Table 8.1). Two isolates were of cytological race B. Race A and B were distinguished by studying the meiosis, using Hoechst 33258 for staining of the chromosomes (Van der Beek *et al.*, G; Chapter 9). For *M. chitwoodi*, five isolates originated from different sites in the Netherlands and three from the USA (Table 8.1), represented the three host races: race 1, race 2 and race 3 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 a cellulase and pectolytic enzyme (20 volume % Celluclast with 20 volume % Pectinex in 60 volume % phosphate buffer pH 4.5) for 24 to 48 hours at

Table 8.1. Origin of the *M. hapla*, *M. chitwoodi* and *M. fallax* isolates used in this study

Isolate	Species	Country[1]	Last host	Sampling year	Obtained from[2]
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
Cb	*M. chitwoodi*	NL	wheat	1990	PD
Ck	*M. chitwoodi*	NL	tomato	1989	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

[1] Country of origin; NL = The Netherlands

[2] 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.

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.

8.3.2. 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 (1988a). 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.*

(1992a) with some minor modifications (Folkertsma *et al.*, 1996). One gel was produced of 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 from 50 females was prepared, resulting in only one gel. After staining, the gels were dried on a vacuum dryer (model 543, Bio-Rad Laboratories, Richmond, California USA) in order to store the gels for a prolonged period of time.

8.3.3. Data analysis

Two types of variants were discerned: isoelectric point (IP) and present-absent (PA) variants (Bakker & Bouwman-Smits, 1988a). IP variants are characterized by small differences in isoelectric point, similar molecular weight and by the same colour when stained with silver (Figure 8.2). 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 (Figure 8.2). 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. Concentrations 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 by 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 (1988a) for *Globodera* spp., because of the different biology of the employed *Meloidogyne* spp.

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 be 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 values 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.

Table 8.2. Estimated frequencies of the nine putative isoelectric point variants (IP1 to IP9), found in the six *M. hapla* isolates with two replicates numbered 1 and 2. Each IP-variants consisted of two to three spots, denoted as a, b and c (see Figure 1). The frequecies are presented as density ratios for each spot and for each of the two samples of the isolates, as measured by image analysing of the protein profiles.

	six *M. hapla* isolates, each with two replicates											
IP-variants	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

8.4. Results

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

8.4.1. M. hapla

Comparison of the six *M. hapla* isolates revealed 418 monomorphic and 22 polymorphic protein spots, corresponding to 5.0% polymorphisms (Figure 8.1). The similarity coefficient F ranged from 0.97 to 0.99 for this species. Of these 22 polymorphisms, nine were IP-variants (Table 8.2) and 13 were PA-variants (Figures 8.1 and 8.2). A similarity dendrogram was constructed of the nine IP-variants,

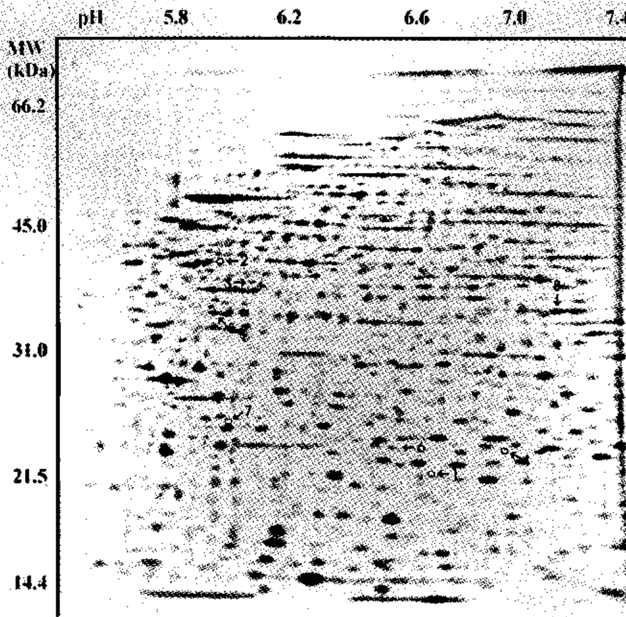


Figure 8.3. Two dimensional gel of total soluble protein extracts from *M. chitwoodi* isolate Ca1. PA-variants are numbered 1 to 8.

variation within these three *Meloidogyne* species is in contrast to the relatively large interspecific variation in protein patterns between these species, showing 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.*, B; Chapter 8). These data show that there is no overlap 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.

Figure 8.2 shows that *M. hapla* race B isolates were clustered apart from race A isolates. This result is in concordance with Esbenshade and Triantaphyllou (1987), who obtained similar results 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. Solely based on these small distances between *M. hapla* race A and B, there would be no reason to exclude the occurrence of successful crosses between isolates of these two races of *M. hapla*. However, the apomictic way of reproduction of race B isolates makes

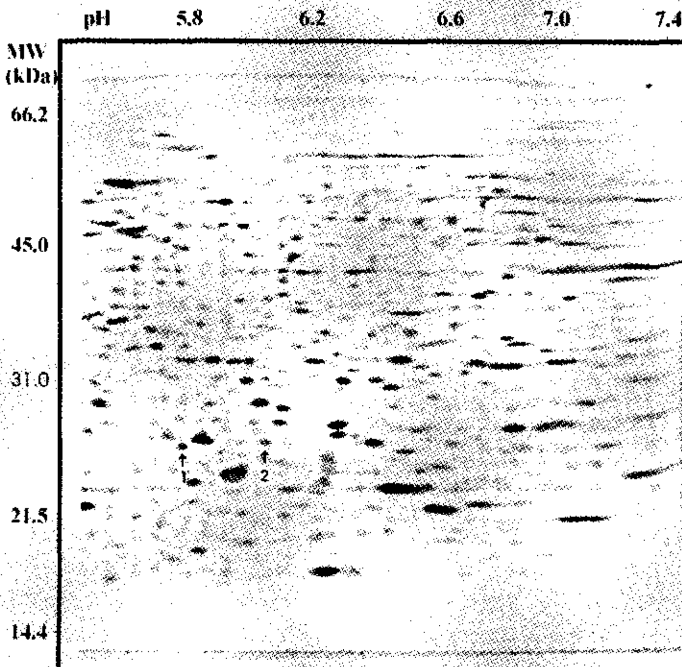


Figure 8.4. Two dimensional gel of total protein extracts from *M. fallax* isolate Fe1. The two PA-variants are numbered 1 and 2.

recombination improbable. Despite these small distances, suggesting a recent divergence, the distinct differences in reproduction remain remarkable.

Although the three species showed small intraspecific variation, considerably more genetic differentiation could be demonstrated in *M. hapla* than in *M. chitwoodi* and in *M. fallax*. In the latter 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: 13 among six *M. hapla* isolates and eight and two among eight *M. chitwoodi* and five *M. fallax* isolates, respectively. In comparing the observed intraspecific variation of *M. hapla* with *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 is demonstrated in Figure 8.2. However, when deleting the two *M. hapla* race B isolates Hh and Hbr from the data files, still eight of the 13 PA-variants and five of the nine IP-variants were 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

CHAPTER 9

Cytological observations of oogenesis and spermatogenesis in *M. hapla*, *M. chitwoodi*, *M. fallax* and an unidentified species

9.1. Summary - The cytology in several isolates of meiotic parthenogenetic *Meloidogyne hapla* race A, *M. chitwoodi*, *M. fallax*, mitotic parthenogenetic *M. hapla* race B, *M. javanica* and of isolate Xa of an unidentified *M.* species was investigated using fluorescent staining techniques with Hoechst 33258 for the oogenesis and ethidium-acridine for the spermatogenesis. Oogenesis was similar in *M. hapla* race A, *M. chitwoodi* and *M. fallax* isolates except for one *M. hapla* race A isolate in which 12 % of the oocytes of inseminated females contained sperm instead of the expected 100 %. In this isolate, prophase I oocytes were found posterior to the spermathecae, where normally prometaphase I oocytes are expected. In oocytes of the meiotic parthenogenetic isolate Xa, with unique esterase and N3-type malate dehydrogenase phenotypes, a second meiotic division only exceptionally occured and in all other cases chromosomal duplication after the first division was suggested. Also in one mono-female line of Xa, embryonic development in the female body occurred, which was genetically controlled. Oogenesis was similar in *M. hapla* race B and *M. javanica*. Spermatogenesis and the three-dimensional arrangements of cellular processes could be investigated *in situ* using confocal laser scan microscopy. Spermatogenesis was similar in *M. hapla* races A and B and *M. chitwoodi*. 69 % of *M. hapla* race B males were monodelphic. *M. fallax* isolates showed in 33 % of the males distortion in the spermatogenesis, ranging from absence of meiosis in some spermatocytes to absence of meiosis in all spermatocytes which prohibited the formation of sperm. This study showed the presence of a large meiotic variation in *Meloidogyne* spp.

9.2. Introduction

Root-knot nematodes (*Meloidogyne* spp.) are plant pathogens that are widely distributed, adapted to a very wide host-range, and cause more economic damage to food crops than any other single group of plant-parasitic nematodes (Lawrence Apple, 1985). Reproduction in plant parasitic *Meloidogyne* spp. is amphimictic or -apomictic. Apomicts of *Meloidogyne* are geographically more widespread, ecologically less restricted and more polyphagous than their amphimictic relatives (Triantaphyllou, 1985a). Comparable information has been collected about other apomictic groups, for example in fishes (Schultz, 1977) and insects (Lokki, 1983). Apomixis is common in the animal kingdom and various classifications have been made according to mode of reproduction and meiotic behaviour (Suomaleinen, 1950, White, 1973, Suomalainen *et al.*, 1987). Root-knot nematodes have undergone extensive cytogenetic diversification, leading to several reproduction systems, such as obligate cross-fertilization and facultative meiotic and obligate mitotic parthenogenesis (Triantaphyllou, 1985a, Eisenback & Hirschmann Triantaphyllou, 1991), which serve as characteristics for classification. Additionally, the chromosome numbers in

Meloidogyne spp. vary considerably, from n = 7 in the amphimictic species *M. spartinae* to 2n = 51-56 in some populations of the mitotic parthenogenetic species *M. arenaria*. Males play a facultative role in reproduction of meiotic parthenogenetic populations. In mitotic parthenogenetic populations males may be formed, but their function is as yet unclear. It is reported that sperm of these males contain the somatic chromosome number (Goldstein & Triantaphyllou, 1978).
A thorough knowledge of the reproductive system is a prerequisite in studying the genetic variation in *Meloidogyne*. The oogenesis of mitotic *M. arenaria, M. hapla* race B, *M. incognita*, *M. javanica* and of meiotic *M. hapla* race A were studied by Dalmasso (1973), Dalmasso and Bergé (1975) and Triantaphyllou (1962,1963, 1966, 1981). The spermatogenesis of mitotic *M. incognita, M. oryzae*, *M. arenaria,* of meiotic *M. hapla* race A, *M. graminicola* and of amphimictic *M. acronea* was described by Goldstein and Triantaphyllou (1980) and Shepherd and CLark (1983), by electron microscopic analysis.

In a series of studies on the reproduction of *Meloidogyne* spp. we investigated the oogenesis and sperm development of *M. hapla* race A, race B, *M. chitwoodi* and the recently described *M. fallax* (Karssen, 1996). These species are pathogenic to many field crops in moderate climates. Oogenesis was further examined in *M. javanica* and in an isolate of a species probably belonging to the *M. arenaria* complex. We studied several isolates of most of these species to detect possible intraspecific cytological variation. Because DNA fluorescent staining techniques on both oogenesis and spermatogenesis, and confocal laser scan microscopy on spermatogenesis were used, additional information on the cytogenetics was obtained. These results are presented and their relevance to reproduction of *Meloidogyne* spp. is discussed.

9.3. Materials and methods

9.3.1. Nematode isolates

Several nematode isolates were used in this study of *M. chitwoodi*, *M. fallax, M. hapla* race A, *M. hapla* race B, *M. javanica* and from an unidentified *Meloidogyne* species an isolate called Xa (Table 9.1). All isolates were collected from various sites in the Netherlands, except for the *M. javanica,* one of the *M. hapla* race A isolates and one of the race B isolates. Isolate Xa was extracted from a mixture of at least two *Meloidogyne* species and its origin is unknown. All isolates were maintained on *Lycopersicon esculentum* cv. Moneymaker. Eggs and females were reared on plants infected with 1 to 3 second-stage juveniles (J2) per ml silver sand. Males were obtained after inoculating seedlings growing in silversand in 96 ml plastic tubes, with J2 in high concentrations of 200 to 300 J2 per ml sand. High population densities cause an increase in production of sex-reverse males from fourth-stage juveniles. The inoculated plants were placed in a growth cabinet at 20 °C and 70 % relative humidity with 16/8 h light/dark. The isolates were found to be true to

Table 9.1. Origin of the isolates of *Meloidogyne* spp.

Isolate	Experiment[1]	Country	Last host	Obtained from[2]
M. chitwoodi				
Ca	o/s	NL	maize	PD
Cb	o/s	NL	wheat	PD
Cd	s	NL	black salsify	CPRO
Ce	s	NL	potato	CPRO
Ch	s	NL	wheat	PD
Ci	s	NL	tomato	PD
Cj	o/s	NL	unknown	PD
Ck	s	NL	tomato	PD
Cn	s	NL	carrot	IPO
Co	o/s	NL	black salsify	PAGV
Caq	o	NL	potato	CPRO
M. fallax				
Fa	s	NL	early primrose	PAGV
Fb	o/s	NL	beet	PD
Fc	s	NL	potato	PD
Fd	s	NL	potato	PD
M. hapla race A				
Ha	o	NL	peony	PD
Hb	o	NL	*Astilbe*	PD
Hc	o/s	NL	*Aconitum*	PD
Hd	s	NL	carrot	PAGV
He	o/s	NL	potato	PAGV
Hf	o/s	NL	carrot	PAGV
Hg	s	NL	fallow (weed)	PAGV
Hi	o/s	NL	imortelle	PAGV
Hj	o/s	NL	carrot	PAGV
Hk	o/s	NL	chicory	PAGV
Hl	s	NL	tomato	IPO
Hp	s	NL	potato	HLB
Hr	s	NL	carrot	CPRO
Hak	o/s	NL	carrot	HLB
Haw	o	France	grape	INRA
M. hapla race B				
Hh	o/s	NL	fallow (weed)	PAGV
Han	o/s	South Korea	*Hosta*	PD
Hbt	o	NL	unknown	BLGG

legend on p 130

Table 9.1. (cont.)

Isolate	Experiment[1]	Country	Last host	Obtained from[2]
M. javanica				
Ja	o	China	bonsai	PD
unidentified *Meloidogyne* sp.				
Xa	o	unknown	unknown	

[1] isolate used to study oogenesis (o) or spermatogenesis (s)
[2] PD = Plant Protection Service, Wageningen, The Netherlands. CPRO = Centre for Plant Breeding and Reproductional Research, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands. HLB = Hilbrands Laboratory for soilborne Diseases and Pests, Assen, The Netherlands. INRA = Institut National de Recherche Agronomique, Montpellier, France. BLGG = Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.

species by isozyme pattern (Esbenshade & Triantaphyllou, 1990) and by Internal Transcribed Spacer region of rDNA (Zijlstra *et al.*, 1995). For the study of spermatogenesis, roots of the infected plants were transferred to a moist chamber with mist of 20 °C at four to five weeks after inoculation. Adults which were outside the roots for one to two days could be collected during several weeks.

9.3.2. Mono-female isolates

In order to study the parthenogenetic nature of isolate Xa, 29 J2 were inoculated singly on root tips of *Solanum tuberosum* cv. Eigenheimer, grown on 2 % water agar in 29 Petri-dishes at 20 °C under dark conditions. Seven weeks after inoculation, egg-masses of two selected females, forming two so-called mono-female isolates, were hatched in tap water at 20 °C, and 60 J2 were inoculated similarly on root tips in Petri-dishes. Seven females were obtained from this culture. Several females of the "original" isolate and the two and seven females from the mono-female isolates were studied.
J2 from the two mono-female isolates were also inoculated on tomato plants in a climate chamber, under conditions as mentioned above. Young egg-producing females were collected seven weeks after inoculation and used for isozyme electrophoresis.

9.3.3. Isozyme analyses of individual females

Malate dehydrogenase and esterase patterns were derived from *M. javanica* and *M.* spec. isolate Xa, using PhastSystem electrophoresis according to the procedure as described by Karssen *et al.* (1995).

9.3.4. Preparation of microscopic slides of adult females

Seven weeks after inoculation, infected roots were brought in a Petri-dish with a 0.9 % NaCl solution and examined under a binocular (6.5 X) on a dark background. Females were collected from the roots by removing the surrounding tissue carefully. White, undamaged females were collected in another Petri-dish containing the same salt solution. Slides were prepared according to the method described by Triantaphyllou (1985b). Four females were transferred to a slide and placed in line, about 0.5 cm from the side edge. Adherent water was removed with a dry pencil or tissue and an incision made with a scalpel at the neck region. The body content was smeared on the slide by applying slight pressure on the female, while drawing it, to make a uniform smear. After drying to ambient air, the smears were ready for fixation. If desirable, the slides can be preserved for several months in a dark and dry place at moderate temperature, prior to fixation.

9.3.5. Preparation of microscopic slides of eggs

After removal from the roots, egg-masses were agitated vigorously during three minutes in a solution of 5 % NaCl, in order to remove the matrix. The egg suspension was poured over a 20 μm mess micro-sieve and rinced several times with tap water. The eggs were rinsed from the sieve into a small narrow container and allowed to settle down. With a micro pipet, eggs were taken from the bottom of the container and in small quantities of the suspension transferred to a slide. After drying the slides in ambient air, they could either be preserved or immediately be used for fixation and staining of the eggs.

9.3.6. Fixation and staining of eggs and smears of females

Triantaphyllou (1985b) stained the females of *Meloidogyne* spp. with propionic-orcein stain. We used Hoechst 33258, which was introduced into nematode cytology by Albertson *et al.* (1978) to study DNA in *Caenorhabditis elegans*, and which gave more contrast between cytoplasm and chromosomes for our purpose. The following fixation and staining procedure is an adapted combination of those described by Triantaphyllou (1985b) and Albertson *et al.* (1978).

The smears of females or the eggs were hydrolysed in 1 N HCl for ten minutes at ambient temperature to extract RNA. The slides were then emerged for fixation in Carnoy's solution (3 glacial acetic acid : 1 ethanol 100%) for 30 minutes, and dried in ambient air. The procedure could be interrupted at this point. The well-dried slides were soaked in 0.1 M PBS at pH 7.2 for five minutes and immediately stained in 1 μg/ml Hoechst 33258 in 0.1 M PBS at pH 7.2 for five minutes and finally rinsed for one minute in either tap water or 0.1 M PBS at pH 7.2. After absorbing most of the water or PBS and drying in ambient air, the smears were embedded in 50 % glycerin and the coverslips sealed with nail polish. Stored in a dark place, the slides kept their quality for at least one year.

The slides were examined under a Zeiss Axioplan UV microscope with excitation

filter G365, beam splitter FT395 and barrier filter LP420.

9.3.7. Fixation and staining of males

Males were stained *in vivo* with 100 μg/ml ethidium-acridine heterodimer (Molecular Probes, E-667) during a five-day-incubation, and fixed in a 4 formalin : 1 glycerin solution during one day. The normally impermeable dye was taken up by the living nematodes and stained the nuclei and cellular contents with high affinity. The nematodes were transferred to object slides which were covered with coverslips and sealed with nail polish.
The testes were visualized with a confocal laser scanning microscope unit (Bio-Rad MRC 500), attached to a Zeiss ICM 405 inverted microscope, using the BHS filter set (excitation at 488 nm and long pass emission at 515 nm), if desired combined with transmitted light observation. For optimal imaging, a 63X Neofluar lens was used in combination with oil immersion, and the pinhole was adjusted for optimal brightness at highest possible resolution. Eventually aniti-fading was used to extend the duration of the fluorescence. The laser was operated at 25 mW output power, and reduced by neutral density filter to 2.5 mW. The images were recorded in Kalman filtering mode, with 20 images averaged by the Som software, provided by Bio-Rad. Images were stored on a Panasonic optical disk, and recorded on film.

9.4. Results

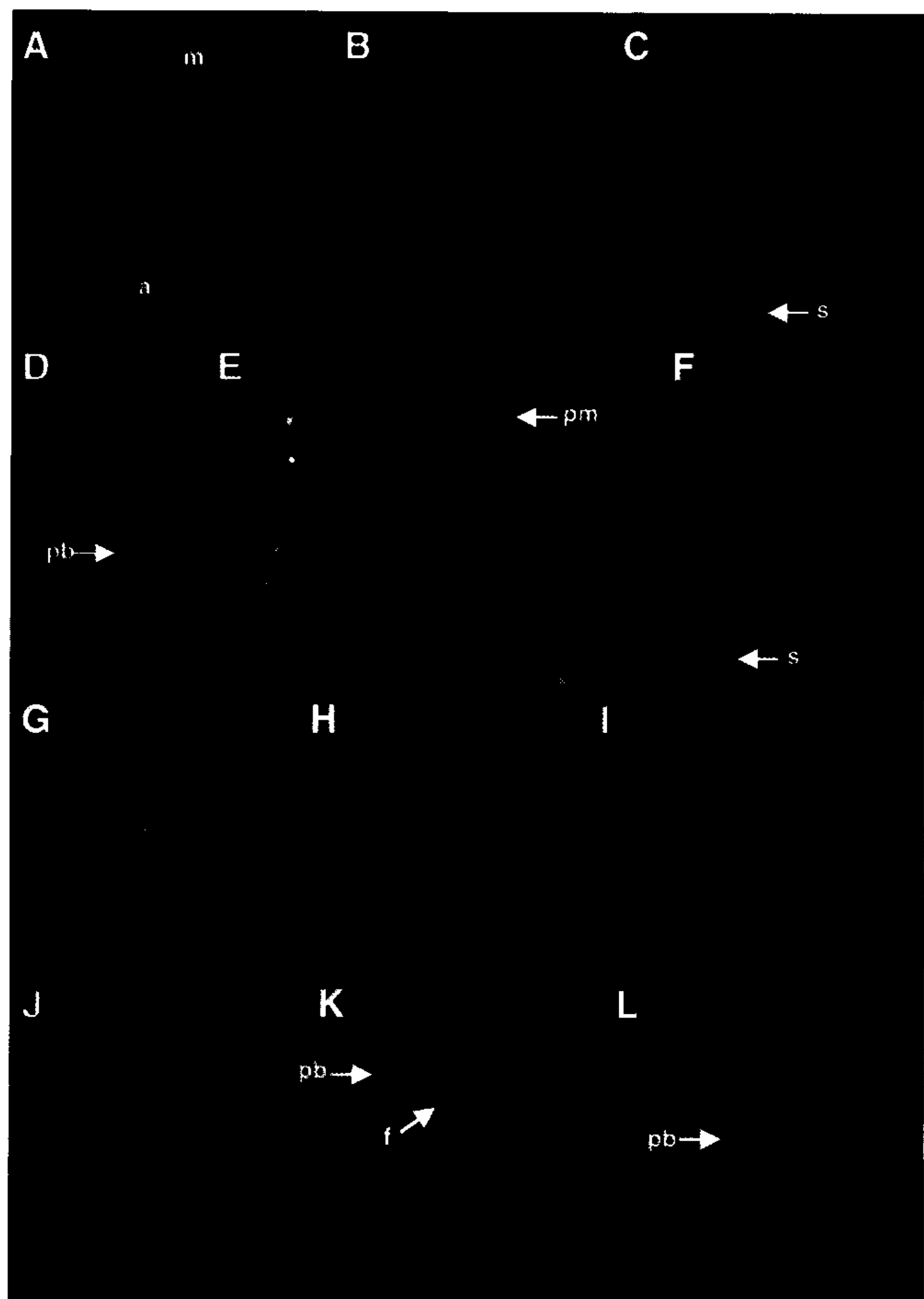
9.4.1. Oogenesis in M. hapla, M. chitwoodi *and* M. fallax

The female gonad of the representative isolates Hj of *M. hapla* race A (2n=2x=34), Ca of *M. chitwoodi* (2n=2x=36) and Fa of *M. fallax* (2n=2x=36) is folded in the female body and covers approximately one half of the total body content. The ovarium is protracted. In the gonad subsequent stages of oogenesis are distinguished: from a primary oogonium in the anterior part of the gonad to eggs after the second division posterior in the gonad. There is only one primordial germ cell, the cap cell. The oogonia occur in the germination zone which is marked by mitotic divisions. Subsequently, primary oocytes start to develop in the synapsis zone in which chromatin is heavily condensed. The first maturation division (Figure 9.1A and B) takes place in the centre of the oocyte with a spindle length-wise to the oocyte. The haploid number of elements with four chromatids, observed during diakinesis and (pro-)metaphase I, indicates a normal pairing of all pairs of homologous chromosomes. During first anaphase, which is found posterior to the spermatheca, a haploid number of elements, consisting of two parallel chromatids, move towards each pole in a perpendicular position towards the equatorial plane (Figure 9.1C). Following this segregation, one of the haploid sets, becoming the first polar body, moves towards the cell surface in the middle of the oocyte where it disintegrates after egg deposition and before the second cleavage division. The second maturation division (Figure 9.1D) takes place, in general, perpendicular to the cell surface. The fate of the resulting haploid second polar body and pronucleus, which

takes an interphase structure, is dependent on the presence of a spermatozoon. In metaphase I oocytes of females that were inseminated by males, one spermatozoon enters at the rostral end at the moment the oocyte passes from the oviduct through the spermatheca and remains remote from the bivalents (Figure 9.1C). This spermatozoon does not change morphology until egg deposition. Then it migrates towards the oocyte centre where it takes an interphase structure. A fusion of the female and male pronuclei likely takes place shortly after deposition, since one nucleus is observed before the first cleavage division, implying a degeneration of both polar bodies. In case no spermatozoon enters the oocyte, fusion of the two interphase nuclei occurs in the centre of the egg after deposition. Each of the two fusing nuclei contains one set of one-chromatid chromosomes resulting from the second maturation division. A second polar body is not formed then. In the other examined *M. hapla* race A isolates Ha, Hb, Hc, Hf, Hi, Hk, Hak, Haw, in *M. chitwoodi* isolates Cb, Cj, Ck, Co, Caq and in *M. fallax* isolate Fb, the oogenesis, with or without the presence of sperm, was similar. Isolates of *M. hapla* race A and *M. chitwoodi* were likely to show some variation in chromosome numbers.

Oogenesis in *M. hapla* isolate He was found to be in general as described above. However, it was noticed that in inseminated females a large number of oocytes did not contain sperm. Oocytes in the uterus directly posterior to the spermatheca were frequently found to be in late prophase I, in contrast to (pro)metaphase I in other isolates of *M. hapla* (Figure 9.1M). Sperm was sometimes observed in the lumen of that region of the uterus. Of 17 inseminated females, collected at two different times and containing clear sperm-filled spermathecae, a total number of 379 oocytes were counted of which only 12 % with sperm, ranging from 0 to 50% per female.

Figure 9.1. (pp 134 and 135) Oogenesis in *Meloidogyne* spp. Magnification 100X objective, except for B: 200X objective. **A**. Metaphase I (m) and early anaphase I (a) in *M. hapla* race A isolate Hi. **B**. Metaphase I in *M. hapla* race A isolate Hj. **C**. Early telophase I with intruded sperm (s) in *M. hapla* race A isolate Hj. **D**. Telophase II with the first polar body (pb), in *M. chitwoodi* isolate Cz. **E**. Prometaphase I (pm) in *M. hapla* race B isolate Hh, passing through the spermatheca in which sperm is visible. **F**. Metaphase I in *M. hapla* race B isolate Hh with penetrated sperm (s). **G**. and **H**. Interkinese in *M. hapla* race B isolate Hh in different focussing. **I**. Egg outside the female body just prior to first cleavage division in *M. hapla* race B isolate Hh. **J**. First cleavage division in *M. hapla* race B isolate Hh. **K**. Oocyte after the first maturation division in isolate Xa of unidentified *M*. sp., showing first polar body (pb) and the second set chromosomes in flocculated appearance (f). **L**. A rare late anaphase II in isolate Xa of unkown *M*. sp. (pb=first polar body). **M**. Spermatheca and the adjacent anterior part of the uterus in *M. hapla* race A isolate He showing successively a spermatheca (st) filled with sperm, two oocytes in prophase I, of which one clearly visible (p) and an oocyte in metaphase I (o) without sperm in the oocytes.


A
m
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M

st

p

o

In mitotic parthenogenetic *M. hapla* race B isolate Han (2n= about 42) only univalents are observed at the end of prophase I and during (pro-)metaphase I, indicating absence of chiasmata-formation and consequently of genetic crossing over. The first maturation division is mitotic and results in two sets of chromosomes each with the somatic number of one-chromatid chromosomes (Figure 9.1G and H). The orientation and position of the spindle is as described above. One set of chromosomes migrates towards the cell surface, where it disintegrates as first polar body after egg deposition, and the other set starts embryonic development after deposition (Figure 9.1J), after having taken first an interphase structure(Figure 9.1I). In females, inseminated by male parthenogenones of expected sex-reversed origin, a spermatozoon enters the metaphase I oocyte as described above for meiotic parthenogenetic isolates, but it does not interfere with the parthenogenesis and likely degenerates without any migration (Figure 9.1F). Maturation of the oocytes was similar with and without a spermatozoon in *M. hapla* race B isolate Hh and Hbt (both 2n= about 42) and in *M. javanica* Ja (2n = about 46).

9.4.2. Oogenesis and isozyme analyses in isolate Xa

Oogenesis of isolate Xa of an unidentified *Meloidogyne* species (2n= about 18) shows a different pattern because of the presence of a normal first maturation division and the absence of a second maturation division. Bivalents are formed and at first anaphase the reduced number of elements, consisting of two chromatids, go to each pole. The chromatids are oriented parallel to the spindle, as was described for *M. hapla*. At telophase, one set of these elements takes a flocculated appearance, while the other set remains contracted, moves towards the surface (Figure 9.1K) and degenerates as first polar body after egg deposition. The flocculated set enters into mitosis of the first cleavage division early after egg deposition. Females may become inseminated by males and the spermatozoon enters the oocyte normally when in prometaphase I. However, in inseminated females no spermatozoon could be found in oocytes in stages beyond metaphase I.

In three oocytes out of more than a hundred, a second maturation division was observed similar to the one in *M. hapla* race A (Figure 9.1L). It concerned two oocytes from an inseminated and one oocyte from a non-inseminated female, neither of them containing a spermatozoon. This type of meiosis occurred also rarely in two subsequent parthenogenetic generations of mono-female lines generated from isolate Xa and the meiotic parthenogenetic reproduction could be established by the offspring of single females.

Although cleavage divisions start, in general, after egg deposition, all eggs in one female from isolate Xa showed embryonic development inside the female body. It appeared that all the parthenogenetic mono-female progeny of this female showed this trait.

Isozyme patterns for malate dehydrogenase of isolate Xa were similar as in *M. arenaria* (N3-type in Esbenshade & Triantaphyllou, 1990) and were unique for esterase showing two bands comparable to the two lower bands in *M. javanica*

(Figure 9.2). Morphology of the second-stage juveniles indicated similarity to *M. arenaria* (G. Karssen, pers. comm.).

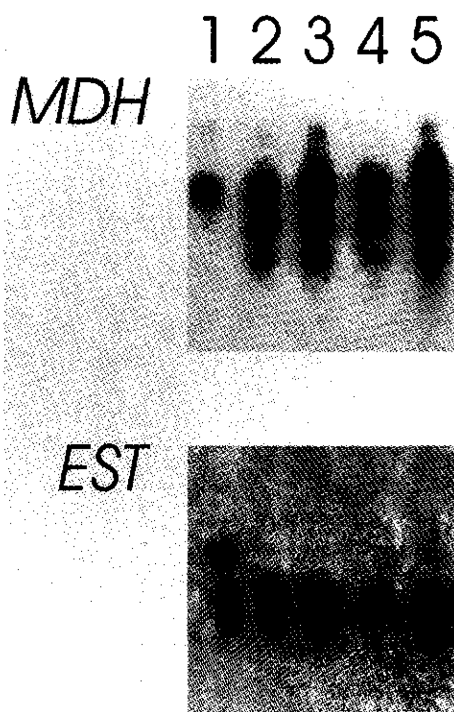


Figure 9.2. Zymogram showing malate dehydrogenase (*MDH*) and esterase (*EST*) phenotypes for single females of *M. javanica* isolate Ja (♀ 1) and unknown *Meloidogyne* sp. isolate Xa (♀ 2 to 5)

9.4.3. Spermatogenesis in M. hapla *and* M. chitwoodi

It appeared to be difficult to obtain a good labeling of the nuclei of cells in the testis using the more common nucleic acid stains such as DAPI or Hoechst 33258, even in the case of fixed nematodes. The inclusion of stains in the culture medium did not result in any significant and reproducible staining. However, by using the normally cell-impermeable dye ethidium-acridine heterodimer in the culture medium, a good nuclear staining was obtained. By using this heterodimer as a vital stain, it became possible to study the spatial relationship of the various cell types during spermatogenesis by confocal laser scanning microscopy. The staining showed good specificity for nuclei, and also revealed several other cellular aspects, as can be seen in Figure 9.3.

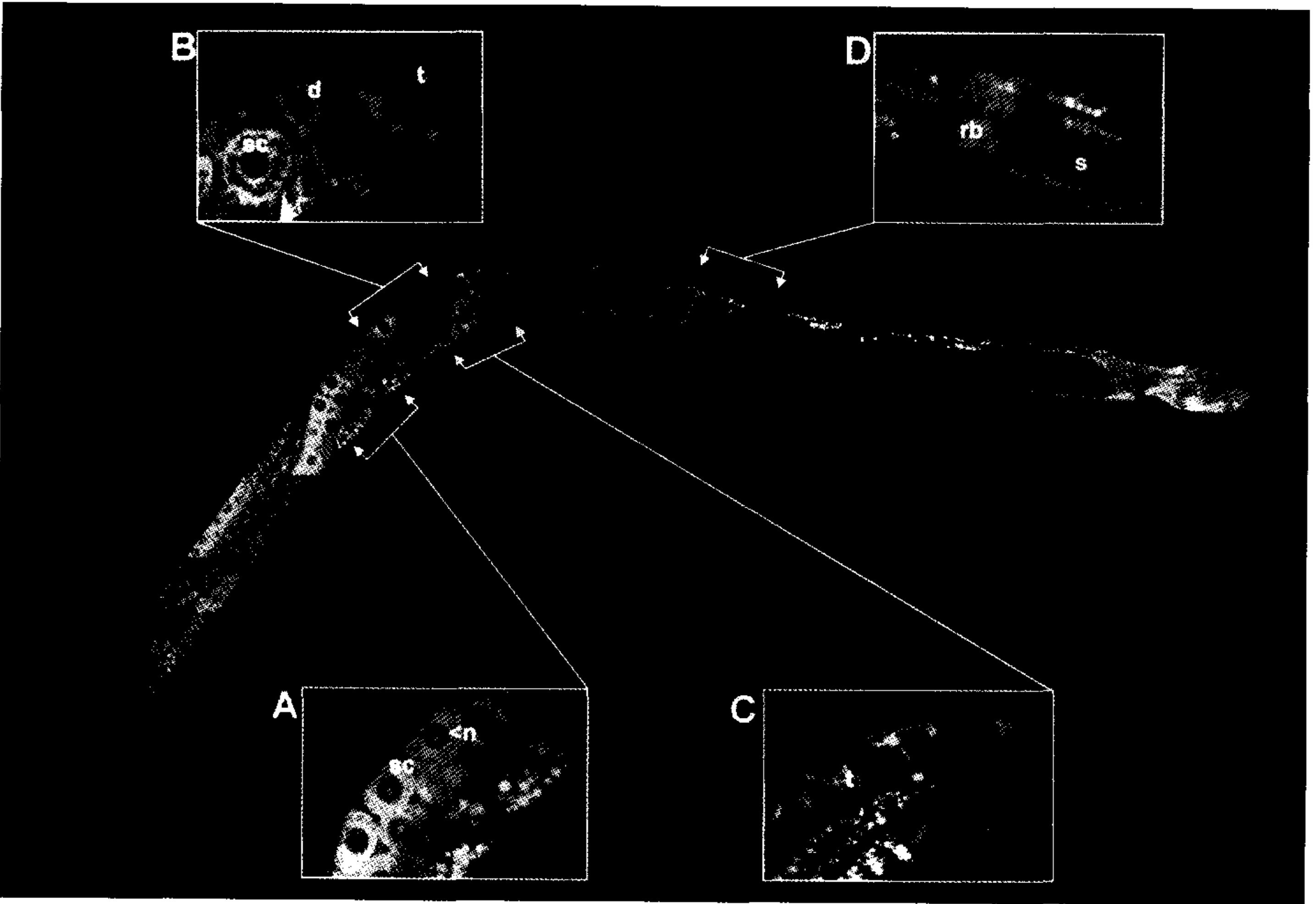
The male gonad of the representative isolate Hk of *M. hapla* race A and Ck of *M. chitwoodi* covers one half to one third of the total nematode body and is monodelphic, but didelphic forms were observed, which occur under certain conditions of stress (Triantaphyllou, 1973; Papadopoulou & Triantaphyllou, 1982). The testis is protracted, although occasionally it is found bended at the zone with spermatogonia and sometimes twisted around its axis. In the male gonad subsequent stages of spermatogenesis are distinguished: from a primary spermatogonium in the anterior part of the gonad to spermatozoa (sperm) in the posterior part. The lengths of the zones corresponding to the different stages, especially to the secondary spermatogonia and the spermatozoa, are related to the age of the male.

There is only one primordial germ cell: the cap cell, of approximately 5 x 8 μm,

which is clearly visible at the rostral end of the testis, with the long side of the cell perpendicular to the axis of the testis. From this cell, two to three strings of secondary spermatogonia are generated, consisting of closely linked, angular cells with equal lengths and widths between 4.7 and 7.2 μm. These cells contain oval-shaped nuclei of 3.4 X 4.4 μm, with spherical nucleoli of 1.9 μm in transversal section containing one nucleolar vacuole. Mitoses were not observed, indicating low division activity. The cytoplasm is stained bright white and some granular structure is visible equally distributed through the cell, suggesting the presence of large amounts of RNA.

The transition from spermatogonium to spermatocyte is marked by the enlargement and separation of the cells. The cells round to an oval shape and increase in size till about 13.5 X 8.5 μm (Figure 9.3A). The separation of cells is caused by the rounding of the cells. The nuclei do not change during the cell enlargement and nucleoli remain visible. In the cytoplasm, a large number of structures of 0.6 μm in diameter become visible, which form a circle around the nucleus (Figure 9.3A and B). These structures become darker, and remain arranged around the nucleus. Progressively more cytoplasmic accumulation, visible as intensely white stained material, is occurring immediately outside the nuclear envelope. Just prior to the disappearing of the nuclear envelope, the nucleoli disappear (Figure 9.3B). The chromosomal material becomes visible in the cell-centre as a light-coloured blot. The first maturation division is quickly followed by the second maturation division. Although the different meiotic stages were difficult to distinguish, first and second maturation divisions were clearly recognizable as two and four nuclei, respectively, surrounded by cytoplasm (Figure 9.3B). Tetrads are formed as semi-circles of spermatids of about 6.0 X 8.0 μm (Figure 9.3C), which in a more advanced stage sometimes become visible as oblong strings of spermatids, transversal to the body wall. During spermatid development, accumulation of the cytoplasm continues until the condensed cytoplasm is concentrated into residual bodies. These residual bodies consist of extruded cytoplasm and become visible as round, brightly white structures as large as 8.0 to 14.0 X 8.0 μm, with some gray spots (Figure 9.3D). The residual bodies are discarded and engulfed by cells in the testis wall. The chromosome

Figure 9.3. (p 139) Spermatogenesis in *Meloidogyne hapla* race A isolate Hk, showing a composition of the latter half of a sex-reversed male with two strings of spermatogonia (left). Magnification 63X objective. **A.** Detail of enlarged spermatocytes (sc) with one nucleolus per nucleus (n). **B.** Detail of the transition to maturation divisions in a different male of the same isolate: regularly arranged dark-coloured structures around the nucleus of the spermatocyte (sc), a dyade (d) after the first maturation division and a tetrad (t) after the second with three nuclei visible. **C.** Detail of tetrad (t) arrangement with the beginning of the formation of residual bodies. **D.** Detail of the transition from spermatids to sperm (s), showing residual bodies (rb) which are apart from the nuclear material and being engulfed into the testis wall.


B
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material is becoming condensed into two to six nucleolar bodies.

The transition of spermatid to spermatozoa is marked by the completion of the engulfment of the residual bodies in the testis wall. Mature spermatozoa are visible as cells of 5.0 X 6.0 μm, excluding filopodia. The appearance of various nucleolar bodies remains visible in mature sperm and is also observed in spermathecae and oocytes after insemination (Figures 9.3D and 9.1E). Darkly coloured structures are spread over the cytoplasm of the spermatozoa. The full width of the male nematode is occupied by the seminal vesicle, observed with a length up to 115 μm, filled with up to 200 spermatozoa, depending of the stage of development of the male.

In other examined isolates of *M. hapla* race A Hc, Hd, He, Hf, Hg, Hi, Hj, Hl, Hp, Hr, Hak and *M. chitwoodi* Ca, Cb, Cd, Ce, Ch, Ci, Cj, Cn, Co the spermatogenesis was similar, and in mitotic parthenogenetic *M. hapla* race B isolates Hh and Han, spermatogenesis showed similar morphology and histology as described above.

In four of 13 studied males of *M. hapla* race B isolate Han didelphic testes were observed with equal lenghts of the gonads. The remaining nine males had single testes and no rudiments of the second gonad were observed.

9.4.4. Spermatogenesis in M. fallax

Sperm development in the representative isolate Fb of *M. fallax* ($2n=2x=36$) is often distorted. Spermatogonia were similar as described above for *M. hapla* and *M. chitwoodi*, but after the transition to primary spermatocyte, irregularities often occur. In extreme cases, the nucleolus and nuclear envelope are invisible in a very early stage and fibrillar bodies extend in size to about 1.6 μm in diameter, leaving almost no cytoplasm between them. Consequently, the cells increase in size up to 13 X 17.5 μm. In some male nematodes, the whole vas deferens is filled with these cells, which often decrease slightly in size and occasionaly disintegrate towards the posterior end of the testis. However, in some of these cells in other males nuclei remain visible but seem unable to enter into meiosis or complete meiosis to produce spermatids. In less extreme cases, some apparently vital spermazoa could be distinguished between the big distorted cells. However, most males were found with an apparently normal spermatogenesis, completing all subsequent stages as described above for *M. hapla* and *M. chitwoodi*. Sperm development in *M. fallax* isolates Fa, Fc and Fd showed identical patterns of anomalies as described for Fb. Of the *M. fallax* isolates Fb and Fd, 33 % of a total of 118 males that were investigated showed some degree of distortion in spermatogenesis.

9.5. Discussion

9.5.1. Oogenesis

These studies on the meiotic divisions of meiotic parthenogenetic *M. hapla* race A and *M. chitwoodi*, and of mitotic parthenogenetic *M. hapla* race B and *M. javanica* confirm earlier work on the cytology and oogenesis of these *Meloidogyne* spp. (Dalmasso, 1973; Dalmasso & Bergé, 1975; Triantaphyllou, 1962,1963, 1966,

1981). The oogenesis of *M. fallax* appeared to be similar to that of the other investigated meiotic parthenogenetic species.

In all examined isolates of meiotic parthenogenetic species, end-to-end orientation of parallel chromatids during first metaphase was followed by a movement of ends of exchanged parts of the chromatids towards the poles during first anaphase, implying cohesion between chromatids at non-exchanged ends (Van der Beek *et al.*, H; Chapter 11).

Some odd observations were made. Firstly, in *M. hapla* race A isolate He, prophase chromosomes of maturing oocytes were observed in the anterior part of the uterus, in contrast to all other investigated *M. hapla* isolates (Figure 9.1M). This observation resembles comparable behaviour of oocytes of *M. incognita* (Triantaphyllou, 1981). However, in *M. incognita*, most oocytes of inseminated females were reported to contain sperm nuclei, in contrast to our *M. hapla* isolate in which mostly no sperm entered. In nearly all prometaphase I oocytes of the other eight tested *M. hapla* isolates, when passing through a sperm-filled spermatheca, a sperm was penetrated resulting in almost 100 % fertilized eggs. The possible reason of this aberration in the female reproduction is that the egg shell, and may be also the cell membrane and the yolk, are not yet morphologically and functionally prepared to receive male gametes in a stage younger than prometaphase I. The low percentage of oocytes with penetrated sperm in *M. hapla* isolate He implies only limited possibilities for sexual recombination. Consequently, the genetic composition of this isolate will be more homogeneous, but not necessarily more homozygous, as post-reduction during meiosis will cause conservation of heterozygosity in a meiotic parthenogenetic population of *Meloidogyne* (Van der Beek *et al.*, H; Chapter 11). Further research will reveal the importance of this phenomenon in population genetics of *M. hapla*.

Secondly, in isolate Xa of an unidentified *Meloidogyne* sp., the majority of oocytes showed only one maturation division, which is reductional (Figure 9.1K). However, the few times that oocytes with a second maturation division were observed point to the ability of completing occasionally a full meiosis (Figure 9.1L). Consequently, it includes possibilities for sexual reproduction. The occurrence of meiotic parthenogenesis and suppression of a second maturation division points towards an intermediate type of parthenogenesis, in between the meiotic form with two maturation division and mitotic parthenogenesis (Figure 9.4). The question how the chromosome number is maintained in this isolate is not fully answered yet. The flocculated appearance of one set of chromosomes after the first maturation division of the majority of oocytes indicates chromosomal duplication. This meiotic behaviour could be related to the ability of this isolate for embryonic development before egg deposition, being the first step in an apomict towards the first cleavage division. In contrast, chromosomes after first maturation division in the other investigated parthenogenetic *Meloidogyne* isolates showed no changes before egg deposition. To our knowledge, no other example of this type of meiosis has been published until now. Possibly a mechanism of endo-reduplication of first division products may

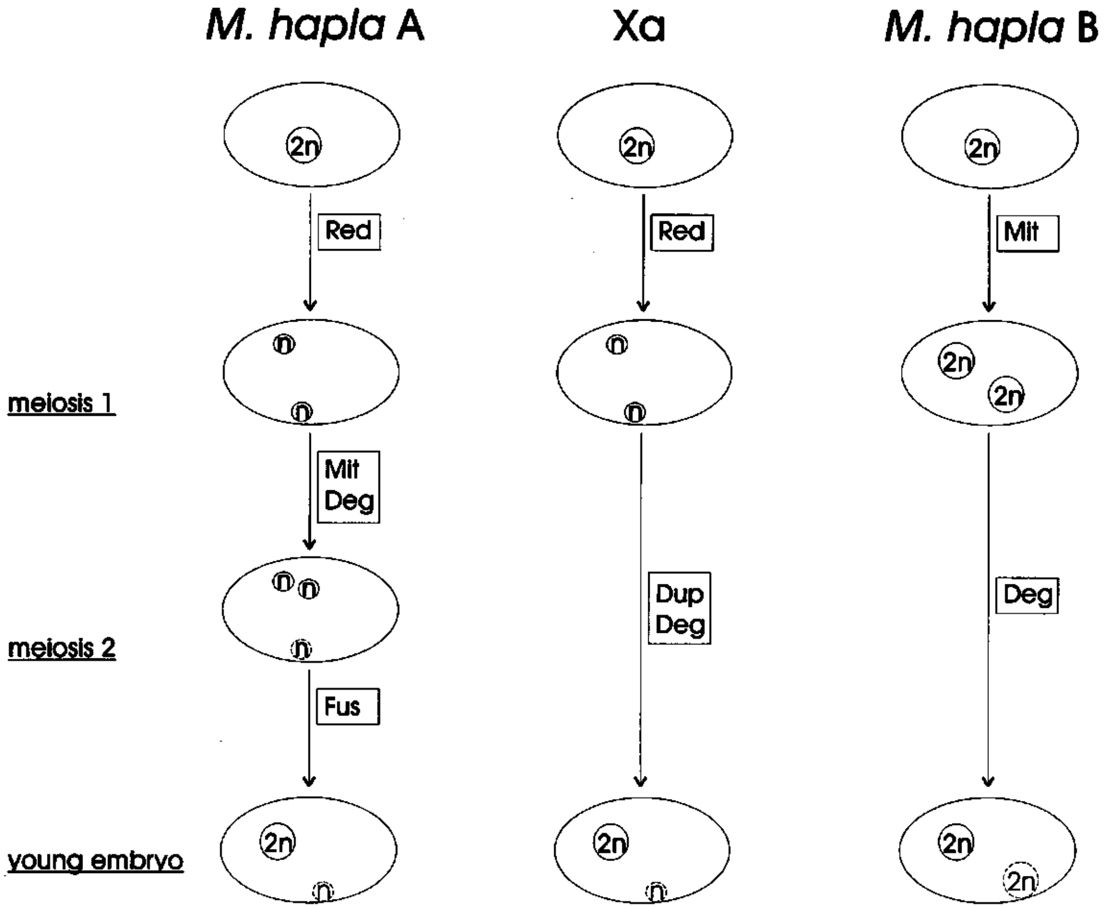

Figure 9.4. Schematic representation of parthenogenetic pathways in meiotic and mitotic parthenogenones of *Meloidogyne*, *M. hapla* races A and B, respectively, compared to that of isolate Xa

Legenda: 2n=set of chromosomes with the somatic number; n=set of chromosomes with the haploid number; Deg=degeneration of the second polar body; Dup=chromosome duplication; Fus=fusion of second polar body with the egg pronucleus; Mit=mitotical division; Red=reductional division.

occur to avoid reduction, similar to that found in *Hysibius dujardini* (Ammermann, 1967).

The occurrence of embryonic development inside the females of the unidentified *Meloidogyne* sp. isolate Xa has so far not been recorded in *Meloidogyne*. Cyst-forming nematodes show this phenomenon, which is considered to be less advanced in terms of evolution. In *Heterodera avenae* for example, a species with a short vulval slit, embryonic development remains inside the female body and a remnant of an egg-mass may be found. In *H. schachtii*, a species with a long vulval slit, the egg-mass may contain even varying numbers of eggs (Golden, 1986). Whether the described phenomenon would be homologous with the observations in cyst nematodes remains unanswered.

Genetically, this aberrant type of meiosis has similar genetic consequences as the described meiosis in meiotic parthenogenetic *Melodoidogyne* spp., because fusion of the second polar body with the egg pronucleus after the second maturation division restores the genetic information from before the second division. It indicates the presence of a large variation in the meiotic system in *Meloidogyne* spp.

The species identity of isolate Xa is as yet unclear. Although the esterase pattern of the females of isolate Xa is unique, and thus suggests a yet undescribed form of *Meloidogyne*, morphology and malate dehyrodrogenase pattern indicate association with the *M. arenaria* group. This would imply the first description of a meiotic parthenogenetic isolate belonging to this group, so far consisting of mitotic parthenogenones. Triantaphyllou (1970) mentioned a *M. arenaria* isolate with 2n=36 chromosomes, but he listed that isolate as mitotic.

9.5.2. Spermatogenesis

Sperm development could be investigated *in situ* by the employed technique, using ethidium-acridine staining and confocal laser scan microscopy. The high specificity of the heterodimer for DNA, in combination with RNA specificity, allows to study the three-dimensional arrangement of the cellular processes involved in spermatogenesis. A detailed overview of the different stages and the transitions between them was obtained (Figure 9.3). The origin and development of residual bodies and changes in position of fibrillar bodies could be followed with great precision. Fixation of the nematodes was desirable for relaxation, but did not appear to be essential for optimal staining with ethidium-acridine. Therefore, this technique allows *in vivo* investigation of males.

The present studies on the histology of spermatogenesis in *M. hapla* races A and B confirm earlier work by Goldstein and Triantaphyllou (1980) and Shephard and Clark (1983). The spermatogenesis of *M. chitwoodi* appeared to be similar to that of *M. hapla*, but in *M. fallax* various degrees of distortions occurred. Our study showed the cohesion and transitions of the different stages of sperm development and provided a detailed overall view. Although residual bodies, which contain ribosomes, Golgi bodies and cytoplasmic spherical bodies (Shepherd & Clark, 1983), became clearly visible at the transition of spermatids to sperm, the present

results indicate that the first visible signs of their formation start at the maturation divisions. The first sign of residual bodies is marked by a white band to the cell wall, increasing in intensity with progression of spermatogenesis. The observation of one residual body to one tetrad in *Meloidogyne* spp. is different from conclusions by Shepherd *et al.* (1973) of one residual body to each spermatid in *Globodera rostochiensis* and *Heterodera schachtii*.

Males of *M. hapla* race B isolate Han, being mitotic parthenogenones, are supposed to have developed from sex-reversed juveniles. These males have didelphic testes comprizing of two gonads of various lengths depending on the developmental stage in which the sex reversal occurs, according to Papadopoulou and Triantaphyllou (1982). Data in this study showed that 70 % of the investigated males possesed only one testis, without rudiments of the second gonadal arm, suggesting that the majority of sex-reversal took place during the first five days following initiation of feeding of juveniles. This suggestion is supported by the high inoculum density of 200 to 300 J2 per ml sand, causing a favourable condition for sex-reversal.

The darkly coloured structures, observed in oogonia, oocytes and sperm, could be attibuted to mitochondria and/or fibrilar bodies, as the topography of these structures is similar (Shepherd & Clark, 1983). The function of fibrilar bodies remains unknown.

M. fallax showed aberrant spermatogenesis by distortion in the spermatocyte development at different stages in different degrees. Despite this condition, *M. fallax* isolate Fa was able to produce viable sperm since hybrids were obtained (Van der Beek & Karssen, accepted; Chapter 10). It is evident that a decreased production of viable sperm implies a decreased potential for sexual recombination, but this is not expected to have much influence on the heterozygosity in populations of *M. fallax*, assuming post-reductional meiosis in *Meloidogyne* spp. (Van der Beek *et al.*, H; Chapter 11). The cause of this distortion is unknown.

CHAPTER 10

Interspecific hybridization of meiotic parthenogenetic *M. chitwoodi* and *M. fallax*

10.1. Summary - Hybridization between two meiotic parthenogenetic species of root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, was investigated in two different crossing experiments on tomato plants growing in sand. The first experiment was a controlled cross between the two species. The second experiment was by bulk mating in a 1:1 mixture of two isolates. The haploid chromosome number of the parental isolates was n=18. Successful interspecific hybridization was obtained and the resulting hybrids produced egg-masses. In eggs, cell division was observed but most of them were without clear differentiation and consequently sterile. Hatched F_2-juveniles were small in number, not viable and showed morphological distortions. In the progeny of the isolate mixture of the bulk mating experiment, parental-type females of the two isolates were present in equal numbers and 10 % of all females were non-viable hybrids. Similar ratios of parental-type and hybrid females were detected in roots of test plants growing in soil from a field sample, containing a mixture of populations of *M. chitwoodi* and *M. fallax*. In the controlled cross experiment, isozyme electrophoresis of malate dehydrogenase was applied to distinguish the two species and their hybrids. In the bulk mating experiment, malate dehydrogenase, esterase and glucose-6-phosphate dehydrogenase were used as markers, two by two simultaneously on the same individual females, providing conclusive evidence for the occurrence of hybrids. This is the first report on interspecific hybridization in *Meloidogyne*. The possible role of interspecific hybridization in species differentiation and in the interspecific exchange of genetic material within *Meloidogyne* is discussed.

10.2. Introduction

Reproduction in the root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* (Golden *et al.*, 1980; Karssen, 1996) is characterized by facultative meiotic parthenogenesis (Triantaphyllou, 1985a), i.e., amphimixis and meiotic parthenogenesis can occur in these species. Amphimixis may result when insemination by males occurs. Until recently, *M. chitwoodi* and *M. fallax* were considered as one species and *M. fallax* has been referred to as *M. chitwoodi* type Baexem (Van Meggelen *et al.*, 1994; Karssen, 1996). During the past few years, significant differences between the two species have been reported. Van Meggelen *et al.* (1994) mentioned enzymatic differences between *M. chitwoodi* and *M. chitwoodi* type Baexem and suggested a race status. Karssen *et al.* (1995) briefly described morphological and biochemical differences, and Zijlstra *et al.* (1995) showed differences in internal transcribed spacer (ITS) regions of ribosomal DNA. Finally, additional morphological differences between the two species and specific hosts were described (Karssen, 1996). These species are distinct from each other in morphological, morphometrical, biochemical and biological characters, but their reproductive

isolation has not been demonstrated.

In a few other nematode species, interspecific hybridization has been proven: in *Heterodera* spp. (Potter & Fox, 1965; Fox,1967; Yeates, 1970; Miller, 1983), in *Globodera* spp. (Franco & Evans, 1978; Mugniéry, 1979; Miller, 1983; Thiéry *et al.*, 1996) and in *Pratylenchus* spp. (Perry *et al.*, 1980). The objective of this study was to investigate the reproductive isolation of *M. chitwoodi* and *M. fallax*, the results of which may explain the role of interspecific hybridization in genetic divergence in the genus and in speciation (Mayr, 1969; King, 1993). To examine this, hybridization between the two above mentioned *Meloidogyne* species was studied in a strictly controlled and a bulk mating experiment.

10.3. Materials and methods

10.3.1. Nematode history and identification

Crossing experiments were conducted with isolates Ca and Ci of *M. chitwoodi* and isolate Fa of *M. fallax*, all three originating from geographically different sites in the Netherlands (Table 10.1). *M. javanica* isolate Ja, originating from China, was used in this study as a reference phenotype in isozyme electrophoresis (Table 10.1). The isolates were obtained from separated field populations maintained in the greenhouse on tomato (*Lycopersicon esculentum* cv. Moneymaker) and regularly checked for species identity. These isolates were identified as true species by the combination of morphological characteristics (Jepson, 1987; Karssen, 1996) isozyme patterns (Esbenshade & Triantaphyllou,1985) and DNA patterns of ITS regions (Zijlstra *et al.*, 1995) (Table 10.1).

10.3.2. Controlled crossing experiment

Reciprocal crosses were made between *M. chitwoodi* and *M. fallax*. Isolates Ca and Fa were allowed to hybridize by the following procedure, which was modified from Triantaphyllou (1993). Two-week-old tomato seedlings, growing on silversand enriched with 3 % slow release fertilizer Osmocote in open-ended transparent plastic tubes of 96 ml content, were inoculated with pre-hatched second-stage juveniles (J2). In order to obtain the paternal line, plants were inoculated on June 23, 1995 with isolates Ca and Fa to a final density of approximately 45 J2 cc^{-1} soil. This high density enhanced the number of J2 developing into males, as the sex ratio in *Meloidogyne* is strongly influenced by nematode density (Davide & Triantapyllou, 1967; Triantaphyllou, 1973). One week later, roots of additional seedlings were inoculated with nematode suspensions of the same two isolates, at a rate of approximately 2.5 J2 cc^{-1} soil, to generate the maternal line. This low density caused most of the J2 to develop into females. The plants were placed in a growth cabinet at 20 °C, 70 % relative humidity and 16/8 h light/dark. Fourteen days after inoculation, roots from the paternal line were placed on sieves in a mist cabinet at 100 % relative humidity, mist temperature of 28 °C and ambient temperature of 18 °C. This high humidity caused a thin water film running from the roots by which males, leaving the roots,

Table 10.1. Specific characteristics of the isolates of *Meloidogyne* spp.

Species	Isolate	Sampling year	Original code[1]	*EST*[2]	*MDH*[3]	*HinfI*[4]	*RsaI*[5]
M. chitwoodi	Ca	1991	C3022 (PD)	S1	N1a	440, 270	760
M. chitwoodi	Ci	1991	C5273-C (PD)	S1	N1a	440, 270	760
M. fallax	Fa	1992	CHB (PAGV)	F3[7]	N1b[8]	430, 270	620
M. javanica	Ja	1990	C3059 (PD)	J3	N1	440, 320	760

[1] PD = Plant Protection Service, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands.

[2] *EST* = Isozyme phenotype for esterase (Esbenshade & Triantaphyllou, 1985; Karssen, 1996). F3 = Unique *EST* phenotype for *M. fallax*.

[3] *MDH* = Isozyme phenotype for malate dehydrogenase (Esbenshade & Triantaphyllou, 1985). N1b = Unique MDH phenotype for *M. fallax*.

[4] *HinfI* = Discriminating restriction products (in bp) upon restriction with *HinfI* of the 760-bp polymerase chain reaction-amplified internal transcribed spacer (ITS) regions (Zijlstra *et al.*, 1995).

[5] *RsaI* = Discriminating restriction products (in bp) upon restriction with *RsaI* of the 760-bp polymerase chain reaction-amplified internal transcribed spacer (ITS) regions (Zijlstra *et al.*, 1995).

accumulated at the bottom of the receptacle. After ten days, males were collected and added to the plants containing the maternal line. This procedure was repeated eight times during a period of 17 days, each time adding 10 to 50 males.

Females and their corresponding egg-masses were collected separately from roots on August 24, 1995. To examine whether females were inseminated, spermatothecae and oocytes of these females were checked for the presence of sperm by making smear preparations (Triantaphyllou, 1985b) which were stained with Hoechst 33258, modified from Albertson *et al.* (1978). In inseminated females, male gametes are accumulated in the spermatothecae which is a specialized structure in the female gonad. Into an oocyte that passes the spermatotheca, normaly in metaphase I, a male gamete enters. In order to detect the required interspecific first generation, hatched J2 of egg-masses of inseminated females were individually inoculated on September 8, 1995 on *Solanum tuberosum* cv. Eigenheimer root tips, growing in Petri-dishes on water agar, with only one J2 per Petri-dish. These Petri-dishes were incubated for seven weeks under dark conditions in a growth cabinet at 20 °C. Females with their corresponding egg-masses were collected separately, and used for electrophoresis and a hatching test, respectively.

10.3.3. Bulk mating experiment

A second experiment with isolates Ci and Fa was conducted from June to October

1995 to test the occurrence of spontaneous mating between *M. chitwoodi* and *M. fallax*. The *M. chitwoodi* isolate Ci was different from the isolate used in the controlled cross to investigate whether possibilities for hybridization with different isolates do exist. Two-week-old tomato seedlings were transplanted to 3.2 l clay pots containing sterilized sandy soil, 33 % (v/v) water-retaining granules (Hydrocorn) and 3 % (w/v) Osmocote. One- to two-day-old pre-hatched J2 were used as inoculum. Three weeks after sowing, four tomato seedlings were inoculated, each with approximately 3.3 J2 cc^{-1} soil in which a 1:1 mixture of Ci and Fa juveniles, and placed in a greenhouse at 20 °C (18-24 °C). To complete approximately two nematode generations, the infected plants were maintained for 14 weeks as based on known population development (Pinkerton *et al.*, 1991). The sex ratio was expected to develop towards a female majority during the first generation, with only a low frequency for possible interspecific hybridization. To detect hybrids and to test the viability of the progeny of these hybrids, the experiment was terminated after about the second generation, which approximated the first hybrid generation. From each tomato plant, 40 females with their corresponding egg-masses, were collected separately and used for electrophoresis and a hatching test, respectively.

10.3.4. Chromosome counting

The chromosomes of the parental lines were examined. Approximately 25 young egg-producing females were collected from infected roots of each parental line and smear preparations were made (Triantaphyllou, 1985b; Chapter 9), which were stained with Hoechst 33258. The chromosomes were counted in oocytes in early metaphase I and just after the first maturation division.

10.3.5. Hybrid detection and F_1 progeny viability

Collected females and corresponding egg-masses from both experiments were kept at -80 °C and 20 °C respectively. Due to the facultative parthenogenetic nature of the isolates, females can produce parthenogenetic or sexual offspring. The latter may be the result of insemination of females by either males of the maternal or of the paternal line. Moreover, more than one male may be involved in the fertilization of one female. Consequently, parthenogenetically and sexually produced eggs may occur in an egg-mass of such an inseminated female, by which a progeny of a crossing may contain both parthenogenetic and sexual offspring. In such a progeny, true hybrid females could be distinguished from a crossing with a male from the maternal line and from a parthenogenetic offspring by a differentiating marker between the two species. Three independant isozyme markers were used: malate dehydrogenase (*MDH*; E.C. number 1.1.1.37), esterase (*EST*; E.C. number 3.1.1.1) and glucose 6-phosphate dehydrogenase (*G6PD*; E.C. number 1.1.1.49). *MDH* was applied in the controlled crossing experiment and *MDH*, *EST* and *G6PD* in the bulk mating experiment. In order to provide conclusive evidence for hybridization, individual females were subjected to isozyme eletrophoreses of *MDH* and *EST*, and other females to electrophoresis of *MDH* and *G6PD*, resulting in two phenotypes for

one female, using independent isozyme markers. Isozyme electrophoresis and staining were carried out on micro-gels according to Karssen *et al.* (1995).
Egg-masses were individually collected in micro-vials with 10 ml water and placed in a incubator at 20 °C to hatch. During a period of eight weeks, hatching was checked weekly.

10.4. Results

10.4.1. Hybrids in the controlled crossing experiment

The haploid chromosome number of both parental isolates in the controlled crossing experiment, Ca and Fa, appeared to be the same, namely n=18. No F_1-hybrids were obtained from the crossing *M. fallax* x *M. chitwoodi* with *M. fallax* as the female parent, because the *M. chitwoodi* male parent did not produce sufficient males for the experiment. This has often been noticed during maintenance of *M. chitwoodi* isolates. F_1-hybrids were obtained from the crossing *M. chitwoodi* x *M. fallax*, with *M. chitwoodi* as the female parent. Seven females were collected from two plants, of which one female contained sperm, which was detected in the spermatotheca and in oocytes. Originating from the egg-mass of this particular inseminated female, 60 hatched J2 were inoculated separately on root tips, resulting in 21 females with egg-masses. Electrophoresis for *MDH* resulted in two different isozyme patterns: eight females exhibited only the maternal (*M. chitwoodi*) isozyme band (Figure 10.1, lanes 4 and 6) and four females showed patterns with three regularly spaced bands corresponding to the two parental bands and an intermediate (Figure 10.1, lanes 1 and 3). For nine females no clear bands were found (Figure 10.1, lane 5), probably due to loss of female material during preparation of the electrophoresis samples.

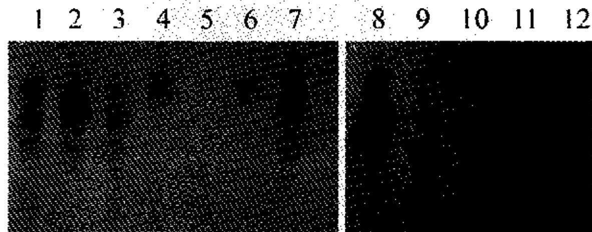

Figure 10.1. Malate dehydrogenase zymograms showing representative samples of parental and cross hybrid phenotypes of individual females of the controlled crossings between *M. chitwoodi* Ca and *M. fallax* Fa. ♀1 and ♀3 show hybrid phenotypes; ♀4 and ♀6 show maternal *M.chitwoodi* Ca phenotypes; ♀5 is absent or null; ♀9 and ♀10 show parental reference phenotypes for *M. fallax* Fa; ♀11 and ♀12 show parental reference phenotypes for *M. chitwoodi* Ca. *M. javanica* Ja is used as a reference phenotype (♀2, ♀7 and ♀8).

Although a successful crossing was obtained, none of the four hybrid females, with clear intermediate bands, produced normal fertile offspring, in contrast to all females showing maternal isozyme patterns. From the nine females without clear isozyme bands, two produced offspring and seven did not. Assuming that these seven were also true hybrids, a total of 11 interspecific hybrid females were obtained and ten parthenogenetic progeny. Egg-masses of hybrid females contained up to 100 eggs each. Most eggs were able to develop into a multi-cellular undifferentiated structure and in some eggs a juvenile was visible (Figure 10.2). Some juveniles were even able to hatch at 20 °C, but were distorted, showing deviating head and tail shapes (Figure 10.3) and died shortly after hatching.

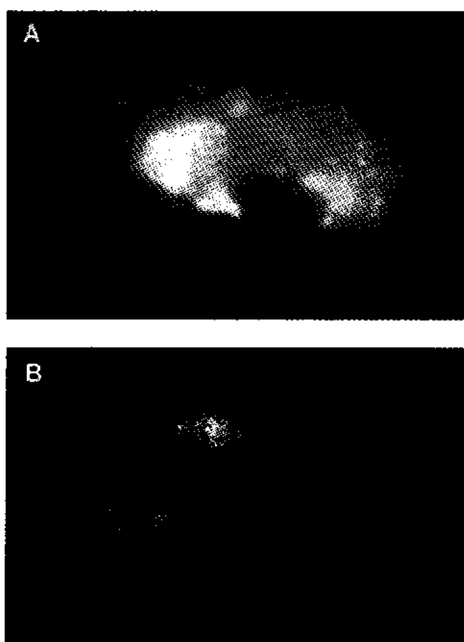

Figure 10.2. Eggs of (*M. chitwoodi* x *M. fallax*)-hybrids at about one month after hatching, stained with Hoechst 33258. **A**: Undifferentiated mass of nuclei. **B**: Malformed juvenile.

10.4.2. Hybrids in the bulk mating experiment

The haploid chromosome number of isolate Ci, used as one parent in the bulk mating experiment of *M. chitwoodi* and *M. fallax*, was n=18, like the isolates Ca and Fa. As a result from this crossing experiment 77 and 67 parental-type females of *M. chitwoodi* and *M. fallax*, respectively, for *MDH* phenotype were obtained at 14 weeks after inoculation (Table 10.2; Figure 10.4). Additionally, 16 F_1-hybrid females were detected, which was 10 % of the total number of examined females. None of the hybrid females produced viable offspring, while all parental-type females produced normal progeny. Parental-type and hybrid females showed corresponding parental and hybrid patterns for the two isozymes *MDH* and *EST* (Figure 10.5) as well as for *MDH* and *G6PD* (Figure 10.6) on the zymograms, comparable to *MDH* (Figure 10.1) in the controlled crossing experiment.

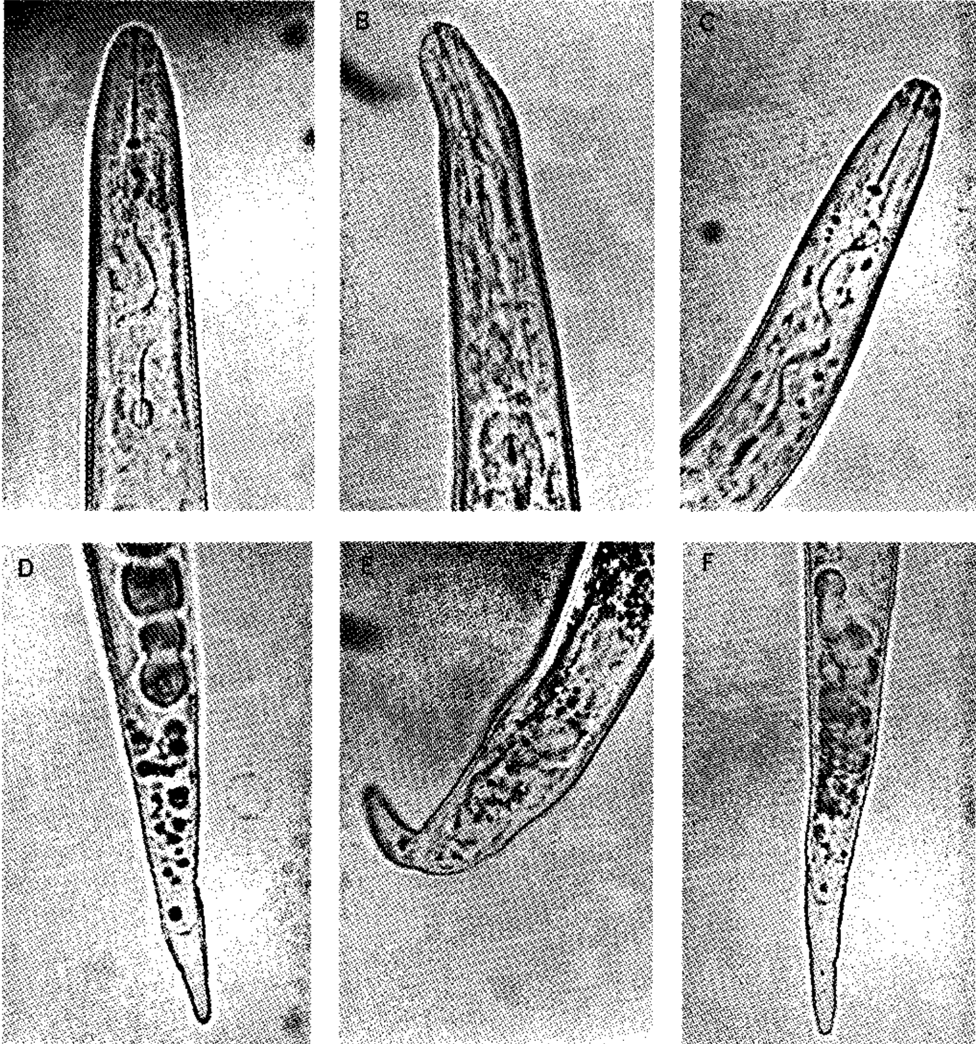

Figure 10.3. Characteristic head and tail structures of a juvenile of a (*M. chitwoodi* x *M. fallax*)-hybrid, compared with its crossing parents. **A, D**: *M. chitwoodi*. **B, E**: Progeny of hybrid. **C, F**: *M. fallax*.

Tomato plant	Ci[1]	Fa[2]	Hybrid[3]	Total ♀♀
1	16	20	4	40
2	24	12	4	40
3	17	18	5	40
4	20	17	3	40
Total	77	67	16	160

[1] Parental-type females, possessing the N1a band (*MDH*-phenotype of Ci)
[2] Parental-type females, possessing the N1b band (*MDH*-phenotype of Fa)
[3] Hybrid-type females, possessing the N1a, N1b and an intermediate band

Table 10.2. Number of parental-type and hybrid females in the bulk mating experiment of *M. chitwoodi* isolate Ci and *M. fallax* isolate Fa

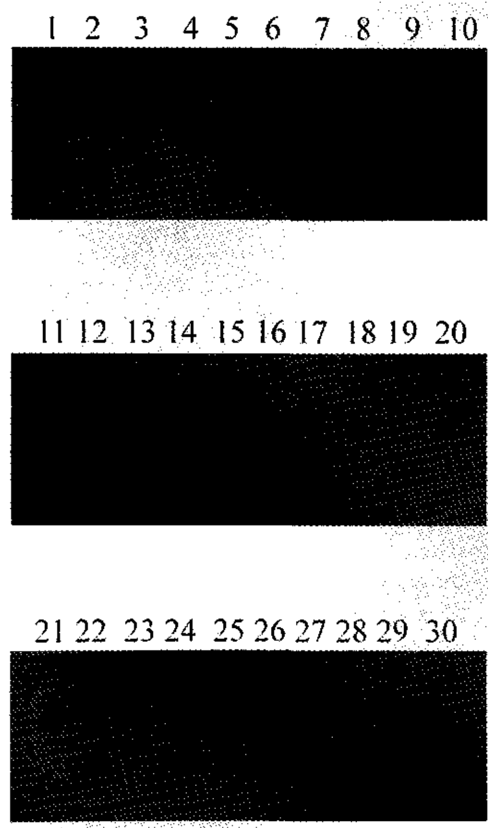

Figure 10.4. Malate dehydrogenase zymograms showing representative samples of parental and cross hybrid phenotypes of the bulk mating experiment between *M. chitwoodi* (Ci) and *M. fallax* (Fa), and phenotypes of mixtures of females of the two spp. Phenotypes of *M. chitwoodi*: ♀2, 4, 10, 18, 20, 21, 23, 28 and 29, *M. fallax*: ♀1, 7, 8, 11, 12, 13, 17, 19, 27 and 30, hybrids: ♀9, 16 and 26, and species mixtures: ♀22, 24 and 25. *M. javanica* (Ja) is used as a reference phenotype (♀5, 6, 14 and 15).

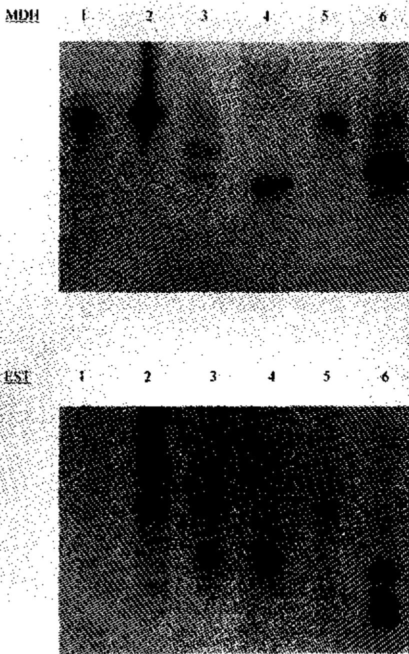

Figure 10.5. Zymograms of malate dehydrogenase *(MDH)* and esterase (*EST*), showing representative samples of parental and cross hybrid phenotypes for both isozymes on the same females of the the bulk mating between *M. chitwoodi* (Ci) and *M. fallax* (Fa). Phenotypes of *M. chitwoodi*: ♀4, *M. fallax*: ♀1, 2 and 5, and hybrid: ♀4 *M. javanica* (Ja) is used as a reference: ♀6. *MDH* and *EST* phenotypes for *M. fallax* are designated N1b and F3 respectively.

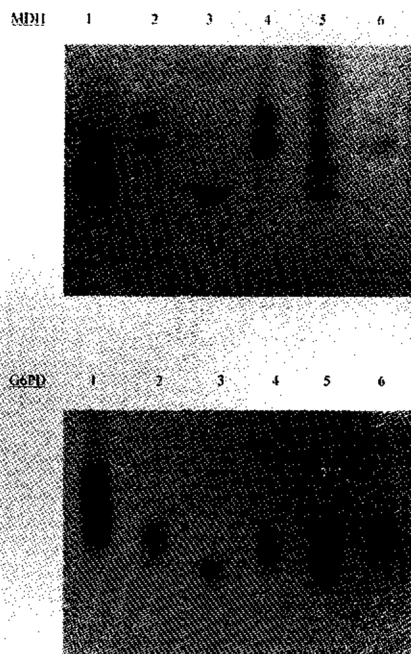

Figure 10.6. Zymograms of malate dehydrogenase (*MDH*) and glucose-6-phosphate dehydrogenase (*G6PD*), showing representative smaples of parental and cross hybrid phenotypes for both isozymes on the same females of the bulk mating between *M. chitwoodi* (Ci) and *M. fallax* (Fa). Phenotypes of *M. chitwoodi*: ♀3, *M. fallax*: ♀2, 4 and 6, and hybrid: ♀5. *M. javanica* (Ja) is used as a reference: ♀1.

10.5. Discussion

This is the first report of successful interspecific crossing within the genus - *Meloidogyne*, demonstrating hybridization between *M. chitwoodi* and *M. fallax*. Ample evidence for interspecific hybridization was obtained in this study from controlled and bulk crossings. Triantaphyllou successfully hybridized *M. hapla* isolates n=15 (♀) and n=17 (♂) chromosomes (A.C. Triantaphyllou, pers. comm.). Hybrid progeny had n=16 or 17 chromosomes. Pairing of homologous chromosomes was not perfect because loops were observed in some bivalents during diakinesis; nevertheless, hybrid progeny was propagated successfully for many generations.

Attempts to hybridize *M. chitwoodi* with *M. hapla*, however, were unsuccessful as detected by using chromosome and biochemical markers (Triantaphyllou & Hirschmann, 1980; A.C. Triantaphyllou, pers. comm.). In this study, the isolates used for crossings appeared to possess identical chromosome numbers. No data are available on crossings between *M. chitwoodi* and *M. fallax* with different chromosome numbers. Differences in chromosome number between parental isolates are expected to interfere with hybridization.

The present study supports the separate species status of *M. chitwoodi* and *M. fallax* by the non-viable progenies of their interspecific hybrids. Triantaphyllou (1985a) stated that ".. conceptually they (facultative meiotic parthenogenetic species) can be considered as true biological species if they are really reproductively isolated from each other. However, tests for demonstrating reproductive isolation are technically very difficult because of the facultative nature of parthenogenetic reproduction and the lack of genetic markers that would verify intercrossing." By demonstrating reproductive isolation between the two meiotic parthenogenetic species in this study, it is now evident that they are true biological species.

Isozyme electrophoresis was proven to be a convenient tool in detecting interspecific hybridization by using isozymes as genetic markers, as has been shown before in certain insects, for example waterflea *Daphnia* spp. (Wolf, 1987), stick-insects *Bacillus* spp. (Bullini & Nascetti, 1989) and planthopper *Muellerianella* (Booij, 1982). Van Meggelen *et al.* (1994) found that *M. chitwoodi* and *M. fallax* have different *MDH* phenotypes. The hybrid was expected to possess three distinct bands in a zymogram: both parental bands and an intermediate band, as *MDH* consists of two polypeptide chains and a heterodimer is expected to be formed in a heterozygote (Pasteur *et al.*, 1988), in contrast to a mixture of species showing only the two parental bands (Figure 10.4). The three different *MDH* patterns found in this study correspond to genotypes for the two parental species and their hybrid, confirming the codominant nature of locus *MDH*. *MDH* is a stable marker in distinguishing species of *Meloidogyne*, showing identical isozyme patterns within one species, with a few exceptions in *M. arenaria*, *M. incognita* and *M. javanica* (Esbenshade & Triantaphyllou, 1985). *MDH* genotypes of approximately 100 females of *M. chitwoodi* and 60 of *M. fallax*, representing various isolates, were characteristic for species, showing identical phenotypes as described in this chapter (non-published results). The genotypes of the hybrid females in this study, demonstrating hybrid patterns for the independent markers *MDH* and *EST*, and for *MDH* and *G6PD* in the same individuals, provide conclusive evidence for hybridization between *M. chitwoodi* and *M. fallax* (Figures 10.5 and 10.6). *G6PD*, distinguishing *M. chitwoodi* from *M. fallax* (Daher *et al.*, 1996), is also a heterodimer and in the hybrid the middle band is predominantly visible. *EST* is a monomer and its phenotype for *M. fallax* has been described as a so-called null (Karssen, 1996). However, stained for 90 minutes, a faint three-band pattern appears, which has not yet been descibed and is denoted as F3 (Figure 10.5, lanes 2 and 5). The hybrid *EST* pattern shows a strong band corresponding to the *M. chitwoodi* S1 phenotype and the slower

band of the *M. fallax* F3 phenotype (Figure 10.5). The *MDH* phenotype of *M. fallax* is described for the first time and designated N1b.

Not all females were expected to be fertilized by males. Mating in parthenogenetic *Meloidogyne* spp. is reported to occur by chance (Santos, 1972). But even if females were inseminated, it could have been entirely or partly by males of the maternal isolate. Additionally, the egg-mass from an inseminated female was expected to contain an unknown portion of unfertilized, parthenogenetic eggs. This explains the occurrence of maternal-type females in the progeny of the inseminated female in the controlled crossing experiment. Furthermore, it explains why a Mendelian segregation in not expected in crossings between meiotic parthenogenetic *Meloidogyne* spp.

Interestingly, the two species used in this study, are either allopatric or sympatric, both being prevalent in the South East of the Netherlands. Because of their similarity in ecological niche, but also in total protein patterns (Chapter 7) and in sharing many host plants including monocotyledons, it could well be that both species share common ancestry. In our experiments with mixtures of isolates, hybrids between *M. chitwoodi* and *M. fallax* occurred regularly. Although deformed and non-viable, a very few juveniles were found in the offspring of these hybrids. Speculatively, hybrids may occasionally produce fertile progeny, being able to backcross to one of the parents, as is shown for example in interspecific hybrids in *Daphnia* (Hebert, 1985; Wolf, 1987). For *Meloidogyne* this would signify that exchange of DNA might be possible, including genes for (a)virulence. If so, successful breeding for durable crop plant resistance should include testing of isolates of all potentially aggressive species that can interbreed.

Besides broadening the genetic base of a species by backcrosses of the hybrid to a parental population, hybridization may also play an important role in speciation in certain organisms (King, 1993). Two steps are required for a species to arise from interspecific hybridization (Bullini & Nascetti, 1989): firstly, interspecific hybridization, and secondly, changes in the maturation divisions allowing the transmission of the hybrid genome to the next generation. The latter changes are undoubtedly rare. Dalmasso and Bergé (1983) suspected that *M. javanica*, a polyploid mitotic parthenogenetically reproducing species, may have arisen in this way, because of its high level of heterozygosity for some isozymes. One of its parents could have been *M. hapla* as testified by similarity in nucleotide sequences between *M. javanica* and *M. hapla* (Hyman & Powers, 1991).

Besides the results described in this chapter, more evidence for hybridization between *M. chitwoodi* and *M. fallax* was derived from observations on lettuce roots in which parental and hybrid females were detected, by using *MDH* as genetic marker (results not shown). These lettuce plants were grown on soil which originated from a field in which mixed populations of the two species were detected.

Mixtures of *Meloidogyne* species are found more frequently under natural conditions. In those situations, hybridization may be feasible, and under environmentally favorable conditions for host plant rotation, intensity of land use and soil characteristics, it is likely that new forms of *Meloidogyne* may appear.

CHAPTER 11

Heterozygosity and post-reduction in parthenogenetic isolates of root-knot nematodes

11.1. Summary - Differences in Amplified Fragment Length Polymorphisms (AFLP) between isolates and between mono-female lines of facultative meiotic parthenogenetic *Meloidogyne hapla* race A and obligate mitotic parthenogenetic *M. incognita* were studied. These data were used in a similarity study. The genetic distance between three mono-female lines after one generation of parthenogenesis of three juveniles from one *M. incognita* egg-mass appeared to be very small ranging from 0.000 to 0.004. In contrast, the genetic distance between two mono-female lines after six generations of parthenogenesis of one juvenile of *M. hapla* with one consecutive generation of parthenogenesis was larger, being 0.083. This strongly suggests genotypically identical offspring of an *M. incognita* nematode in contrast to the variation in offspring of an *M. hapla* nematode. The genetic distance between two mono-female lines from one juvenile of *M. hapla* did not appear to be necessarily smaller than that between mono-female lines from different juveniles of the same isolate, indicating maintenance of heterozygosity during subsequent generations of parthenogenesis. The maintenance of heterozygosity is likely to be caused by the combination of post-reductional meiosis, due to the holokinetic nature of the chromosomes, and the fusion of sets of chromosomes after the second meiotic division. Levels of heterozygosity appeared to be equally variable between mono-female lines within one *M. hapla* isolate as between various isolates of *M. hapla*.

11.2. Introduction

Parthenogenesis, being the production of an embryo from a female gamete without the participation of a male gamete (Rieger *et al.*, 1976), has long been considered to be a "dead end" strategy from an evolutionary point of view (Darlington, 1937, White, 1948, Suomalainen, 1950). However, more recently, several authors described mechanisms which enhance genetic variation in parthenogenetic organisms, like in frogs (Nace *et al.*, 1970), in several insects (Bullini & Nascetti, 1989) and in mites (Wrensch *et al.*, 1994).
In the root-knot nematode genus *Meloidogyne*, most species possess a parthenogenetic mode of reproduction: only six of the 23 examined species mentioned by Eisenback and Hirschmann Triantaphyllou (1993) are amphimictic. (Cyto-)genetics have been studied in *Meloidogyne* spp. by Triantaphyllou (1971, 1985, 1987). In mitotic parthenogenetic *Meloidogyne* species, polyploidy can lead to the maintenance of heterozygosity. In meiotic parthenogenetic species the presence of male gametes, originating either genetically or by sex reverse, provides possibilities for sexual reproduction. However, if male gametes are absent, the combination of two mechanisms, interacting during the reproductive phase of maturing oocytes, enables the maintainance of genetic variation: the occurrence of post-reductional meiosis and

fusion of the second polar body with the egg pronucleus.

Post-reductional meiosis, in which the actual reduction takes place during second maturation division, has been demonstrated cytologically in a number of organisms with holokinetic chromosomes, for instance in the plant *Luzula purpurea* (Nordenskiöld, 1962), in the aphid *Tamalia coweni* (Ris, 1942), in several coccids (Hughes-Schrader, 1948), and in the spider mite *Tetranychus urticae* (Feiertag-Koppen, 1980). In *Meloidogyne* spp., also possessing holokinetic chromosomes (Goldstein & Triantaphyllou, 1980; Triantaphyllou, 1983), conclusive cytological evidence for the occurrence of post-reductional meiosis is hampered by the extremely small chromosome size (Van der Beek *et al.*, G; Chapter 9). The aim of the present research is to provide indirect experimental evidence for the occurrence of post-reductional meisosis in *Meloidogyne* spp. Therefore, levels of genetic variation are investigated in meiotic parthenogenetic mono-female lines and isolates of *M. hapla*, which restore their somatic chromosome number by fusion of the egg-pronucleus and the second polar body, and in mono-female lines of mitotic parthenogenetic *M. incognita*, in which meiosis is characterized by one mitotic division (Figure 11.1). It is expected that mono-female lines of *M. incognita* are genetically homogeneous, in contrast to those of *M. hapla*. Moreover, post-reductional meiosis will cause a maintenance of heterozygosity even after repeated parthenogenesis of mono-female lines of *M. hapla*. Significant differences between such lines will point towards the occurrence of post-reductional meiosis in *Meloidogyne* spp. The genetic consequences of this type of meiosis for meiotic parthenogenetic populations are discussed.

11.3. Materials and methods

11.3.1. Isolates and mono-female lines of M. hapla *and* M. incognita

Five isolates of meiotic parthenogenetic *M. hapla* race A and one isolate of mitotic parthenogenetic *M. incognita* were studied (Table 11.1). All these isolates were maintained and propagated on *Lycopersicon esculentum* cv. Moneymaker. From *M. incognita* isolate Ia and *M. hapla* isolate Ha, three (Ia1 to Ia3) and six (Ha1 to Ha6) mono-female lines, respectively, were developed by inoculating second stage juveniles (J2) on root tips of *Solanum tuberosum* cv. Eigenheimer growing in Petri-dishes containing 2.0 % agar technical nr. 3 (Oxoid). To ensure parthenogenetic offspring, only one J2 was inoculated per Petri-dish. The progeny of such a female is called a mono-female line (Figure 11.2). This procedure was repeated once for Ia-lines and seven times consecutively for Ha-lines. The mono-female lines Ia1 to Ia3 originated from one-egg mass, while the lines Ha1 to Ha6 each originated from a different egg-mass, except Ha2 and Ha3. These two lines were derived from the same egg-mass after six generations of compulsary parthenogenesis after which they were separately multiplied for one generation. Finally, one single egg-mass of these nine lines was separately multiplied on plants of 'Moneymaker'. The resulting

Table 11.1. Origin of the five isolates of *M. hapla* race A and one of *M. incognita*

Isolate	Origin	Host of origin	Year of sampling	Obtained from[1]
Ha	Lisse (NL[2])	peony	1990	PD
He	Drouwenerveen (NL)	potato	1992	PAGV
Hf	Borgers (NL)	carrot	1992	PAGV
Hi	Smilde (NL)	immortelle	1992	PAGV
Hbj	Canada	unknown	<1994	WM
Ia	unknown	unknown		PD

[1] PD = Plant Protection Service, Wageningen, The Netherlands. PAGV = Research Station for Arable Farming and Field Production of Vegetables, Lelystad, The Netherlands. WM = University of Wisconsin-Madison, Madison, USA.
[2] NL = The Netherlands

individuals were preserved in liquid nitrogen (Van der Beek *et al.*, 1996; Chapter 2). After thawing, two more generations followed on 'Moneymaker' in order to obtain proper amounts of J2 for DNA extraction. During these generations on plant, males were observed.
All isolates and mono-female lines were found to be true to species by rDNA analysis of ITS regions (Zijlstra *et al.*, 1995).

11.3.2. Amplified Fragment Length Polymorphism (AFLP)
DNA extraction from hatched juveniles was performed as described in Zijlstra *et al.* (1995) and AFLP procedure was according to Van der Beek *et al.* (B; Chapter 7). The primer combinations E11M19, E19M12 and E11M12 were employed.
For each mono-female line and isolate, two independent sub-samples of the extracted DNA were subdued to AFLP analysis.

11.3.3. Data analysis
AFLP autoradiograms were evaluated visually by superimposing them on a bench viewer. On the autoradiograms, DNA fragments for each isolate or line which were present in both sub-samples, were marked, resulting in a data matrix with presence-absence data. The similarity in AFLP patterns of the *M. hapla* isolates and lines was determined by converting the data to similarity indices using the Dice coefficient (Aquadro & Avise, 1981) and using the SIMQUAL procedure of NTSYS-pc (Rohlf, 1994). The similarity indices (F-values) between AFLP patterns were calculated using the formula $2n_{xy}/(n_x + n_y)$, with n_x and n_y being the number of fragments observed for genotypes x and y respectively, and n_{xy} the number of fragments scored in both patterns. Cluster analysis was performed on the resulting similarity matrix using the SAHN procedure of NTSYS-pc according to the UPGMA method.
The genetic distance is calculated as 1-F.

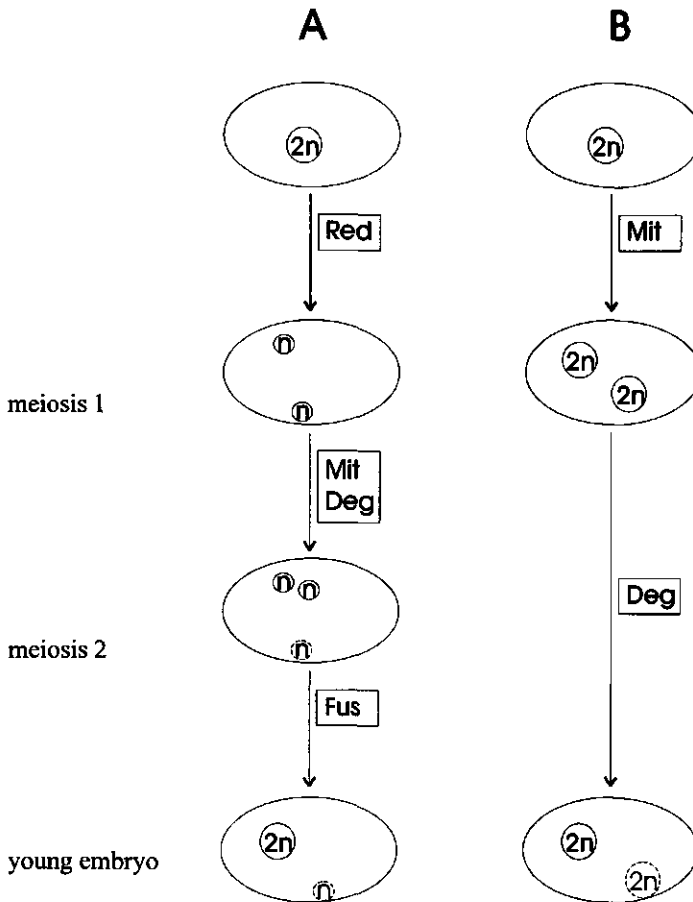

Figure 11.1. Meiotic (A) and mitotic (B) parthenogenetic pathways in *Meloidogyne hapla* race A and *M. incognita*, respectively.
Legenda: 2n=set of chromosomes with the somatic number; n=set of chromosomes with the haploid number; Red=reductional division; Mit=mitotical division; Fus=fusion of second polar body with the egg pronucleus; Deg=degeneration of the polar body.

11.4. Results

Between the three *M. incognita* mono-female lines, 222 monomorphic and four polymorphic markers were observed, corresponding to 1.8 % polymorphisms. Mono-female lines I1 and I3 were identical and the four polymorphisms occurred between these two and line I2 (Figure 11.3). The F-values between these lines

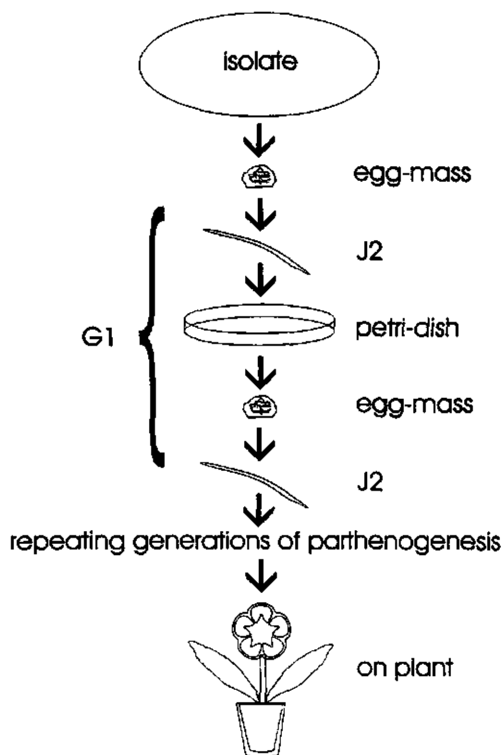

Figure 11.2. Schematic representation of the procedure of obtaining mono-female lines in parthenogenetic *Meloidogyne* spp.

ranged from 1.000 to 0.996.
Between the *M. hapla* mono-female lines and the isolates, 53 monomorphic and 128 polymorphic markers were observed (Figure 11.3). Between pairs of lines of isolates, the number of polymorphisms ranged from 11 between Ha2 and Ha5 to 68 between Ha and He. F-values in the corresponding matrix with similarity coefficients ranged from 0.933 to 0.746 between the mono-female lines, and from 0.892 to 0.670 between the isolates (Table 11.2). The largest similarity was found between Ha2 and Ha5, with an F-value of 0.933. The F-value was 0.917 between the two most related lines Ha2 and Ha3, based upon 8.8 % polymorphisms. The corresponding similarity dendrogram is presented in Figure 11.4.
The number of observed fragments varied among the *M. hapla* isolates and mono-female lines, and ranged from 75 to 128 between the isolates and from 88 to 110 between the mono-female lines (Table 11.3). For the *M. incognita* mono-female lines this number ranged from 222 to 226.

11.5. Discussion

11.5.1. Genetic distances between isolates and between mono-female lines

The extremely small genetic distances of 0.000 and 0.004 between the three *M. incognita* mono-female lines strongly suggest that they are genotypically identical, resulting from a parthenogenetic offspring of one egg-mass of a mitotic parthenogenone. The differences in DNA pattern could be due to mutation in mono-female line I2. Although males were observed during the multiplication phase on plants, this did not result in large genetic variation, which confirms that males do not take part in the reproduction of *M. incognita*. The extremely low number of polymorphisms between the lines also indicates high reliability of the produced AFLP patterns.

The genetic distances between the *M. hapla* isolates ranged from 0.108 to 0.330 (Table 11.2), which are similar to those found in other AFLP studies between *M.*

Table 11.2. F-values for six mono-female lines and five isolates of *M. hapla* race A, based upon Dice-similarity coefficients of AFLP-data

	Ha1	Ha2	Ha3	Ha4	Ha5	Ha6	Ha	He	Hi	Hf	Hbj
Ha1	1										
Ha2	0.870	1									
Ha3	0.811	0.917	1								
Ha4	0.848	0.787	0.784	1							
Ha5	0.846	0.933	0.884	0.746	1						
Ha6	0.800	0.870	0.896	0.836	0.838	1					
Ha	0.812	0.789	0.809	0.844	0.778	0.815	1				
He	0.698	0.744	0.707	0.683	0.718	0.698	0.676	1			
Hi	0.686	0.764	0.723	0.655	0.736	0.703	0.670	0.892	1		
Hf	0.686	0.802	0.784	0.622	0.778	0.772	0.727	0.747	0.743	1	
Hbj	0.755	0.847	0.780	0.643	0.833	0.768	0.712	0.778	0.813	0.849	1

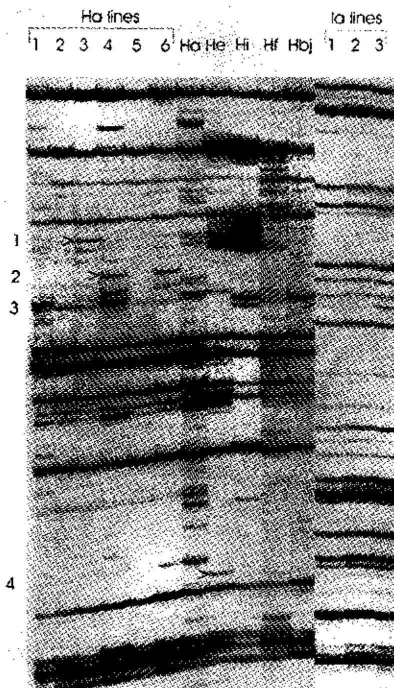

Figure 11.3. AFLP-pattern, showing polymorphic DNA fragments amongst the six mono-female lines (Ha-lines) and five isolates of *M. hapla* and the three mono-female lines of *M. incognita* (Ia-lines). Examples of specific fragments are indicated by arabic characters: **1** for Ha3; **2** for Ha4 and Ha6; **3** for Ia1 and Ia2; **4** for He.

hapla races A and B, being 0.16 (Van der Beek *et al.*, B; Chapter 7). The genetic distances between the isolates were approximately within the same range as those between the mono-female lines of isolate Ha, from 0.067 to 0.254 (Figure 11.4; Table 11.2). Consequently, the genetic variation within isolates can be as large as the variation between isolates.

The relatively large variation in genetic distances between the mono-female lines of *M. hapla* Ha conveys genetic differences between the egg-masses from which they originated. Lines Ha4 and Ha5 for instance showed a large genetic distance and Ha2 and Ha5 appeared to be genetically extremely similar (Figure 11.4; Table 11.2), which would reflect similar differen-

Table 11.3. Number of observed DNA fragments of a total of 181 for *M. hapla* isolates and lines, and of 226 for *M. incognita* lines for three enzyme combinations

Isolate/Line	# observed fragments
Ha1	-[1]
Ha2	90
Ha3	102
Ha4	-[1]
Ha5	88
Ha6	110
Ha	128
He	82
Hi	75
Hf	92
Hbj	80
Ia1	226
Ia2	222
Ia3	226

[1] missing observations

ces between the juveniles they were raised from. The distance between the most related lines Ha2 and Ha 3 is 0.083, which is larger than that between Ha2 and Ha5, and also larger than the differences between the patterns of the mono-female lines of *M. incognita*. Consequently, Ha2 and Ha3 are genetically not necessarily less distant from each other than one of these two from any other mono-female line. It is concluded that a mechanism is active, which is able to conserve heterozygosity, despite six generations of forced parthenogenesis. The only convincing interpretation for this phenomenon is by a combined occurrence of two processes during maturation divisions: post-reductional meiosis and a mechanism of restoration of the somatic chromosome number, which does not result in homozygosity, i.e. fusion of the egg pronucleus and the second polar body (Figures 11.5 and 11.1, respectively).

The differences between Ha2 and Ha3 can only be explained by the occurrence of males. Seven generations of parthenogenesis will predominantly result in genetically identical nematodes in one line, which are heterozygous for a certain number of loci. The two multiplication cycles of these lines on plants allowed males to be produced, having the same genotypes as the females. If no male would have been produced, no differences between Ha2 and Ha3 would have occurred, and the situation would not have been distinguishable from that in organisms with monocentric chromosomes. However, in such a case, all individuals of a mono-female line, including the males, are expected to be genetically identical and homogeneous for nearly all loci. Therefore, the occurrence of males would not provoke heterozygosity. Consequently, the amount of heterozygosity found between Ha2 and Ha3 can be explained by the occurrence of males in the presence of post-reductional meiosis.

11.5.2. Post-reductional meiosis and fusion of 2-nd polar body with egg-pronucleus

Goldstein and Triantaphyllou, (1980), Albertson and Thomson (1982) and Triantaphyllou (1983) provided evidence for the holokinetic nature of chromosomes in the taxon Nematoda. Triantaphyllou (1983) wrote: "At prometaphase I, the bivalent chromosomes ... are in an end-to-end orientation and each one consists of two

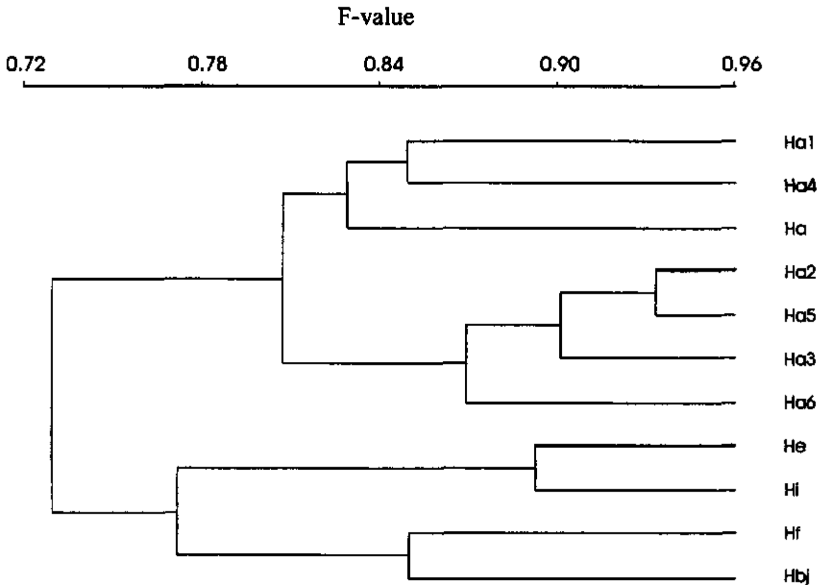

Figure 11.4. Similarity dendrogram of the five *Meloidogyne hapla* isolates and the six mono-female lines of isolate Ha

distinct chromatids oriented parallel to each other." Ultrastructural research on males of *M. hapla* provided evidence for the occurrence of holokinetic chromosomes, which are characterized by a diffuse centromere with microtubuli inserted at many places into the chromatin (Goldstein & Triantaphyllou, 1980). Sister chromatids are not associated at any point by centric connections and a complete spatial separation is possible which may provoke the chromatid to behave like a chromosome. This chromosomal behaviour during meiosis allows post-reduction meiosis to occur during maturation (Battaglia & Boyes, 1955). Restoration of the somatic chromosome number after reduction in meiosis occurs in meiotic parthenogenetic *Meloidogyne* spp. by fusion of the second polar nucleus with the egg pronucleus (Figures 11.1 and 11.5). The same restoration mechanism is observed in a limited number of other parthenogenetic species from the Homoptera, Thysanoptera, Diptera and Hymenoptera (Suomaleinen *et al.*, 1976). Genetic consequences following this mechanism of fusion were discussed by Asher (1970) and Suomalainen *et al.* (1987), but without considering organisms with non-localized centromeres. It is the first time that genetic evidence is given for the combined occurrence of the above mentioned mechanism of fusion and post-reductional meiosis.

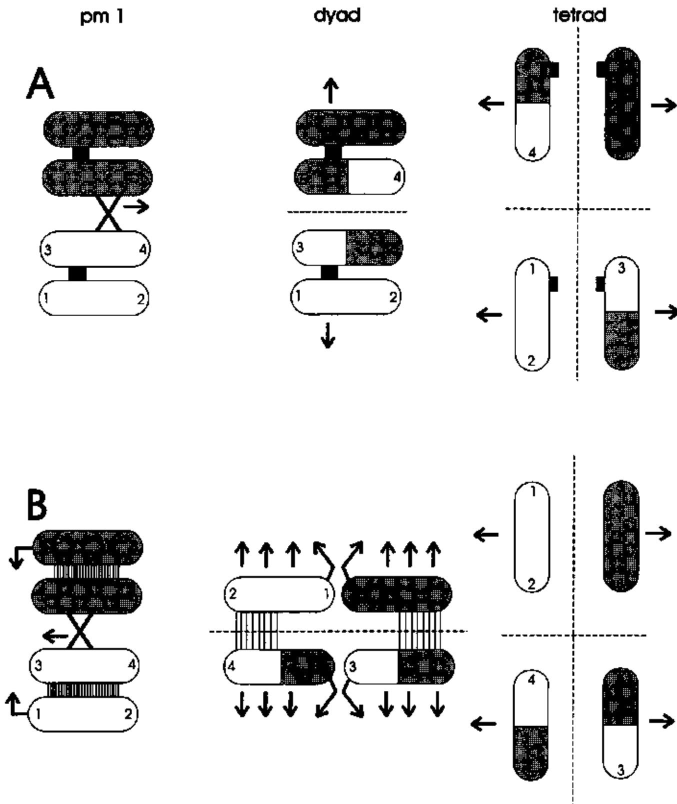

Figure 11.5. A. Schematic representation of meiosis in organisms with monocentric chromosomes. **B.** A proposed explanation of the course of meiosis in meiotic parthenogenetic *Meloidogyne* spp. Legenda: pm1 = prometaphase I; dyad = early anaphase I; tetrad = telophase II; lllllll = protein connection between non-exchanged chromatids; ▬ = centromere

11.5.3. Variation in heterozygosity

The numbers of DNA inserts on the AFLP patterns for the three enzyme combinations varied among isolates and lines (Table 11.3). These numbers were considerably higher for the three *M. incognita* mono-female lines compared to the *M. hapla* isolates and lines, indicating either a larger haploid genome or substantially more heterozygosity in *M. incognita*. Although at present we can not exclude that *M. incognita* isolates do have a larger genome than *M. hapla* isolates, a high level of heterozygosity is expected, because of the obligate mitotic parthenogenetic nature of *M. incognita*, which also favors polyploidy.

The varying numbers of fragments between the *M. hapla* isolates also indicate differences in heterozygosity between the isolates. Variation in haploid genome sizes seems unlikely, not even between aneuploids, because two *M. hapla* isolates with different chromosome numbers appeared to have the same total DNA content (Lapp & Triantaphyllou, 1972). The relatively high heterozygosity of isolate Ha is reflected in its proceeded mono-female lines: the heterozygosity of these mono-female lines is larger than that of the other four *M. hapla* isolates used in this study

(Table 11.3). Also within isolate Ha varying levels of heterozygosity were found between the mono-female lines. This variation is not expected to be caused by different genomic sizes as all are from one isolate.

11.5.4. Maintenance of heterozygosity

The occurrence of post-reductional meiosis (Figure 11.5) together with the fusion of the egg pronucleus and second polar body has important consequences for the genetic variation in meiotic parthenogenetic populations of *Meloidogyne* spp. We will now consider the change in heterozygosity during successive generations of parthenogenesis of genotypes with heterozygous loci, not taking into consideration the effect of mutation. In the simple case of absence of crossing-over during meiosis, females which are heterozygous for a given locus *A/a* will always produce parthenogenetic offspring with genotype *Aa*: 1 *Aa* -> 1 *Aa*. Homozygous offspring would possibly occur if we consider interchromosomal recombination. Suppose the probability of chiasmata terminalization, resulting in a recombination for locus *A/a*, is p. Chiasmata at holokinetic chromosomes may hypothetically terminalize towards both bivalent ends. During the first meiotic division, alleles between which recombination took place, can move towards the same pole, but also towards different poles. Suppose the probability that they move towards the same pole is q. Chiasmata between the holokinetic chromatids will favor a metaphase I orientation of the two recombined alleles on the same side of the equatorial plate. Therefore probability q will be 1 or close to 1. In some publications it is suggested that organims with holokinetic chromosomes could show reduction at either the first or the second meiotic division (Suomalainen, 1950, White, 1973, Wrensch *et al.*, 1994). Parallel orientation of the chromosomes during first metaphase and anaphase (equatorial orientation) would indicate post-reductional meiosis. This has been found in *Luzula purpurea*, *Tamalia coweni*, several coccids, and *Tetranychus urticae*, as mentioned above. However, in *Caenorhabditis*, axial orientation is reported to be present (Albertson & Thomson, 1993). In other nematodes, equational orientation would be present in *Meloidogyne* spp. and *Heterodera* spp., based upon parallel first metaphase and anaphase orientation (Triantaphyllou, 1966). Recent cytological observations in *Meloidogyne* spp., however, revealed parallel orientation during metaphase and axial orientation during anaphase (Van der Beek *et al.*, G; Chapter 9). This anaphase orientation could possibly be caused by coherence of the ends of those sister chromatids towards which the chiasmata did not terminalize (Figure 11.5). It would imply that probability q equals 1. One female individual which is heterozygous for locus *A/a* will generate descendants of genotypes *Aa* and *AA+aa* in proportions (1-p+pq) and p(1-q), respectively. For example, if p=0.5 and q=0.9 or 1.0, then the proportion of heterozygous genotypes in the offspring of genotype *Aa* is 95 % or 100%, respectively. The proportion of heterozygous genotypes in generation N (Het_N) is expressed in the following equation:

$$\text{Het}_N = \{1 - p(1\text{-}q)C\}^n.(\text{Het}_{G0}),$$

in which C is the probability of occurrence of a chiasma, n is the number of parthenogenetic generations and Het_{G0} is the proportion of heterozygous genotypes in generation 0.

At least one chiasma will always occur at each chromosome, so probability C will be almost or equal to 1. If we consider the probability that a chiasma will result in a recombination for locus *A/a* equals 0.5, then the increase in homozygosity will be different for that locus after a certain number of generations for values of q of 0.8, 0.9 and 1.0 (Figure 11.6). Still 63.1 % of the population will be heterozygous for locus A/a after 10 generations, under the assumption of p=0.5, q=0.9 and O=1.0.

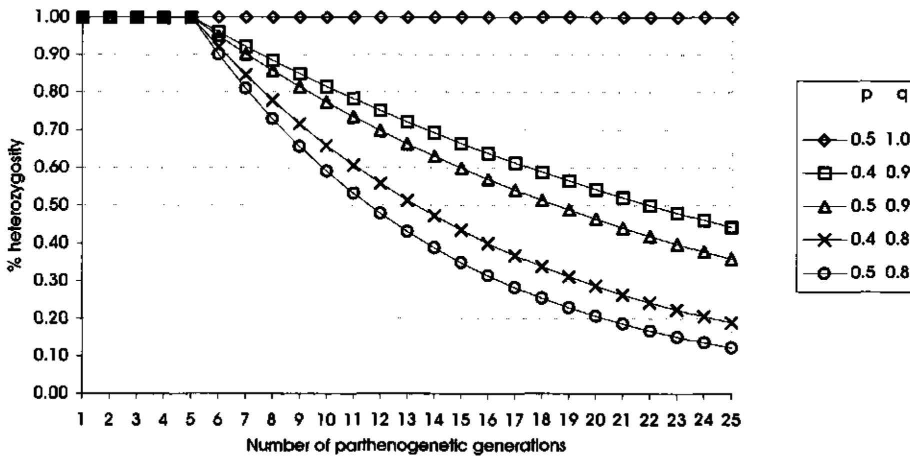

Figure 11.6. Simulated decrease in heterozygosity during successive generations of compulsary parthenogenesis of a heterozygous nematode of a meiotic parthenogenetic *Meloidogyne* spp., with values for p= 0.4 and 0.5 and q=0.8 and 0.9 (see text). Under q=1.0, heterozygosity stays 100 %.

Heterozygosity in meiotic parthenogenetic *Meloidogyne* spp. is maintained, which is explained by the occurrence of post-reductional meiosis. Consequently, populations of these species will possess high levels of genetic flexibility. The particular combination of meiotic parthenogenesis and post-reductional meiosis allows possibilities for wide adaptation of these pathogens and may well be responsible for their world-wide success. Males, which can appear in abundancy in these populations, will guarantee high levels of heterozygosity as the facultative nature of the parthenogenesis allows fertilization of eggs. The genetic consequences of meiotic parthenogenesis and post-reductional meiosis in *Meloidogyne* are comparable to those in many fungi and other lower organisms showing a clonal reproduction with occasionally sexual recombination.

CHAPTER 12

General discussion

12.1. Species concept in relation to genetic variation

According to the biological species concept species are distinguished by their reproductive isolation. They are the largest and most inclusive reproductive communities of sexual and cross-fertilizing individuals that share a common gene pool (Mayr, 1942). Additional morphological, physiological and ecological differences are involved in species distinction. Other species concepts have been developed, such as the recognition concept, cohesion concept, evolutionary concept, ecological concept and the phylogenetic concept (King, 1993). This illustrates the difficulties encountered in distinguishing species, especially when it concerns organisms with uniparental reproduction. Parthenogenetic *Meloidogyne* species were initially separated by host preference and morphological differences, for instance in perineal pattern, but large overlaps in this and other characters hamper a clear distinction (Netscher & Taylor, 1979). Well defined biological units including a good species classification is a prerequisite for a succesfull crop protection strategy, especially in plant resistance. In experimentation, adequate knowledge of the taxonomic status of the employed isolates is essential. It is a requirement to know the species composition in field populations, for many species differ in host preference and virulence. In this respect, Netscher's (1978, 1983) suggestion to re-classify *M. incognita*, *M. arenaria* and *M. javanica* as sub-species of one tropical species would only complicate the systematics of *Meloidogyne* because it creates a need for a more complex classification to describe the variability in this group of species. In fact, these three species are, despite high similarity, clearly distinguished for isozyme patterns, DNA patterns, 2-DGE patterns of total protein and morphological characters (Chapter 7), which support separate species status.
The ultimate species distinction between *M. chitwoodi* and *M. fallax* was proven by reproductive isolation (Chapter 10). However, to supply similar conclusive evidence for all meiotic parthenogenetic *Meloidogyne* spp. would be laborious and impossible for mitotic parthenogenetic *Meloidogyne* spp. Comparison of the species at the molecular level provides a reliable tool in classification based upon similarities, as intraspecific variation is considerably smaller than interspecific variation (Chapters 7 and 8). This would support the use of molecular data for the distinction between species, besides morphological characterization. *Meloidogyne* spp., as other lower organisms, show a tendency in displaying more variation at the molecular level than on higher organisational levels, in contrast to higher organisms, like in primates (Chapter 7). In the nematode *Xiphinema* for instance, molecular variation was useful for species determination (Vrain, 1993) while adequat biological and morphological differentiation was lacking (Heyns, 1983).

A special case is represented by the species *M. hapla* which occurs as two

cytological races, A and B, with different chromosome numbers. Race A reproduces in an amphimictic or meiotic parthenogenetic way and race B by mitotic parthenogenesis. The two races are incompatible (Triantaphyllou & Hirschmann, 1980), and are consequently reproductively isolated suggesting separate species status. Despite significant differences in their reproductive systems (Triantaphyllou, 1966) and in certain morphometrical characters (Chapter 7), the races resemble each other strongly in morphology and isozyme phenotypes (Eisenback, 1993; Esbenshade & Triantaphyllou, 1985), on the basis of which they are considered to belong to the same species. Supportive evidence for this was obtained by the occurrence of limited variation in total protein content and genomic DNA (Chapter 7), although the molecular level isolates of the two races can be clustered per race (Chapter 8). The low overall genetic variation between the two races indicates that the triploid *M. hapla* race B (2n=approximately 42; Chapter 9) is the result of an autopolyploidisation. The small differences in genetic variation between the two races may possibly be the result of a continuous production of race B genotypes from race A genotypes. An alternative explanation may be that mutation from race A to race B has occurred only once and recently. Because of their reproductive isolation, divergence may gradually be building up and could lead to two separate species. Untill now the occurrence of both meiotic and mitotic parthenogenetic variants within one species is only demonstrated in *M. hapla*. However, it may well occur more widely in this genus, given the large cytological variation: mechanisms of chromosome duplication occur in an isolate of an unknown *Meloidogyne* sp., in which a mixture of two meiotic pathways in one female is observed (Chapter 9). Meiotic flexibility, such as this mechanism of chromosome duplication, could possibly lead to polyploid mitotic parthenogenetic populations.

12.2. True and sex-reverse males

The sex ratio in *Meloidogyne* spp. is unbalanced. *M. chitwoodi* produces consistently low numbers of males compared to the meiotic parthenogenetic species *M. hapla* race A and *M. fallax* (not published). All three species produce viable sperm, but *M. fallax* showed various degrees of spermatogenesis distortion (Chapter 9). Mitotic parthenogenones like *M. hapla* race B and *M. javanica* were found to produce abundant males producing sperm that was able to intrude oocytes after insemination, but fusion of the male and female sets of chromosomes was not observed (Chapter 9). Besides these differences among species, the sex ratio within one population of a parthenogenetic *Meloidogye* species can be altered by environmental influences (Davide & Triantaphyllou, 1967). Under environmental conditions unfavorable for development, the pathway of female formation is changed, giving rise to sex-reverse males. Depending on the start of the stress period after initiation of feeding of the second-stage juveniles (J2), a range of cytological types is developed: from males with one complete and one strongly reduced gonad to males with two complete gonads (Papadopoulou & Triantaphyllou, 1982; Eisenback & Hirschmann-

Triantaphyllou, 1991), which are all able to produce viable sperm.

By electron microscope studies, oocytes at pachytene stage of *M. hapla* race A were found to have bivalents with synaptonemal complexes (SC) along their entire length (Goldstein & Triantaphyllou, 1978). These SC were absent in oocytes of *M. hapla* race B, showing a mitotic division during meiosis, without chromosome pairing. SC of males and females of *M. hapla* race A were found to differ by the absence and presence, respectively, of decondensed chromatin regions (DCR) in the SC (Goldstein, 1981). On average 16 DCR per nucleus occur, representing approximately 5 % of the karyotype. As this percentage equals that of sex chromosomes in other organisms, it was hypothesized that DCR in *M. hapla* race A could be involved in the sex chromatin. Genetically a female would be homozygous for presence of DCR, and the male would be heterozygous, resulting in absence of DCR (Goldstein, 1981). Females can thus be represented by genotype *aa* and males by *Aa*. Parthenogenetic offpring of *aa* can only result in genotype *aa*, which would be a total female offspring as DCR are present. Goldstein (1981) further suggests that the formation of sex-reverse males, occurring under stress, could be caused by a cessation of the production of a hypothetical substance which is normally produced in homozygous condition and which is essential for female determination. However, these sex-reverse males would then still be of *aa* genotype and consequently posess DCR.

Histological evidence for the formation of sex-reverse males in *M. incognita* was provided by Papadopoulou and Triantaphyllou (1982), who found males with male gonads ranging from normal monodelphic to totally didelphic. They further showed that the gonad primordium in J2, consisting of two germinal cells and two somatic cells, develops differently in males and in females, from six days after feeding initiation onwards. The primordia become either rod-shaped, with one posterior oriented cap cell, resulting in males, or V-shaped, with two anterior oriented cap cells. One of these cap cells may either degenerate at different stages of the J2 development, resulting in sex-reverse males with one gonad and one rudiment, or fully develop resulting in (normal) females or sex-reverse males with two gonads (Chapter 9). The J2 with rod-shaped primordia were called true male juveniles and their development was hypothesized to be of a cytogenetic basis (Papadopoulou & Triantaphyllou, 1982). This would imply that genetic differences would be involved between these and other males from juveniles with V-shaped primordia. This is unlikely as *M. incognita* is mitotically parthenogenetic and offspring are considered to be genetically identical. Therefore, the alternative suggestion by Papadopoulou and Triantaphyllou (1982) i.e. sex-reversal during the first five days of feeding initiation, is a more plausable explanation. This would include that no distinction should be made between true (or normal) and sex-reverse males in mitotic parthenogenetic *Meloidogyne* spp., as is done for example by Eisenback and Hirschmann-Triantaphyllou (1991).

Males in meiotic parthenogenetic populations of *Meloidogyne* are found with monodelphic or with didelphic testes. It is hypothesized that parthenogenetic species and populations originate from amphimictic forms (Triantaphyllou, 1984). The most simple genotypical representation of males and females is by one gene *A/a*, by which *aa* and *Aa* are the female and male genotypes, respectively. Amphimictic populations would thus show a 1 to 1 female to male ratio, which is also mentioned by Eisenback and Hirschmann-Triantaphyllou (1991). In meiotic parthenogenones, sex reverse males, originating from 'female' J2, would have genotype *aa* and true males with genotype *Aa* would be expected to decrease in consequent generations. Therefore the consequence of this model is that in meiotic parthenogenetic populations true males are expected to disappear.

In conclusion, both in meiotic and in mitotic *Meloidogyne* populations no true males would be expected. The males in these populations are all expected to be sex-reverse. Future research will confirm or reject this hypothesis.

12.3. Variation in pathogenicity and new pathotypes

Dutch isolates of *M. hapla* showed remarkably large variation in pathogenicity on three genotypes of *S. bulbocastanum*, *S. hougassi* and *S. sparsipilum* (Chapter 4), while these genotypes were never before exposed to populations of *M. hapla* in the Netherlands. Apparently, these virulence factors are already present in the gene pool without any selection pressure and in relatively large frequencies as shown by the occurrence of high numbers of egg-masses. By such a selection-neutral situation these virulence factors may persist in populations. These persisting factors, even when in low frequencies, may subsequently become apparent on host genotypes possessing resistance genes.

Variation in pathogenicity appeared to be smaller in *M. chitwoodi* than in *M. hapla* when tested on potato cultivars (Chapter 3) and on specific genotypes of wild *Solanum* spp. (Chapters 4 and 5). Nevertheless, in an experiment with three *M. chitwoodi* isolates and two *S. bulbocastanum* genotypes, isolate-by-genotype interactions were shown (Table 6.3), indicating the presence of different pathotypes in *M. chitwoodi* in relation to *S. bulbocastanum*. The high similarity in 2-DGE protein patterns between these three and other *M. chitwoodi* isolates from the USA and from the Netherlands, would suggest a relatively small overall genetic variation in this species (Chapter 8). The reported differences between the American and the Dutch isolates in host races and the occurrence of different pathotypes suggest that selection on pathogenicity occurs in genetically fairly homogeneous genotypes of *M. chitwoodi*. This would suggest that specific virulence may arise relatively fast, indicating perhaps small genetic differences between different virulent specificities. Because of the high levels of heterozygosity in *Meloidogyne* species (Chapter 11), the fact that *M. chitwoodi* can grow two to three generations per year on one potato

crop and because of the polyphagous nature of this pathogen, new interactions can be expected to develop in favourable growing areas, giving rise to new pathotypes.

In introgression programmes for resistance to *M. chitwoodi* and *M. fallax*, using *S. bulbocastanum*, *S.cardiophyllum*, *S. branchistrotrichum*, *S. fendleri* and *S. hougasii* (Janssen, 1997), attention should therefore be paid to the variation in pathogenicity. It is as yet unkown whether these resistances will be as durable as the *Mi* gene, introgressed from *Lycopersicon peruvianum* into the tomato (Smith, 1944) and effective to *M. incognita* during several decennia.

12.4. Heterozygosity

Overall genetic variation based upon AFLP patterns appeared not necessarily smaller within isolates than between isolates, and even within a mono-female line heterozygosity was preserved by post-reductional meiosis (Chapter 11). Due to the preservation of heterozygosity, the distinction between isolates is arbitrary. For instance, genetic distances and the level of heterozygosity were lower between certain *M. hapla* isolates than between certain mono-female lines of one isolate (Tables 11.2 and 11.3).
Heterozygous isolates will be composed of genotypically identical nematodes, under parthenogenesis. However, during maintenance and multiplication of isolates, males may occur, causing isolates to be heterogeneous by recombination. Consequently, no genetically stable isolates are to be expected in meiotic parthenogenetic *Meloidogyne* spp. and the development of homozygous isolates or lines is considered to be virtually impossible.

12.5. Testing for virulence and the selection for virulent isolates

Testing for virulence by studying isolate-by-genotype interaction requires reliable experimental methods. Obviously, a high repeatibility of test results, which depends on test conditions and plant and nematode genotypes, is essential. It is appropriate to state that the repeatibility of the nematode material is often largely overestimated. This is probably an important reason why results of different tests are contradictory, as illustrated by the significant isolate-by-genotype interaction on *Solanum* genotypes of *M. hapla* isolates originating from the same root samples but maintained by different labs (Chapter 4). Likewise, in an experiment with *M. fallax* isolates originating from one root sample and maintained by different labs and on different host plants, significant interaction for isolate-by-plant genotype was obtained (data not shown; in cooperation with G.J.W. Janssen, CPRO-DLO, Wageningen, The Netherlands and J.J. Van de Haar, RZ-Research, Metslawier, The Netherlands).
Possibly, the genetic variation within isolates may be an important cause of contradicting results in testing for virulence. As the development of homozygous lines is impossible, isolates should be developed which are homozygous only for

virulence factors. A set of such isolates, homozygous for different virulence factors, could thus be developed. The development of such isolates requires the following steps. Firstly, differences in virulence between isolates of a *Meloidogyne* species on genotypes of a host species need to be detected. Secondly, selection for homozygosity of virulence factors should be established for interesting isolates. A possible way to achieve this homozygosity would be by producing mono-female lines of a virulent isolate. Mono-female lines of *M. hapla* isolate Ha (Table 4.4.) and of *M. chitwoodi* isolate Cb (Table 5.3.) can be clustered in two groups for aggressiveness, in which the more aggressive lines (Ha1, Ha6, Cb5) could be homozygous for the virulence factor(s). Marker-assisted selection by DNA-polymorphism analysis will be a great advantage: lines with two alleles for virulence can be detected by bulk segregant analysis followed by the analysis of each separate line, using a codominant marker such as AFLP. These lines need to be re-tested on differentiating host genotypes. Thirdly, a pathotype scheme needs to be established, by grouping isolates with identical virulence patterns (Chapter 6). Finally, the isolates need to be maintained and multiplied separately to avoid every risk of contamination. Storage of the obtained isolates may be done in liquid nitrogen, guaranteeing long term preservation of the genetic material (Chapter 2).

The genetic variation within an isolate may not only be influenced by post-reductional meiosis (Chapter 11), but also by contamination with other isolates. Despite maximum precautions, undesirable and inexplicable interspecific contaminations may occur (Chapter 4 and 5) and it is unknown of the extent to which isolates are contaminated with other isolates of the same species. The sudden appearances of interspecific contaminations may indicate their presence in concentrations below the detection limit of 1 to 2 % with analysis of ITS regions of rDNA (Zijlstra *et al.*, 1997). More sensitive DNA based detection techniques are being developed (C. Zijlstra, pers. com.) and will further help to detect contaminations. Frequent occurrence of interspecific contaminations emphasizes the need to produce 'pure' cultures from mono-female lines.

12.6. Breeding perspectives

The large degree of variation within isolates and the occurrence of pathotypes need special attention in breeding programmes. Application of the above described approach in virulence testing (12.5.) will prevent as much as possible that plant genotypes with introgressed resistance genes are compatable with current isolates of pathotypes. This requires a combined approach involving the host species and the pathogen species in an early stage of the breeding programme for resistance. For example, a breeding programme for resistance of the potato to *M. chitwoodi* and *M. fallax* by the introgression of resistance genes from *S. bulbocastanum,* needs a parallel research programme for virulence, including selection for stable virulent isolates and the development of a pathothype scheme. In this way, maximum

durability of the resistance will be ensured. These programs are laborious and the feasibility of such programmes for resistance and virulence should be investigated for potato as well as for other crops in the Dutch rotation scheme for potato. A combined effort of plant breeders and nematologists will contribute to an adequate approach to this variable pathogen.

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Summary

"Interaction between root-knot nematodes and *Solanum* spp.: Variation in pathogenicity, cytology, proteins and DNA"

Root-knot nematodes (*Meloidogyne* spp.) are world wide among the most wide-spread pests in agriculture. In potato (*Solanum tuberosum*), the most important arable crop in the Netherlands, damage is caused by yield reduction and tuber deformations. *M. hapla*, *M. chitwoodi* and *M. fallax* are polyphagous parasites of many arable crops in temperate regions. These three species show a facultative meiotic parthenogenetic mode of reproduction, except for *M. hapla* race B, which, unlike race A, is reproducing in an obligate mitotic parthenogenetic fashion. During the last decade legal regulations in the Netherlands accentuate the need for a stronger emphasis on environmentally safe measures to control these pathogens. Among these measures host plant resistance is one of the most effective strategies. However, no resistance of an acceptable level is found in cultivars of potato, nor in those of other rotation crops. Therefore, sources for resistance need to be found in related species. The successful development of resistant cultivars requires also a thorough knowledge of the variation in the pathogen.
The aim of this PhD-thesis is to contribute to a better understanding of the general biology and the genetic variation of *M. hapla*, *M. chitwoodi* and *M. fallax*. This was investigated at various levels: preservation of genetic variation, variation in pathogenicity on *Solanum* spp., variation in proteins and DNA among and within *Meloidogyne* spp., cytogenetic variation, interspecific hybridisation and maintenance of heterozygosity.

In order to preserve the pathogenicity and other characteristics of *Meloidogyne* germplasm, in the form of mono-female lines and original field samples, a suitable method for long-term preservation in liquid nitrogen was optimized (Chapter 2). The procedure is based on a two-step treatment of second-stage juveniles with cryoprotectant ethanediol and provided an average survival rate of 75 %, ranging from 45 to 98 %. One *M. hapla* isolate revealed a significant higher survival than the other, indicating isolate specific variation, possibly related to variation in lipid reserves. Viable juveniles were obtained in 1997 from samples that were frozen for the first time in 1994, indicating successful long-term storage with this method. Isolates and mono-female lines from storage in liquid nitrogen were used for experimentation, after at least one generation of multiplication on *Lycopersicon esculentum* cv. Moneymaker.

Variation in pathogenicity of *Meloidogyne* spp. on *Solanum* spp. is discussed in Chapters 3 to 6. Virulence and aggressiveness of various isolates of *M. hapla*, *M. chitwoodi* and *M. fallax* were evaluated on potato cultivars, showing a significant isolate-by-cultivar interaction effect for number of egg-masses in *M. hapla* but not in

the other two species (Chapter 3). This indicates variation in virulence in *M. hapla* and suggests the occurrence of different genetic factors for virulence in *M. hapla* and for resistance in the employed potato cultivars. Testing these isolates on potato roots in Petri-dish culture resulted in similar conclusions for the reproduction rate (Pf/Pi). However, in a greenhouse experiment results for reproduction rate were different, probably due to a shorter life cycle of *M. fallax* isolates compared to *M. hapla* and *M. chitwoodi*. This indicates that the Petri-dish method is accurate in testing for virulence in root-knot nematodes in potato cultivars. *M. fallax* was most aggressive on potato, followed by *M. chitwoodi*, *M. hapla* race A and finally *M. hapla* race B.
When tested on genotypes of the wild *Solanum* spp. *S. chacoense*, *S. hougasii* and *S. sparsipilum*, a significant isolate-by-genotype interaction for number of egg-masses of *M. hapla* was observed (Chapter 4). These results indicate the occurrence of different genetic factors for virulence and for resistance in the employed *Solanum* genotypes. The factors for virulence appeared to be distributed throughout *M. hapla*, regardless the parthenogenetic nature of the isolates. No significant interaction term was observed when testing different mono-female lines of one *M. hapla* isolate on the *Solanum* genotypes, indicating a similar virulence factor in all lines. However, variation in aggressiveness was observed between these lines. In testing for resistance to *M. hapla*, attention should be paid to the variation in virulence. Mono-female lines are to be preferred in virulence testing, to avoid testing with isolates possessing several virulence factors in different genotypes.
Testing various isolates of *M. chitwoodi* and *M. fallax* on genotypes of the wild *Solanum* spp. *S. bulbocastanum*, *S. chacoense*, *S. hougasii* and *S. stoloniferum*, did not reveal significant isolate-by-genotype interaction for number of egg-masses (Chapter 5). These results indicate absence of intraspecific variation for virulence in the isolates tested to the employed *Solanum* genotypes. Differences in aggressiveness between isolates and between mono-female lines of one of the *M. chitwoodi* isolates were observed. Despite absence of variation in virulence, *M. chitwoodi* juveniles in a species mixture with *M. fallax* were able to break the resistance of the *S. bulbocastanum* and *S. hougasii* genotypes. Because of the limited variation in virulence of *M. chitwoodi* and *M. fallax* isolates on genotypes of *S. bulbocastanum* and *S. hougasii*, these species are promising for introgression of resistance into commercial potato cultivars. Incomplete resistances such as in *S. stoloniferum* might also be promising as no data are available on the durability of the resistance.
The occurrence of considerable variation in virulence in *M. hapla* isolates and the virulent *M. chitwoodi* juveniles on *S. bulbocastanum*, indicate that potentially new virulent isolates can be formed, resulting in new pathotypes. The usefulness of the formulation of a pathotype scheme for each interesting interaction between a root-knot nematode species and a host species is discussed (Chapter 6). Three pathotypes were identified in *M. chitwoodi* on *S. bulbocastanum* genotypes, suggesting at least two different genetic factors for virulence and resistance. Such a pathotype scheme is recommended for virulence studies. The host race concept, which describes intraspecific variation in pathogenicity in a root-knot nematode species to a specific set

of host genotypes belonging to different plant genera, is useful for isolate identification but not for virulence studies. Experimental data show deviations from the expected host race reactions of isolates to the different hosts. No evidence for the existance of host race 2 in the Netherlands was obtained after testing eight *M. chitwoodi* isolates.

Total genetic variation as revealed by two dimensional gel electrophoresis of total soluble protein (2DGE) and Amplified Fragment Length Polymorphisms (AFLP) among and within *Meloidogyne* spp. is discussed in Chapters 7 and 8. Based upon more than 100 protein spots, 192 AFLP fragments and 21 morphological characters, UPGMA dendrograms were constructed for the mitotic parthenogenetic species *M. arenaria*, *M. hapla* race B, *M. incognita* and *M. javanica*, and for the meiotic parthenogenetic species *M. chitwoodi*, *M. fallax*, *M. hapla* race A and *M. naasi*. The dendrograms based on 2DGE and AFLP data were highly congruent, showing clustering of *M. hapla* races A and B, of the tropical species *M. incognita*, *M. arenaria* and *M. javanica*, and of *M. chitwoodi* and *M. fallax*. The dendrogram of the morphological data deviated from those of the molecular data, particularly for *M. incognita* and *M. naasi*, possibly due to selection on morphological characters.
Between six isolates of *M. hapla*, four of race A and two of race B, between eight of *M. chitwoodi*, three from North America and five from the Netherlands, and between five of *M. fallax*, limited polymorphisms were revealed with 2DGE (Chapter 8), in contrast with the distinct variation between these species (Chapter 7). This confirms that these species are clearly distinct biological groups. Percentages of polymorphisms of 5.0, 2.2 and 0.6 % were found among the isolates of *M. hapla*, *M. chitwoodi* and *M. fallax*, repectively. This order corresponds with the order of variation in virulence of these species on *Solanum* spp. (Chapters 3, 4 and 5).

Meiotic variation and reproductive isolation are discussed in Chapters 9, 10 and 11. Oogenesis and spermatogenesis were studied in several isolates of *M. hapla*, *M. chitwoodi* and *M. fallax* (Chapter 9). Oogenesis was similar in these species, except for *M. hapla* isolate He, in which only 12 % of the oocytes of inseminated females contained sperm in stead of the expected 100 %. This was caused by a prolonged first prophase in oocytes which were found posterior to the spermatheca. Spermatogenesis was similar in *M. hapla* as in *M. chitwoodi*. In *M. fallax* various degrees of distortion were observed in about one-third of the males, ranging from absence of meiosis in some spermatocytes to a complete absence of meiosis which resulted in the total degeneration of the spermatocytes. In oocytes of an unidentified meiotic parthenogenetic species, probably related to the *M. arenaria* group, only exceptionally a second meiotic division occurred and mostly one meiotic division was observed after which chromosome duplication was suggested to take place. In successive generations of one mono-female line of this unknown species, embryonic development within the female body occurred, which appeared to be genetically controlled. These studies show that in *Meloidogyne* a large meiotic variation exists.

Molecular evidence was obtained for a clear species dinstinction of *M. hapla*, *M. chitwoodi* and *M. fallax* (Chapter 8), as limited overall genetic variation was shown for total proteins. Hybridization between the closely related species *M. chitwoodi* and *M. fallax* was attempted to test the biological species concept in meiotic parthenogenetic *Meloidogyne* spp. (Chapter 10). Successful interspecific hybridization was obtained in controlled and bulk mating experiments, but the obtained hybrids failed to produce viable offspring. The possible role of interspecific hybridization in species differentiation and in the interspecific exchange of genetic material is discussed.
Genetic variation between mono-female lines of *M. hapla* race A and between mono-female lines of *M. incognita* was investigated by AFLP analysis (Chapter 11). The genetic distances between three mono-female lines, originating from three juveniles of *M. incognita* after one generation of parthenogenesis, ranged from 0.000 to 0.004, and were considerably smaller than that between two mono-female lines from one juvenile of *M. hapla* after six generations of parthenogenesis, being 0.083. This strongly suggests genotypically identical offspring of mitotic parthenogenetic *M. incognita* in contrast to variation in offspring of meiotic parthenogenetic *M. hapla* race A. The genetic distances between mono-female lines from one juvenile of *M. hapla* appeared not to be necessarily smaller than that between mono-female lines from different juveniles of the same isolate, indicating maintenance of heterozygosity during subsequent generations of parthenogenesis. The maintenance of heterozygosity is likely to be the result of the combination of two mechanisms in meiotic parthenogenones: post-reductional meiosis and the fusion of the second polar body with the egg pronucleus. Levels of heterozygosity between mono-female lines of *M. hapla* and between different isolates appeared to be equal. No completely homozygous lines are to be expected by repeated parthenogenesis of meiotic parthenogenetic isolates. For studies in virulence, isolates need to be developed which are stable for the virulence factor(s) present. An approach is presented to develop these isolates and how they can be applied in a plant breeding programme (Chapter 12).

In conclusion, a better understanding is attained in the genetic variation in pathogenicity in root-knot nematodes on *Solanum* spp., in the genetic variation in cytology, in proteins and in DNA. The consequences of the variation for the species concept, the origin of males, the distinction of isolates, the occurrence of new pathotypes, the testing for virulence and the plant breeding perspectives are discussed (Chapter 12).

Account

The chapters 2 to 11 are based on the following publications:

Chapter 2:
Van der Beek JG, Veldhuis WBJ, Zijlstra C, Van Silfhout CH, 1996. Preservation of *Meloidogyne hapla* and *M. chitwoodi* in liquid nitrogen: differences in response between populations. Fundamental and applied Nematology 19:227-234.
Chapter 3:
Van der Beek JG, Vereijken PFG, Poleij LM, Van Silfhout CH. Isolate-by-cultivar interaction in root-knot nematodes *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax* on potato. Canadian Journal of Botany, submitted.
Chapter 4:
Van der Beek JG, Janssen GJW, Poleij LM, Zijlstra C, Janssen R. Variation in virulence within *Meloidogyne hapla* on *Solanum* spp. Phytopathology, submitted.
Chapter 5:
Van der Beek JG, Janssen GJW, Poleij LM, Zijlstra C. Variation in virulence within *Meloidogyne chitwoodi* and *M. fallax* on *Solanum* spp. Phytopathology, submitted.
Chapter 6:
Van der Beek JG, Maas PWTh, Zijlstra C, Janssen GJW, Van Silfhout CH. Pathotypes and host races to describe intraspecific variation in pathogenicity in *Meloidogyne* spp.: three pathotypes identified in *M. chitwoodi* on *Solanum bulbocastanum*. Journal of Nematology, submitted.
Chapter 7:
Van der Beek JG, Folkertsma R, Zijlstra C, Van Koert PHG, Poleij LM, Bakker J. Genetic variation among parthenogenetic *Meloidogyne* species revealed by AFLPs and 2D-protein electrophoresis contrasted to morphology. Fundamental and applied Nematology, submitted.
Chapter 8:
Van der Beek JG, Folkertsma R, Poleij LM, Van Koert PHG, Bakker J. Molecular evidence for *Meloidogyne hapla*, *M. chitwoodi* and *M. fallax* being distinct biological entities. Fundamental and applied Nematology, accepted.
Chapter 9:
Van der Beek JG, Los JA, Pijnacker LP. Cytology of parthenogenesis in *Meloidogyne hapla*, *M. chitwoodi*, *M. fallax* and an unidentified species. Genome, to be submitted.
Chapter 10:
Van der Beek JG, Karssen G. Interspecific hybridization of meiotic parthenogenetic *Meloidogyne chitwoodi* and *M. fallax*. Phytopathology, accepted.
Chapter 11:
Van der Beek JG, Zijlstra C, Poleij LM, Pijnacker LP. Heterozygosity and post-reduction meiosis in parthenogenetic isolates of root-knot nematodes. Genome, submitted.

Samenvatting

"De interactie tussen wortelknobbelnematoden en *Solanum* spp.: variatie in ziekteverwekkend vermogen, cytologie, eiwitten en DNA"

Wortelknobbelnematoden (*Meloidogyne* spp.) behoren wereldwijd tot de meest verspreide plagen in de landbouw. Schade in aardappel (*Solanum tuberosum*), het belangrijkste gewas in de open teelt in Nederland, komt tot uitting in opbrengstverliezen en kwaliteitsverlies door knolmisvormingen. *M. hapla*, *M. chitwoodi* en *M. fallax* zijn parasieten met meerdere waardplanten als gastheer in open teelten in gematigde gebieden. Deze drie soorten tonen een facultatief, meiotisch ongeslachtelijke wijze van vermenigvuldiging, behalve *M. hapla* race B, die zich uitsluitend mitotisch ongeslachtelijk vermenigvuldigt. Tevens zijn *M. chitwoodi* en *M. fallax* quarantaine-organismen in pootaardappelen in de Europese Unie. Het streven naar reductie van chemische middelen ter bestrijding van nematoden hebben de noodzaak benadrukt van milieuvriendelijke maatregelen om deze ziekteverwekkers te beheersen. Van deze maatregelen is waardplantresistentie een van de meeste doeltreffende strategieën. Er zijn echter geen effectieve resistentieniveaus bekend noch in gebruiksrassen van aardappel, noch in die van andere gewassen in het rotatieschema van aardappel. Daarom moet er in verwante soorten gezocht worden. Tevens is voor het succesvol ontwikkelen van resistente rassen een gedegen kennis van de variatie in de ziekteverwekker nodig.
Het doel van dit proefschrift is een bijdrage te leveren aan een beter inzicht in de biologie en de genetische variatie van *M. hapla*, *M. chitwoodi* en *M. fallax*. Dit werd onderzocht op verschillende niveaus: instandhouding van genetische variatie, variatie in ziekteverwekkend vermogen op *Solanum* spp., variatie in eiwitsamenstelling en DNA tussen en binnen *Meloidogyne* spp., cytogenetische variatie, soortskruisingen en behoud van heterozygotie.

Om pathogeniteit en andere eigenschappen te behouden van isolaten van *Meloidogyne* spp., zoals van lijnen afkomstig uit één vrouwtje (monofemale lijnen) en oorspronkelijke monsters van veldpopulaties, werd een methode geoptimaliseerd die geschikt is voor lange termijn bewaring in vloeibare stikstof (Hoofdstuk 2). De procedure is gebaseerd op een tweestaps behandeling van tweede stadium larven met ethaandiol als invriesbeschermend middel. Deze methode bewerkstelligde een gemiddelde overleving van 75 %, variërend van 45 tot 98 %. Eén *M. hapla* isolaat toonde een significant hogere overleving dan de andere, wat duidt op isolaatspecifieke variatie die mogelijk gerelateerd is aan de variatie in vetreserves. Levensvatbare larven werden verkregen in 1997 van monsters die voor het eerst ingevroren werden in 1994, hetgeen duidt op succesvolle lange termijn bewaring met deze methode.

Vóór gebruik in proeven werden de isolaten uit vloeibare stikstof tenminste gedurende een generatie vermeerderd op tomaat.

Variatie in ziekteverwekkend vermogen van *Meloidogyne* spp. op *Solanum* spp. wordt besproken in de Hoofdstukken 3 tot en met 6.Virulentie en agressiviteit van verschillende isolaten van *M. hapla*, *M. chitwoodi* en *M. fallax* werden beoordeeld op aardappelrassen, waarbij een significante isolaat-cultivar interactie werd verkregen voor aantal eiproppen van *M. hapla* maar niet voor die van de twee andere soorten (Hoofdstuk 3). Dit is een aanwijzing voor variatie in virulentie in *M. hapla* en het voorkomen van verschillende genetische factoren voor virulentie en voor resistentie in de gebruikte rassen. Bij het toetsen van deze isolaten op aardappelwortels in petrischalen, werd dezelfde conclusie getrokken voor de vermeerderingsfactor (Pf/Pi). Echter, in een kassenproef waren de resulten ten aanzien van de vermeerderingsfactor verschillend, hetgeen hoogstwaarschijnlijk veroorzaakt werd door een kortere generatieduur van *M. fallax* in vergelijking met *M. hapla* en *M. chitwoodi*. Dit geeft aan dat de petrischalenmethode een nauwkeurige toetsmethode is om virulentie aan te tonen in wortelknobbelnematoden op aardappelrassen. Op aardappel bleek *M. fallax* het meest agressief, gevolgd door *M. chitwoodi*, *M. hapla* race A en tenslotte *M. hapla* race B.
Bij proeven met genotypen van de wilde aardappelsoorten *S. chacoense*, *S. hougasii* en *S. sparsipilum*, werd een significant isolaat-genotype interactie verkregen voor aantal eiproppen van *M. hapla* (Hoofdstuk 4). Deze resultaten tonen de aanwezigheid aan van verschillende genetische factoren voor virulentie en voor resistentie in de gebruikte *Solanum* genotypen. De factoren voor virulentie bleken willekeurig verspreid voor te komen in *M. hapla* race A en race B. Geen significante interactie werd waargenomen bij het testen van monofemale lijnen van een *M. hapla* isolaat op de *Solanum* genotypen, hetgeen duidt op dezelfde factor voor virulentie in al deze lijnen. Wel was er variatie in agressiviteit tussen deze lijnen. In resistentieprogramma's tegen *M. hapla* zal rekening gehouden moeten worden met de relatief grote variatie voor virulentie. Het gebruik van isolaten die stabiel zijn voor virulentie, zoals de gebruikte monofemale lijnen, lijkt veelbelovend in het effectief toetsen van aardappelrassen en -soorten.
Bij het toetsen van diverse isolaten van *M. chitwoodi* en *M. fallax* op genotypen van de wilde *Solanum* soorten *S. bulbocastanum*, *S. chacoense*, *S. hougasii* en *S. stoloniferum* werd geen significante isolaat-plantgenotype interactie waargenomen (Hoofdstuk 5). Dit duidt op afwezigheid van binnen-soort variatie voor virulentie in de gebruikte isolaten op de *Solanum* genotypen. Verschillen in agressiviteit werden waargenomen tussen isolaten en tussen monofemale lijnen afkomstig van een van de *M. chitwoodi* isolaten. Hoewel in het algemeen geen virulentieverschillen werden gevonden, waren *M. chitwoodi* larven, die gemengd met *M. fallax* voorkwamen, in staat de resistentie van de *S. bulbocastanum* en *S. hougasii* genotypen te doorbreken. Vanwege de lage variatie in virulentie van *M. chitwoodi* en *M. fallax* op genotypen van *S. bulbocastanum* en *S. hougasii* zijn deze soorten veelbelovend voor

introgressieprogramma's voor resistentie in de cultuuraardappel. Incomplete resistentie zoals die in *S. stoloniferum* kan echter ook veelbelovend zijn want er is nog niets bekend over de duurzaamheid van resistentie.
Het voorkomen van veel variatie in virulentie in *M. hapla* isolaten en het virulente *M. chitwoodi* isolaat op *S. bulbocastanum* zijn aanwijzingen dat potentieel isolaten kunnen ontstaan met nieuwe virulentie, die nieuwe pathotypen vormen. Het nut van het opstellen van een pathotypenschema voor elke interessante waard-pathogeen interactie tussen wortelknobbelnematodensoorten en waardplantsoorten wordt besproken (Hoofdstuk 6). Drie pathotypen werden onderscheiden in *M. chitwoodi* op *S. bulbocastanum*, hetgeen minstens twee verschillende genetische factoren veronderstelt voor virulentie en resistentie. Ten behoeve van virulentiestudies wordt zo'n pathotypenschema aanbevolen. Het waardplanten-'race' concept, dat tussen-soort variatie in pathogeniteit beschrijft in wortelknobbelnematodensoorten op een specifieke set waardplantgenotypen die behoren tot verschillende plantengeslachten, is nuttig voor de identificatie van isolaten maar niet voor virulentiestudies. Proefgegevens toonden afwijkingen van de verwachte reacties op de verschillende waardplanten. In acht Nederlandse *M. chitwoodi* isolaten kwam waardplanten-'race' 2 niet voor, hetgeen er op duidt dat dit 'race' waarschijnlijk niet voorkomen in Nederland.

Totale genetiche variatie, aangetoond door middel van twee dimensionele gelelectroforese van totaal oplosbaar eiwit (2DGE) en 'Amplified Fragment Length Polymorphisms' (AFLP) tussen en binnen *Meloidogyne* spp., wordt besproken in de Hoofdstukken 7 en 8.
Uitgaande van meer dan 100 eiwitvlekken, 192 AFLP fragmenten en 21 morfologische eigenschappen werden UPGMA dendrogrammen vervaardigd voor de mitotisch ongeslachtelijk vermenigvuldigende soorten *M. arenaria*, *M. hapla* race B, *M. incognita* en *M. javanica*, en voor de meiotisch ongeslachtelijk vermenigvuldigende soorten *M. chitwoodi*, *M. fallax*, *M. hapla* race A en *M. naasi*. De dendrogrammen die gebaseerd zijn op 2DGE en AFLP waren in hoge mate congruent en vertoonden clustering van *M. hapla* race A met *M. hapla* race B, van de tropische soorten *M. incognita*, *M. arenaria* en *M. javanica*, en van *M. chitwoodi* met *M. fallax*. Het dendrogram van de morfologische gegevens week af van die van de moleculaire gegevens, in het byzonder voor *M. incognita* en *M. naasi*. Mogelijke oorzaken van dit verschil tussen moleculaire en morfologsiche gegevens worden besproken.
Tussen zes isolaten van *M. hapla*, vier van race A en twee van race B, tussen acht van *M. chitwoodi*, drie uit de Verenigde Staten en vijf uit Nederland, en tussen vijf van *M. fallax* werden weinig polymorfismen gevonden met behulp van 2DGE (Hoofdstuk 8), in tegestelling tot de variatie tussen deze soorten (Hoofdstuk 7). Dit bevestigt dat deze soorten duidelijk onderscheidbare biologische groepen vormen. Percentages polymorfismen van 5,0, 2,2 en 0,6 % werden gevonden tussen de isolaten van respectievelijk *M. hapla*, *M. chitwoodi* en *M. fallax*. Deze volgorde

komt overeen met de volgorde in variatie voor virulentie van deze soorten op *Solanum* spp. (Hoofdstukken 3, 4 en 5).

Variatie in meiose en kruisingsbarrière worden besproken in de Hoofdstukken 9, 10 en 11. Eicel- en spermavorming werden bestudeerd in diverse isolaten van M. *hapla*, *M. chitwoodi* en *M. fallax* (Hoofdstuk 9). De eicelvorming verliep in deze soorten gelijk, behalve voor *M. hapla* isolaat He waarbij slechts in 12 % van de oocyten in geïnsemineerde vrouwtjes sperma was binnengedrongen, in plaats van de verwachte 100 %. Dit werd veroorzaakt door een verlengde profase I in oocyten die de spermatotheca reeds gepasseerd waren. De spermavorming verliep in *M. hapla* op gelijke wijze als in *M. chitwoodi*. In *M. fallax* werden in wisselende mate afwijkingen geconstateerd in ongeveer een-derde van de onderzochte mannetjes die wisselden van afwezigheid van meiose in sommige spermatocyten tot een volledige afwezigheid van meiose hetgeen leidde tot een volledige afwezigheid van sperma. In een onbekende meiotisch ongeslachtelijk vermenigvuldigende soort, vermoedelijk behorende tot de *M. arenaria* groep, trad slechts sporadisch een tweede meiotische deling op en in alle andere oocyten wordt chromosoomverdubbeling na de eerste meiotische deling verondersteld. Ook werd in een monofemale lijn van deze soort embryo-ontwikkeling binnen het vrouwtje waargenomen. Deze eigenschap bleek genetisch bepaald te zijn. Dit onderzoek toont aan dat in *Meloidogyne* een grote meiotische variatie bestaat.
Met moleculaire technieken werd bevestigd dat *M. hapla*, *M. chitwoodi* en *M. fallax* onderscheidbare biologische eenheden vormen (Hoofdstuk 8). Kruisingen werden uitgevoerd tussen de nauw verwante *M. chitwoodi* en *M. fallax* om het biologische soortsconcept in meiotisch ongeslachtelijk vermenigvuldigende *Meloidogyne* spp. te bestuderen (Hoofdstuk 10). De kruising tussen deze soorten was geslaagd in proeven met gecontroleerde en spontane kruisingen, maar de verkregen hybriden waren onvruchtbaar. De mogelijke rol van soortskruisingen in de soortsvorming en in de uitwisseling van genetisch materiaal op soortsniveau wordt besproken.
Genetische variatie tussen monofemale lijnen van *M. hapla* race A en van *M. incognita* werd bestudeerd met behulp van AFLP analyse (Hoofdstuk 11). De genetische afstanden tussen drie monofemale lijnen, afkomstig van drie larven van *M. incognita* na één generatie ongeslachtelijke vermeerdering, varieërde van 0,000 tot 0,004, en waren beduidend kleiner dan die tussen twee monofemale lijnen uit één larve van *M. hapla* na zes generaties ongeslachtelijke vermeerdering, namelijk 0,083. Dit is een sterke aanwijzing voor een genotypisch identieke nakomelingschap van een *M. incognita* nematode in tegenstelling tot de variatie in de nakomelingschap van een *M. hapla* race A nematode. De genetische afstanden tussen monofemale lijnen afkomstig van één *M. hapla* larve bleek niet noodzakelijkerwijs kleiner dan die tussen monofemale lijnen afkomstig van verschillende larven uit hetzelfde isolaat, hetgeen duidt op behoud van heterozygotie gedurende achtereenvolgende generaties ongeslachtelijke vermenigvuldiging. Het behoud van heterozygotie is hoogstwaarschijnlijk het resultaat van de combinatie van twee mechanismen in

meiotisch ongeslachtelijk vermenigvuldigende populaties: postreductionele meiose en het versmelten van het tweede poollichaampje met de eivoorkern. Niveaus van heterozygotie tussen monofemale lijnen van *M. hapla* en tussen verschillende isolaten bleken vergelijkbaar te zijn. Geen volledig homozygote lijnen kunnen worden verwacht na herhaalde ongeslachtelijke vermenigvuldiging van meiotisch ongeslachtelijk vermenigvuldigende isolaten. Voor het bestuderen van virulentie is het noodzakelijk om isolaten te ontwikkelen die stabiel zijn voor de aanwezige virulentiefactor(en). Een aanpak daartoe wordt voorgesteld en aangegeven hoe deze stabiele isolaten gebruikt kunnen worden in een veredelingsprogramma.

Concluderend kan gesteld worden dat een beter inzicht is verkregen in de cytologische variatie, in eiwitsamenstelling, in DNA en in de genetische variatie in ziektewekkend vermogen van wortelknobbelnematoden op *Solanum* spp. De gevolgen van de variatie voor het soortsconcept, de oorsprong van mannetjes, het onderscheid tussen isolaten, het voorkomen van nieuwe pathotypen, het testen op virulentie en veredelingsperspectieven worden besproken (Hoofdstuk 12).

Nawoord

Dit proefschrift is tot stand gekomen uit onderzoek dat met uitzondering van enkele onderdelen op het DLO Instituut voor Plantenziektenkundig Onderzoek is uitgevoerd vanaf 1 januari 1993. Ik heb mijn aanstelling destijds als iets heel bijzonders ervaren en ik ben de directie van het IPO-DLO erkentelijk voor het in mij gestelde vertrouwen en de geboden mogelijkheid voor een promotie-onderzoek. Tijdens dit onderzoek ben ik begeleid geweest door prof. dr. R.F. Hoekstra als mijn promotor en ir. P.W.Th. Maas en dr. C.H. van Silfhout als co-promotoren. Onze regelmatige bijeenkomsten om 4 uur 's-middags "op-de-kamer-van-Cor" waren plezierig en constructief en liepen meestal uit tot ver na ambtenarensluitingstijd. Rolf, Paul en Cor, bedankt voor jullie vele bemoedigingen, correcties, uitdagende opmerkingen en de geboden vrijheden ten aanzien van de invulling van het onderzoek.

Dit onderzoek is uitgevoerd in het kader van een project met als doel het bestuderen van genetische variatie tussen en binnen populaties van wortelknobbelnematoden die voorkomen in de vollegrond in Nederland. Hierbij bedank ik de anderen van het onderzoeksteam op het IPO-DLO die zich met dit thema bezighouden: Th.H.M. Donkers, L.M. Poleij en C. Zijlstra, voor hun collegialiteit en steun. In dit verband wil ik Leo speciaal bedanken. Je begon, evenals trouwens ikzelf, min of meer als een groentje in de Nematologie, maar je hebt je ontwikkeld tot een waardig nematoloog. Jij bent voor dit onderzoek onmisbaar gebleken. Door jouw aandeel in de realisatie van de figuren in dit proefschrift verdien je tevens het predikaat "CorelDraw-specialist".

In 1993/94 heeft W.B.J. Veldhuis in het kader van zijn vervangende dienstplicht meegeholpen aan de experimenten, met name aan de "cryoproeven" en het isozymwerk. In 1994/95 heeft B.J. Uenk in hetzelfde kader voor een gedeelte van zijn tijd meegeholpen aan het isozymwerk en de "petrischalenproef". Eveneens in 1994/95 heeft J.A. Los als toegevoegd onderzoeker cytologisch onderzoek verricht. Wim, Bert en Jan, bedankt voor jullie inzet en de goede samenwerking. Hoewel de werkzaamheden tijdens die perioden tot wederzijds voordeel strekten moet ik constateren dat dat uiteindelijk bij geen van jullie geresulteerd heeft in een baan in het landbouwkundig onderzoek!

Dat dit proefschrift het resultaat is van de samenwerking van velen heeft al mogen blijken uit het voorafgaande. Het moge des te meer blijken uit het aantal mede-auteurs voor de publicaties die uit het onderzoek voortkwamen. Hier komen ze in alfabetische volgorde: J. Bakker, R. Folkertsma, G.J.W. Janssen, R. Janssen, G. Karssen, P.H.G. van Koert, J.A. Los, P.W.Th. Maas, L.P. Pijnacker, L.M. Poleij, C.H. van Silfhout, W.B.J. Veldhuis, P.F.G. Vereijken en C. Zijlstra. Iedereen bedankt.

During some weeks in July-August 1994 I visited prof. A.C. Triantaphyllou at the North Carolina State University in Raleigh. I was priviledged to exchange ideas with one of the leading scientists in (cyto)genetics in nematodes. Thank you, Tasso, for

sharing your expertise and for working with me at the department, despite the fact being emeritus professor. This thesis shows traces of the precious time that I spent with you.

Niet alle werkzaamheden vermeld in dit proefschrift zijn door mij uitgevoerd op het IPO-DLO. De electroforese-runs voor de 2-D experimenten in de hoofdstukken 7 en 8 zijn uitgevoerd door R. Folkertsma en P.H.G. van Koert van de Vakgroep Nematologie van de Landbouwuniversiteit. De spermatogenese (hoofdstuk 9) is onderzocht op de confocaal laser scan microscoop van het DLO Centrum voor Plantenveredelings- en Reproductieonderzoek onder begeleiding van J. Blaas en H.A. Verhoeven. En de gegevens voor het experiment van de spontane kruising tussen *M. chitwoodi* en *M. fallax* (hoofdstuk 10) alsmede de morfometrische gegevens uit hoofdstuk 7 zijn afkomstig van G. Karssen van de Plantenziektenkundige Dienst. Rolf, Paul, Jan, Harry en Gerrit, bedankt voor jullie bijdrage.

Het idee dat de combinatie van postreductie meiose en parthenogenese genetische consequenties heeft (zie hoofdstuk 11) was de aanleiding tot een contact met L.P. Pijnacker van de Vakgroep Genetica, Rijksuniversiteit te Groningen. Dat eerste contact heeft geleid tot telefoontjes en bezoekjes over en weer. Laas, het was elke keer weer stimulerend met je te discussiëren over meiose en parthenogenese. Vooral je kennis over de afwijkende vormen daarin hebben mij weer eens laten zien dat biologische processen nooit geheel in bepaalde wetmatigheden zijn onder te brengen. Bedankt voor al je input en de vele corrigerende opmerkingen n.a.v. de hoofdstukken 9 en 11.

Statistische begeleiding voor dit onderzoek is gegeven door P.F.G. Vereijken en in de beginfase door J. de Bree. Pieter, dankzij jouw inbreng weet ik van het bestaan van gegeneraliserende lineaire, AMMI en GAMMI modellen. De vele discussies over plant-pathogeen interacties zijn, denk ik, niet alleen tot mijn maar tot wederzijds nut geweest. Bedankt, Pieter en Joop, voor jullie statistische bijdrage.

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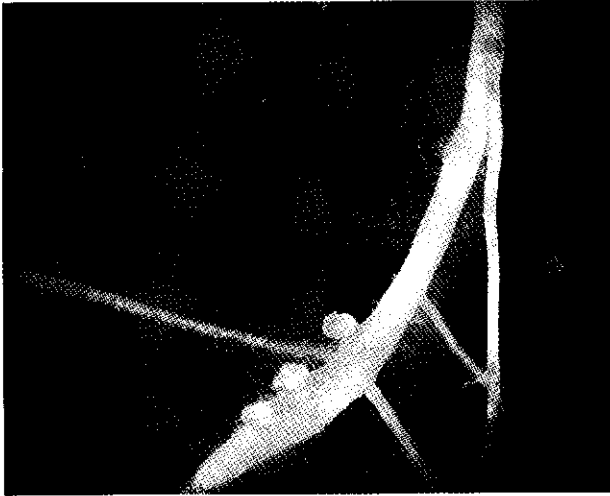
Tot slot: ik geloof in God de Schepper van hemel en aarde. Alle eer kom toe aan Hem die mij de kracht en de gezondheid gaf om dit onderzoek te doen.

Curriculum vitae

Johan Gerrit van der Beek werd geboren op 22 juni 1952 te Lisse. Na het behalen van het HBS-B diploma aan het Augustinus College te Groningen begon hij zijn studie biologie aan de Rijksuniversiteit te Groningen in 1970. Na twee jaar werd de ommezwaai gemaakt naar de Landbouwuniversiteit te Wageningen, waar hij plantenveredeling studeerde met erfelijkheidsleer, plantenziektenkunde en tropische plantenteelt als bijvakken. Ook werd een eerstegraads onderwijsbevoegdheid gehaald. De studie werd onderbroken door het vervullen van de vervangende diensplicht van 1976 tot 1977 op de toenmalige SVP, nu het CPRO-DLO, als assitent onderzoeker aan *Phytophthora infestans* in aardappel. Aansluitend heeft hij zijn praktijktijd vervuld in Suriname, werkende aan synthetische maïsrassen, adaptatie van drooglandrijstrassen en *Pyricularia oryzae* in rijst. Na het afstuderen begon in 1980 in het kader van de ontwikkelingshulp voor hem en zijn gezin een periode in Noord Afrika. Achtereenvolgens werkte hij aan *Puccinia hordei* en *Erysiphe graminis* f.sp. *hordei* in gerst als assistent deskundige voor de FAO in Marokko, aan wortelknobbelnematoden in tomaat als assistent deskundige voor de FAO in Algerije, en in de veredeling van peper (*Capsicum annuum*) als deskundige voor DGIS in Tunesië. Terug in Nederland heeft hij van 1990 tot 1992 als toegevoegd onderzoeker op het CPRO-DLO gewerkt aan de localisatie in tomaat van het *Cf*-9 gen voor *Cladosporium fulvum* en het *Ol*-1 gen voor *Oidium lycopersicum* m.b.v. RFLP en RAPD technieken. Sinds 1 januari 1993 is hij als onderzoeker in dienst van het IPO-DLO bij de afdeling Virulentie en Resistentie en sinds 1 januari 1997 bij de afdeling Entomologie en Nematologie werkzaam aan variatie in virulentie tussen en binnen populaties van wortelknobbelnematoden.



Egg-masses of *Meloidogyne fallax* on roots of potato cv. Bintje on Petri-dish culture, 8 weeks after inoculation

Tubers of potato cv. Eigenheimer, infested by *Meloidogyne chitwoodi* (below) compared to the control (above), 12 weeks after inoculation